Skin thickness as a potential indirect trait for genetic improvement of lamb survival

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Abstract

Lamb survival, as a trait of great economic importance with low heritability, might show more response to indirect selection for traits of higher heritability that are genetically correlated with lamb survival, as a trait of high economic importance. The main objective of this thesis was to explore if ultrasonographically measured skin thickness (ST) at about nine months of age could be considered as an alternative to direct selection for lamb survival from birth to weaning (SBW). For this purpose, in the first step, the reliability of ultrasonography as an accurate and noninvasive method for measurement of ST was validated using plicometry and histometry. In the second experiment, the heritability of ultrasonographically measured ST at an age of about 9 months was estimated to be 0.21 ± 0.03 and 0.20 ± 0.03, respectively from analyses with and without adjustment for live weight at scanning (LWS), implying that the trait would respond to genetic selection. Estimates of genetic correlation of ST with SBW from the analyses with LWS considered as a covariate for ST ranged from 0.16 to 0.35 depending on the minimum number of progeny per sire for each trait, while the corresponding estimates from the analyses with LWS excluded ranged from 0.08 to 0.27. When correction was made for LWS, ST showed genetic correlations of 0.21 ± 0.07, -0.13 ± 0.09, -0.32 ± 0.12, -0.23 ± 0.09, -0.10 ± 0.10, 0.02 ± 0.11, and 0.20 ± 0.11 with fat depth (FD), eye muscle depth (EMD), weights at weaning (WWT), 8 months (LW8), scanning (LWS), and 12 months (LW12), and fleece weight at 12 months (FW12), respectively. The corresponding estimates when no adjustment was made for LWS, were respectively 0.24 ± 0.08, -0.08 ± 0.10, -0.01 ± 0.12, 0.09 ± 0.09, 0.19 ± 0.09, 0.30 ± 0.10, and 0.20 ± 0.11. In the third experiment, the role of skin thickness in thermoregulation through its effect on surface heat loss and a few other indices of cold resistance was explored in two groups of new-born lambs with the thickest skin (thick-skinned category) and the thinnest skin (thin-skinned category) exposed to cold-stress. As a result of lower skin surface temperature (as an indicator of

heat loss) in thick-skinned lambs compared to thin-skinned lambs, the first group had higher rectal temperature and were more likely to maintain body temperature during cold stress, even though they produced significantly less heat (W Kg^{-1}). This means there is less need to consume body reserves as a source of energy and consequently better conservation of body reserves in the thick-skinned lambs. In the fourth experiment, skin thickness measured at six to eight months of age was revealed to be a moderately reliable indicator of skin thickness at birth. This is of high importance from both practical and economic points of views. Measuring skin thickness at six to eight months of age is much easier than at birth for sheep farmers/breeders. Furthermore, ultrasound measurement of skin thickness at these ages facilitates simultaneous recording of other traits of importance like fat depth and eye muscle depth, which are normally taken at these ages. In the final study, the effects of genetic variation in the uncoupling protein 1 (*UCP1*), prolactin (*PRL*), and prolactin receptor (*PRLR*) genes on the indices of cold resistance were tested in new-born lambs exposed to cold stress. Although significant effects on some of the indices were observed at/during some time-points/periods of the cold stress, they seem to be mostly due to biases resulting from low number of lambs rather than being real. Considering all the findings, it could be generally concluded that ultrasonographically measured skin thickness at about nine months of age could be considered as a supplement to direct selection for lamb survival in genetic improvement programs. Nevertheless, the large standard errors of the correlations of ST with SBW as well as the unfavorable correlation of ST with other traits should also be considered.

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Introduction

Lamb mortality is a major issue to sheep producers both in New Zealand and worldwide, not only due to economic losses but also as an animal welfare and management problem. For New Zealand sheep, it has been demonstrated that the economic value of ewe prolificacy is greater for flocks with high rates of lamb survival (Amer *et al.*, 1999). In a study of economic weights for hill sheep in the United Kingdom, Conington *et al.* (2004) have reported that lamb survival has one of the main influences on overall productivity.

Lamb mortality rates of up to 40% have been reported on some farms in New Zealand (Fisher, 2004). Such a reduction in the number of lambs raised to slaughter can dramatically reduce the annual income earned by New Zealand sheep producers (Everett-Hincks *et al.*, 2007). According to the reduced number of lambs raised to slaughter and sold, lamb mortality rates in 2007 have been estimated to cost New Zealand farmers over $580 million annually (Everett-Hincks *et al.*, 2007). Hence, even a small improvement in lamb survival rates would increase annual profit remarkably (Everett-Hincks *et al.*, 2007). Reducing lamb deaths would also improve animal welfare perceptions and diminish the risk of non-tariff barriers imposed by international markets (Everett-Hincks *et al.*, 2007). Apart from this cost, the reduction in productivity of hypothermic lambs that manage to survive and the reduced selection potential incurred by having fewer lambs surviving until selection impose additional costs (Forrest *et al.*, 2006). Starvation/exposure is the second most common cause of lamb deaths in the neonatal period after dystocia (Kerslake *et al.*, 2005, Everett-Hincks *et al.*, 2007). In countries like New Zealand, the UK and Australia, where lambing mostly takes place outdoors, thermoregulatory capacity of new-born lambs plays a major role in lamb survival due to its effect on reducing sensitivity to cold exposure, which is a main contributor to starvation-exposure mortality rates (Mellor and Stafford, 2004). Starvation/exposure is especially important in multiple-born lambs, since lambs of larger litters would have larger surface area to weight ratio which impose

much more heat loss on them compared with single-born ones (Samson and Slee, 1981). Hence with the current focus on increasing lambing percentage (Bray, 2005, Kenyon *et al.*, 2005) and consequently an associated decline in single-born lambs and an increase in multiple-born ones, the situation could worsen. It has been shown that as average litter size increases above 1.7, the decline in single-born lambs is offset by an increase in triplets (Davis *et al.*, 1983).

Although some studies have shown the positive effects of providing shelter (Smart and Marchant, 2013) and improved dam nutrition (Hatcher *et al.*, 2009) on reduction of lamb mortality, genetic improvement of lamb survival could be relatively cost-effective and permanent compared with environmental improvements. On the other hand, lamb survival has been reported to have a low direct and maternal heritability (Lopez-Villalobos and Garrick, 1999, Morris *et al.*, 2000, Riggio *et al.*, 2008, Brien *et al.*, 2010) and is a trait of importance that is impossible to measure for all animals. Therefore, indirect selection, based on selection for other easy-to-measure traits of higher heritability which are genetically correlated with survival is an alternative to direct selection for the trait itself. Cold tolerance as a component of lamb survival, is a trait of moderate to high heritability (Wolff *et al.*, 1987, Slee *et al.*, 1991). Apart from heat production through non-shivering thermogenesis using energy derived from brown fat (Alexander and Williams, 1968, Symonds and Lomax, 1992), reducing heat loss from the skin surface is vital for maintaining core body temperature and increased cold tolerance (Alexander, 1978).

Samson and Slee (1981) have revealed an effect of skin thickness on increased cold resistance in new-born lambs. Similarly, it has been shown that selection for improved cold resistance results in lambs with increased skin thickness and an overall increase in total body insulation which conserve body energy reserves in new-born lambs (Stott and Slee, 1987). Hence, selection for skin thickness might be a potential alternative to selection for cold resistance and consequently lamb survival. Skin thickness is a trait of moderate to high heritability (Gregory,

1982a, Slee *et al.*, 1991) and is anticipated to respond to selection. Furthermore, unlike cold resistance whose assessment needs laboratory-based techniques which are not feasible for breeders, skin thickness could be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000). However, before implementing this trait in selection for lamb survival, it is inevitable to first evaluate its heritability and genetic association with other economic traits. Although a limited number of studies have been undertaken for estimating these parameters (Gregory, 1982a, Slee *et al.*, 1991, Ponzoni *et al.*, 1995, Hynd *et al.*, 1996), the sample size in those experiments have been small. Furthermore, complementary experiments are required to examine the effect of skin characteristics on thermoregulation through reduction in heat loss in new-born lambs.

Finding a genetic relationship between skin thickness and lamb survival and thermoregulation could assist sheep breeders in selection of animals of greater thermolegulatory capacity through selection for skin traits in order to generate lambs with high cold resistance. Increased cold resistance in turn will lead to more lambs surviving to weaning whose sale is the main source of income for New Zealand sheep farmers.

Therefore, to meet this goal, a study was undertaken with six main objectives:

1. To validate the accuracy of ultrasonically measured skin thickness by plicometry and histometry
2. To estimate heritability for ultrasonographically measured skin thickness (ST), fat depth (FD), and eye muscle depth (EMD) at an average age of about 9 months, lamb survival from birth to weaning (SBW), fleece weight at 12 months (FW12), and live weights at weaning (WWT), 8 months (LW8), scanning (LWS), and 12 months (LW12).

3. To estimate genetic, environmental, and phenotypic correlations of skin thickness (as the proposed trait influencing lamb survival) with other traits of interest in New Zealand Romney sheep as the most predominant dual-purpose breed in New Zealand.
4. To explore the possible role of skin thickness in thermoregulation through its effect on surface heat loss and a few other indices of cold resistance in new-born lambs exposed to cold-stress.
5. To explore whether skin thickness at an older age is an appropriate indicator of the trait at birth.
6. To test the possible effect of genetic variation in three possible candidate genes (uncoupling protein 1 (*UCP1*), prolactin (*PRL*), and prolactin receptor (*PRLR*)) on cold resistance in new-born lambs.

Chapter 1

Literature review

1.1. Lamb survival

Lamb survival is a matter of great interest to sheep producers both in New Zealand and worldwide, due to its economic importance and also as an animal welfare issue. Amer *et al.* (1999) have shown that the economic value of ewe prolificacy is greater for flocks with higher rates of lamb survival in New Zealand. Also, Conington *et al.* (2004) have reported that lamb survival has one of the main influences on overall productivity of sheep farms in the United Kingdom.

Mortality rates of up to 13.1% and 25.8% have been reported in singleton and twin lambs, respectively, in New Zealand farms (Hight and Jury, 1970, Wiener *et al.*, 1973, Nicoll *et al.*, 1999). However, higher lamb mortality rates of 20 to 37% have been observed in high fecundity flocks (Hinch *et al.*, 1985), which is likely to be a result of high mortality rates of triplet lambs ranging from 35 to 41% (Hinch *et al.*, 1983, Amer *et al.*, 1999, Morel *et al.*, 2008, Kerslake *et al.*, 2009). High mortality rates have been also observed in many other countries around the world including Australia (Alexander and Peterson, 1961, Alexander, 1964), the U.K. (Purser and Karam, 1967, Wiener *et al.*, 1973), and the U.S.A. (Vetter *et al.*, 1960). A large range of 5 to 70% has been reported for lamb mortality rate in Australia (McHugh and Edwards, 1958, Smith, 1962). Therefore, the necessity of improving lamb survival seems clear.

1.1.1. Costs of lamb mortality

The New Zealand red meat sector is a principal driver of the New Zealand economy. Accounting for about 47% of the world's trade in lamb, New Zealand is the world largest lamb exporter (Morris, 2009). Lamb export revenues for the year ending 30 September 2019 were estimated at $3.21 billion (Beef + Lamb New Zealand 2020). According to the reduced number of lambs raised to slaughter and sold, lamb mortality rates have been estimated to cost New Zealand farmers over $580 million annually (Everett-Hincks *et al.*, 2007). Hence, even a small

improvement in lamb survival rates would increase annual profit remarkably (Everett-Hincks *et al.*, 2007).

High lamb mortality rates due to cold exposure is a major issue not only in New Zealand, but also in other areas of the world like Australia (Alexander, 1964), the U.S.A. (Vetter *et al.*, 1960), and the U.K. (Purser and Karam, 1967), where lambs are born outdoors. It has been estimated that lamb mortalities would cost the Australian sheep industry more than $1 billion each year, and that improving the survival of single lambs by 5% and twin lambs by 20% would improve farm profit across the industry by $100 M and $350 M per annum, respectively (Hancock *et al.*, 2019).

Poor lamb survival may also hinder attempts to improve net reproductive rates, with techniques increasing the number of lambs born often resulting in decreases in overall lamb survival particularly due to an increased proportion of multiple births (Slee *et al.*, 1991). Young *et al.* (2014) concluded that research on improving survival of twin-born lambs is likely to have a much higher payoff than research on improving the number of lambs conceived.

The cost of lamb mortality caused by cold exposure has been estimated at approximately $40 million per year in New Zealand (Gudex, 2001). Also, the reduction in productivity of hypothermic lambs that manage to survive impose other additional costs (Forrest *et al.*, 2006).

Reducing lamb deaths would also improve animal welfare perceptions and diminish the risk of non-tariff barriers imposed by international markets (Everett-Hincks *et al.*, 2007). The loss of selection potential incurred by lamb mortality is another cost which is usually overlooked. As the number of dead lambs increases, there would be smaller numbers of potential animals to be selected as the parents of next generation which means reduced selection intensity and consequently decreased genetic gain.

1.1.2. Causes of lamb mortality

Dystocia (difficult birth) and starvation/exposure are the two main causes of lamb mortality (Meyer and Clarke, 1978, Kerslake *et al.*, 2005, Everett-Hincks *et al.*, 2007) depending on the post mortem diagnostic procedure used. Since in some cases the cause of lamb death is diagnosed as starvation/exposure which has been triggered by dystocia (Dwyer and Morgan, 2006). Haughey (1983) has suggested that between 20 to 60% of neonatal lamb deaths pathologically categorized as starvation or exposure are actually consequences of birth stress. If a lamb suffering from difficult birth can survive the birth process, it would be probably more susceptible to starvation/exposure compared with those who have undergone a normal birth, since they have trouble maintaining body temperature, teat searching and suckling (Eales *et al.*, 1982).

1.1.2.1.Dystocia

Dystocia has been reported as the primary cause of lamb deaths particularly in single- born lambs (Hartley and Boyes, 1964, Joyce *et al.*, 1976, Dalton *et al.*, 1980, Tarbotton and Webby, 1999, Kerslake *et al.*, 2005). Birth weight has been reported as the predominant factor leading to dystocia in single-born lambs, and as birth weight increases from an optimum weight, there would be a rise in the rate of mortality resulting from dystocia (Scales *et al.*, 1986, Fogarty *et al.*, 1992). Dystocia can be influenced by many factors including lamb birth weight, sire breed, dam pelvic conformation (Fogarty and Thompson, 1974), malpresentation, maternal overfeeding, or prolonged parturition (Sargison, 1997, Everett-Hincks *et al.*, 2007). Apart from its direct effect on lamb mortality, dystocia can affect lamb death indirectly through impairment in thermoregulation. Hypoxia as a result of the birth process has been suggested to be associated with depressed heat production in new-born lambs (Stott and Slee, 1987). Also, Alexander *et al.* (1980) have reported that birth injury can lead to increased susceptibility to hypothermia as a result of impaired thermoregulation in new-born lambs.

1.1.2.2.Starvation/exposure

Starvation/exposure has been determined as the second most common cause of lamb mortality (Gumbrell and Saville, 1986, Kerslake *et al.*, 2005). Starvation/exposure has been reported to be responsible for approximately 30% of new-born lamb losses under New Zealand conditions (Hartley and Boyes, 1964, McCutcheon *et al.*, 1981). Post-mortem studies have shown that the percentage of all neonatal deaths resulting from starvation-exposure are highly variable (ranging from 15.1% to 46%), which is probably due to different weather conditions (Hartley and Boyes, 1964, Hight and Jury, 1970, McCutcheon *et al.*, 1981, Cloete and Scholtz, 1998, Kerslake *et al.*, 2005). Starvation/exposure is particularly important in multiple born lambs due to their lighter birth weight (Dalton *et al.*, 1980).

Therefore, increased birth weight is an advantage to the survival of twin and triplet lambs. Hight and Jury (1970) have reported that lambs of heavier birth weights are better equipped to survive conditions predisposing them to exposure and starvation because they have more energy stored as brown fat reserves and maintain their suckling drive for a greater duration than lighter lambs.

Severe weather conditions are a major contributor to death from starvation/exposure (Slee, 1981, Mellor and Stafford, 2004). Cold resistance is another factor largely influencing lamb mortality from starvation/exposure, which will be discussed in detail in the section related to thermoregulation in new-born lamb.

1.1.3. Important environmental factors affecting lamb survival

1.1.3.1.Birth weight and body size

For both single- and multiple-born lambs, birth weight is the dominant factor affecting lamb survival (Hight and Jury, 1970, Hinch *et al.*, 1985). Birth weight in relation to lamb survival is so important that some authors (Morris *et al.*, 2000, Gudex *et al.*, 2005) have suggested that it

would be fruitless to study lamb mortality without considering lamb birth weight. Burfening and Carpio (1993) have reported that lamb birth weight is 2 to 3 times more important than birth date and live weight of the dam. A curvilinear relationship between lamb birth weight and survival was first described by Hight and Jury (1970) and subsequently by many others (Dalton *et al.*, 1980, Purser and Young, 1983, Hinch *et al.*, 1985). In general, it can be mentioned that lambs with birth weight around average tend to have greatest survival rate at birth (Morris *et al.*, 2000, Sawalha *et al.*, 2007), and deviations from average birth weight, especially toward a reduced birth weight, increases lamb loss (Petersson and Danell, 1985, Burfening and Carpio, 1993). Lambs with birth weights between 3 kg and 5.5 kg have been reported to have the highest survival rates (Dalton *et al.*, 1980), 4.7 kg being the suggested optimal weight that is almost similar (4.6 kg) to that reported by Knight *et al.* (1988). As birth weight decreases under the average, lamb mortality caused by starvation/exposure would increase (Scales *et al.*, 1986, Yapi *et al.*, 1990), due to insufficient body reserves (Alexander, 1984). On the other hand, a deviation toward an increased birth weight could lead to decreased lamb survival caused by dystocia or birth injury (Dalton *et al.*, 1980, Fogarty *et al.*, 1992, Nowak and Poindron, 2006). Morris *et al.* (2000) have reported that larger sized lambs have a good chance to survive once they get through the birth process.

Lamb size is also a major factor influencing lamb survival because small lambs are characterized by a greater surface area to body weight ratio (Sykes *et al.*, 1976), which causes them to have lower heat production relative to their surface area (Stott and Slee, 1987). Small lambs also have poorer insulation, less cold resistance and a reduced ability to recover from hypothermia compared to larger lambs (Stott and Slee, 1987). Furthermore, small lambs are at a disadvantage in terms of thermoregulation owing to their relative low energy reserves (Alexander, 1984).

1.1.3.2.Birth rank

It has been shown that lamb mortality generally increases with litter size (Hinch *et al.*, 1983, Petersson and Danell, 1985, Nicoll *et al.*, 1999, Riggio *et al.*, 2008, Hatcher *et al.*, 2009). In a study conducted on seven ram breeding flocks in New Zealand, Nicoll *et al.* (1999) showed mortality rates of 9.9%, 12.1% and 26.4% for singles, twins and triplets, respectively. In a large longitudinal study in Australia, even higher rates of 20%, 34% and 54% was observed for singleton, twin and triplet or higher order litters, respectively (Hatcher *et al.*, 2009).

Cundiff *et al.* (1982) have reported evidence for an antagonistic genetic correlation between litter size and lamb survival. However, Bradford (1972), Dalton *et al.* (1980), and Purser and Young (1983) have suggested that litter size does not affect lamb survival directly but through its effects on birth weight. However, Hinch *et al.* (1985) have reported that the effect of litter size on lamb mortality remains significant even after accounting for lamb birth weight. Stevens *et al.* (1981) and Hinch *et al.* (1983) have demonstrated that lambs born as triplet or quadruplet have a lower chance of survival than singletons and twins of the same birth weight and postulated that this was owing to hypoxia and competition.

The metabolic rate of single and twin lambs has also been shown to differ. After adjusting for live weight, it has been shown that singletons have a greater metabolic rate compared to twin lambs at both thermoneutral and cold conditions (Stott and Slee, 1987). They calculated that singleton lambs had a proportionately greater cold resistance than twin lambs (Stott and Slee, 1987) and concluded that this was due to higher metabolic rates, a longer duration to reach summit metabolism, longer sustained summit metabolism, greater cold resistance and slower decrease in rectal temperature. On the contrary, in a comparison of twin and triplet lambs, Kerslake *et al.* (2010) found no effect of litter size after an adjustment was made for birth weight.

On the other hand, litter size can also influence the distribution of the ewe's grooming behavior. O'Connor *et al.* (1992) have shown that twin-born lambs receive less overall grooming attention compared to singleton lambs.

1.1.3.3.Sex

While Atkins (1980) found no significant association between sex of lamb and survival rate, there are many other studies reporting that male lambs have a higher rate of mortality compared to females (Mullaney, 1969, Hight and Jury, 1970, Dalton *et al.*, 1980, Johnson *et al.*, 1982, Southey *et al.*, 2001, Riggio *et al.*, 2008, Hatcher *et al.*, 2009), which is in contrast with the fact that male lambs have on average more birth weight compared with female lambs (Hight and Jury, 1970, Jopson *et al.*, 2000). So there might be another factor other than birth weight which causes the difference in survival of males and females. Johnson *et al.* (1982) have reported that the difference in survival to weaning of male and female lambs is more significant amongst single-born lambs than multiples. Also, Alexander *et al.* (1955) and Gunn and Robinson (1963) showed that the higher rate of male lamb mortality could be a result of a greater number of difficult births in males compared with ewe lambs.

On the other hand, McCutcheon *et al.* (1983b) have reported that male lambs show a greater resistance to cold stress, which has been largely attributed to the greater body weight in males which is again in disagreement with the fact that ram lambs have higher rates of mortality than ewe lambs. Contrary to these results, Stott and Slee (1987) reported greater total body insulation and metabolic rate for female lambs compared to males, whether weight adjusted or not, which support the higher survival rates of female lambs than male lambs. Therefore, they concluded that the higher mortality rates of male lambs may be due to differences in thermoregulation.

1.1.3.4.Age of dam

It is a common observation that very young ewes have lower lamb survival than older ewes (Hight and Jury, 1970, Atkins, 1980, Petersson and Danell, 1985, Knight *et al.*, 1988, Sawalha *et al.*, 2007, Riggio *et al.*, 2008, Everett-Hincks *et al.*, 2014). Usually, the highest lamb survival rate is found for 4 to 6 year old dams (Knight *et al.*, 1988, Lopez-Villalobos and Garrick, 1999). Also, Hatcher *et al.* (2009) reported that the poorest lamb survival was seen in case of 2-year old as well as aged dams (6 year or older), while maximum survival was among progeny of 4-year-old dams. Increased lamb mortality observed in older ewes could be due to a higher potential for udder damage in this group of dams, which can negatively affect lamb survival (Hatcher *et al.*, 2009). It seems that an increase in lamb survival corresponding to increased dam age could be contributed to more experienced dams and higher birth weights of lambs born to the more mature ewes (Dalton *et al.*, 1980).

1.1.4. Genetic selection for lamb survival

Although some studies have shown improvement in lamb survival through manipulating environmental factors like dam nutrition (Hatcher *et al.*, 2009) and providing shelter at lambing (Smart and Marchant, 2013), genetic improvement of this trait could be relatively cost effective and permanent compared with environmental improvements. An important factor for a trait in order to respond to genetic selection is to be heritable, so that the larger the heritability, the more the genetic gain would be (Falconer and Mackay, 1996).

Therefore, direct genetic selection for lamb survival would not seem promising due to its low heritability (Lopez-Villalobos and Garrick, 1999, Morris *et al.*, 2000, Riggio *et al.*, 2008, Brien *et al.*, 2010). On the other hand, lamb survival is a trait of importance that is impossible to measure for all animals. Therefore, indirect selection, based on selection for other easy-to-measure traits of higher heritability that are genetically correlated with survival, is an alternative to direct selection for this trait itself. Cold resistance as a major component of lamb

survival, is a trait of moderate to high heritability (Wolff *et al.*, 1987, Slee *et al.*, 1991). But in practice it is not feasible, from both economic and animal welfare points of view, to measure cold tolerance of individual lambs through physiological responses to progressive cooling in a water-bath systems (Stott and Slee, 1987, Wolff *et al.*, 1987).

Hence, selection for skin thickness, as a trait suggested to affect cold tolerance (Samson and Slee, 1981, Slee *et al.*, 1991), might be a potential alternative to selection for cold resistance and consequently lamb survival. Skin thickness is a trait of moderate to high heritability (Gregory, 1982a, Slee *et al.*, 1991) and is anticipated to respond to selection. Furthermore, unlike cold resistance, whose assessment needs laboratory-based techniques that are not feasible for breeders, skin thickness could be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000).

1.1.4.1.Statistical models for genetic evaluation of lamb survival

Unlike many production traits such as body weight, carcass weight and growth rate, which are generally expressed on a continuous scale and are assumed to be normally distributed, there are many other traits of importance in animal production such as littler size, degree of calving difficulty, and survival that present a discontinuous distribution of phenotypes.

Estimation of variance components through mixed model methodology used for continuous traits typically assumes that the variance is not a function of the mean, and that the random effects follow normal distributions. These assumptions may be violated for binomial traits. For example, lamb survival has a binomial distribution and therefore phenotypic variances and means are not independent (Lush *et al.*, 1948). Due to these violations, linear models used for analyzing continuous responses are often believed to be inadequate for categorical responses (Thompson, 1979, Gianola, 1982, Ramirez-Valverde *et al.*, 2001). An alternative to overcome this problem is to transform data. Logit and probit are two common forms of transformation

used for categorical traits in animal breeding. Wright (1934) proposed a transformation for discontinuous data to a continuous scale.

An alternative is to consider traits like survival as threshold characters in which these traits are assumed to be categorical on an observed scale but believed to be continuous on an underlying (unobserved) scale (Falconer and Mackay, 1996). For a threshold trait to be exposed, a threshold point on the underlying continuous scale (liability scale) must be crossed (Wright, 1934). The liability is influenced by both environmental factors and many genes that have small additive effects (Dempster and Lerner, 1950).

Generalized linear mixed models (GLMM) based on threshold theory have been studied for the analysis of categorical traits and have been shown to be theoretically better than linear statistical models (Gianola, 1980, Gianola, 1982). The threshold model (TM) is more demanding than the linear model (LM) from a computational standpoint. According to this method, for survival as a binary trait, individuals are scored 1 (alive) if they exceed a certain threshold value, otherwise are scored 0 (dead). A binomial distribution with a probit link function is assumed in the threshold model (Gianola and Foulley, 1983). Many studies have also adopted threshold models for categorical traits in practice (McGuirk *et al.*, 1998, Van Tassell *et al.*, 1998, Boettcher *et al.*, 1999, Riggio *et al.*, 2008).

Some studies have suggested that TM do not offer advantages over LM in genetic evaluation of discrete traits. Amer and Jopson (2003) have suggested that routine analyses of lamb survival would not require computationally expensive transformations to a logit or probit scale. Ron *et al.* (1990) also concluded that TM do not offer advantages over LM in genetic evaluation of discrete traits. Also, Weller *et al.* (1988) compared LM and TM for binary traits in Holsteins and did not find differences between these two models.

However, there are many other studies that have confirmed advantages of using TM over LM in analyzing discrete traits. In a study on genetic parameters for disease and fertility (as binary traits) in cattle, Kadarmideen *et al.* (2000) reported higher estimates of heritability from a threshold model compared with those from a linear model, though accuracy was lower with the TM than the LM for the same heritability. In another study (Matos *et al.*, 2000) that employed both models to estimate the direct and maternal heritabilities of lamb survival in low fecund Rambouillet and prolific Finn sheep, TM yielded higher heritability estimates than those obtained by LM (0.03 vs 0.06 and 0.03 vs 0.04 for direct and maternal heritabilities in Rambouillet, and 0.09 vs 0.17 and 0.19 vs 0.26 in Finn sheep). Also, Welsh *et al.* (2006), using a TM, reported relatively high estimates for direct and maternal heritabilities of survival (0.106 and 0.082, respectively) in New Zealand Romney lambs. Similarly, Riggio *et al.* (2008) showed that lamb survival heritability from the LM was somewhat lower compared to TM (0.09 versus 0.33 for direct heritability of perinatal survival) and it was recognized that the assumption of the trait as being continuous with normally distributed residuals was violated for this trait. It can be concluded that TM analysis is able to detect mentionable levels of additive and maternal genetic variation in lamb survival.

1.2. Skin thickness as a trait affecting lamb survival

1.2.1. Skin histology

The external lining of the body consists of skin and its different specializations, which among various domestic species include claw, hoofs, pads, and horns. The integument, as the largest organ in the body, functions to protect the body against exposure to the continual changes occurring in the external environment. Furthermore, the skin contributes to external sensory awareness, thermoregulation, and immunologic defense (Samuelson, 2007).Skin is composed of two main layers of epidermis and dermis whose developments vary remarkably among different species depending on the protective needs of the animal. In addition to the epidermis

and dermis there is another structure associated with skin called hypodermis, which lies internally to the dermis. The hypodermis, also referred to as subcutaneous tissue, can be rich in adipose tissue and loose connective tissue, which serves to anchor the skin to adjacent structures, such as skeletal muscle (Samuelson, 2007).

1.2.1.1.Epidermis

As the outermost layer of the skin, epidermis is a keratinized stratified squamous epithelium which mainly acts as a seal that prevents the body from rapid dehydration. The epidermis is composed of two cell populations: keratinocytes (those that solely constitute the epithelium) and non-keratinocytes (those cells that have migrated into the keratinized stratified layer, including melanocytes, macrophages, and lymphocytes). The epidermis and dermis are thicker in those regions that routinely receive the most abrasion. These layers are connected to each other by the basement membrane (Samuelson, 2007).

1.2.1.2.Dermis

The dermis, or corium, lies beneath the basement membrane and extends to the hypodermis. This layer of skin is of mesodermal origin and consists of dense irregular connective tissue composed of collagen, elastic, and reticular fibers embedded in an amorphous ground substance. The dermis can be divided into a superficial papillary layer which blends into a deep reticular layer without a clear line of demarcation (Dellmann, 1993).

As the most superficial layer of dermis, the papillary layer consists of loose connective tissue which may protrude into the epidermis resulting in the formation of the dermal papillae, which appear saw-toothed in shape and lead to a strong attachment between dermis and epidermis. Each dermal papilla has a capillary bed or loop that not only provides nutrition to the epidermis layer, but also has a role in regulating body temperature (Samuelson, 2007).

The reticular layer of dermis is characterized generally by the size and density of the extracellular fibers which are larger and more compact than those of the papillary layer. Animals with thick skin have very well-developed reticular layers consisting mainly of collagen type I and proteoglycans that are dominant in dermatan sulfate. The elastic fibers in this layer have large diameter as opposed to those in the papillary layer which are remarkably of smaller diameter. The nervous elements, glands (sweat glands and sebaceous gland), and hair follicles (referred to as fleece or fiber in sheep) of skin lie mostly within this layer. Adipocytes can be observed in this layer in scattered clusters especially in the border of the reticular layer and adjacent tissues such as bone or muscle (Samuelson, 2007).

1.2.1.3.Hypodermis

Beneath the dermis is a layer of loose connective tissue, the hypodermis or subcutaneous tissue (subcutis) which is not part of the skin but rather the superficial fascia seen in gross anatomic dissections (Dellmann, 1993). Adipose tissue is present in this layer and may form small clusters of fat cells or large masses that result in the formation of a cushion or pad of fat called panniculus adiposus, which contributes to body contour, in addition to fat storage and heat insulation (Samuelson, 2007).

1.2.2. Skin thickness measurement

Before skin thickness could be used in selection programs for improving other traits of importance, its accurate measurement is important. While many studies have applied skin-fold calipers as a tool for measuring the thickness of skin (Gregory, 1982a, Slee *et al.*, 1991, Williams and Thornberry, 1992), it seems that differences in the subcutaneous fat thickness and also pinching force could lead to inaccuracy in measurement (Alexander and Miller, 1979). Real time ultrasonography is another technique for measuring skin thickness whose application has attracted increasing attention because of its advantages over skin-fold thickness measurement. Many countries around the world have incorporated the ultrasound

measurements into genetic programs for lamb carcass quality improvement due to the relatively low cost and portability of ultrasound equipment (Stanford *et al.*, 1998). Ultrasound scanning was first used as an objective method for estimating subcutaneous fat thickness in cattle and is frequently used in both sheep and cattle (Dicker *et al.*, 1988). Ultrasound scanning could provide an objective, non-invasive and simple way to measure fat and muscle depths and potentially skin thickness (Brown *et al.*, 2000). Another benefit of this method is that the wool generally does not need to be clipped before measurement, which would damage the skin and decrease the commercial value of the animal (Teixeira *et al.*, 2008). Clear images could be obtained simply by parting the wool and applying a small amount of vegetable oil (Brown *et al.*, 2000). Ultrasound also provides a real-time image of the skin, its structure and characteristics (Zanna *et al.*, 2012). Ultrasonography is the detection of reflected sound waves through tissues that have inherently different acoustic characteristics. Particularly, echoes in the dermis are the result of the reflection of the ultrasonography waves at the boundaries between dermal components, including collagenous and reticular fibers, dermal ground substance, sebaceous and sweat glands, and the surrounding water-rich ground substance (Gniadecka and Quistorff, 1996). Therefore, the resulting ultrasonography image consists of regions of different echogenicity, corresponding to different histologic layers of the skin (Aspres *et al.*, 2003). One important aspect in soft tissue ultrasonography is the selection of an appropriate transducer.

Another aspect of ultrasonography is the position from which measurement is taken. Ultrasound measurements at the 13th thoracic vertebra have been widely used both in cattle and sheep because this position can be clearly detected and measurements taken from this area are valuable for evaluating carcass quality (McEwan *et al.*, 1989, Young and Deaker, 1994).

Using ultrasound technique operated at 3.5 MHz, Brown *et al.* (2000) measured skin thickness in sheep and offered many potential advantages over the caliper technique. Although, they

found some discrepancies between their measurements made by calipers and real time ultrasound, it seems that the lack of correlation between the measurements could be due to using a low frequency transducer. While subcutaneous fat measurements can be made using conventional scanners operating at 5 to 7 MHz (Brown *et al.*, 2000), ultrasound equipment operating at higher frequency and resolution are required to produce adequate images to identify features of the skin (Alexander and Miller, 1979, Dines *et al.*, 1984). Butler and Head (1993) showed that although some difficulties are apparent in identifying the skin surface and therefore measuring tissue thicknesses, a medium frequency (7.5 MHz) is suitable in detecting the papillary and reticular layers of cattle skin, about 2 and 4 mm thick, respectively. Nevertheless, higher frequencies would be required for imaging the skin of smaller animals like pigs and sheep with thinner layers.

Using ultrasonography at 8 to 10 MHz, Ripoll *et al.* (2010) successfully measured skin thickness taken over the 10th to 11th and 12th to 13th thoracic vertebrae and over the first to second and third to fourth lumbar vertebrae in suckling lambs, light lambs and wethers, which ranged from 1.5 to 1.8 mm. In a study conducted on dogs, a positive correlation was shown between histologic histometric analysis results and results of ultrasonography by use of a 13-MHz linear-array transducer (Zanna *et al.*, 2012).

1.2.3. Skin thickness changes affected by environmental factors

Changes in the thickness of the skin, attributable to differences in nutrition, have been reported by Hutchinson (1957), who suggested that measurable changes in skin thickness, weight or protein content may be due to changes in nutrition, providing a useful measure of the incidence and magnitude of seasonal nutritional stresses that commonly occur under grazing conditions.

Williams and Thornberry (1992) have reported that the average thickness of skin did not differ between two flocks of adult medium wool Merino sheep assigned a high or low level of

nutrition. They observed a reduction in thickness of skin of sheep under low dietary treatment, which was attributed to a reduction in live weight. However, those sheep fed high diet, which resulted in a substantially increased live weight, did not show a change in the skin thickness. These results suggest the relationship between skin thickness and nutrition may be complex. Wodzicka (1958b) observed a thickening of the skin, with no change in fat or protein content, following shearing and this was attributed to cold stress (Wodzicka-Tomaszewska, 1960). Scobie *et al.* (1998) demonstrated a greater thickness of leather from shorn animals compared with that from non-shorn group.

In a long-term study (Wodzicka, 1958a) that monitored skin thickness of twelve Romney wether lambs from 10 days of birth until five months of age at monthly intervals, skin thickness was found to increase by some 14% until the lambs were 5 to 10 weeks of age. However, by 13 weeks it decreased to the original thickness at birth and remained at this value until 5 months of age. From the results of this experiment they suggested that within 10 days of birth, the skin of lamb is as thick as at five months of age. On the other hand, since no measurement was obtained at birth, they also suggested that the high value obtained at the first sampling was the result of a rapid increase in skin thickness in early stages. The significant correlation they obtained between skin thickness and age at first sampling supported this suggestion.

1.2.4. Selection for skin thickness

1.2.4.1.Heritability

Heritability defined as the proportion of phenotypic variation attributable to additive genetic variation (Falconer and Mackay, 1996), plays a major role in the rate of genetic progress gained by selection for quantitative traits. Gregory (1982a) estimated heritability of skin thickness in South Australian Merinos, using half-sib correlation and dam-offspring regression methods to be 0.60 and 0.25, respectively. Also, in a study by Slee *et al.* (1991), the heritability of skin thickness measured by skin fold calipers at tagging has been estimated as 0.35. Heritabilities

of 0.36 and 0.24 have been estimated for skin quality (graded as 1 for worst, very tight to 5 for best, pliable) in South Australian Merino sheep of 10 and 16 months age, respectively (Ponzoni *et al.*, 1995).

1.2.4.2.Relationship of skin thickness with other economic traits

1.2.4.2.1. Wool traits

Wool has always been considered as an important trait of interest in sheep breeding programs both in New Zealand and worldwide. As deeper growing crops generally yield higher yielding crops, sheep with thick skin will likely produce heavier fleeces (Williams and Thornberry, 1992). This association has been investigated in a few studies.

Gregory (1982b) found a significant genetic correlation of 0.39 between skin thickness and clean fleece weight in South Australian Merino sheep. Furthermore, skin thickness was found to be genetically correlated (0.20) with average fiber diameter in the same population. Contrary to this, Hynd *et al.* (1996) indicated a slight negative correlation between skin weight (as an indicator of thickness) and clean fleece weight. Also, they found that sheep with heavy skin tended to genetically have higher fiber diameter, staple length and strength. In addition, crimp definition showed a moderate negative association with skin weight so that sheep with heavier skin possessed poorer crimp definition. On the other hand, in a study by Ponzoni *et al.* (1995), clean fleece weight was shown to be genetically correlated with subjectively assessed skin quality (graded as 1 for worst, very tight to 5 for best, pliable) in South Australian Merino. They also found very low to low genetic and phenotypic correlations between skin quality and wool traits like yield, clean fleece weight, fiber diameter, standard deviations of fiber diameter, coefficient of variation of fiber diameter, staple strength, handle and crimp definition, though high values were obtained for wool condition and lock.

All these studies addressing the association of skin thickness with wool traits have been carried out in South Australian Merino sheep. Hence, it would be worth investigating these relationships in New Zealand Romney sheep, the most predominant dual-purpose breed experiencing different environmental conditions.

1.2.4.2.2. Meat traits

There is only one published study (Ripoll *et al.*, 2010) looking at the association of skin thickness with meat and carcass traits. Ultrasound measured skin thickness was found to be poorly correlated (r = 0.19 to 0.33) with cold carcass lean, subcutaneous fat, intermuscular fat and bone, in 3 commercial categories of lamb of Churra Tensina breed.

1.2.4.3. Indirect selection

Indirect selection is particularly promising for economic traits with low heritability or those whose measurement is impossible or costly. Skin thickness, as a moderately heritable trait (Gregory, 1982a, Slee *et al.*, 1991), could be measured with a relatively low cost and non-invasive ultrasound technique (Brown *et al.*, 2000) and has a great potential to be included in breeding programs as an indirect trait correlated with other economic traits. The impact of this inclusion will depend on its heritability and its genetic associations with the main traits in the breeding objective. Apart from its association with wool and carcass traits, skin thickness has also been suggested as a character influencing thermoregulation and as a result lamb survival in new-born lambs whose heritability is low.

1.3. Thermoregulation in new-born lamb

Thermoregulation is the ability of keeping the body temperature within certain boundaries, even when the ambient temperature is very different. Within a temperature tolerance range, known as the thermal neutral zone (TNZ), the basal rate of heat production is in equilibrium with the rate of heat loss to the external environment. Within the TNZ, an animal can maintain

homeostasis through normal physiological and metabolic processes, which may require minimal expenditure of energy (Hahn, 1985). An animal does not have to use large amounts of energy to control its temperature within the TNZ, while any deviation from this range would impose an extra expenditure of energy to return the body temperature into the range. For a new-born lamb, the lower limit of the TNZ is about 29°C in still air (Alexander, 1961) and 30 to 33°C with the inclusion of wind (0.55 m/sec) (Alexander, 1962a). Below this temperature thermoregulatory mechanisms are invoked to cause the animal to increase metabolism to produce heat.

New-born lambs have a well-developed thermoregulation system to maintain their rectal temperature, as an indicator of core body temperature (Alexander, 1961, McDonald, 1962). A lamb can enhance its rate of heat production by up to five times the basal metabolic rate required under thermoneutral conditions in response to the body heat loss to the environment (Alexander, 1962b). If the heat loss of the lamb exceeds its maximum sustainable metabolic rate, or the lamb fails to regulate the body temperature, the deep body temperature will decrease which results in hypothermia and consequently death if the core body temperature drops below 30°C (Alexander and McCance, 1958).

For measuring the ability of the lamb to generate heat in response to cold an index called summit metabolic rate is used (SMR), which is the maximum sustainable rate of heat production per unit body weight (McDonald, 1962, McCutcheon *et al.*, 1981). SMR is approximately 17 kcal per kg body weight per hour (or 200-300 kcal per m^2 of surface area per hour) for all lamb body weights, and therefore heat production per unit area increases with increasing body weight (Alexander, 1962b, McDonald, 1962). Under cold conditions, a new-born lamb can reach SMR half an hour after birth (Alexander, 1962b, McDonald, 1962). The high rates of heat production are usually maintained for about 2 h after birth to balance high rates of heat loss resulting from poor insulation (due to the wet fleece) and evaporation as the

fleece dries (Mellor and Cockburn, 1986). If the ambient temperature continues to drop after lamb has reached its SMR, the metabolic rate will decrease and the lamb will die of hypothermia (Cannon and Nedergaard, 2004).

New-born lambs can produce heat from shivering as well as non-shivering thermogenesis (Mellor and Cockburn, 1986). A principal tissue involved in non-shivering thermogenesis and metabolic adaptation to low ambient temperature is brown adipose tissue (BAT), which has the ability to rapidly generate large amounts of heat and also converts thyroxine to triiodothyronine (T3), the dominant hormone regulating metabolic rate (Symonds and Lomax, 1992). In new-born lambs BAT comprises 1-2% of birth weight (Alexander and Bell, 1975a) and is found predominantly in the perirenal-abdominal area (Alexander, 1978). McDonald (1962) reported that lambs born to well-fed ewes can have up to twice as much fat than lambs from poorly fed ewes. In another study by Gate *et al.* (1999), however, it has been observed that fetal BAT stores can be affected by both maternal nutrition and chronic cold exposure of the ewe and an interaction of the two. On the other hand, chronic cold exposure in conjunction with sub-optimal feeding has been shown to have a beneficial effect on BAT development (Symonds and Lomax, 1992, Clarke *et al.*, 1997a).

Most lambs can afford cold exposure due to generally having adequate reserves of liver and muscle glycogen and sufficient brown adipose tissue to generate about 60-70 kJ of heat per kg of body weight, from both shivering and non-shivering thermogenesis (Alexander, 1979, Eales and Small, 1980b, McCutcheon *et al.*, 1983a). Nevertheless, in smaller lambs and especially those which may have suffered intrauterine growth retardation, there are smaller fat reserves (Mellor and Murray, 1985) resulting in a smaller margin of safety for maintaining homeothermy (Mellor and Cockburn, 1986).

Apart from heat production through non-shivering thermogenesis using energy derived from brown fat (Alexander and Williams, 1968, Symonds and Lomax, 1992), a reduction in heat loss from the skin surface by increasing body insulation (Alexander, 1978) could be considered as an alternative for improving thermoregulation in new-born lambs. The latter is especially important for multiple born lambs whose number is rising with a focus on increasing lambing percentage in New Zealand (Bray, 2005, Kenyon *et al.*, 2005). Lambs of low birth weight are more susceptible to hypothermia firstly due to having less body reserves (Alexander, 1962b) and secondly due to having a greater surface area to body weight ratio (Alexander, 1962a, McCutcheon *et al.*, 1983b).

1.3.1. Role of skin thickness in thermoregulation

Skin as an organ covering the body can have a potential effect on thermoregulation through its role in increasing body insulation and reducing heat loss from the body surface. There are a few studies reporting the positive association of skin thickness with cold resistance. Samson and Slee (1981) showed that cold resistance was positively correlated with skinfold thickness in new-born lambs of 10 different breeds. It is unclear how exactly skin thickness affects body insulation and cold resistance. Samson and Slee (1981) suggested that the relationship of skinfold thickness and cold resistance may have been due to an association with nutritional status, or changes in skin histology for example subcutaneous fat deposition, while Alexander (1978) found that new-born lambs had virtually no subcutaneous fat. Furthermore, Slee *et al.* (1991) estimated a significant genetic correlation of 0.51 ± 0.27 between skin thickness and cold resistance in new-born Merino lambs. Also, Stott and Slee (1987) found that increased cold resistance was genetically associated with increased skin thickness and increased total body insulation. In their study upwards selection for increased cold resistance resulted in lambs with thicker skin. On the other hand, in a study conducted on Coopworth lean and fat selection lines, Jopson *et al.* (2000) showed that the line selected for fat had significantly thicker skin

and higher survival rate from tagging to weaning compared with the lean line, so that for every one millimeter increase in skin thickness there was a 2.7% increase in lamb survival.

1.3.2. Calorimetric measurement of heat production/dissipation

As mentioned before, heat production increases in response to the body heat loss into the environment at low ambient temperatures in new-born lambs, in order to return their core body temperature into the TNZ. Therefore, as criteria in assessing thermoregulatory capacity of a new-born lamb, it is important to measure the amount of heat production.

The science that quantifies the heat release from metabolism is termed calorimetry. In general, calorimetry could be divided into two main approaches: direct and indirect calorimetry (Levine, 2005).

In direct calorimetry, heat production is measured based on the same general principle as the bomb calorimeter, in which the heat produced is used to increase the temperature of a surrounding medium (Rodríguez *et al.*, 2007). These instruments are extremely expensive and complex to build and run, and require enormous expertise to establish and maintain, while offering little to the majority of investigators beyond much cheaper and simpler indirect calorimeters. Therefore, application of direct calorimetry is limited to highly specialized laboratories where direct heat production measurements are of specific value (Levine, 2005).

In indirect calorimetry, which could be categorised into two general methods of closed-circuit calorimetry and open-circuit calorimetry, heat production is estimated by determining the consumption of oxygen and production of carbon dioxide using appropriate formulae (Weir, 1949, Cunningham, 1990).

According to the Brouwer equation (Brouwer, 1965);

$$\text{Heat production (kcal)} = (3.866 \times O_2) + (1.200 \times CO_2) - (0.518 \times CH_4) - (1.431 \times N)$$

where O_2, CO_2 and CH_4 represent volumes of oxygen consumed and of carbon dioxide and methane produced (l) and N is the quantity of urinary nitrogen excreted (g).

The first two terms in the equation contribute approximately 75 and 25 %, respectively to the total heat production. The other two terms each contribute about 1 % (McLean, 1972).

In an open-circuit system, the subject inspires air with continuous flow and the expired gases are then analyzed. In this method of calorimetry, the direct measurements are ventilation rate and the composition of inlet and outlet air, and it is common to use these values to calculate the quantities of gases consumed or produced. For greatest possible accuracy it is of course necessary to measure oxygen, carbon dioxide and methane concentrations as well as urinary nitrogen excretion rate. But as McLean (1972) has suggested, in open-circuit calorimetry heat production can be predicted solely from the measurement of oxygen consumption with estimates of heat production only affected by ± 2 %. According to McLean (1972), it is assumed that 20.46 KJ of heat is produced per liter of oxygen consumed. Hence,

Heat production (W) = [(oxygen consumption (l/h) x 20.46)/3.6]

Division of oxygen consumption converts from KJ h^{-1} to W.

In closed-circuit calorimetry the subject is placed in an air-tight chamber in which a stream of oxygen enters the chamber from a spirometer. Carbon dioxide and water given off by the subject are absorbed in external containers, through which the air from the chamber is circulated, and then oxygen can be reintroduced into the air stream. Heat produced by the subject can be calculated from the quantities of carbon dioxide absorbed and oxygen re-introduced (Levine, 2005).

Closed-circuit calorimeters for small animals are cheap to construct and operate. The main running cost involves the supply and regeneration of absorbents for carbon dioxide and water which increase in proportion to animal size. Also, the animal can freely eat and drink during

calorimetry, which makes this method suitable for experiments of long period. However, any fluctuation in environmental temperature or atmospheric pressure during the experimental period will introduce errors by creating an artificial pressure gradient between the chamber and free atmosphere, resulting in artificially high or low values for oxygen consumption (Miller *et al.*, 1981). On the other hand, a hood/canopy/mask indirect open-circuit calorimeter can be purchased for a relatively modest cost (US$10,000–20,000). These instruments are fully automated and simple for a technician with moderate skill to use, validate and calibrate. Measurements to within 1% of chemical standards can be obtained using these instruments. The instruments are often configured as a metabolic cart and therefore are transportable although often not portable, which can be used for measurements of basal metabolic rate and resting energy expenditure (Levine, 2005). However, this method of calorimetry is not appropriate for continuous measurements, since the animals under experiment are not able to eat and drink and tend to become irritable when restricted by a mask for a long period. Due to their relatively less cost and simplicity compared with direct calorimetry (Levine, 2005), both open- and closed-circuit calorimetry approaches have been popularly used in farm animals (Hickey, 1960, McCutcheon *et al.*, 1983a, Revell *et al.*, 2002, Kerslake *et al.*, 2009, Nielsen *et al.*, 2014).

1.3.3. Infrared thermography

As mentioned earlier, apart from maximizing heat production through non-shivering thermogenesis using energy derived from brown fat (Alexander and Williams, 1968, Symonds and Lomax, 1992), the ability of lambs to thermoregulate effectively is dependent on minimizing heat loss from the skin surface by increasing body insulation (Alexander, 1978). Thermogenesis studies have traditionally focused on measuring core body temperature but not the contribution of radiated heat loss at the skin surface (McCoard *et al.*, 2014). It should be

remembered that heat production equals heat loss unless deep body temperature increases or decreases (Mellor and Cockburn, 1986).

All bodies with the surface temperatures higher than absolute zero emit energy at all wavelengths of the electromagnetic spectrum. Radiation is a form of heat loss through infrared rays involving the transfer of heat from one object to another without physical contact. The largest part of radiation energy is emitted with wavelength of 7–14 μm, which is referred to as infrared (IR) radiation. In case of animals 40–60% of heat loss is within this range (Kleiber, 1975).

Infrared thermography (IRT) or thermal vision is a modern, contactless, non-invasive and safe technique for measuring remotely the temperature of objects and to represent it through images (Vadlejch *et al.*, 2010). One major advantage of this method is the fact that it does not require direct physical contact with the surface monitored, thus allowing remote reading of temperature distribution (Speakmen and Ward, 1998). Thermal cameras collect infrared radiation emitted by the surface, convert it into electrical signals and create a thermal image showing the distribution of the body superficial temperature (Speakmen and Ward, 1998). Different colors in the thermal image correspond to a specific temperatures and not to the “true” color of the object (Figure 1.1).

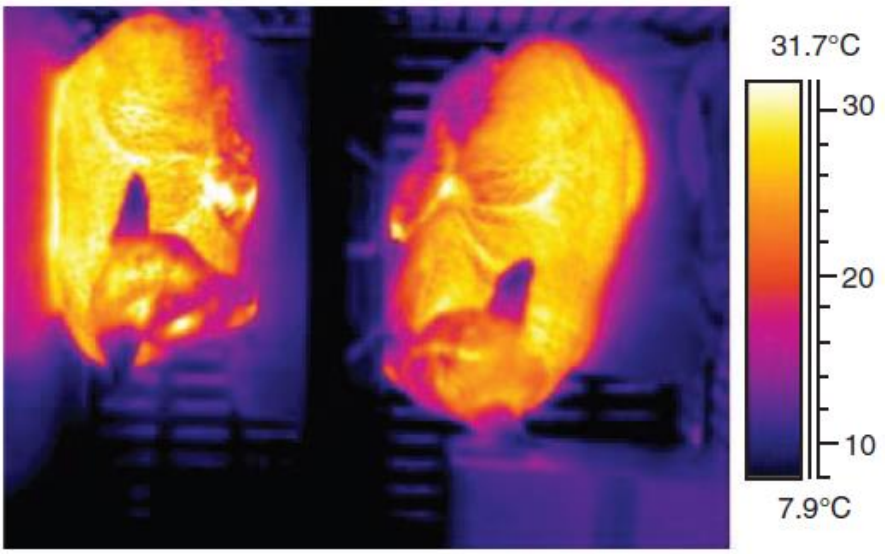


Figure 1.1. Thermal image of a pair of twin lambs, adapted from McCoard et al. (2014).

Apart from the temperature of the object, the radiation measured by thermographic camera is also a function of its emissivity (Kunc *et al.*, 2007). In living organisms, changes in the vascular circulation lead to changes in tissue temperature, which is used for assessment of the area (Harper, 2000). Hence, skin emissivity is an important factor in determining the true skin temperature. When using IRT on live animals, it is important to note that the water concentration in the skin makes very high emissivity values (between 0.95 and 0.98). Therefore, the presence of reflected radiation has little effect on a correct reading of images. But, the presence of metal objects or particular keratin materials (scales, bones, nails, etc.) has to be considered because their presence in the thermal image, mixes the energy reflected with the signal emitted by the area being examined, providing incorrect perceptions of temperature and thermal anomalies (Redaelli *et al.*, 2014). The main limitations of IRT have been mentioned as: thermal images must be collected out of direct sunlight and wind drafts; hair coats of animals should be free of dirt, moisture or foreign material (Stewart *et al.*, 2005).

Sensitivity of infrared thermal imaging to estimate heat loss from the skin in the new-born lamb using infrared thermography has been successfully tested by McCoard *et al.* (2014). They captured continuous thermal images of new-born lambs for 30 min each during the baseline (11-18°C), cold-exposure (0°C) and recovery (11-18°C) periods by using an infrared camera. During the baseline period, infrared heat loss was relatively stable, followed by a rapid decrease during cold exposure, and a rapid increase to baseline levels in the recovery period.

Using infrared thermography, Knizkova *et al.* (2005) have compared thermal insulation of birth coat in 3-day-old lambs in four genetic types exposed to a cold environment and to rain simulation. They showed the cold resistance of postnatal lambs to be influenced by breed as well as birth coat character.

Infrared thermography has been used as an early and reliable tool for neonatal thermal monitoring in humans (Abbas and Leonhardt, 2014). Infrared thermography has also been used in livestock species including cattle for diagnosis of mastitis (Berry *et al.*, 2003, Polat *et al.*, 2010) and laminitis (Nikkhah *et al.*, 2005), screening for feed utilization efficiency (Montanholi *et al.*, 2008), and early detection of foot-and-mouth disease (Rainwater-Lovett *et al.*, 2009).

1.3.4. Genetic aspects of cold resistance

Cold resistance, defined as the ability to resist hypothermia during a standard cold exposure, was first measured in adult sheep by Slee (1966). Based on the observed high repeatability (r = 0.73) in the study, it was suggested that a genetic component could be involved. Direct evidence for genetic components of cold resistance is also available from studies conducted on lambs. Slee (1978) and Samson and Slee (1981) have reported significant breed differences for cold resistance in new-born lambs using climate chambers, and water-bath cooling, respectively.

Wolff *et al.* (1987) estimated paternal half-sib heritability of cold resistance as 0.44 ± 0.18 with adjustment for lamb weight and wool depth, and 0.36 ± 0.16, ignoring lamb weight and wool depth. Similarly, using paternal half-sib analysis, Slee *et al.* (1991) estimated a heritability of 0.70 ± 0.25 for cold resistance. After adjustment for the effects of birthweight, birthcoat grade and depth, and skin thickness as covariates, the estimated heritability decreased to 0.55 ± 0.23. Also in the same study, a moderately high, positive and significant genetic correlation was seen between birth coat grade and cold resistance, while the phenotypic correlation was much lower and the corresponding environmental correlation negative. Accordingly, they concluded that some genes contributing to hairy birthcoat also contribute to high cold resistance, but not directly through improved insulation, since this would have produced a higher phenotypic correlation.

This conclusion was based on their previous study (Slee, 1978), which showed that differences in coat type that were associated with differences in cold resistance, both between and within breeds, were still present to some extent after the coat was removed by clipping. This evidence suggests that genes affecting birthcoat type might also have pleiotropic effects on cold resistance or are in linkage with other genes which have such effects (Slee *et al.*, 1991).

Similar findings were observed with regard to skin thickness, except that the phenotypic relation with cold resistance was somewhat higher, which made them suggest that skin thickness may exert part of its effect on cold resistance through insulation, not completely through linkage or pleiotropic effects (Slee *et al.*, 1991). An earlier study by the same group (Stott and Slee, 1987) also reported that increased cold resistance is genetically associated with increased skin thickness and increased total body insulation. In that study, upwards selection for increased cold resistance was found to result in lambs with thicker skin.

A study (Slee *et al.*, 1987) on two Scottish Blackface lines selected for high or low resistance to body cooling tested new-born lambs for their metabolic response to cold exposure in a water bath. The high line lambs showed significantly greater cold resistance and metabolic response to cold compared to low line lambs. Also, they found a significant sire effect on non-shivering thermogenesis, indicating evidence of genetic variation for cold resistance.

Recently, a polymorphism at the ovine β3-adrenergic receptor gene has been found to be associated with cold-related mortality in Merino lambs (Forrest *et al.*, 2003, Forrest *et al.*, 2006). β3-adrenergic receptor plays a pivotal role in the regulation of energy balance as mediators of the lipolytic and thermogenic activities in brown and white adipose tissue. Therefore, it would not be unreasonable if we postulate that this gene might be one of the genes that determined genetic cold resistance in new-born lambs reported in previous studies.

1.4. Candidate gene studies

A candidate gene is a gene which is presumed to be associated with a particular disease or phenotypic trait, whose biological function(s) is derived either directly or indirectly from other studies including animal model studies with other species (using comparative genomic studies), genome- wide association studies (GWAS), classical map-based positional cloning method, and a more recent next-generation sequencing (NGS) method (Giri and Mohapatra, 2017).

Candidate gene studies are focused on the selection of genes that have previously been shown to be related in some way to the disease (trait) and thus come with prior knowledge about gene function (Patnala *et al.*, 2013). They are relatively cheap and quick to perform.

The first critical step in conducting candidate gene studies is the choice of a suitable candidate gene that may plausibly play a relevant role in the mechanisms underlying the disease (trait) of interest (Kwon and Goate, 2000). This is followed by assessing and selecting polymorphisms, that might have a functional consequence, either by affecting gene regulation or its protein

product (Collins *et al.*, 1997, Kwon and Goate, 2000). Finally, the association of gene variant with disease (trait) of interest is tested by observing its occurrence in random test subjects (cases) having the disease and the selected control subjects which do not (Patnala *et al.*, 2013). In other words, this type of association study tries to answer the question, "Is one allele of a candidate gene more frequently seen in subjects with the disease than in subjects without the disease?". In case of quantitative traits, the association would be assessed by comparing different genotypes of the gene variant using appropriate statistical analysis.

Single nucleotide polymorphisms (SNPs) are the most widely tested markers in association studies, but microsatellite markers, insertion/deletions, variable-number tandem repeats (VNTRs), and copy-number variants (CNVs) are also used (Lewis and Knight, 2012).

While genome wide association studies (GWAS) investigate genetic variants spanning the entire genome, candidate gene studies limit the analysis to a relatively few number of genes. As a result, candidate gene studies have increased statistical power to detect differences (Modena et al., 2019). When a quantitative trait is considered, the power of an association study could be increased by ascertaining individuals only from the extremes of the distribution (Slatkin, 1999).

A significant genetic association may be interpreted as either (1) direct association, in which the genotyped SNP is the true causal variant conferring disease susceptibility; (2) indirect association, in which the genotyped SNP is in linkage disequilibrium (LD) with the true causal variant; or (3) a false-positive result due to chance or bias (Lewis and Knight, 2012). Distinguishing between direct and indirect association is challenging and may require resequencing of the candidate region, dense genotyping of all available SNPs, or functional studies to confirm the role of a putative mutation in disease/trait (Lewis and Knight, 2012).

1.4.1. Possible candidate genes for cold resistance

1.4.1.1. Uncoupling protein 1 (*UCP1*) gene

Non-shivering thermogenesis in BAT is a major source of heat production in the new-born lamb (Alexander and Williams, 1968). It accounts for approximately one-half of heat generated during summit metabolism in new-born lambs exposed to cold stress (Stott and Slee, 1985, Slee *et al.*, 1987).

Uncoupling protein 1 (UCP1) as the defining feature of brown fat is responsible for the unique thermogenic properties of the tissue (Matthias *et al.*, 2000, Golozoubova *et al.*, 2001, Nedergaard *et al.*, 2001). This protein catalyzes the leak of protons across the mitochondrial inner membrane, dissipating the electrochemical proton gradient that would otherwise drive adenosine triphosphate (ATP) production by ATP synthase. As a result, instead of ATP production, the energy from the oxidation of respiratory substrates is released as heat (Crichton *et al.*, 2017).

UCP1 belongs to a family of mitochondrial integral Membrane Carrier Proteins (Ricquier and Bouillaud, 2000). The ovine *UCP1* gene is located on chromosome 17 and is approximately 6.7 kb in length, with a coding sequence of about 1621 bp (Yuan *et al.*, 2012). It is composed of six exons separated by five introns (Yang *et al.*, 2014).

UCP1 is first activated at birth following cold exposure to the extra-uterine environment and intense endocrine stimulation (Symonds, 2013, Symonds *et al.*, 2015). UCP1 is activated by long-chain fatty acids that are produced within brown adipocytes by the lipolysis of cytoplasmic lipid droplets upon adrenergic stimulation of BAT (Cannon and Nedergaard, 2004). Enerback *et al.* (1997) indicated that *UCP1*-deficient mice consume less oxygen after treatment with a beta3-adrenergic-receptor agonist and are sensitive to cold due to their defective thermoregulation. A failure to utilize non-shivering thermogenesis during neonatal

development is associated with unexpected death (Symonds *et al.*, 1989). Therefore, rapid activation of UCP1 is critical in preventing hypothermia in the new-born (Clarke *et al.*, 1997b).

1.4.1.2.Prolactin (*PRL*) gene

Prolactin (PRL) is a single-chain polypeptide hormone that is synthesized mainly in the anterior pituitary of all vertebrates (Wang et al., 2011). According to its genetic, structural, binding and functional properties, prolactin belongs to the prolactin/growth hormone/placental lactogen family (group I of the helix bundle protein hormones (Boulay and Paul, 1992, Horseman and Yu-Lee, 1994)). The ovine *PRL* gene has been mapped on chromosome 20 (Jawasreh et al., 2019).

Although PRL is usually known as a hormone crucial for mammary gland functioning and so the regulation of lactation, it is involved in the regulation of numerous processes including reproduction, growth, development, fat metabolism, hair shedding, cellular differentiation, osmoregulation and immune response (Freeman et al., 2000, McMurray, 2001).

It has been shown that PRL administration throughout gestation to the mother, which results in substantial transfer of PRL into the fetus (Yang et al., 2002), could promote UCP1 abundance in the rat fetus (Budge *et al.*, 2002). Furthermore, it has been reported that administration of PRL can elicit a thermogenic effect in neonatal sheep through an increase in lipolysis occurring in conjunction with a rise in the thermogenic potential of UCP1, suggesting a potential role for PRL in the regulation of UCP1 expression and function (Pearce *et al.*, 2005b). So, PRL can act as a potential thermogenic hormone over the first few days of postnatal life. Therefore, PRL might have an effect on cold resistance in new-born lambs due to its role in BAT thermogenesis.

On the other hand, there are some reports demonstrating the role of PRL in hair/wool growth and development (McCloghry *et al.*, 1993, Pearson *et al.*, 1996, Nixon, 2002). Therefore,

PRL might also have an effect on cold resistance in new-born lambs due to its plausible effect on body surface heat loss through its role in wool properties.

Circulating PRL and thermal stress have been reported to be associated in various mammals including humans (Mills and Robertshaw, 1981), though a direct modulatory role for PRL in thermoregulation has not been proved. Therefore, apart from its possible role in thermoregulation through an effect on thermogenesis, PRL might also have an effect on cold resistance in new-born lambs due to its plausible effect on body surface heat loss through its role in wool properties.

1.4.1.3.Prolactin receptor (*PRLR*) gene

Prolactin receptor (PRLR), through which prolactin exerts its physiological roles, is a single pass membrane-spanning protein belonging to the growth hormone/cytokine receptor superfamily (Goffin and Kelly, 1997). There are two distinct PRLR isoforms (long and short forms) that are produced by alternative splicing mechanism (Bignon et al., 1997). The ovine *PRLR* gene has been mapped on chromosome 16 (Jenkins et al., 2000).

The stage of fetal development at which PRLR mRNA becomes abundant coincides with the first appearance of UCP1 in BAT (Symonds et al., 1998). Increase in UCP1 abundance in fetuses of well-fed sheep has also been reported to be closely associated with increased abundance of PRLR-1 (Budge et al., 2000). Furthermore, the abundance of UCP1 and PRLR peak at birth (Casteilla *et al.*, 1989, Clarke *et al.*, 1997a) coincides with maximum expression of other mitochondrial membrane proteins within adipose tissue, including VDAC (Mostyn et al., 2003), which has a role in regulating the supply of mitochondrial ATP and ADP (Gottlieb, 2000). Also, the results of a study on new-born lambs has shown that the disappearance of UCP1 from BAT is very closely correlated with loss of the PRLR in the tissue (Pearce *et al.*, 2005b). Hence, in addition to UCP1, PRLR can also be considered as a marker of BAT function

(Pope et al., 2014). Therefore, PRLR might have an effect on cold resistance in new-born lambs due to its role in BAT thermogenesis.

On the other hand, since PRL exerts its physiological roles through its receptor, PRLR can possibly have an effect on lamb birth coat, according to some studies reporting the role of PRL in hair/wool growth and development (McCloghry *et al.*, 1993, Pearson *et al.*, 1996, Nixon, 2002). In addition, PRLR has been shown to be highly expressed and regulated in follicle cell populations known to play a key role in controlling follicle output and hair cycles (Nixon, 2002). Accordingly, PRLR might also have an effect on cold resistance in new-born lambs due to its plausible effect on body surface heat loss through its role in wool properties.

Due to the effects of UCP1, PRL, and PRLR on BAT and consequently on thermogenesis, as well as the possible effects of PRL and PRLR on body surface heat loss, the *UCP1*, *PRL*, and *PRLR* genes can be considered as possible candidate genes affecting thermoregulation in new-born lambs.

Chapter 2

Is ultrasound scanning an accurate technique for measuring skin thickness in sheep?

2.1. Abstract

The relationship of skin thickness with a few other economically important traits has introduced it as a trait that could be potentially used in indirect selection for those traits. So, finding a feasible method that can accurately measure the skin thickness is important. Ultrasonography is a technique for measuring skin thickness whose application has attracted increasing attention because of its advantages over skin-fold thickness measurement. This study aimed to evaluate the accuracy of ultrasonically measured skin thickness by plicometry and histometry and also to understand the histological characteristics corresponding to the structural differences in subcutaneous tissue, observed in images taken by ultrasonography. Skin thickness in 35 ewe lambs of 9-11 months of age was measured by ultrasonography during 2015 (n = 15) and 2016 (n=20) at the 12^{th} rib, and biopsy samples (skin and subcutaneous tissue) were taken from the same region. Skin thickness was also measured by skinfold caliper in 2016. Sections from biopsy specimens were stained by the Hematoxylin and Eosin and Oil Red O methods and were observed under microscope and their images used for histometric measurements of skin thickness and histological examination of subcutaneous tissue. The results of our analysis performed using PROC CORR procedure in SAS software, showed a significant ($P<0.05$) correlation of 0.52 between skin thickness measured by ultrasonography and the corresponding measurement made by histometry. However, no significant ($P>0.05$) correlation was found between skin thickness measured by ultrasound and plicometry. Also, there was no significant ($P>0.05$) correlation between skin thickness measured by plicometry and histometry. Structural differences in subcutaneous tissue evident in the ultrasound images were found to be the result of variations in the amount of connective tissue within the subcutaneous tissue. The results of this study showed that ultrasonography using a frequency 7.5 MHz could be used as an accurate, objective, non-invasive and simple method for measuring skin thickness in sheep.

2.2. Introduction

Due to its association with a few other economically important traits like cold resistance in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991), lamb survival (Jopson *et al.*, 2000), wool traits (Gregory, 1982b, Williams and Thornberry, 1992, Ponzoni *et al.*, 1995, Hynd *et al.*, 1996), and carcass composition (Ripoll *et al.*, 2009), and being moderate to high in heritability (Gregory, 1982a, Slee *et al.*, 1991), skin thickness could be potentially used in indirect selection for these traits. However, before skin thickness could be used in selection breeding programs for improving other traits of importance, its accurate measurement is required.

While many studies have used skin-fold calipers as a tool for measuring skin thickness (Gregory, 1982a, Gregory, 1982b, Slee *et al.*, 1991, Williams and Thornberry, 1992), it seems that differences in the subcutaneous fat layer and also pinching force during measurement could lead to inaccuracy in measurement (Alexander and Miller, 1979). Real time ultrasonography is another technique for measuring skin thickness whose application has attracted increasing attention because of its advantages over skin-fold thickness measurement (Alexander and Miller, 1979, Brown *et al.*, 2000, Zanna *et al.*, 2012). Ultrasound scanning could potentially provide an objective, non-invasive and simple way for measuring skin thickness (Brown *et al.*, 2000). Another benefit of this method is that the wool generally does not need to be clipped before measurement, which would damage the skin and decrease the commercial value of the animal (Teixeira *et al.*, 2008). Clear images could be obtained simply by parting the wool and applying a small amount of vegetable oil (Brown *et al.*, 2000). Ultrasound also provides a real-time image of the skin, its structure and characteristics (Zanna *et al.*, 2012). Many countries around the world have incorporated the ultrasound measurements of traits like fat depth and eye muscle depth and area into their breeding programs for the improvement of carcass quality due to the relatively low cost and portability of ultrasound equipment (Stanford *et al.*, 1998).

Therefore, it would be practical and cost-effective if skin thickness can be measured at the same time with these traits. Ultrasonography has been successfully used and its accuracy has been proven for the measurement of skin thickness in human (Alexander and Miller, 1979), dogs (Zanna *et al.*, 2012), and mice (Tedstone *et al.*, 2008). However, to our knowledge, there is no published literature addressing the accuracy of ultrasonically measured skin thickness in sheep.

On the other hand, in a study (chapter three) where we aimed to estimate genetic parameters like heritability for skin thickness and its correlation with a few other traits of interest, we had access to a dataset including skin thickness of lambs measured by ultrasonography at around 9 months of age. Hence, prior to using that data to estimate genetic parameters, we needed to validate the accuracy of the skin thickness measurements taken by ultrasound. Furthermore, the images from the ultrasonography showed differences in the structure of subcutaneous fat layer among lambs that have not been addressed in the literature, as far as we know. Therefore, the aims of the current study were to:

1. Validate the accuracy of ultrasonically measured skin thickness by plicometry and histometry.
2. Understand the histological differences corresponding to the structural differences in subcutaneous tissue, revealed by ultrasonography.

2.3. Materials and methods

2.3.1. Ethics statement

The study protocol was approved by the Massey University Animal Ethics Committee (Protocol # 15-47 and 16-34). Animals were all closely monitored throughout the experiment and no animal health or welfare issues were observed during or after the experiment.

2.3.2. Ultrasonic measurement

As a routine farm operation, a total of 754 ewe Romney lambs of 9-11 months of age were subjected to ultrasonography on a farm (Brookfield) located in Kiwitea, New Zealand during August 2015 and July 2016. Skin thickness, subcutaneous fat depth, and eye muscle depth of the lambs were measured ultrasonically. A commercial operator took measurements using an ultrasound scanning machine (Sonosite M Turbo, FUJIFILM Sonosite, Inc., Bothell, Washington, United States) with a 38 mm probe at 7.5 MHz set at a depth of 40 mm on the left side of the lambs around the 12th rib. Based on the structure of subcutaneous tissue and the amount of bubble-like structures evident in the ultrasound images (Figure 2.1), lambs were categorized into 5 groups (from 1 to 5). In the first year, 15 lambs (3 from each category) were randomly selected for the subsequent plicometry and histology experiment so that in addition to testing the accuracy of skin thickness measured by ultrasound scanning, the differences in the structure of subcutaneous tissue evident in the ultrasound images (Figure 2.1) could be examined histologically. However, possible histological differences between subcutaneous fat from five categories corresponding to the structural differences in subcutaneous tissue revealed by ultrasonography could not be examined in the first year due to not having enough depth of subcutaneous fat sampled in the biopsy collection. Therefore, the study was repeated in the second year with collecting biopsies of more depth in 20 lambs. Also, in the second year, only lambs with low (0 or 1) and high (4 or 5) bubble category scores with larger sample size per category (10 lambs for low and 10 lambs for high bubble categories) were used to increase the possibility of detecting any possible histological differences between the subcutaneous tissues from different categories.

2.3.3. Plicometry

Plicometry was performed only for the 20 lambs that were used in the second year. Lambs were appropriately restrained by experienced farm staff, during plicometry and biopsy in a standing

position. Using an electric animal clippers, 10 x 10 cm patches were close-clipped on the left side of each animal around the 12th rib region, where ultrasonography was done. The position where ultrasonography was done on the left sit was encircled with a permanent marker to be clear for plicometry. A double fold of skin was measured by a dial-gauge Harpenden skinfold caliper using a constant pressure of 1250 g/cm^2 (Lyne, 1964, Brown *et al.*, 2000). Two measurements were made, one with the callipers facing in anterior/posterior direction relative to the body of the sheep and a second measurement in a dorsal/ventral direction while an assistant held the skin at the marked site between thumb and forefinger. Average skin thickness was calculated by dividing the sum of these measurements by 4 (2 measurements x 2 layers).

2.3.4. Collection of biopsy specimen

Skin and subcutaneous samples at the marked sites were obtained using an 8 mm (diameter) biopsy punch, under local anaesthesia (2% lignocaine Hydrochloride) with subcutaneous injection. Also, the animals were sedated with acepromazine 0.05-0.1 mg kg^{-1} IV, 20 min prior to biopsy. A flat, wrinkle-free area in the encircled area was selected as the biopsy site. Biopsy specimens were taken applying a gentle downward pressure to the punch and cutting through the skin and subcutaneous fat to the eye muscle by manual rotation. While samples to a depth of around 8 mm were collected during the first year, a depth of at least 1.5 cm was exerted for the biopsy collection to make sure that the whole subcutaneous tissue was sampled. The circular piece of specimen was finally removed with forceps and surgical scissors and placed in a labelled specimen tube container and put in a container of ice until they were transferred to the laboratory at Massey University. The biopsy sites were treated with a broad spectrum topical antiseptic (povidone-iodine) spray and left unsutured for healing by second intension. The animals were observed for 4-5 days to ensure that the surgical sites healed properly. Upon arriving in the laboratory, the biopsies were fixed for further processing required for histologic and histometric examinations.

2.3.5. Fixation of specimens

In order to preserve cells and tissues as they naturally occur, the specimens were fixed upon arriving in the laboratory. For this purpose, the biopsy samples from the first year were put in 10% buffered formalin in labeled cassettes at room temperature for 48 hours. The biopsies from the second year were placed in labeled molds and enough optimal cutting temperature (OCT) inert medium was added to cover the specimen and frozen as quickly as possible in liquid nitrogen in order to avoid ice crystal formation that might result in any artifact. After the freezing was done, the samples were kept in a freezer at a temperature of -20° C.

2.3.6. Paraffin infiltration

In this procedure, which was used for the biopsy samples that were fixed in 10% buffered formalin, specimens were dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with wax.

Once fixed, the specimens were processed using a tissue processer with the following program:

1. 50% ethanol for 2½ hours.

2. 70% ethanol for 2½ hours.

3. 90% ethanol for 3 hours.

4. 90% ethanol for 3 hours.

5. Absolute ethanol 1 for 3½ hours.

6. Absolute ethanol 2 for 3½ hours.

7. Xylol 1 for 2½ hours.

8. Xylol 2 for 2½ hours.

9. Paraffin wax 1 (63°C) for ½ hour.

10. Paraffin wax 2 (63°C) for ½ hour.

2.3.7. Embedding in paraffin

The wax-infiltrated tissues were then embedded into blocks. Once the tissue was embedded, it would remain stable for many years. Small amounts of molten paraffin were put in labeled molds, dispensing from a paraffin reservoir. Using forceps, specimens were transferred into the molds, with skin surface perpendicular to the mold surface. When a specimen was in the desired orientation, the labeled tissue cassette was placed on top of the mold, then hot paraffin was added to the mold from the paraffin dispenser until it covered the face of the cassette and then molds were placed on a cooling plate to allow the paraffin to solidify. At this stage the specimen and paraffin attached to the cassette formed a block ready for sectioning.

2.3.8. Sectioning specimens

Specimens embedded in paraffin were sectioned perpendicular to the surface of the skin in 6 µM thickness using a microtome. A fresh blade was place on the microtome and was replaced whenever sectioning became problematic. The blocks to be sectioned were placed on an ice block in order to keep them solid for sectioning. Then the paraffin blocks of each specimen were inserted into the microtome chuck so that the wax block faced the blade and was aligned in the vertical plane. The dial was set to cut 10 µM sections in order to make sure that the blade is cutting smoothly and is reaching the specimen. Once it was cutting smoothly, 6 µM was set. When the block was ribboning well, four sections were cut and picked up with a fine paint brush and floated on the surface of a 35°C water bath. Then the sections were floated onto the surface of clean glass labeled slides. If a block was not ribboning well then it was placed back on the ice block to cool and become firm. Slides with paraffin sections on them were placed in a 65°C oven for 20 minutes so that the specimen bonded to the glass and got ready for subsequent staining.

Frozen specimens were sectioned perpendicular to the surface of the skin in 6 µM thickness using a cryostat. The blocks of OCT containing specimens were placed in the microtome object holder and the set screw was tightened. The machine was set to cut sections in 6 µM thickness. Cutting continued until a full specimen section was obtained. One edge of the section was held flat with a fine paint bush and the other with the knife edge of cryostat. The first two or three sections were discarded to make sure that the blocks were sectioning well. Clean glass labeled slides were carefully lowered onto the blade, keeping the slide parallel to the section. As the section came into contact with the slide, the OCT and specimen melted causing the specimen to adhere to the slide. Then, the slides with the specimens on them were air dried to get ready for subsequent staining.

2.3.9. Hematoxylin and Eosin (H&E) staining for paraffin embedded sections

Paraffin embedded sections cut in 6 µM thickness from previous step were deparafinized by 2 changes of xylene, 10 minutes each, then re-hydrate in 2 changes of absolute alcohol, 5 minutes each and 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Then they were washed briefly in distilled water and stained with Gill's Haematoxylin for 3 minutes, rinsed in running tap water, blued with Scott's tap water for 30 seconds and rinsed in running tap water for 30 seconds. After that, sections were stained with Eosin/Phloxine for 1 minute, then rinsed in running tap water for 1 minute, dehydrated, cleared and mounted.

2.3.10. Hematoxylin and Eosin (H&E) staining for frozen sections

Frozen specimens cut in 6 µM thickness from previous step were air dried at room temperature, then fixed with methanol for 3 minutes. Then they were stained with Gill's Haematoxylin for 3 minutes, rinsed in running tap water, blued with Scott's tap water for 30 seconds, rinsed in running tap water for 30 seconds. After that, sections were stained with Eosin/Phloxine for 1 minute, then rinsed in running tap water for 1 minute, dehydrated, cleared and mounted.

2.3.11.Oil Red O staining of frozen sections

Oil Red O staining method was applied for the frozen sections. For this purpose, slides of frozen sections were placed directly into filtered 0.5% Oil Red O in Dextrin, stained for 20 minutes, and rinsed with running water briefly. Then, the specimens were counter stained with Gill's haematoxylin for 20-30 seconds, rinsed with water, dehydrated, cleared and mounted.

2.3.12.Histometry of skin thickness

The resulting slides were observed under a microscope (Zeiss Aziophot, Carl Zeiss AG, Oberkochen, Germany) and photographed by a camera (Olympus DP72, Olympus Corporation, Tokyo, Japan) connected to the microscope. The images were collected and histometric measurements of skin thickness were performed using the Olympus CellSens Dimension processing software (Olympus Corporation, Tokyo, Japan). The distance from the surface of epidermis layer to the bottom of dermis layer was considered as the skin thickness. Since the border between dermis bottom and underlying subcutaneous fat was not clearly evident in all of the images of the specimens stained with the Oil Red O method, skin thickness measurement was performed using specimens stained with the H&E method. Five measurements were taken for each image and then averaged to estimate the skin thickness of each animal.

2.3.13.Histological examination of subcutaneous tissue

The subcutaneous tissue of specimens from different categories corresponding to the structural differences in subcutaneous tissue revealed by ultrasonography were examined by observing and comparing the specimens stained by both methods.

2.3.14. Statistical analysis

The CORR procedure of the SAS software (SAS, 2011) was used to calculate Pearson correlation coefficients between skin thickness measurements taken by ultrasound, plicometry and histometry.

2.4. Results and discussion

As displayed in Figure 2.1, skin thickness, subcutaneous fat tissue, and eye muscle depth were clearly distinguished in the images taken by ultrasonography. While conventional scanners operating at 5 to 7 MHz are appropriate for subcutaneous fat depth measurement, higher frequencies have been suggested for detecting thin layers like skin (Alexander and Miller, 1979, Dines *et al.*, 1984). Our results indicate that the frequency of 7.5 MHz used in the present study was appropriate for detecting even the thin layer of skin in the image. Ripoll *et al.* (2009) reported successful measurement of skin thickness in lambs using frequencies of 8 to 10 MHz. Skin thickness has been measured successfully in dogs using a 13-MHz transducer (Zanna *et al.*, 2012). Due to cattle skin being thicker compared to sheep, Butler and Head (1993) showed that a medium frequency of 7.5 MHz is even suitable in detecting the papillary and reticular layers of cattle skin though with some difficulties.

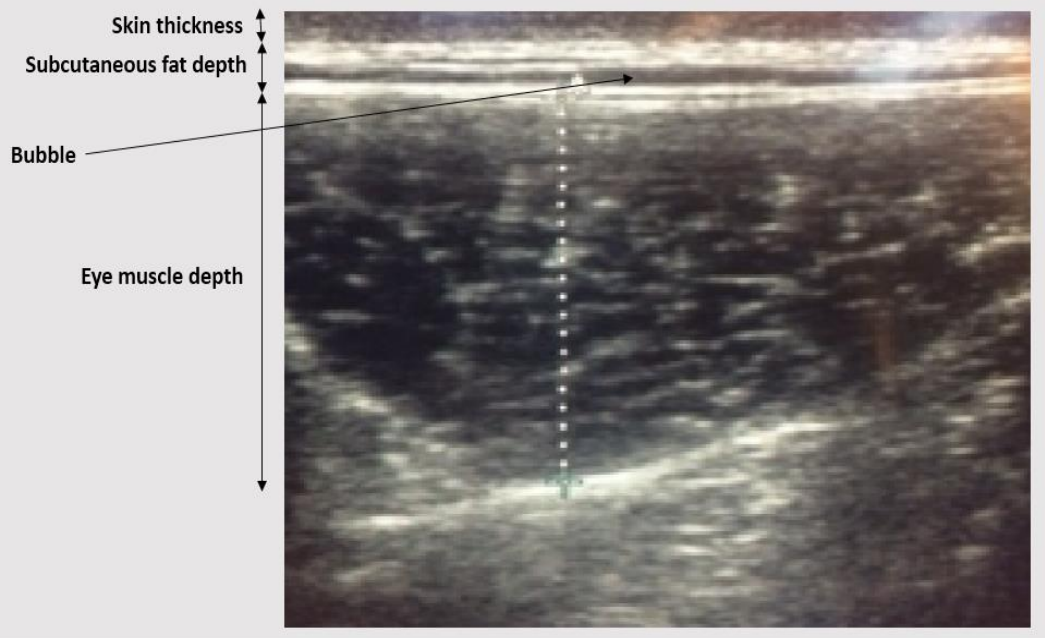


Figure 2.1. Image of skin, subcutaneous fat, and eye muscle depth taken by ultrasonography.

The biopsy specimens were successfully stained by the methods H&E and Oil Red O (Figures 2.2 and 2.3). However due to the higher quality of the images of the specimens stained with H&E method, the border between dermis bottom and underlying subcutaneous fat was clearly evident in those images. So, they were used for the skin thickness measurement by histometry.

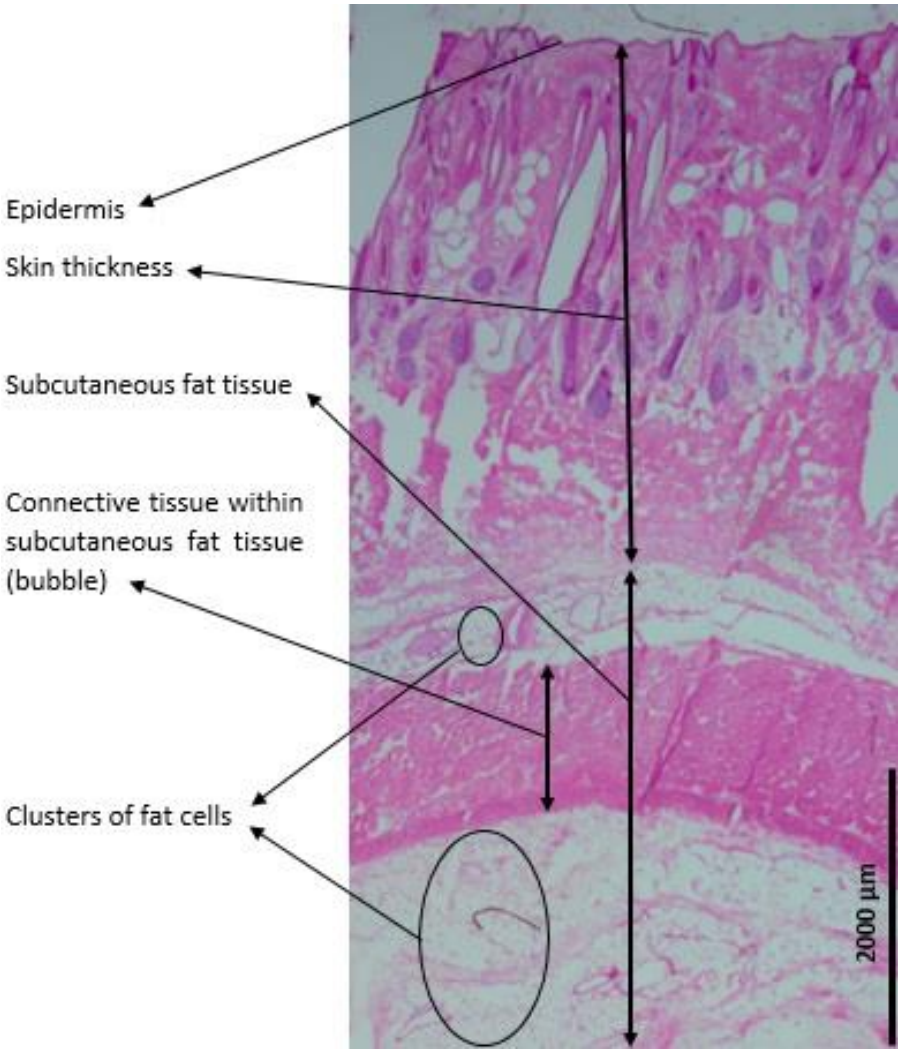


Figure 2.2. Photomicrograph of the skin biopsy section stained by H&E method.

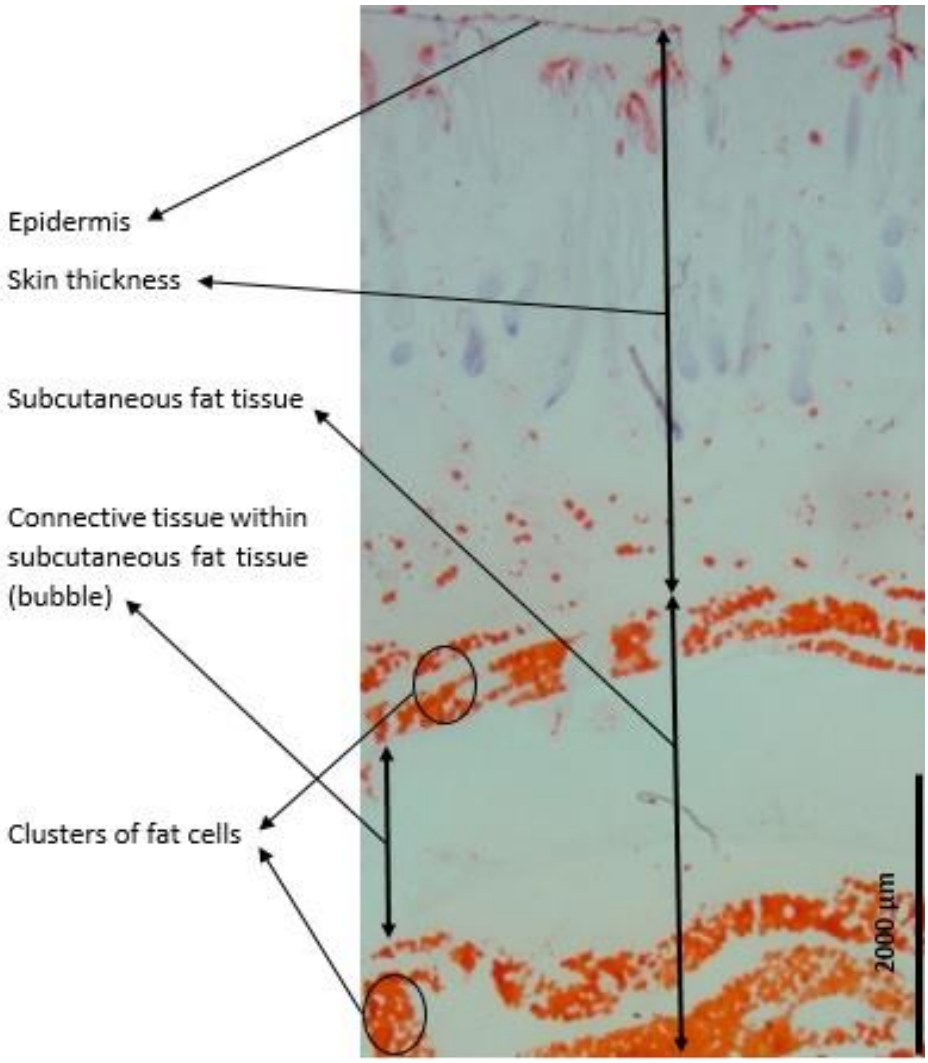


Figure 2.3. Photomicrograph of the skin biopsy section stained by Oil Red O method.

As shown in Table 2.1, while the means of skin thickness measurements taken by ultrasound and histometry were relatively close together, the skin thickness measured by plicometer had a mean almost double these values. This could be contributed to the fact that the skinfold thickness measurements include subcutaneous fat other than skin.

Table 2.1. Descriptive statistics and number of records for the skin thicknesses measured by ultrasonography, histometry, and plicometry.

Measurement time (month)	No. of records	Mean (mm)	SD	Min.	Max.	CV (%)
Ultrasonography	35	3.17	0.60	2.1	4.5	18.87
Histometry	35	3.67	0.50	2.73	4.84	13.74
Plicometry	20	6.45	0.60	5.50	8.00	9.38

In the present study, the skin thickness measurements taken by ultrasound were significantly ($P<0.01$) correlated with those measured by histometry, with a Pearson correlation coefficient of 0.52 (Figure 2.4). This result is consistent with the result from a study by Zanna *et al.* (2012), who found a positive correlation between skin thickness measured by ultrasonography and histometric analysis in Shar-Peis and Beagle dogs.

It should be noted that the histometric measurement of skin thickness as a gold standard method was not perfectly done in this experiment. Skin biopsy samples tend to shrink after collection that could have probably affected the accuracy of skin thickness measurement taken by histometry in this study. This might have consequently influenced the observed correlation between skin thickness measured by ultrasonography and histometric analysis. To prevent shrinkage artifacts, the biopsy samples must have been suspended in an appropriate buffered solution immediately after biopsy collection. Following this precaution might have improved the correlation between skin thickness measured by ultrasonography and histometry, in the present study.

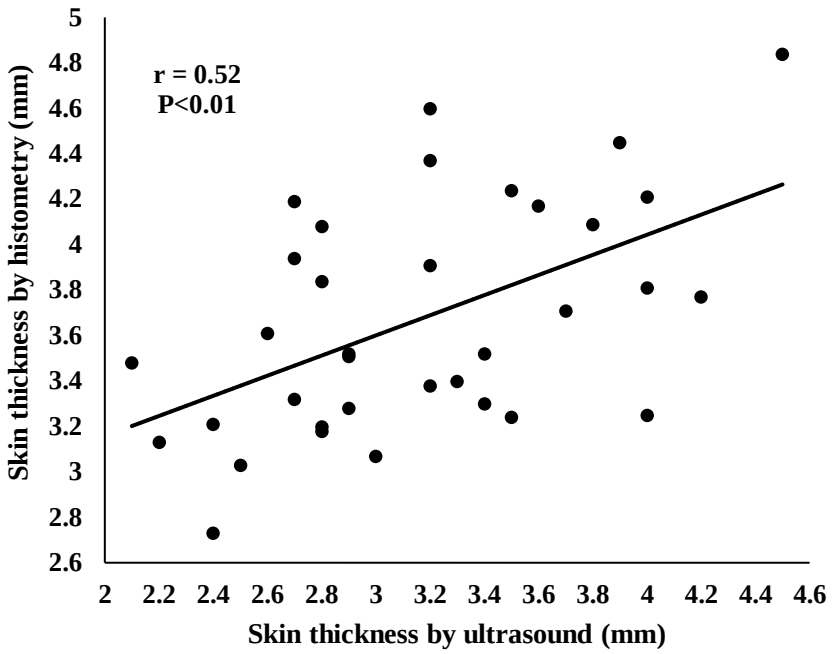


Figure 2.4. The correlation of skin thickness measured by ultrasonography (mm) and histometry (mm). Pearson correlation coefficient has been indicated by r.

On the other hand, there was no significant (P>0.05) correlation between measurements made via ultrasonography and plicometery (Figure 2.5). Contrary to this, Zanna *et al.* (2012) reported a significant correlation between ultrasonography and plicometery measurements in Shar-Peis dogs. However, in accordance with our results, they did not find such a correlation in Beagles. Also, in a study conducted on Merino sheep, the relationship between the measurements made using ultrasonography and plicometery, were only moderately to highly correlated in 6 out of the 12 measurements made throughout a year (Brown *et al.*, 2000). Furthermore, no significant correlation was evident between skin thickness measured by histometry and those obtained by plicometer (P>0.05) in the present study (Figure 2.6). This might be, to some extent, due to the possible problem of shrinkage artifact occurring in this experiment. Contradictory results obtained for reliability of plicometry measured skin thickness in different studies could be attributed to small number of samples and/or due to differences in subcutaneous fat depth included as part of skinfold thickness measurement.

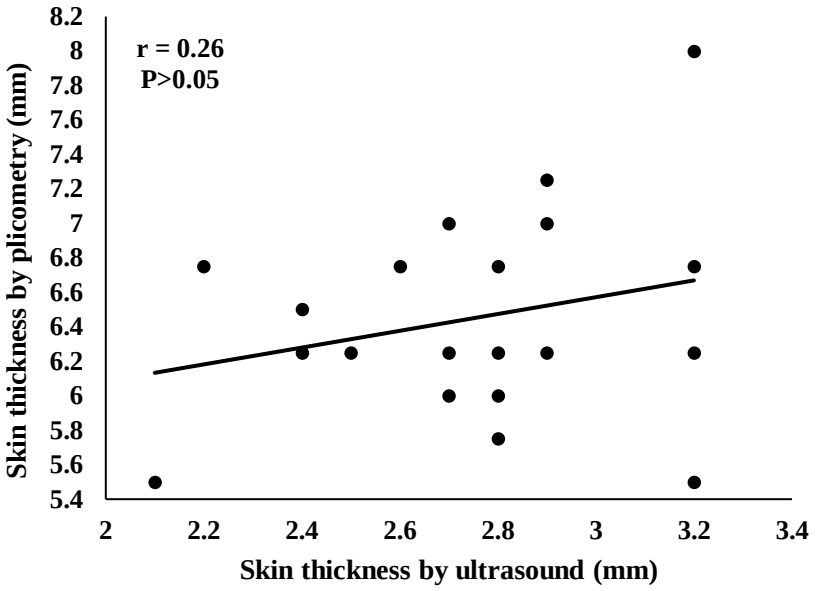


Figure 2.5. The correlation of skin thickness measured by ultrasonography (mm) and plicometry (mm). Pearson correlation coefficient has been indicated by r.

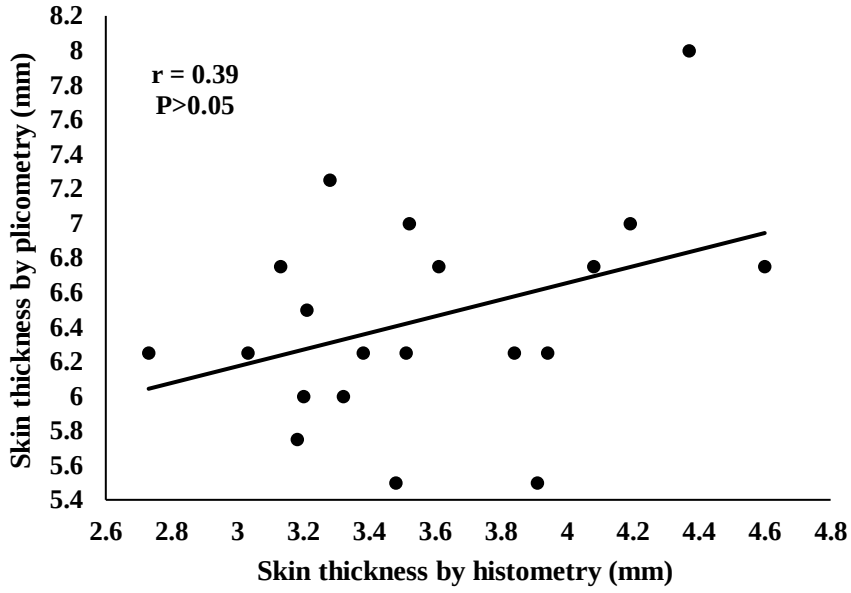


Figure 2.6. The correlation of skin thickness measured by histometry (mm) and plicometry (mm). Pearson correlation coefficient has been indicated by r.

Histological differences between subcutaneous fat from five categories corresponding to the structural differences in subcutaneous tissue revealed by ultrasonography could not be examined in the first year due to not having enough depth of subcutaneous fat sampled in the biopsy collection. However, the histological examination of the stained biopsy sections from lambs with low bubble scores (0 or 1) and high bubble scores (4 or 5) in the second year, with deeper biopsy samples taken, showed that the differences in the structure of subcutaneous tissue, revealed by ultrasonography (Figure 2.7) were due to variations in the amount of connective tissue within the subcutaneous tissue (Figure 2.8). In other words, lambs of low bubble category scores had less connective tissue within the subcutaneous tissue, so a higher proportion of the tissue was occupied by fat deposition compared to lambs of higher bubble category scores. Further investigation on the reason and factors that cause the variation in fat deposition, and also any possible association this could have with carcass composition would be of interest.

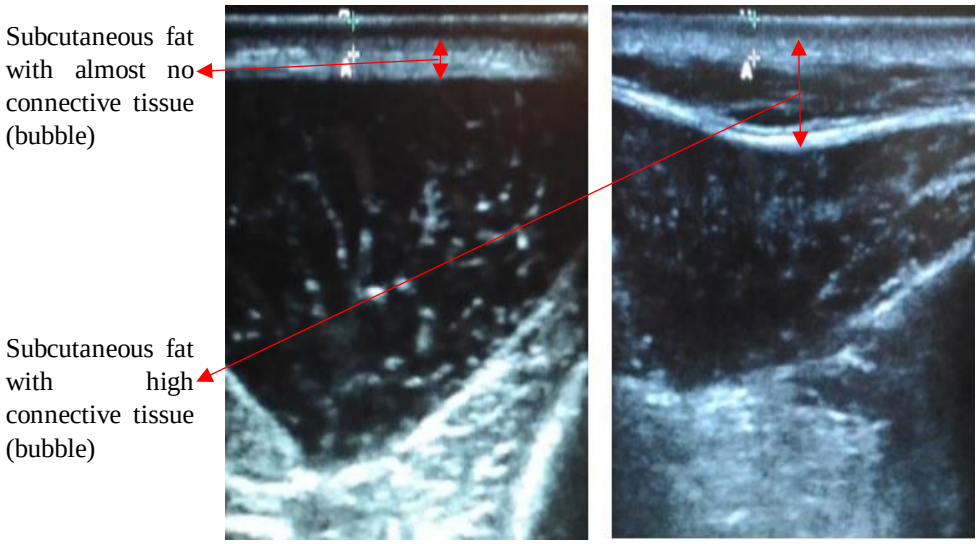


Figure 2.7. Ultrasound images showing samples from bubble categories 0 (left) and 5 (right).

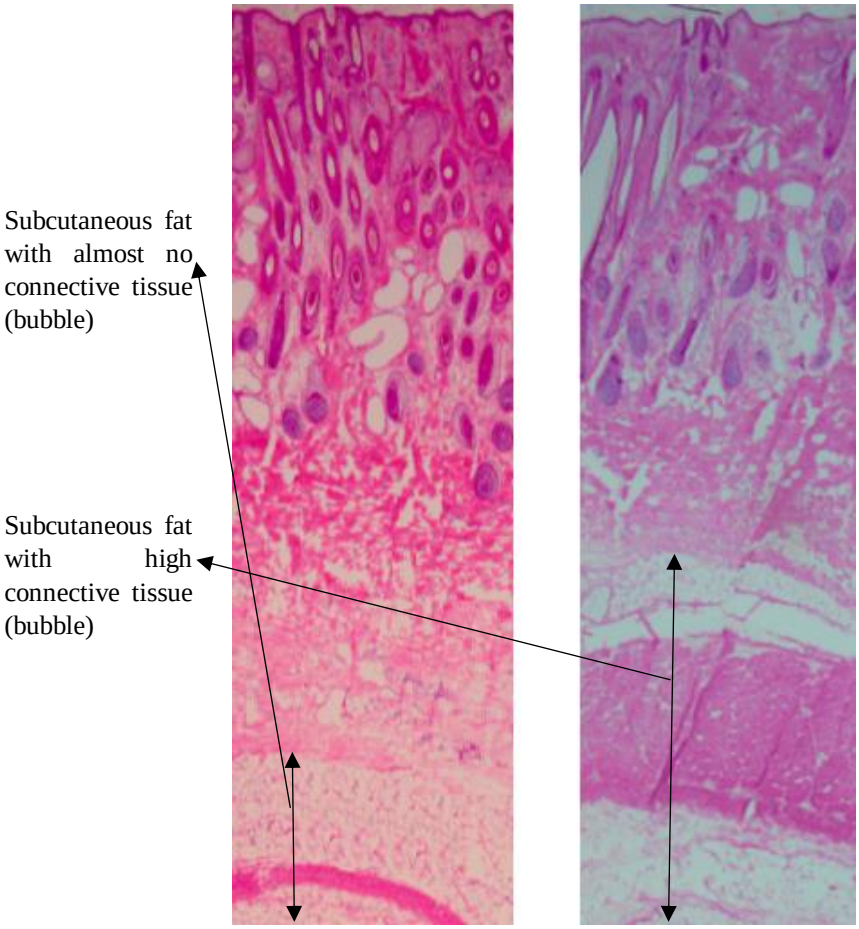


Figure 2.8. Photomicrographs of the biopsy sections stained by H&E method, showing bubble categories 0 (left) and 5 (right).

2.5. Conclusions

In conclusion, this study validates the reliability of ultrasonography as an accurate and non-invasive method for measurement of skin thickness in lambs. Therefore, we can rely on skin thickness data obtained by ultrasonography, and hence, use them in our subsequent quantitative analysis of this trait (estimation of genetic parameters). The part of reliability of skin thickness measured by ultrasound, which is associated with operator has been discussed in chapter five. Also, the structural differences in subcutaneous tissue, revealed by ultrasonography were due to variations in the amount of connective tissue within the subcutaneous tissue, which might be an indicator of fat deposition in subcutaneous tissue. The results obtained from this study might be beneficial to other animal species.

Chapter 3

Heritability estimate for skin thickness measured at nine months of age and its genetic, environmental, and phenotypic correlations with other traits in New Zealand Romney sheep

Part of this chapter has been published in the conference proceedings cited below.

Soltani-Ghombavani, M., Dukkipati, V. S. R., & Blair, H. T. (2017). Genetic association of skin thickness with lamb survival from birth to weaning, and growth and wool traits in New Zealand Romney sheep. In Proceedings of the 22nd Conference of the Association for the Advancement of Animal Breeding and Genetics, Townsville, Queensland, Australia, 2-5 July 2017, (pp. 589-592).

3.1. Abstract

Lamb survival, as a trait of high economic importance with low heritability, might show more response to indirect selection for traits of higher heritability that are genetically correlated with survival. This study aimed to estimate heritability and genetic association of skin thickness (ST; as a potential trait in indirect selection for lamb survival) with lamb survival from birth to weaning (SBW), and a few growth and wool traits including fat depth (FD), eye-muscle depth (EMD), weaning weight (WWT), 8-month live weight (LW8), live weight at scanning (LWS), 12-month live weight (LW12), 12-month fleece weight (FW12) in New Zealand Romneys. Data for ST, FD, and EMD were collected using ultrasound scans on hoggets at an average age of nine months. Appropriate animal and sire models were applied to estimate the genetic parameters using the ASReml software. The estimates of direct heritability for ST were 0.21 ± 0.03 and 0.20 ± 0.03, respectively from analyses with and without adjustment for LWS. Also, a maternal heritability of 0.02 ± 0.01 was estimated either including or excluding LWS as a covariate. Interestingly and unlike ST heritability estimates that were not affected by adjustment for LWS, the correlations (genetic, phenotypic and environmental) of ST with other traits, particularly live weight traits at different ages, were highly influenced by the covariate. When correction was made for LWS, ST showed genetic correlations of 0.21 ± 0.07, -0.13 ± 0.09, -0.32 ± 0.12, -0.23 ± 0.09, -0.10 ± 0.10, 0.02 (± 0.11, and 0.20 ± 0.11 with FD, EMD, WWT, LW8, LWS, LW12, and FW12, respectively. The corresponding estimates when no adjustment was made for LWS, were respectively 0.24 ± 0.08, -0.08 ± 0.10, -0.01 ± 0.12, 0.09 ± 0.09, 0.19 ± 0.09, 0.30 ± 0.10, and 0.20 ± 0.11. When LWS was included as a covariate, genetic correlation of ST with SBW ranged from 0.16 to 0.35 (SE ranging from 0.20 to 0.24) depending on the minimum numbers of progeny per sire considered for each trait, while the corresponding estimates from the analyses with LWS excluded ranged from 0.08 to 0.27 (SE ranging from 0.20 to 0.24). The estimates of heritability and positive genetic correlation of skin

thickness with lamb survival, obtained in this study, suggest the idea of considering this trait as a supplement to direct selection for lamb survival in selection programs. Nevertheless, the large standard errors of the correlations of ST with SBW as well the unfavourable correlation of ST with other traits should also be considered.

3.2. Introduction

Lamb survival is a trait of high economic importance which has a major effect on overall productivity of ewes (Amer *et al.*, 1999, Conington *et al.*, 2004). Several studies in the literature have reported low estimates of direct and maternal heritability for this trait (Lopez-Villalobos and Garrick, 1999, Morris *et al.*, 2000, Everett-Hincks *et al.*, 2005, Riggio *et al.*, 2008). This implies that direct genetic selection for this trait is unlikely to be promising. Hence, indirect selection, based on selection for other traits of higher heritability that are genetically correlated with survival can be considered as an alternative to direct selection for the trait itself.

Starvation/exposure has been reported to be responsible for approximately one third of lamb mortalities (Hartley and Boyes, 1964, McCutcheon, 1981) especially in outdoor lambing which is mostly common in countries like New Zealand, the UK and Australia. A few studies have reported a positive association between increased skin thickness and increased cold resistance in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991). Hence, selection for skin thickness might be a potential alternative to selection for lamb survival through its effect on cold resistance as a main component of lamb survival. Skin thickness could be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000) at the same time when routinely-measured ultrasound traits like fat depth and eye muscle depth are recorded. However, prior to considering this trait in selection for lamb survival, it is important to first estimate its heritability and genetic association with other economic traits.

A limited number of studies have estimated the heritability of skin thickness to be moderate to high (Gregory, 1982a, Slee *et al.*, 1991). Also, skin thickness has been reported to be positively genetically correlated with clean fleece weight and mean fiber diameter (Gregory, 1982b). In contrast, however, Hynd *et al.* (1996) demonstrated a negative correlation between skin weight (as an indicator of thickness) and clean fleece weight. Nevertheless, the sample size in those experiments was small and on the other hand, they have all been done only in Australian Merinos. Also, to our knowledge, there is no published report on the correlation of skin thickness with other traits of importance in sheep. Therefore, the objectives of this study were to:

1. Estimate heritability for ultrasonographically measured skin thickness (ST), fat depth (FD), and eye muscle depth (EMD) at an average age of about 9 months, lamb survival from birth to weaning (SBW), fleece weight at 12 months (FW12), and live weights at weaning (WWT), 8 months (LW8), scanning (LWS), and 12 months (LW12).
2. Estimate genetic, environmental, and phenotypic correlations of skin thickness (as the proposed trait influencing lamb survival) with other traits of interest in New Zealand Romney sheep as the most predominant dual-purpose breed in New Zealand.

3.3. Material and methods

3.3.1. Data collection

Data were collected from four Terminal Romneys for Increased Genetic Gain (TRIGG) farms in the Manawatu region of New Zealand for the lambing years 2010 to 2016. Data on date of birth, sex, flock, birth type, rearing type, dam age, dam and sire identities, status of lamb at weaning (alive or dead), weaning weight, 8-month live weight, 12-month live weight, fleece weight at 12 months, and dates of measurements were obtained from the Sheep Improvement Limited (SIL) database. Data for ultrasound traits including skin thickness, fat depth, and eye

muscle depth were collected as part of routine farm operations using ultrasound at approximately 8 months of age, during 2011 to 2017. A commercial operator took measurements using a Sonosite M Turbo (FUJIFILM Sonosite, Inc., Bothell, Washington, United States) ultrasound scanning machine with a 38mm probe at 7.5 MHz set at a depth of 40 mm on the left dorsal loin region of the lambs, around the 12th rib. Live weight was also recorded at scanning. The definition of the traits analysed in this study were as follows:

Survival from birth to weaning (SBW): This trait was recorded as a binary trait (all or none trait). If weaning weight was recorded, then lamb was assumed to be alive and coded 1, otherwise it was considered as dead and coded 0.

Skin thickness (ST): The thickness of skin layer (mm) measured by ultrasound scanning at an average age of 279 days with a range of 173 to 360 days.

Fat depth (FD): The depth of subcutaneous fat layer (mm) measured by ultrasound scanning at an average age of 279 days with a range of 189 to 360 days.

Eye muscle depth (EMD): The depth of eye muscle (mm) measured by ultrasound scanning at an average age of 277 days with a range of 183 to 351 days.

Weaning weight (WWT): Lamb live weight (kg) recorded at an average age of 99 days with a range of 39 to 158 days.

Live weight at 8 months of age (LW8): Lamb live weight (kg) recorded at an average age of 167 days with a range of 100 to 218 days.

Live weight at scanning (LWS): Lamb live weight (kg) recorded at ultrasound scanning of lambs at an average age of 276 days with a range of 189 to 360 days.

Live weight at 12 months of age (LW12): Lamb live weight (kg) recorded at an average age of 316 days with a range of 211 to 367 days.

Fleece weight at 12 months of age (FW12): Fleece weight (kg) recorded at an average age of 322 days with a range of 220 to 379 days.

3.3.2. Data editing

The normality of the data was checked using the PROC UNIVARIATE in SAS 9.3 (SAS, 2011) and all outlier observations (determined visually from distribution plots) were removed from the data. Also, records with missing fixed effects that were significant for each trait were removed from the data. For all the traits, data with less than 5 records in each level of significant fixed effects were excluded from the analysis (n=61). Records with dam ages of 8 years or more were all considered as 7 because of their small numbers (n=104). For the same reason, lambs with birth ranks of 4 and 5 (n=52) were considered as triplet-born lambs. Similarly, lambs with rearing rank of 4 (n=16) were classified as lambs with rearing rank of 3. Lambs of unknown parents in the pedigree (n=439) were excluded from the analysis. After data cleaning and editing, the data set had 30,535 lambs born to a total of 245 sires and 7,485 dams. Records available for each trait based on year, flock and sex have been presented in Table 3.1.

Table 3.1. Records available for each trait based on year, flock and sex.

Year	Flock	Trait								
		SBW	ST	FD	EMD	WWT	LW8	LWS	LW12	FW12
2010	1	B	-	-	-	B	B	-	-	M
	2	B	B	-	M	B	B	B	F	B
	3	B	-	-	M	B	B	M	B	B
	4	B	B	B	M	B	B	B	B	M
2011	1	B	-	-	-	B	B	-	-	M
	2	B	B	M	M	B	B	B	B	B
	3	B	-	-	-	B	B	-	B	B
	4	B	B	B	-	B	B	B	B	B
2012	1	B	M	M	M	B	B	M	-	M
	2	B	B	B	B	B	B	B	B	B
	3	B	B	B	B	B	B	B	B	B
	4	B	B	B	B	B	B	B	B	-
2013	1	B	-	-	-	B	B	-	-	M
	2	B	F	B	B	B	B	B	B	B
	3	B	-	-	-	B	B	-	M	B
	4	B	B	B	B	B	B	B	B	M
2014	1	-	-	-	-	-	-	-	-	-
	2	B	-	-	-	B	B	-	-	-
	3	B	-	-	-	B	B	-	-	-
	4	B	B	B	B	B	B	B	-	-
2015	1	-	-	-	-	-	-	-	-	-
	2	B	-	-	-	B	B	-	-	-
	3	B	-	-	-	B	B	-	-	-
	4	B	B	B	B	B	B	B	-	M
2016	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-
	3	B	-	-	-	B	-	-	-	-
	4	B	B	B	B	B	B	B	B	M

SBW: survival from birth to weaning, ST: skin thickness, FD: fat depth, EMD: eye-muscle depth, WWT: weaning weight, LW8: 8-month live weight, LWS: live weight at scanning, LW12: 12-month live weight, FW12: 12-month fleece weight.
B: data were available for both males and females, F: data were available only for females, M: data were available only for males.

3.3.3. Statistical analysis

After data editing and cleaning, the final data were analysed, separately for each trait, to identify significant fixed effects and covariates to be included in final models for each trait. The 'PROC GLIMMIX' of SAS 9.3 (SAS, 2011) was used for the analysis of SBW, assuming

a binomial distribution for the trait using logit and probit link functions. For the remaining traits, the 'PROC MIXED' of SAS 9.3 (SAS, 2011) was used, assuming a normal distribution for the traits.

The initial models for all the traits contained the effects of year-flock (13-24 levels depending on the trait), sex (male and female), birth rank (single, twin, and triplet) for SBW and birth-rearing type for other traits (6 levels: born as single, twin, or triplet, and reared as single, twin, or triplet), dam age (7 levels, 1-7 years and older), and their two-way interactions as fixed effects and weight (for ST, FD, and EMD) or age (for WWT, LW8, LWS, LW12, and FW12) at each trait measurement, and date of birth and date of birth within each year-flock for SBW as covariates. All non-significant fixed effects ($P>0.05$), interactions ($P>0.05$) and covariates ($P>0.05$) were excluded from the models in the subsequent analyses.

(Co) variance components were estimated by Restricted Maximum Likelihood (REML) procedure using the ASREML software (Gilmour *et al.*, 2015). Because survival was coded as a binary trait, a generalized linear mixed model (GLMM) analysis was performed for this trait, assuming a binomial distribution using both logit and probit link functions. For all other traits, linear mixed models were used assuming normal distribution for the traits. To identify significant random effects to be included in the final model for the traits of normal distribution, several models with different combinations of random effects were compared to determine the most suitable model to use for each trait. The random effects included in the models were direct additive genetic effect (σ^2_A), maternal additive genetic effect (σ^2_M), direct-maternal genetic covariance (σ_{AM}), and maternal permanent environmental effect (σ^2_{PE}). The different models ran for each trait had the same fixed effects and differed only in random effects included, and were compared using the likelihood ratio test. The increase in log-likelihood was calculated when a random effect was added to a model. If the increase timed by 2 was greater than the critical valve of the chi-square distribution for one degree of freedom at a significance level of

α = 0.05 (3.841), the included random effect was considered as significant ($p<0.05$) and retained in the model. Otherwise, the random effect was considered as non-significant and excluded from the final model.

Since a GLMM was used for the analysis of SBW, and LogL values obtained from GLMMs are not suitable for comparing different models (Gilmour *et al.*, 2015), the likelihood ratio test could not be used to identify significant random effects to be included in the final model for this trait. Therefore, all the random effects were included in the final model for the analysis of SBW. All significant effects (fixed and random effects and covariates) included in the final models for each trait has been summarised in Table 3.2.

Table 3.2. Fixed effects, covariates, and random effects included in the final univariate analysis for each trait.

	Trait								
Factor	**SBW[1]**	**ST[2]**	**FD[3]**	**EMD[3]**	**WWT**	**LW8**	**LWS**	**LW12**	**FW12**
Fixed effects									
Year-flock	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sex	✓	✓	✓	✓	✓	✓	✓	✓	✓
Birth type	✓								
Birth-rearing type			✓	✓	✓	✓	✓	✓	✓
Dam age	✓		✓	✓	✓	✓	✓	✓	✓
Year-flock × Sex	✓	✓	✓	✓	✓	✓	✓	✓	✓
Year-flock × Birth type	✓								
Year-flock × Birth-rearing type					✓	✓	✓		
Year-flock × Dam age			✓		✓	✓	✓	✓	✓
Sex × Birth-rearing type			✓		✓				
Sex × Dam age	✓		✓		✓	✓	✓		
Birth type × Dam age	✓								
Birth-rearing type × Dam age					✓	✓	✓		
Covariates									
Weight at measurement		✓	✓	✓					
Age at measurement					✓	✓	✓	✓	✓
Date of birth	✓								
Date of birth × Year-flock	✓								
Random effects									
σ^2_A	✓	✓	✓	✓	✓	✓	✓	✓	✓
σ^2_M	✓	✓			✓	✓	✓	✓	
σ_{AM}	✓				✓	✓			
σ^2_{Pe}	✓				✓	✓	✓	✓	
σ^2_E	✓	✓	✓	✓	✓	✓	✓	✓	✓

σ^2_A: direct additive genetic effect, σ^2_M: maternal additive genetic effect, σ_{AM}: direct-maternal genetic covariance effect, σ^2_{PE}: maternal permanent environmental effect, σ^2_E: residual (error) effect. SBW: survival from birth to weaning, ST: skin thickness, FD: fat depth, EMD: eye-muscle depth, WWT: weaning weight, LW8: 8-month live weight, LWS: live weight at scanning, LW12: 12-month live weight, FW12: 12-month fleece weight.[1]For SBW, the effect year-flock × birth type was replaced by year × birth type and flock × birth type, and the effect year flock × sex was replaced by year × sex and flock × sex due to a failure in running the models with the initial effects included. [2]For ST, sex and birth-rearing type were included in the model when ST was analysed without including scanning weight as a covariate. [3]For FD and EMD, scanning weight was replaced by scanning age in the models where these traits were analysed without including scanning weight as a covariate.

Genetic, environmental, and phenotypic correlations were estimated using appropriate bivariate analyses for all combinations of the traits using the ASREML software (Gilmour *et al.*, 2015). In the bivariate models where survival was included as a trait, sire models were used

applying the same effects used in univariate animal models for each trait, except for the random effect of animal (direct additive genetic) that was replaced by sire effect. In those models, SBW was considered as a binary trait with binomial distribution and all other traits as continuous with normal distribution. Since models with logit link function considered for SBW did not converge, the analyses were only performed using probit link function. A few bivariate sire analyses were performed for SBW and ST with the same effects used in each model but with different numbers of progeny per sire for SBW and ST in different models. This was done to get an idea of the appropriate numbers of progeny per sire for SBW and other traits required in the bivariate sire analyses. Based on the results obtained from these analyses, in the final bivariate sire models, only those sires that had at least 40 records of their progeny for SBW and 20 for each of the other traits were included in the analyses. For estimating the correlations among all other traits with normal distribution, bivariate animal models were used applying the same effects used in univariate analyses for each trait. In some of the bivariate models where SBW was included as one of the traits, when date of birth within year-flock was considered as covariate, the model failed to run in the ASREML software. However, when date of birth within year-flock was included as random, all models could be run successfully. The (Co) variance components estimated from the analyses where date of birth within year-flock was used as a covariate, were almost identical to those estimated by the analyses with date of birth within year-flock considered as a random effect. So, for consistency, in all bivariate analyses with SBW as a trait in the model, the effect of date of birth within year-flock was considered as random instead of covariate. To determine if the correlations were significant, the likelihood ratio test, as explained for the univariate analyses, was used. For the same reason as explained for the univariate analysis of SBW, this test was not suitable for determining the significance of correlations in the bivariate analyses where SBW was involved.

To test if indirect selection for lamb survival (based on selection for skin thickness) is more efficient than direct selection for the trait, the following equation was used (Turner and Young, 1969):

$$Q = r_g * h_Y / h_X$$

where Q is the relative efficacy of indirect selection compared to direct selection, rg is the genetic correlation between the two traits, and hY and hX are the square roots of the heritabilities of the indirect and direct traits, respectively.

When Q > 1, then indirect selection is more efficient.

3.4. Results and discussion

Summary statistics for each trait have been presented in Table 3.3. Lamb survival from birth to weaning showed an average of 79% in this study. This average survival was similar to that reported in Romney flocks in New Zealand (Morris *et al.*, 2000), higher than the averages of 72% and 75% respectively reported in Australian Merino (Hatcher *et al.*, 2010) and South African Merino (Cloete *et al.*, 2009), respectively, and lower than 87% in New Zealand Romney reported by Lopez-Villalobos and Garrick (1999). The variations in average lamb survival could be attributed to differences in factors like weather conditions, management and/or breed. The coefficient of variation (CV) of 52% for lamb survival in our study is within the range of 48 to 57% found in the literature (Matos *et al.*, 2000, Safari and Fogarty, 2003, Safari *et al.*, 2005). A high CV of lamb survival is useful for genetic change through selection, although most of the variation is most likely due to variations in non-genetic factors affecting this trait, due to low heritability.

Skin thickness in this study, measured by ultrasound scanning, had a mean of 2.86 mm (Table 3.3), which is slightly lower than mean skin thickness report by Jopson *et al.* (2000) in new-born Coopworth lambs in New Zealand, measured by skinfold calipers. The observed

difference in skin thickness might result from differences in breed, age at measurement, and techniques used for measuring skin thickness. The moderate CV of 17.45% and the wide range of 1.5 to 5mm found for skin thickness in the present study could be considered as a reasonable source of variation required for genetic gain through selection.

Table 3.3. Descriptive statistics and number of records for each trait.

Trait	Number of records	Mean	SD	Minimum	Maximum	CV (%)
SBW (%)	30,535	0.79	0.41	0	1.00	52.01
ST (mm)	7,507	2.86	0.50	1.50	5.00	17.45
FD (mm)	7,579	2.72	1.39	1.00	12.00	51.13
EMD (mm)	5,813	25.67	3.04	16.00	38.00	11.84
WWT (kg)	23,799	28.63	6.07	9.00	57.00	21.20
LW8 (kg)	21,070	36.79	7.08	13.5	73.00	19.25
LWS (kg)	8,143	46.88	6.21	27.00	77.50	13.24
LW12 (kg)	7,048	48.56	6.48	27.5	76.00	13.35
FW12 (kg)	6,099	3.42	0.72	1.60	5.80	20.94

SBW: survival from birth to weaning, ST: skin thickness, FD: fat depth, EMD: eye-muscle depth, WWT: weaning weight, LW8: 8-month live weight, LWS: live weight at scanning, LW12: 12-month live weight, FW12: 12-month fleece weight.

3.4.1. Non-genetic factors affecting skin thickness

Body weight at scanning in the present study showed a positive association ($P<0.0001$) with skin thickness with a 1 kg increase in live weight being associated with 0.02 ± 0.001 mm increase in skin thickness (Figure 3.1). In line with this result, Jopson *et al.* (2000) reported a positive association between skin thickness measured at birth and birth weight in Coopworth lambs in New Zealand. However, the amount of increase in skin thickness per kg increase in birth weight was much higher in their study (0.37 ± 0.06 mm increase in skin thickness with a 1 kg increase in body weight). This could be attributed to the fact that the range for birth weight would be much smaller compared to that for LWS, while skin thickness would not change

much from birth onwards. Also, as reported in chapter five, a study on new-born Romney-type lambs, every 1 kg increase in birth weight was associated with 0.1 ± 0.02 mm increase in skin thickness taken by ultrasound. These findings show a decrease in the magnitude of the association of skin thickness with body weight as lambs grow.

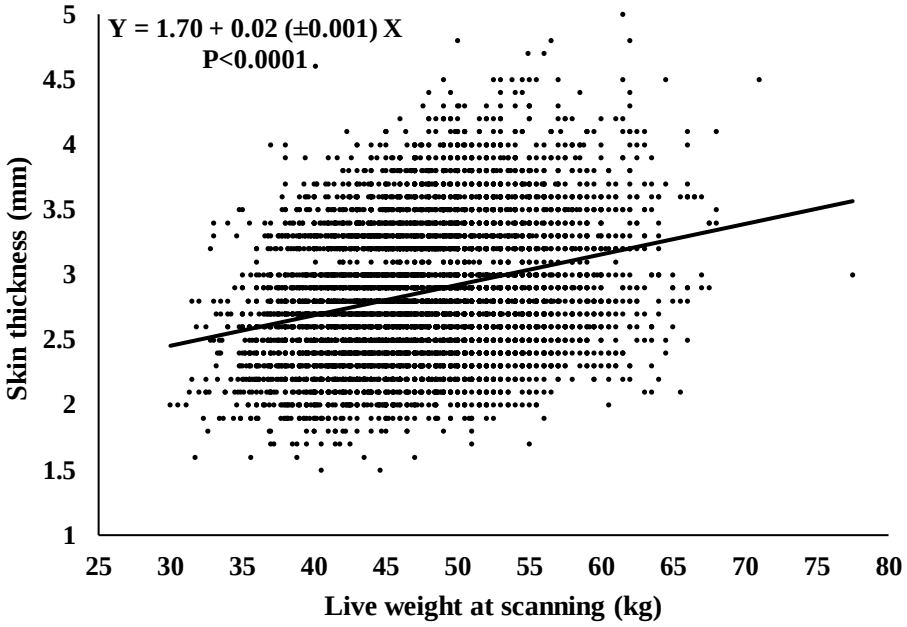


Figure 3.1. The association of live weight at scanning (kg) with skin thickness (mm) after making adjustment for all other significant effects influencing skin thickness.

In addition, birth-rearing type had a significant effect on skin thickness before making adjustment for LWS so that lambs born and reared as single had thicker skin (P<0.001) compared to lambs born and reared as either twins or triplets (Figure 3.2). However, there was no significant difference (P>0.05) between lambs of different birth-rearing types after adjustment was made for LWS (Figure 3.3). The difference in skin thickness observed before adjustment for body weight could be a result of greater scanning weight in lambs born and reared as single compared to lambs born and reared as twins or triplets. This implies that birth rank would not have any effect on skin thickness *per se*. Consistent with our result, in a study

by Jopson *et al.* (2000), birth rank had no effect on skin thicknesses, though measured at birth, when adjustment was made for birth weight.

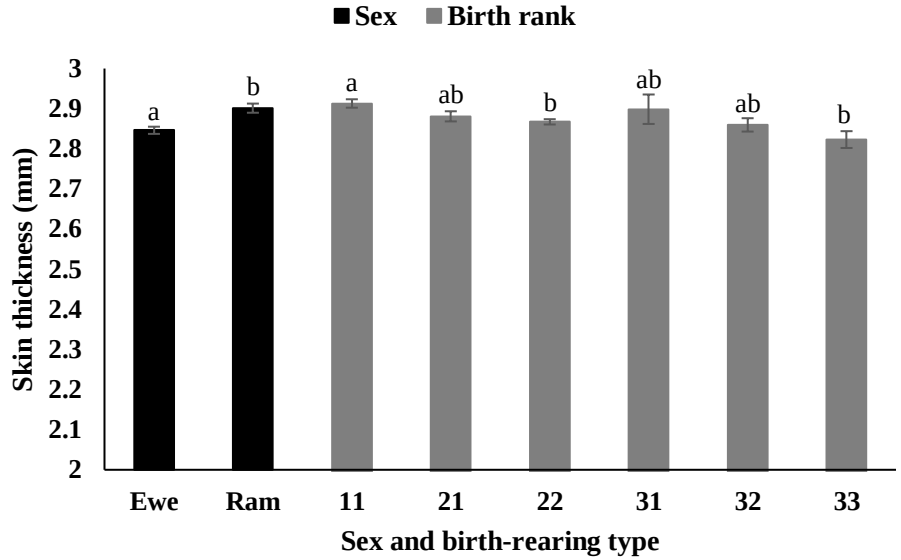


Figure 3.2. The effects sex and birth-rearing type on skin thickness (mm) before making adjustment for live weight at scanning. All values are presented as Least Square Means ± standard errors. Values with different letters are significantly different (P<0.001).

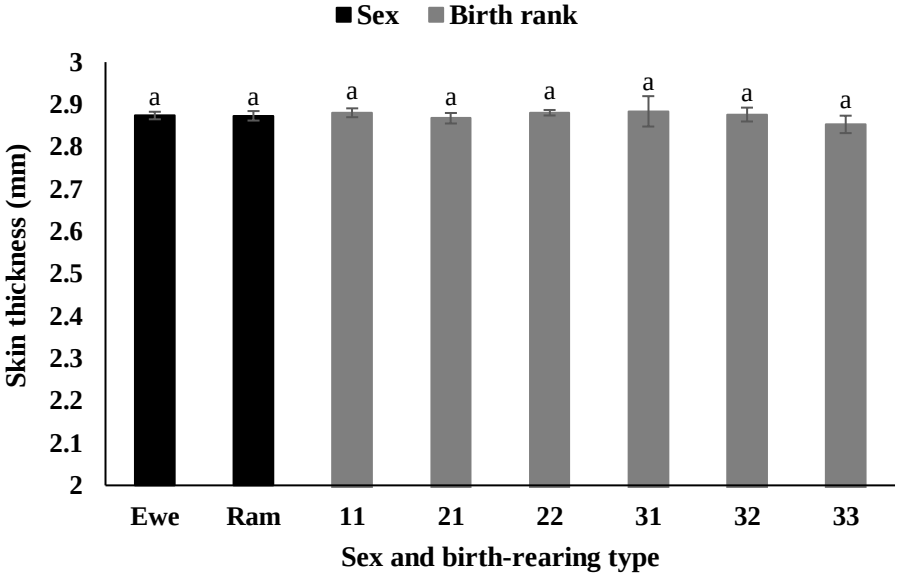


Figure 3.3. The effects sex and birth-rearing type on skin thickness (mm) after making adjustment for live weight at scanning. All values are presented as Least Square Means ± standard errors. Values with the same letters are not significantly different (P>0.05).

Also, ram lambs showed a significantly thicker skin ($P<0.001$) than ewe lambs before making adjustment for LWS (Figure 3.2). However, after correction was made for LWS, the effect of sex on skin thickness depended on year-flock (Figure 3.4), while the overall effect of sex was not significant ($P>0.05$, Figure 3.3). As displayed in Figure 3.4, ram lambs had significantly higher ($P<0.05$) skin thickness compared to ewe lambs in flock-years 12, 14, 22, and 64, while in flock-years 32, 34, 44 and 54, ewe lambs showed significantly thicker skin than rams, and no difference (P.0.05) was evident in skin thickness of ram and ewe lambs in the other flock-years. Since correction was made for weight at scanning, the effect of sex on skin thickness being different in different year-flocks cannot be simply justified by ewe and ram lambs having different body weights. The reason behind the observed interaction between sex and flock-year for skin thickness is not clear. However, part of that might be due to different behaviours male and female lambs showed in response to possible differences in weather and/or nutritional conditions they experienced in different year-flocks, thought there is no evidence in literature supporting the idea that male and female lambs react differently (in terms of skin thickness change) to changes in weather and/or nutritional conditions. In line with our result, in a study by Jopson *et al.* (2000), sex had no effect on skin thicknesses measured at birth when adjustment was made for birth weight, however, they did not report any interaction between sex and any other effect in their study.

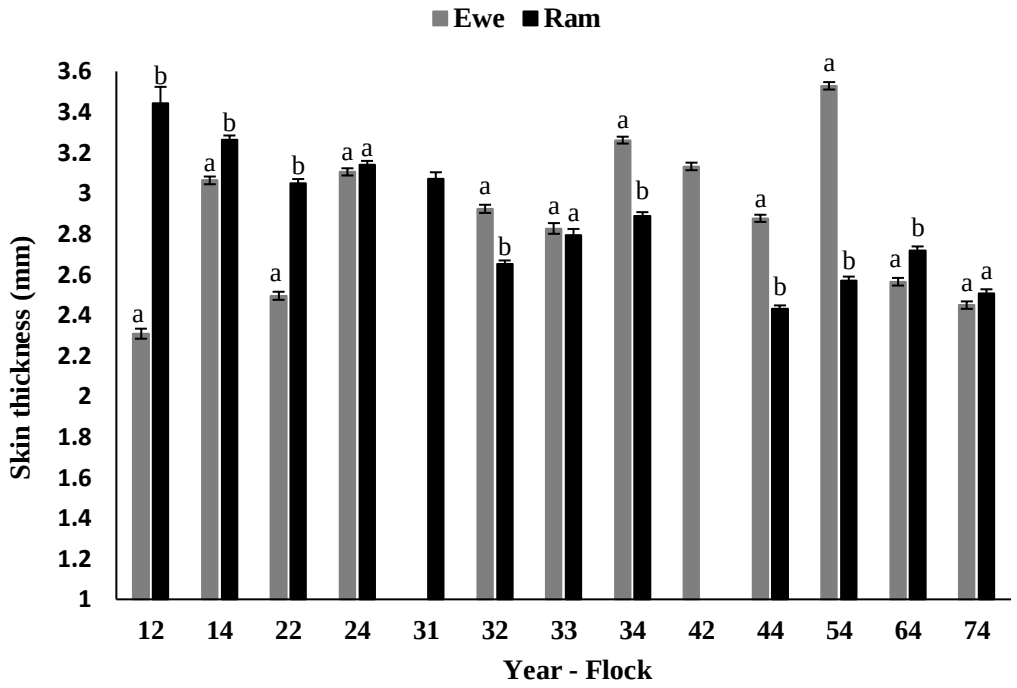


Figure 3.4. The effect of sex on skin thickness (mm) within different Year - Flocks after making adjustment for live weight at scanning. The first and second digits in each of the numbers along X-axis indicate year and flock, respectively. There were seven (1 to 7) birth years (2010 to 2016). Skin thickness was recorded for only one sex in case of year-flocks 31 and 42. All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

As presented in Figure 3.5, during the first, second and fourth years of ultrasound scanning, skin thickness was only measured in flocks 2 and 4. For years 1 and 2, lambs in flock 4 had significantly higher (P<0.05) skin thickness than those in flock 2, but the trend was exactly opposite i.e. flock 4 sheep had significantly (P<0.05) thicker skin than flock 1 sheep. In the third year of scanning where skin thickness was recorded for all the four flocks, lambs of flocks 1 and 4 had similar (P>0.05) skin thicknesses that were significantly higher (P<0.05) than those in flocks 2 and 3; the sheep in flocks 2 and 3 had similar skin thickness (P>0.05). For the third last years (5, 6 and 7), skin thickness was only recorded for flock 4, so no comparison could be made between flocks in these years.

Also, as displayed in Figure 3.5, for flock 2 where skin thickness was measured in the first four years, skin thickness measured in each year was significantly higher than that in the preceding

year ($P<0.05$), except for third year where the thickness was similar ($P>0.05$) to that in year 2. For flock 4 where skin thickness was measured in all the seven years, no particular trend was observed; skin thickness remained constant during the first three years, while it significantly ($P<0.05$) decreased in the fourth year, before significantly ($P>0.05$) increasing in year 5 to the level in years 2 and 4 but was still significantly lower ($P<0.05$) than that seen in the first year. However, in the sixth year, it decreased to an average equal ($P>0.05$) to that of the fourth year, followed by another significant decrease ($P<0.05$) reaching to a mean similar ($P>0.05$) to that of the first year. These changes in skin thickness observed in different year-flocks could reflect possible differences in known and/or unknown non-genetic factors like weather and nutritional conditions lambs experienced in each year-flock. Consistent with this, Hutchinson (1957) reported changes in skin thickness, attributable to differences in nutrition. On the other hand, these changes might even arise from selection programs exerted, either based on direct selection for skin thickness or based on selection for other traits that might indirectly affect skin thickness due to their possible genetic correlations with skin thickness.

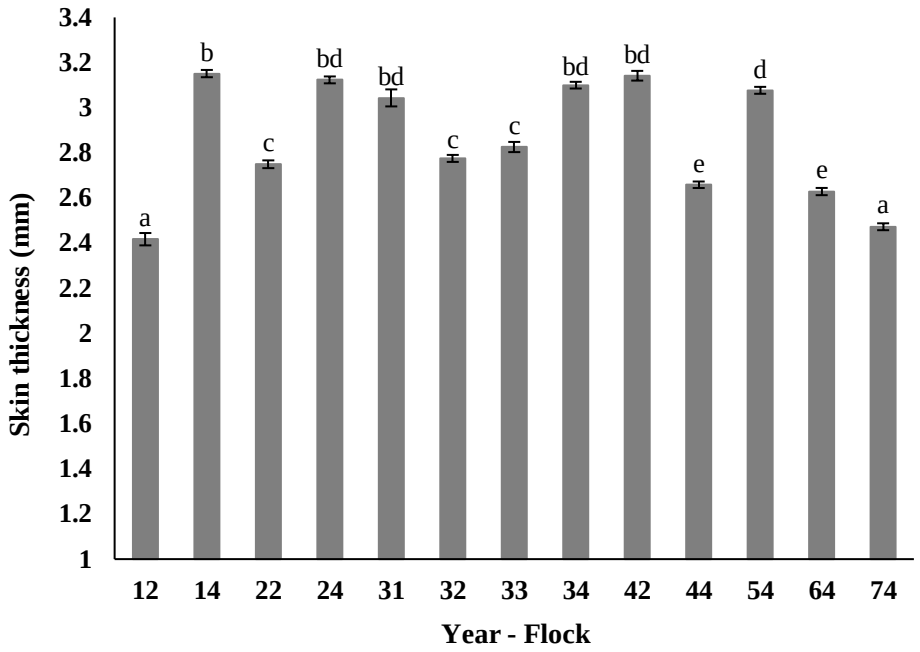


Figure 3.5. The effect of year-flock on skin thickness (mm) after making adjustment for live weight at scanning. The first and second digits in each of the numbers along X-axis indicate year and flock, respectively. There were seven (1 to 7) birth years (2010 to 2016). Skin thickness was not recorded for some year-flocks. All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different ($P<0.05$).

3.4.2. Non-genetic factors affecting lamb survival

All factors that had significant effects on lamb survival have been indicated in Table 3.2. Also, survival percentage and relative risk of death for each sex, birth type, and age of dam have been presented in Table 3.4. Because of their high numbers of classes, survival percentage and relative risk of death have not been shown for all other significant effects.

Table 3.4. Lamb survival percentage and relative risk of death for each sex, birth type, and dam age

Effect	Number of lambs	Survival (%)	Relative risk of death[†]
Sex			
Male	17,204	69	1.000
Female	13,331	91	0.291[*]
Birth type			
Single	5,688	86	0.148[*]
Twin	20,594	80	0.347[*]
Triplet	4,253	62	1.000
Dam age (year)			
1	3,737	69	1.000
2	8,167	79	0.733[*]
3	6,868	82	0.625[*]
4	5,419	80	0.595[*]
5	3,879	81	0.554[*]
6	1,923	77	0.645[*]
7 and more	542	71	0.917

[†] using logit link function
[*] Risk is significantly (P<0.05) different from 1.

Year-flock had a significant effect (P<0.0001) on lamb survival in this study, reflecting the considerable effect of weather, management and nutritional conditions of the ewes, or a combination of all these, on the survival of lambs. Also, sex of lamb had a significant effect on lamb survival (P<0.0001); female lambs showed higher survival and lower risk of death compared to males (Table 3.4). Contrary to this, Atkins (1980) reported that males and females have similar survival rates. Consistent with our result, however, female lambs showed higher survival rates than males at different ages from birth to weaning in other studies (Dalton *et al.*, 1980, Sawalha *et al.*, 2007, Riggio *et al.*, 2008, Maxa *et al.*, 2009). Lower survival in male lambs has been attributed to their greater average birth weight, which causes a greater risk of dystocia (Smith, 1977, Gama *et al.*, 1991). However, this effect could not be examined in our study because no data were available for birth weight of the lambs.

Also, birth type had a significant effect ($P<0.0001$) on survival and relative risk of death, with singles having the highest survival rate and the lowest relative risk of death, triplets having the lowest survival and the highest risk of death while twins were intermediate between these two (Table 3.4). Similar to this finding, several studies have reported general increase in lamb mortality with increased litter size (Petersson and Danell, 1985, Nicoll *et al.*, 1999, Riggio *et al.*, 2008, Hatcher *et al.*, 2009). Bradford (1972) suggested that litter size does not affect lamb survival directly but through its effects on birth weight. The litter size effect on survival can also be attributed to the distribution of the ewe's grooming behaviour (Riggio *et al.*, 2008). Consistent with this, twin-born lambs were demonstrated to receive less overall grooming attention compared to singles (O'Connor *et al.*, 1992). A higher ratio of surface area to body weight in lambs of higher birth rank might have also imposed more heat loss and consequently higher risk of death in multiple-born lambs compared to singles.

Lamb survival was also significantly ($P<0.0001$) affected by dam age. Lambs born to one- and seven-year-old ewes had the lowest survival, while lambs born to ewes of other ages were in the middle (Table 3.4). Lambs born to one-year-old ewes had the same ($P>0.05$) relative risk of death as those born to seven-year-old ewes, while the former showed significantly ($P<0.05$) higher relative risk of death compared to ewes of other ages (Table 3.4). The lower survival of lambs born to one-year-old dams could be attributed to the fact that dystocia is more prevalent in these ewes. Also they are inexperienced mothers and their lambs receive less attention. In line with our results, other studies (Knight *et al.*, 1988, Lopez-Villalobos and Garrick, 1999, Sawalha *et al.*, 2007, Riggio *et al.*, 2008) reported lower survival in lambs born to younger and older ewes, and higher survival in ewes of intermediate age.

3.4.3. Genetic parameters

Estimates of the direct heritability (h^2_a), maternal heritability (h^2_m), maternal permanent environmental (pe^2) effect, direct-maternal genetic correlation (r_{am}), and total heritability (h^2_T) for each trait have been presented in Table 3.5.

A low direct heritability (h^2_a) of 0.023 was estimated for lamb survival in this study using both logit and probit link functions (Table 3.5). Although not exactly the same as our estimates, Lopez-Villalobos and Garrick (1999) also reported low estimates of 0.013 and 0.007 in Romney lambs using the logit and probit link functions, respectively. Similarly, (Morris *et al.*, 2000) reported corresponding estimates that ranged from 0 to 0.02 using the logit transformation in Romney sheep. In contrast, a higher estimate of 0.14 for lamb survival was reported in Coopworth lambs using the logit-transformation (Everett-Hincks *et al.*, 2005). Furthermore, Cloete *et al.* (2009) found an estimate of 0.28 using threshold animal model in a flock of South African Merino, while Riggio *et al.* (2008) reported estimates of 0.05 and 0.08 for survival from birth to 12 weeks in Scottish Blackface sheep by sire models using linear and probit analyses, respectively. The variation observed for the estimates in our study and other studies could be attributed to differences in the population sizes, breeds, models, and analyses used in different studies.

Table 3.5. Estimates (±SE) of the direct heritability (h^2_a), maternal heritability (h^2_m), maternal permanent environmental (pe^2) effect, direct-maternal genetic correlation (r_{am}), and total heritability (h^2_T) for each trait.

Trait	h^2_a	h^2_m	pe^2	r_{am}	h^2_T
SBW (logit)	0.023 ± 0.008	0.021 ± 0.011	0.011 ± 0.012	0.077 ± 0.347	0.036 ± 0.011
SBW (probit)	0.023 ± 0.008	0.024 ± 0.011	0.011 ± 0.013	0.127 ± 0.346	0.040 ± 0.012
ST (adj)	0.21 ± 0.03	0.02 ± 0.01	-	-	0.22 ± 0.03
ST (unadj)	0.20 ± 0.03	0.02 ± 0.01	-	-	0.22 ± 0.03
FD (adj)	0.38± 0.06	0.08 ± 0.02	-	-0.63 ± 0.08	0.25 ± 0.03
FD (unadj)	0.35 ± 0.05	0.08 ± 0.02	-	-0.63 ± 0.08	0.23 ± 0.03
EMD (adj)	0.42 ± 0.03	-	-	-	-
EMD (unadj)	0.39 ± 0.04	0.05 ± 0.02	-	-	0.41 ± 0.04
WWT	0.14 ± 0.02	0.11 ± 0.02	0.14 ± 0.01	-0.39 ± 0.09	0.12 ± 0.02
LW8	0.21 ± 0.03	0.09 ± 0.02	0.10 ± 0.01	-0.33 ± 0.09	0.19 ± 0.02
LWS	0.21 ± 0.03	0.04 ± 0.02	0.06 ± 0.02	-	0.23 ± 0.03
LW12	0.22 ± 0.03	0.04 ± 0.02	0.06 ± 0.02	-	0.24 ± 0.03
FW12	0.48 ± 0.04	-	-	-	-

adj: adjusted for scanning weight.
unadj: unadjusted for scanning weight.
SBW: survival from birth to weaning, ST: skin thickness, FD: fat depth, EMD: eye-muscle depth, WWT: weaning weight, LW8: 8-month live weight, LWS: live weight at scanning, LW12: 12-month live weight, FW12: 12-month fleece weight.

Also, low estimates of 0.021 and 0.024 were found for maternal heritability (h^2_m) of lamb survival in this study using logit and probit link functions, respectively (Table 3.5). Corresponding estimates of 0.035 and 0.036 using logit and probit link functions (Lopez-Villalobos and Garrick, 1999), 0.09 to 0.10 using logit-transformed data (Morris *et al.*, 2000), 0.11 using the logit transformation (Everett-Hincks *et al.*, 2005), and 0.14 using threshold animal model (O'Connor *et al.*, 1992), were reported in other studies. The low estimates of heritability, either direct or maternal, found in this study and several previous reports, shows that direct genetic selection for this trait would not be promising. Therefore, direct selection for other traits of higher heritability that are genetically correlated with lamb survival would be more effective than direct selection for the trait itself.

Direct-maternal genetic correlations (r_{am}) for lamb survival were positive, though with low values of 0.08 and 0.13 using logit and probit link functions, respectively. The positive estimate of the correlations suggests that dams with good genetic mothering ability have good direct genetic potential to produce lambs with higher survival too. However, this conclusion should be made with caution because a large standard error of 0.35 obtained for the estimates does not allow a clear interpretation. Direct-maternal genetic correlations of different signs and magnitudes (-1.04 to 0.83) have been reported for lamb survival in various populations using different types of analyses (Burfening, 1993, Lopez-Villalobos and Garrick, 1999, Morris *et al.*, 2000, Everett-Hincks *et al.*, 2005, Welsh *et al.*, 2006, Sawalha *et al.*, 2007, Maxa *et al.*, 2009). Everett-Hincks *et al.* (2005) suggested that a negative unfavourable direct-maternal genetic correlation is possibly due to an incompatibility between genes that regulate physiological and biochemical processes for survival and genes that enhance ewe-lamb bonding. Burfening (1993) suggested that if there is very little additive genetic variation for direct and maternal effects, the covariance between these two cannot be estimated well. In support of this, using simulated data, Robinson (1996) concluded that negative estimates of correlation between direct and maternal genetic effects might be obtained even in the absence of a true antagonism between them.

The estimates of direct heritability for skin thickness obtained from analyses including and excluding LWS as a covariate, were 0.21 and 0.20, respectively (Table 3.5). Also, a maternal heritability of 0.02 was estimated either with or without adjustment for LWS. So, our results showed that the heritability estimates of skin thickness, either direct or maternal, were not affected by weight at scanning implying that no correction would be required for this covariate.

In an analysis where we removed maternal genetic effect from the model used, higher values of 0.24 ± 0.03 and 0.23 ± 0.03 were obtained for direct heritability estimates, respectively from analyses including and excluding scanning weight LWS as a covariate. However, ignoring

maternal genetic effects when they are significant could lead to inaccurate genetic evaluation and consequently prediction of responses to selection programs (Mortimer *et al.*, 2014). Therefore, both direct and maternal components must be considered in order to achieve optimum genetic progress. It is not clear how additive genetic effect of mother can influence skin thickness at a late age. However, it might arise from carry over maternal effects influencing this trait, left from before weaning or even at birth. As suggested by Robison (1981), even if maternal effects tend to decrease with age, some adult traits will nevertheless be having part of this source of variation.

Gregory (1982a) reported estimates of 0.25 and 0.60 for the heritability of skin thickness in South Australian Merinos, using dam-offspring regression and half-sib correlation methods, respectively. Also, using an intra-class correlation method, an estimate of 0.35 was reported for skin thickness measured by skin fold callipers in new-born Merino lambs (Slee *et al.*, 1991). The observed variation in the heritability estimates could result from differences in the models, analysis methods, breeds, environments in which different breeds were kept, population size, and age of measurement in different studies. The moderate heritability and CV, together with a wide range of 1.5 to 5 mm observed for skin thickness in the present study, imply that this trait would show a reasonable response to genetic selection.

Estimates of direct heritability for the other two ultrasound traits (FD and EMD) were higher when adjustment was made for live weight (Table 3.5), which is consistent with other reports (Mortimer *et al.*, 2014, Mortimer *et al.*, 2017, Cánovas *et al.*, 2018). The estimates of direct heritability of FD and EMD in our study, either corrected or uncorrected for LWS, were higher than other reports (Fernandes *et al.*, 2004, Safari *et al.*, 2005, Huisman *et al.*, 2008, Maximini *et al.*, 2012, Mortimer *et al.*, 2014, Mortimer *et al.*, 2017). However, Kvame and Vangen (2007) reported a higher estimate of 0.54 for FD and similar estimate of 0.40 for EMD. The variation observed in the heritability estimates could be attributed to differences in the models,

effects included in the analyses, breeds, population size, and age of measurement in different studies.

The estimates of direct heritability for live weights at different ages generally increased with age as maternal effects became less important, which is consistent with other reports (Bahreini Behzadi *et al.*, 2007, Pickering *et al.*, 2012, Pannu *et al.*, 2016, Mortimer *et al.*, 2017). The estimates of heritability for the live weight traits were consistent with a review by Safari *et al.* (2005) and later reports for Merinos (Safari *et al.*, 2007, Huisman *et al.*, 2008) and meat sheep (Brown *et al.*, 2015).

The estimated direct heritability of FW12 had a high value of 0.48, which was within the range of 0.37 to 0.57 found in the literature (Safari *et al.*, 2005, Swan *et al.*, 2008, Pickering *et al.*, 2012, Mortimer *et al.*, 2017).

3.4.4. Correlation of skin thickness with other traits

The estimates (±SE) of genetic (r_g), environmental (r_e), and phenotypic (r_p) correlations between skin thickness and other traits (excluding SBW) and also the number of records used for each analysis, have been presented in Tables 3.6 and 3.7, respectively, from analyses with the scanning traits (ST, FD, and EMD) adjusted and unadjusted for LWS. The different correlations of ST with SBW have been presented in the next section.

Unfavourable genetic correlations of 0.21 and 0.24, both significant ($P<0.05$), were estimated between ST and FD, respectively from analyses with and without correction for LWS. These unfavourable correlations indicate that the genes that are in favour of increased skin thickness would lead to an increase in fat depth. Also, unfavourable relevant environmental correlations of 0.03 and 0.12 were observed respectively from the analyses with and without LWS as a covariate, with the latter being significant ($P<0.05$), implying that the environmental factors that increase skin thickness would result in an increase in fat depth too. The corresponding

phenotypic correlations were also unfavourable, with values of 0.08 and 0.15, respectively. In agreement with these, Jopson *et al.* (2000) showed that lambs from lines selected for high back fat depth had thicker skin than those selected for low back fat.

Table 3.6. Estimated (±SE) genetic (r_g), environmental (r_e), and phenotypic (r_p) correlations of skin thickness with other traits (excluding SBW) and number of records used for each analysis, when ultrasound traits were adjusted for scanning weight.

Trait	r_g	r_e	r_p	Number of records
FD	0.21 ± 0.07*	0.03 ± 0.02	0.08 ± 0.01	6,950
EMD	-0.13 ± 0.09	0.09 ± 0.03*	0.02 ± 0.02	5,176
WWT	-0.32 ± 0.12*	-0.02 ± 0.02	-0.06 ± 0.01	7,446
LW8	-0.23 ± 0.09*	-0.04 ± 0.02$	-0.08 ± 0.01	7,435
LWS	-0.10 ± 0.10	0.01 ± 0.02	-0.01 ± 0.02	7,486
LW12	0.02 ± 0.11	-0.03 ± 0.03	-0.01 ± 0.02	5,790
FW12	0.20 ± 0.11$	0.10 ± 0.04*	0.13 ± 0.02	3,928

* significant at P<0.05

$ significant at P<0.1

FD: fat depth, EMD: eye-muscle depth, WWT: weaning weight, LW8: 8-month live weight, LWS: live weight at scanning, LW12: 12-month live weight, FW12: 12-month fleece weight.

Table 3.7. Estimated (±SE) genetic (r_g), environmental (r_e), and phenotypic (r_p) correlations of skin thickness with other traits (excluding SBW) when ultrasound traits were not adjusted for scanning weight.

Trait	r_g	r_e	r_p
FD	0.24 ± 0.08*	0.12 ± 0.02*	0.15 ± 0.01
EMD	-0.08 ± 0.10	0.21 ± 0.03*	0.12 ± 0.02
WWT	-0.01 ± 0.12	0.10 ± 0.02*	0.07 ± 0.01
LW8	0.09 ± 0.09	0.10 ± 0.02*	0.09 ± 0.01
LWS	0.19 ± 0.09*	0.22 ± 0.02*	0.20 ± 0.01
LW12	0.30 ± 0.10*	0.15 ± 0.03*	0.17 ± 0.01
FW12	0.20 ± 0.11$	0.17 ± 0.04*	0.17 ± 0.02

* significant at P<0.05
$ significant at P<0.1
FD: fat depth, EMD: eye-muscle depth, WWT: weaning weight, LW8: 8-month live weight, LWS: live weight at scanning, LW12: 12-month live weight, FW12: 12-month fleece weight.

Genetic correlations of -0.13 and -0.08 were estimated between ST and EMD respectively from analyses with or without correction for LWS with both being non-significant (P>0.05). Unlike the unfavourable negative genetic correlations, favourable positive environmental correlations were obtained from analyses with or without adjustment for LWS, both being significant (P<0.05), though the estimate from the latter was much higher (0.09 vs 0.21). Hence, the environmental factors that cause an increase in skin thickness would favourably result in increased in eye muscle depth as well. Consistent with the genetic and environmental correlations, the corresponding phenotypic correlation were 0.02 and 0.12, respectively when LWS was included or excluded.

Unlike FD and EMD whose genetic correlations with ST were not largely affected by inclusion or exclusion of LWS as a covariate, the correlations of ST with live weights at different ages were highly affected by the covariate. The genetic correlation of ST with WWT estimated from the analysis with LWS as a covariate, was unfavorable and significant (P<0.05) with a value of -0.32. However, the relevant estimate when LWS was excluded, was non-significant

(P>0.05) with a value of -0.01. Similarly, a significant (P<0.05) unfavorable genetic correlation of -0.23 was estimated between ST and LW8, from the analysis with LWS as a covariate, while the relevant estimate when LWS was excluded was non-significant (P>0.05) with a value of 0.09. The negative genetic correlation of ST and WWT, when ST was corrected for LWS, could be attributed to some extent to the compensatory growth that lambs of lesser WWT might have experienced from weaning to scanning time. Unfavourable pre-weaning conditions like poor nutrition could result in lambs having less WWT compared to those who have been under better conditions and nutrition. Lambs that were nutritionally or environmentally deprived during the pre-weaning period can often compensate their low WWT during post-weaning period with gains greater than what the lambs with higher WWT might experience. At a constant scanning weight (due to scanning weight being considered as a covariate for ST in the bivariate analyses of ST with live weights at different ages), compared to lambs of greater WWT, the lighter lambs at weaning that were nutritionally deprived during the pre-weaning period, would experience greater weight gain from weaning to scanning when they get access to better conditions during this time. For instance, a lamb with a weaning weight of 20 kg, would gain 40 kg to get a constant scanning weight of 60 kg, while a lamb with a greater weaning weight of 25 kg would only gain 35 kg to reach the constant scanning weight of 60 kg. The higher growth from weaning to scanning due to better conditions might in turn have led to an increase in skin thickness probably as a result of skin thickness-controlling genes being expressed more by better nutritional conditions. Consistent with this postulation, Hutchinson (1957) suggested measurable changes in skin thickness resulting from changes in nutrition. This might also be the reason for the negative genetic correlation found between ST and LW8 when LWS was included as a covariate for ST. Based on the presented assumption, if true, the negative genetic correlations found between ST and WWT and LW8 are unlikely to be due to an antagonism between genes that control skin thickness and those that control WWT and LW8 *per se*. In fact,

it is probably the pattern of growth before WWT and LW8 compared to the pattern from these points to the scanning time that determines the correlations of ST with WWT and LW8.

On the other hand, a non-significant (P>0.05) genetic correlation of -0.09 was estimated between ST and LWS when weight at scanning was included as a covariate for ST, while a significant (P<0.05) favourable genetic correlation of 0.19 was observed when ST was not corrected for LWS. Similarly, a non-significant (P>0.05) genetic correlation of 0.02 was observed between ST and LW12 when ST was corrected for LWS, while the relevant correlation was significant (P<0.05) and favourable with a value of 0.30 when no correction was made for the LWS. The correlations of ST with LWS are expected to be close to the values of the correlations between ST and LW12 because scanning and LW12 measurements were taken at close time points (276 vs 316 days). However, it should be considered that the animals that were used for estimating the correlations of ST with LWS were not all applied for the estimation of the correlations between ST and LW12 since some of them had no record of LW12. This could have caused the difference observed between the correlations of ST and LWS with the values obtained for the correlations between ST and LW12 (Table 3.7).

The environmental correlations between ST and live weights measured at different ages were generally low (-0.04 to 0.03) and non-significant (P>0.05), when ST was corrected for LWS (Table 3.6). However, when LWS was excluded as a covariate, the corresponding correlations were higher (0.10 for WWT and LW8, 0.22 for LWS, and 0.15 for LW12) and significant (p<0.05). The relevant phenotypic correlations were generally low (-0.08 to 0) when ST was corrected for LWS, with higher corresponding values (0.07 to 0.20) when LWS was excluded as a covariate (Tables 3.7 and 3.8). The positive environmental correlations are favourable since those conditions that result in an increase in ST, would also cause increases in live weights at different ages.

A moderate favorable genetic correlation of 0.20, either when ST was corrected or uncorrected for LWS, was estimated between ST and FW12 that tended ($P<0.1$) to be significant. Also, the relevant environmental correlations were favourably positive and significant ($P<0.05$) with values of 0.10 and 0.17, respectively for analyses with or without adjustment for LWS. Consistent with the positive genetic and environmental correlations, favourable positive phenotypic correlations of 0.13 and 0.17 were obtained using analyses that included or excluded LWS as a covariate for ST. The favourable genetic and environmental correlations imply that the genetic and environmental factors that cause an increase in skin thickness would favourably result in an increase in the amount of wool produced at 12 months of age. Thicker skin might be associated with more wool follicles and/or better nutrient supply for the follicles, as it is the skin that nourishes and supports the massive population of fibre-producing follicles (Hynd *et al.*, 1996). Consistent with this, Hutchinson (1957) reported changes in skin thickness that were attributed to differences in nutrition. Consistent with our results, Gregory (1982b) found a significant genetic correlation of 0.39 between skin thickness and clean fleece weight in South Australian Merino sheep. Also, Williams and Thornberry (1992) reported that sheep selected for high fleece weight had thicker skin than those selected for low fleece weight, which is agreement with our results. Contrary to our result, however, Hynd *et al.* (1996) indicated a negative correlation between skin weight (as an indicator of thickness) and clean fleece weight. Interestingly, and consistent to some extent with our results, they found that sheep with heavy skin tended to genetically have higher fibre diameter, and staple length, both of which being determinants of wool weight.

3.4.5. Correlation of skin thickness with lamb survival

In order to have reliable correlation estimates between ST and SBW, using the bivariate sire models, only sires having at least a minimum number of progeny per sire for SBW and other traits were required to be included in the analyses. For this reason and to determine the

minimum number of progeny needed for each sire, a few analyses each with different numbers of progeny per sire for SBW and ST (both with and without ST being corrected for LWS) were performed, while including the same effects for each trait for all analyses, and the estimated correlations compared. The minimum and average numbers of progeny per sire for SBW and ST in different analyses, together with the correlation estimates from each analysis have been presented in Figures 3.6 and 3.7, respectively.

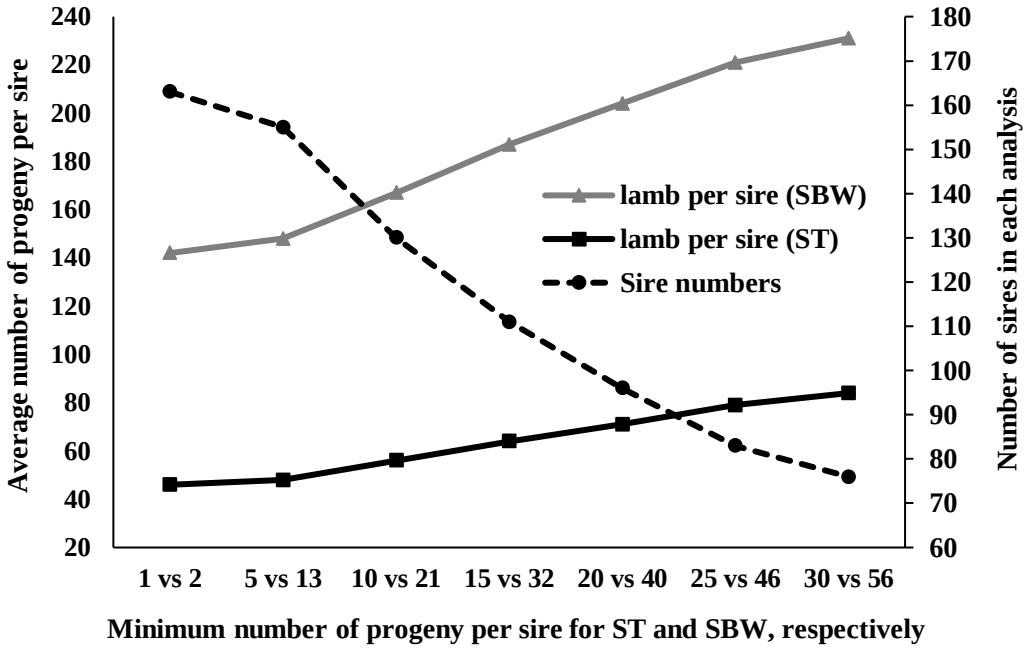


Figure 3.6. Average number of progeny per sire, and number of sires included for estimating genetic correlation of skin thickness and survival at weaning for analyses with different numbers of progeny per sire for each trait. ST: skin thickness, SBW: survival from birth to weaning.

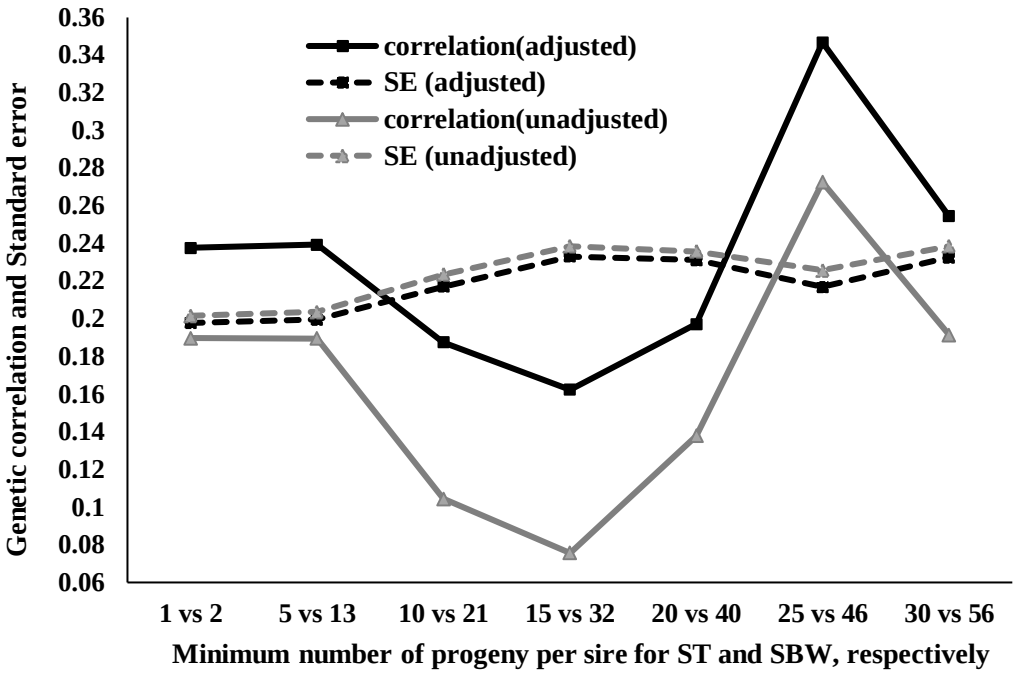


Figure 3.7. Genetic correlations of skin thickness (adjusted and unadjusted for scanning weight) and survival at weaning and their standard errors (SE) estimated from analyses with different numbers of progeny per sire for each trait.

As indicated in Figure 3.6, as the minimum (and consequently the average) number of progeny per sire for each trait increased, more numbers of sires had to be excluded from the analysis. As expected, the reduction in the number of sires generally increased the standard errors of the correlation estimates (Figure 3.7).

Unlike the standard errors that generally increased with decreasing the number of sires included in the analyses, the genetic correlation did not follow a steady trend to reach a stable point (Figure 3.7). A definite minimum number of progeny per sire for each trait to be included in the bivariate sire models could not be determined in this study. However, considering the moderate and low heritability estimates of ST and SBW, respectively, it seems that at least 20 and 40 lambs per sire would be required, respectively, for ST and SBW, in order to have unbiased correlation estimates from the bivariate sire models. Further investigations to

determine the minimum number of progeny per sire with records for any particular trait to get unbiased results using sire model analyses would be of interest.

Estimates of genetic correlation of ST with SBW from the analyses with LWS considered as a covariate for ST ranged from 0.16 to 0.35 depending on the numbers of progeny per sire for each trait, while the corresponding estimates from the analyses with LWS excluded ranged from 0.08 to 0.27 (Figure 3.7). All the genetic correlations of ST with SBW had large standard errors which do not allow a clear interpretation. Our results indicated that including LWS as a covariate for ST in the analyses would result in higher genetic correlation estimates between ST and SBW, which is favourable and could be made use of in selection programs. However, the reason why the inclusion of scanning weight as a covariate for ST increased the genetic correlation of this trait with SBW is not clear. The favourable genetic correlation of ST with SBW, if reliable (due to large SE), might be due to the effect of increased skin thickness on improved thermoregulation at birth as reported by previous studies (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991).

Even if the positive genetic correlation of ST measured at an average age of 279, with SBW is considered as non-significant due to its large standard error, still the possibility of having such a correlation between SBW and ST measured at birth cannot be ruled out. If there is very little additive genetic effect for a trait, which is the case for SBW (indicated by its low heritability), the genetic correlation of that trait with another trait might not be properly estimated (Burfening, 1993). Hence, it is likely that there might be a real genetic correlation between SBW and ST at 279 days of age, while it might be undetectable.

Depending on the minimum numbers of progeny per sire considered for each trait, and also depending on including or excluding LWS as a covariate for skin thickness, the relative efficacy of indirect selection compared to direct selection ranged from 0.22 to 1.02.

Considering the observed range, indirect selection for lamb survival based on selection for skin thickness might not seem promising. However, considering the upper limit of the range of Q (1.02), skin thickness can be considered as an indirect trait or at least as a supplement to direct selection for lamb survival.

It should be noted that the statistical analysis was performed using the skin thickness data only from those animals that were alive until ultrasound scanning, and this might have led to bias in the resulting genetic correlation of lamb survival and skin thickness.

The environmental and phenotypic correlations of ST with SBW from analyses with different numbers of progeny per sire for each trait, were all close to zero (-0.01 to 0) with standard errors of 0.02 to 0.03, either with ST corrected or uncorrected for LWS.

3.5. Conclusions

The following conclusions could be drawn from the results presented in this study:

1) Lamb survival from birth to weaning was lowly heritable, implying that the trait would not easily respond to direct genetic selection.
2) Skin thickness measured by ultrasound at an average age of 9 months was moderately heritable, either with or without correction for weight at scanning, implying that the trait would respond to genetic selection.
3) Skin thickness showed a moderate favourable genetic correlation with lamb survival although with high standard error.
4) Skin thickness had a moderate unfavourable genetic correlation with fat depth, a moderate favourable correlation with fleece weight at 12 months of age, and no significant genetic correlation with muscle depth.

5) Skin thickness had moderate unfavourable genetic correlations with weights at weaning and 8 months of age when skin thickness was corrected for weight at scanning, while the correlations were not evident when scanning weight was not included.
6) Skin thickness showed moderate favourable genetic correlations with weights at scanning and 12 months of age when skin thickness was not corrected for weight at scanning, while the correlations were not evident when scanning weight was included as a covariate for skin thickness.

Taking these findings together, skin thickness could be suggested as a likely trait in indirect selection for lamb survival in selection programs. However, its inclusion should be with caution due to its possible unfavourable genetic correlation with fat depth, and weight at weaning and 8 months of age. Otherwise, although selection of animals with thicker skin might result in lambs with improved survival as well as increased 12-month fleece weight and weights at scanning and 12 months of age, it could also lead to animals with more fat depth, and less weights at weaning and 8 months of age. Also, further experiments to test if increased skin thickness in mature animals affects their performance in warm season will be of interest due to a possible unfavourable effect of thicker skin on heat tolerance.

Chapter 4

The effect of skin thickness on cold resistance in new-born lambs

4.1. Abstract

This study aimed to explore the possible role of skin thickness in thermoregulation through its effect on surface heat loss and a few other indices of cold resistance in new-born lambs exposed to cold-stress. Four hundred and thirty Romney-type ewes (3-4-year-old) were randomly divided into two batches of 215 and estrus synchronized one week apart using Controlled Intravaginal Drug Release (CIDR). Single- and twin-born lambs were scanned for skin thickness measurement within 24 hours of age using ultrasonography on the left dorsal loin region around the 12th rib. Out of 324 lambs, 32 lambs with the thickest skin (thick-skinned category) and 32 lambs with the thinnest skin (thin-skinned category) were selected. These lambs were exposed to a period of cold stress and their heat production was calculated using oxygen consumption measured by indirect open-circuit calorimetry. Skin surface temperature (as an indicator of heat loss) on left dorsal loin region was obtained during the experiment using an infrared thermography (IRT) camera. According to the results of this study, lambs of thick-skinned category showed lower ($P<0.001$) overall average skin surface temperature compared to thin-skinned lambs. Also, single-born lambs had lower ($P<0.05$) skin surface temperature than twins. Ram lambs of thick-skinned group showed lower skin temperatures than ewe lambs of the same skin category ($P<0.05$), whereas no significant difference was observed between ewe and ram lambs categorized as thin-skinned group ($P>0.05$). However, when the overall skin surface temperature was considered, males and females showed the same ($P>0.05$) skin temperatures. Thin-skinned lambs had greater ($P<0.05$) overall average heat production (W Kg^{-1}) compared to thick-skinned lambs as a response to higher heat loss through skin in the first group. However, lambs of different skin categories were the same in terms of maximum heat production (MHP). Furthermore, twins produced more ($P<0.05$) average heat than singles, whereas no significant difference ($P>0.05$) was evident between ewe and ram lambs. Skin category had an effect ($P<0.05$) on time to reach MHP, with thick-skinned lambs taking longer

time to reach MHP compared with thin-skinned lambs, only before making adjustment for birth weight, while sex and birth rank did not show any effect ($P>0.05$) neither before nor after adjustment for birth weight. According to our results, the higher average heat produced by thin-skinned lambs was not able to compensate the higher heat they lost through skin, and consequently resulted in lower ($P<0.05$) overall rectal temperature in these lambs compared to thick-skinned lambs. Consistent with this, thick-skinned lambs were more likely to maintain body temperature than thin-skinned lambs during cold stress period. Sex and birth rank had no effect on rectal temperature and likelihood of maintaining body temperature, while for every one kg increase in body weight, lambs were 6.9 times more likely ($P<0.01$) to maintain their core body temperature during the cold stress. In conclusion, increased skin thickness at birth can positively affect thermoregulation in new-born lambs through decreased heat loss from skin surface, thereby minimizing the heat production required to maintain core body temperature.

4.2. Introduction

Lamb mortality is a major issue to sheep producers both in New Zealand and worldwide, not only due to economic losses but also as an animal welfare and management problem. Lamb mortality rates of 3 to 25% has been reported in New Zealand (Hight and Jury, 1970, Dalton *et al.*, 1980, Gumbrell and Saville, 1986), though mortality rates of up to 40% have been found on some farms (Fisher 2004). Lamb survival has been reported to have one of the main influences on overall productivity of ewes (Amer *et al.*, 1999, Conington *et al.*, 2004). Reducing lamb deaths would also improve animal welfare perceptions and diminish the risk of non-tariff barriers imposed by international markets (Everett-Hincks *et al.*, 2007). Furthermore, the reduction in selection potential incurred by having fewer lambs surviving until selection imposes an additional cost (Forrest *et al.*, 2006). Hence, even a small improvement in lamb survival rates would increase annual profit remarkably (Everett-Hincks *et al.*, 2007).

On the other hand, lamb survival has been reported to have a low direct and maternal heritability (Lopez-Villalobos and Garrick, 1999, Morris *et al.*, 2000, Riggio *et al.*, 2008, Brien *et al.*, 2010) and is a trait of importance that is impossible to measure for all animals. Therefore, indirect selection, based on selection for other easy-to-measure traits of higher heritability which are genetically correlated with survival is an alternative to direct selection for this trait itself.

Starvation/exposure as the second most common cause of lamb deaths in the neonatal period after dystocia (Kerslake *et al.*, 2005, Everett-Hincks *et al.*, 2007), can account for approximately 30% of lamb mortalities (Hartley and Boyes, 1964, McCutcheon, 1981). Starvation/exposure is especially important in multiple-born lambs firstly, due to having less body reserves as a source of heat production (Alexander, 1962b) and secondly, due to a greater surface area to body weight ratio (McCutcheon *et al.*, 1983b), which imposes much more heat loss on them compared with single-born ones (Samson and Slee, 1981). On the other hand, the current focus on increasing lambing percentage (Bray, 2005, Kenyon *et al.*, 2005) and consequently an associated decline in single-born lambs and an increase in multiple-born ones, could worsen the situation.

In countries like New Zealand, the UK and Australia, where lambing mostly takes place outdoor, thermoregulatory capacity of new-born lambs plays a major role in lamb survival due to its effect on reducing sensitivity to cold exposure, which is a main contributor to starvation-exposure mortalities (Mellor and Stafford, 2004). Apart from heat production through both shivering and non-shivering thermogenesis (Alexander and Williams, 1968, Symonds and Lomax, 1992), reducing radiated heat loss from the skin surface is vital for maintaining core body temperature and increased cold tolerance (Alexander, 1978).

Skin as an organ covering the body can have a potential effect on thermoregulation through its role in increasing body insulation and reducing heat loss from the body surface. A limited number of studies have revealed an association of skin thickness with increased cold resistance in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991). Unlike cold resistance, whose assessment requires laboratory-based techniques that are not feasible for breeders at farm level, skin thickness can be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000). On the other hand, skin thickness is a trait of moderate to high heritability (Gregory, 1982a, Slee *et al.*, 1991) and is anticipated to respond to selection. Hence, selection for skin thickness might be a potential alternative to selection for cold resistance and consequently lamb survival.

In a study in New Zealand Romney sheep we found skin thickness measured ultrasonically at approximately eight months of age to be genetically, positively correlated with lamb survival from birth to weaning (chapter three). The correlation was postulated to be due to the effect of skin thickness on improved thermoregulation through its effect on heat loss at birth. However, the mechanism by which the skin might influence thermoregulation is not clear. Also, in the studies reporting an association of skin thickness with increased cold resistance (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991), the significance of each effect was impossible to evaluate independently due to strong inter-correlations between skin thickness and other traits affecting cold resistance like birth weight, coat depth and coat grade (Slee *et al.*, 1991).

Therefore, the objective of this study was to explore the possible role of skin thickness in thermoregulation through its effect on surface heat loss and a few other indices of cold resistance in new-born lambs exposed to cold-stress.

4.3. Materials and methods

4.3.1. Ethics statement

The study protocol was approved by the Massey University Animal Ethics Committee (Protocol#16-21). Animals were closely monitored throughout the experiment and no animal health or welfare issues were observed during or after the experiment.

4.3.2. Estrous synchronization

Four hundred and thirty Romney-type ewes (3-4-year-old) from Massey University Keeble farm managed under commercial conditions were randomly selected for this study. To better manage the experiment in respect to skin thickness measurement and calorimetry of new-born lambs, all the ewes were estrous synchronized to have their lambs born within a short period of time. The ewes were randomly divided into two batches of 215 and synchronized one week apart using Eazi-Breed^{TM} Controlled Intravaginal Drug Release (CIDR), containing 0.3 g progesterone (Zoetis, Auckland, New Zealand). CIDRs were inserted into vagina 12 days prior to mating. On the same day when CIDRs were removed (17 and 24 April 2016 for the first and second batches, respectively), eight rams equipped with harnesses and crayons were introduced and allowed to mate for seven consecutive days. All ewes marked with crayons were kept and the remaining ewes were removed from the study. Pregnancy scanning for all ewes was carried out 50 days after rams were introduced to the first batch and non-pregnant ewes were removed. Triplet-bearing ewes were excluded. Remaining ewes were managed under identical conditions throughout pregnancy.

4.3.3. Categorization of lambs based on skin thickness

During lambing (11-26 September 2016), all lambs were weighed and tagged within 12 hours from birth and their sex, birth rank, and date of birth were recorded. To have two groups of lambs (32 in each group), thin- and thick-skinned categories, ultrasound scanning was

performed within 24 hours of birth. Scanning was conducted every morning for lambs born from the previous 24 hours. A commercial operator did the scanning using a Sonosite M Turbo (FUJIFILM Sonosite, Inc., Bothell, Washington, United States) ultrasound scanning machine with a 38 mm probe at 7.5 MHz set at a depth of 40 mm on the left dorsal loin region of the lambs around the 12th rib. Prior to the commencement of this study, there was no published report on the range of skin thickness variation in new-born Romney lambs. Also, it was not feasible to select the 64 lambs after all lambs had been born and scanned since lamb thermoregulation was to be tested within 24 to 48 hours of age. Although synchronization did narrow down the lambing window, lambs were not all born within two days. Hence, determining the threshold points for the two skin thickness categories was crucial. For this purpose, a skin thickness range was obtained from a previous study (chapter three) on 8-month-old Romney lambs to use as an initial guide for the thresholds, to start with on the first day of scanning. These thresholds were subsequently revised at the beginning of each day, based on the thickness readings observed until the previous day to ensure, as far as possible, two divergent groups of lambs were picked for the study. Based on the determined thresholds, the 64 lambs were selected over 11 days (2 to 8 lambs each day), regardless of their birth rank, sex, and birth weight. The range, mean, and median of skin thickness of thin-skinned vs thick-skinned groups in mm were respectively 1.66-2.62 vs 2.78-4.31, 2.10 vs 3.19 and 2.07 vs 3.08. Thin-skinned group consisted of 16 male and 16 female lambs, while there were 19 male and 13 female lambs in the thick-skinned category.

4.3.4. Cold stress index

In order to make an adjustment for different weather conditions each lamb experienced on the day of birth, an average cold stress index (CSI) was calculated and included in the models used for the statistical analysis.

The CSI on the day of birth was calculated for each lamb using the following equation (Donnelly, 1984):

$$CSI\ (kJ/m^2/h) = (11.7 + 3.1\ W^{0.5}) \times (40 - T) + 481 + 418(1 - e^{-0.04R})$$

where W is the mean wind speed (m/s), T is the mean temperature (°C), and R is the mean rainfall (mm).

The weather data used in the equation were obtained from a weather station at the AgResearch Limited, Grasslands Research Centre, located near the Massey University Keeble farm in Palmerston North, where the lambs were born.

4.3.5. Calorimetry and infrared thermography (IRT)

The lambs selected under the thin- and thick-skinned categories were taken to the Massey University Animal Physiology Unit and kept in pens along with their dams and sibling (if any) for calorimetry and skin temperature measurement. Before calorimetry, lambs were weighed and wool was removed from the back and sides of all lambs using an electric clipper and only a small amount of wool remained on the ventral surface. This removed any possible bias in skin temperature readings (as an indicator of heat loss) obtained by infrared thermography (IRT), resulting from variation in coat properties (depth, type, weight). It would also facilitate heat loss and consequently increase the chance of reaching the summit metabolic rate in lambs. Lambs were then placed in pairs over galvanized mesh in a crate with their legs hanging down and body restrained by rubber strings on their shoulder and hips. This restraint prevented the movement of lambs that could affect the quality of images taken during IRT. Surface temperature was recorded using an IRT camera (Fluke TiX500, Fluke Corporation, Everett, WA, USA) mounted on a tripod at a fixed angle approximately 1 m over the lambs. Continuous thermal images were captured every one minute for a total of 10 minutes, referred to as baseline time (Figure 4.1). During this time the room temperature was within the range of 11 to 16°C.

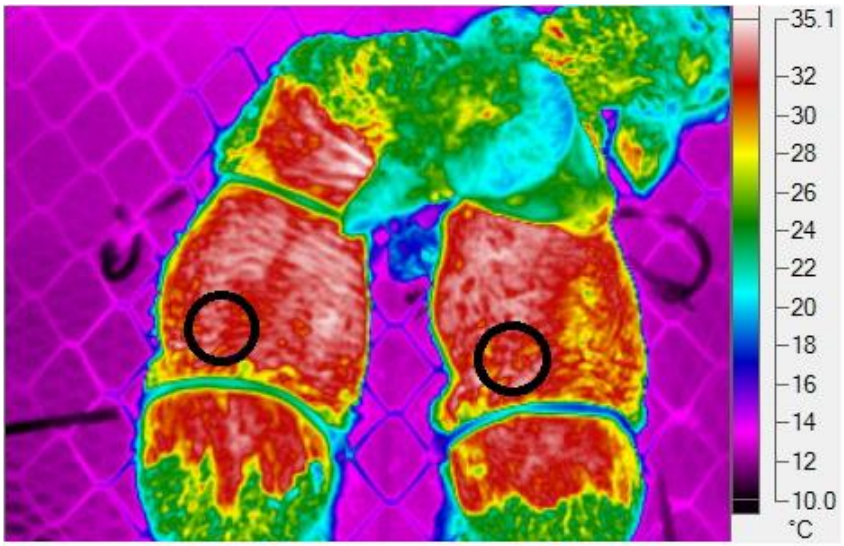


Figure 4.1. Thermal image of a pair of lambs taken during the baseline period (11-16°C). Black circles show the areas used for the calculation of skin temperature on left dorsal loin region.

Immediately after this period, lambs entered the calorimetry chamber to measure metabolic rate using indirect open-circuit calorimetry (McCutcheon *et al.*, 1983a). The lambs were placed in pairs in a crate inside the chamber while being restrained in a fashion similar to the previous step and their head was placed in a plastic hood which was sealed around the neck using a plastic collar (Figure 4.2).

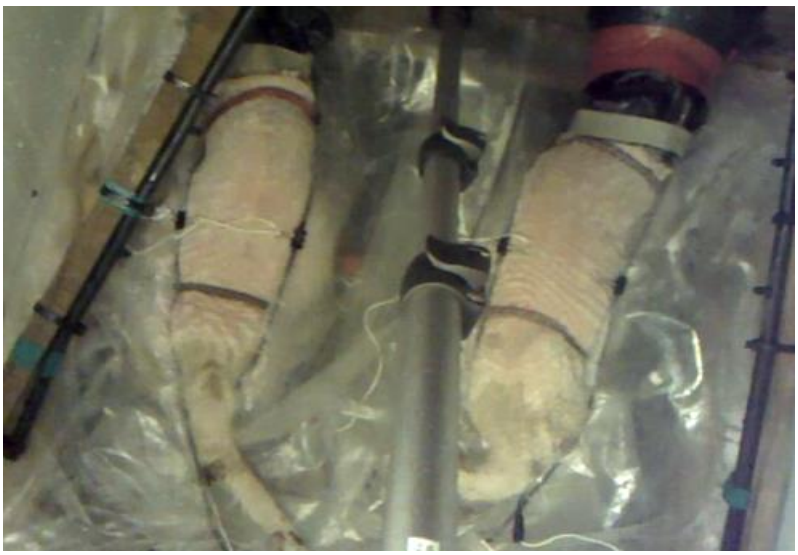


Figure 4.2. A pair of lambs restrained in the cold chamber during the calorimetry and infrared thermography.

A digital temperature probe was inserted into the rectum of the lamb to record body temperature during calorimetry. The lambs were exposed to an initial ambient temperature of 4°C and were allowed to acclimatize to these conditions for 12 minutes. This period of time was referred to as the adjustment period (min -12 to 0). At the end of the adjustment period, artificial chilled rain (on the ventral surface) and moving air (from behind) were applied to induce summit metabolism (maximal heat production), and was referred to as the onset of severe cold stress. The severe cold period was itself divided into four consecutive stages indicated as A-B (min 0 to 18), B-C (min 18 to 36), C-D (min 36 to 54), and D-E (min 54 to 90). At the start of severe cold stress (first stage, min 0), artificial chilled rain (1°C) was applied on the ventral surface through sprinklers at a rate of 0.36 l/min and cold air was blown over the lamb by a fan positioned behind the animal at the rate of 1.0 m/s. Since water drops can interfere with infrared thermography, a plastic cover surrounding the sides of the lamb was used to stop water drops falling on to the dorsal surface of lamb (Figure 4.2). After 18 min (second stage), the water flow and air speed were increased to 0.72 l/min and 1.5 m/s respectively. After another 18 min (third stage, min 36), water flow and air speed were further increased to reach 1.08 l/min and 2.0 m/s respectively. Finally, after another 18 min (fourth stage, min 54), the plastic cover was removed and the cold water was allowed to fall on top of lambs as well in order to stimulate summit metabolism in those lambs that had not yet reached the maximum metabolic rate. These conditions were allowed to continue until the end of calorimetry (min 90) or until the lambs reached maximal heat production, whichever occurred first. Summit metabolism (maximum heat production) was assumed to have been reached when the rectal temperature of the lamb declined at the rate of 1°C/20 min and there was no further increase in the rate of oxygen consumption (Alexander, 1962b). Ambient temperatures were kept at 4, 4, 3, 3, and 2°C during the five stages of calorimetry (adjustment, A-B, B-C, C-D, and D-E, respectively). During calorimetry, rectal temperature and oxygen consumption were recorded at 3 min intervals.

Dorsal body surface temperature (as an indicator of heat loss) was recorded throughout the calorimetry using the IRT camera mounted at a fixed distance of 1 m above the lambs. Thermal images were captured at 1 min intervals during this time.

At the end of the calorimetry, the lambs were thoroughly dried and placed in a heated room (25°C) for one hour before being returned to their dams. Wool covers were placed on lambs after one hour of interaction with mother, and were removed after 5-7 days. The dams and their lambs remained in the pens in the unit for up to 24 hours depending on weather conditions and commercial sheep nuts, chaff and water were available during this period. The ewes and lambs were observed during this period to ensure successful ewe/lamb bonding and that the lambs were suckling.

4.3.6. Heat production calculation

Heat production, as an indicator of metabolic rate, was calculated from oxygen consumption using the following formula (Revell *et al.*, 2002):

$$\text{Heat production (W Kg}^{-1}\text{)} = [(\text{oxygen consumption (l/h)} \times 20.46) / 3.6] / \text{body weight (kg)}$$

Based on the five stages into which the calorimetry period was divided, and parallel to the time-points at which thermal images were analyzed, five time-intervals were defined for heat production calculation as follows: adjustment (min -12 to 0), A-B (min 0 to 18), B-C (min 18 to 36), C-D (min 36-54), and D-E (min 54 to 66). For each time-interval, an average heat production was calculated for each lamb. Since calorimetry was not performed during the baseline period, no heat production was calculated for this time-period. Also, since most of the lambs reached summit metabolism and were removed from calorimeter by min 66 during the period D-E, no heat production was calculated after this time. Maximum heat production was calculated using the same formula when summit metabolism was attained for each lamb.

4.3.7. Thermal image analysis

The images taken by the IRT camera were analyzed using the Fluke SmartView 4.3 software considering an emissivity of 0.95. For each lamb, images captured at the end of the baseline and adjustment periods, and those taken at the start of the four stages of severe cold stress (A (min 0), B (min 18), C (36), and D (min 54)) were used and were considered as repeated measures for the analysis of skin temperature. Because from stage D (min 54) onwards the artificial rain was applied on the top of animals, images taken after this time were not included in the analysis. For each image, the average temperature of a circle (of the same size for all images) on left dorsal loin region surrounding the point at which skin thickness was measured (Figure 4.1), was calculated using the software. Images with low focus due to lamb movement or due to water drops falling on to the area of interest were replaced by the corresponding images taken either before or after the actual time whichever had the required focus. If the selected area had a low focus for any reason (water drops or the area being at an angle greater than 45 degrees to the camera when capture), the same area on the right side of lamb was used.

4.3.8. Power analysis

Assuming an expected difference of 2°C (23°C vs 25°C) in skin temperature between the thick-skinned and thin-skinned lambs, a standard deviation of 2.4 for skin temperature measured during cold stress (McCoard et al., 2014) and a power value of 0.90, power analysis using PROC POWER in SAS software (SAS, 2011) indicated that 64 lambs (32 in each group) were required for detecting the expected difference.

4.3.9. Statistical analysis

Skin surface temperatures on left dorsal loin region taken at the five selected time-points were analyzed by repeated measures ANOVA using the PROC MIXED of the SAS software (SAS, 2011). The time-points included baseline (last min of the 10-min period prior to the calorimetry

at a room temperature of 11-16°C), point A (min 0, the end of cold adjustment period), point B (min 18), point C (min 36), and point D (min 54). The model contained the effects of sex (male or female), birth rank (single or twin), synchronization batch (first or second), skin thickness category (thick or thin), time point (baseline, A, B, C, or D), and their two-way interactions as fixed effects and body weight and average CSI on the day of birth as covariates. All non-significant fixed effects (P>0.05), interactions (P>0.05) and covariates (P>0.05) were excluded from the final model. The final model included the effects of time-point, birth rank, category, sex*category, and time-point*category.

Similarly, for rectal temperature, measurements recorded at the five time-points (baseline, A, B, C, and D) were considered as repeated measures and therefore analysed by repeated measures ANOVA using the PROC MIXED of the SAS software (SAS, 2011). The fixed effects and covariates used in the initial model were the same as those used in the analysis of skin temperature. However, after excluding the non-significant fixed effects (P>0.05), interactions (P>0.05) and covariates (P>0.05), the final model contained the effects of time-point, category, time-point*category, and average CSI.

A repeated measures ANOVA using the PROC MIXED of the SAS software (SAS, 2011) was carried out also for the analysis of heat production with five averages of heat production calculated for each of the five time-intervals (adjustment (min -12 to 0), A-B (min 0 to 18), B-C (min 18 to 36), C-D (min 36-54), and D-E (min 54 to 66)) as the repeated measures of heat production. In addition to the fixed effects and covariates used in the analysis of skin temperature, rectal temperature at the onset of adjustment period was included as a covariate in the initial model. After removing the non-significant fixed effects (P>0.05), interactions (P>0.05) and covariates (P>0.05), the effects of time-point, birth rank, category, time-point*category and rectal temperature at the start of adjustment period remained in the final model. Due to a temporary fault in the calorimeter, oxygen consumption measured in the first

and second readings were not correct for six lambs. Therefore, for those six lambs the averages of heat production calculated for the adjustment period (min -12 to 0) were excluded from the analysis.

For all the three above-mentioned analyses using repeated measures ANOVA, three commonly used covariance structures including unstructured (UN), autoregressive (AR(1)), and compound symmetric (CS), were applied. The type of covariance structure used in the final models was based on model fit that was determined by the Akaike information criterion (AIC), where smaller values indicate better fit. The unstructured (UN) type of covariance structure was used in the final models for the analysis of skin temperature and rectal temperature. However, for the analysis of heat production, autoregressive (AR(1)) type of covariance structure was used in the final model due to an error (convergence criteria met but final hessian is not positive definite) after running the model with the unstructured (UN) type. Also, for the data analyzed by repeated measures ANOVA, the random effect of animal was included in the initial models. But the effect was excluded from the final models since either it did not improved model fitness or the model with the random effect of animal resulted in an error after running the model (convergence criteria met but final hessian is not positive definite or stopped because of infinite likelihood).

The maximum heat production and time to reach maximum heat production were analyzed by the PROC MIXED of the SAS software (SAS, 2011) with sex, birth rank, synchronization batch, skin thickness category, and their two-way interactions as fixed effects and body weight, rectal temperature at the start of adjustment period, and average cold stress index (CSI) on the day of birth as covariates included in the model. After excluding the non-significant fixed effects ($P>0.05$), interactions ($P>0.05$) and covariates ($P>0.05$), the effects of category and rectal temperature at the start of adjustment period remained in the final model for maximum

heat production, and category, body weight and rectal temperature at the start of adjustment period in the final model for time to reach maximum heat production.

A lamb was considered as not being able to maintain its body temperature during the cold stress period if the lamb experienced four consecutive falls of at least 0.2°C in rectal temperature per each 3-minute period. Otherwise, it was considered as being able to maintain its body temperature during the cold stress period. A logistic regression model using PROC LOGISTIC of the SAS software (SAS, 2011) was used to identify factors affecting the likelihood of a lamb maintaining its body temperature during the cold stress period. The fixed effects and covariates used in the initial model were the same as those used in the analysis of maximum heat production. However, after excluding non-significant fixed effects ($P>0.05$), interactions ($P>0.05$) and covariates ($P>0.05$), only skin thickness category and birth weight were retained in the final model.

4.4. Results and Discussion

4.4.1. Skin surface temperature

Skin temperature in the loin region of lambs were recorded while the lambs were at room temperature (11 to 16°C) as well as in the cold chamber (2 to 4°C), during calorimetry. Figure 4.3 shows the average skin surface temperature as an indicator of surface heat loss measured at different time points. The skin temperature measured at the baseline point showed a mean of 33.1°C with a considerably significant decrease ($P<0.0001$) of 4.9°C at the next time point (A). As displayed in Figure 4.3, the decrease in surface temperature continued to the last time point (D), though with decreasing rates for subsequent time points to reach a mean of 24.2°C at the end.

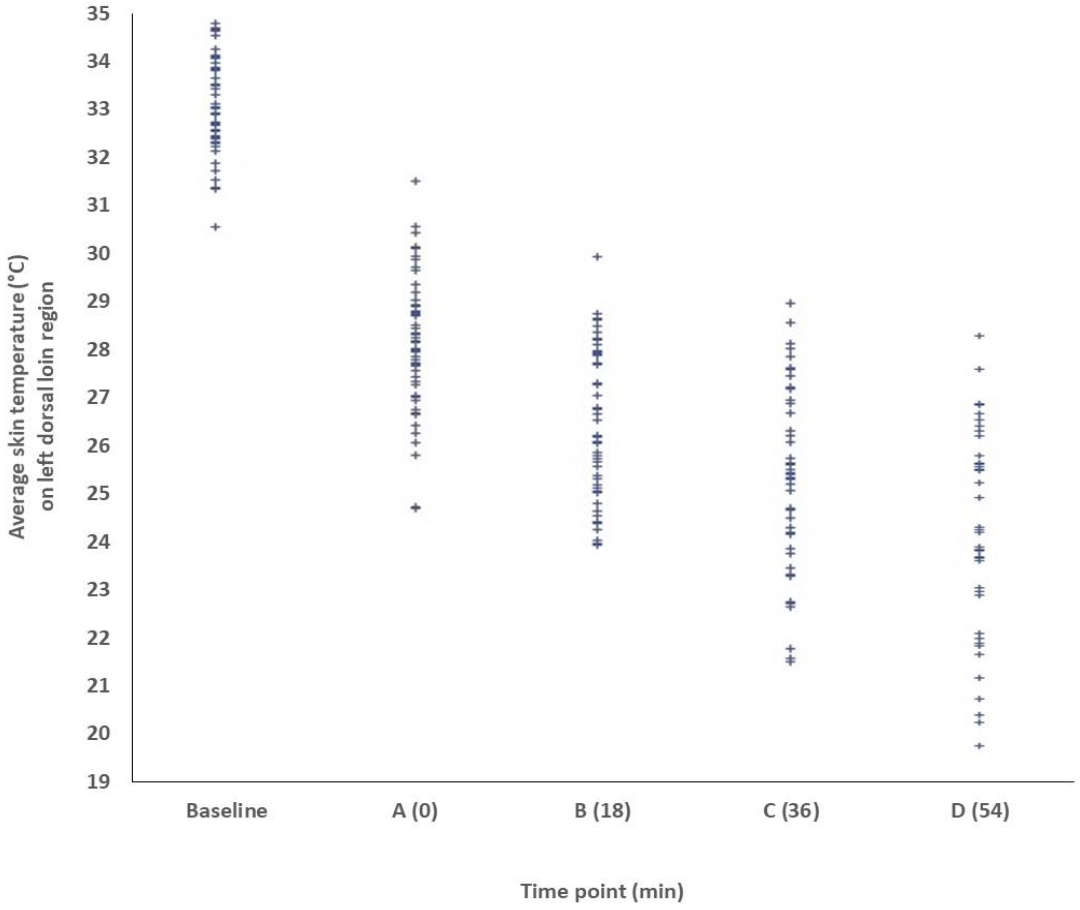


Figure 4.3. Average skin surface temperature measured by infrared thermography (IRT) in the left dorsal loin region of the individual lambs at different time-points. Conditions applied were as follows. Baseline: 11-16°C, with no wind or rain; A-B: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s).

A mean of 29°C was reported for hind-leg skin temperature measured by copper-constant thermocouple in Romney–type new-born lambs exposed to an ambient temperature of 15°C (McCutcheon *et al.*, 1983a). The same lambs showed an average temperature as low as 11°C when exposed to an environmental temperature of 5°C combined with wetness and moving air.

A study using infrared thermal imaging on new-born lambs exposed to an ambient temperature of 11-18 °C, (McCoard *et al.*, 2014), reported an average surface skin temperature of 35°C

which is comparable to the results of the present study. Similarly, they observed a rapid decrease of 5°C in heat loss within 5 min of cold exposure, followed by a further decrease of 10°C by the end of a 30-minute cold-exposure period (0°C). The differences in rates of heat loss reported in these studies (McCutcheon *et al.*, 1983a, McCoard *et al.*, 2014) compared to the present study could be attributed to differences in conditions to which lambs were exposed in each experiment, as well as individual or/and breed variations in peripheral vasoconstriction and skin thickness. The difference in the methods used for skin temperature measurement could also be a possible reason for the observed discrepancies.

As presented in Figure 4.4, the results of this study showed a significant effect ($P<0.001$) of skin thickness category on overall skin surface temperature considering all the time-points, so that lambs of thick-skinned group had lower overall average skin surface temperature compared to thin-skinned lambs. Also, single-born lambs lost less heat from their skin surface than twins ($P<0.05$, Figure 4.4), though no difference was observed between twins and singles in terms of skin thickness. Contrary to our results, in a previous study (Labeur *et al.*, 2017), birth rank had no significant effect on skin temperature measured by infrared thermal imaging in new-born lambs exposed to cold. One plausible reason for the difference observed between singles and twins for skin temperature might be that single-born lambs constricted peripheral blood vessels to a greater extent than the twins. On the other hand, another possible reason for the effect of birth rank on heat loss in our experiment might be the bias resulting from the lower number of singletons compared to twins (12 vs 52).

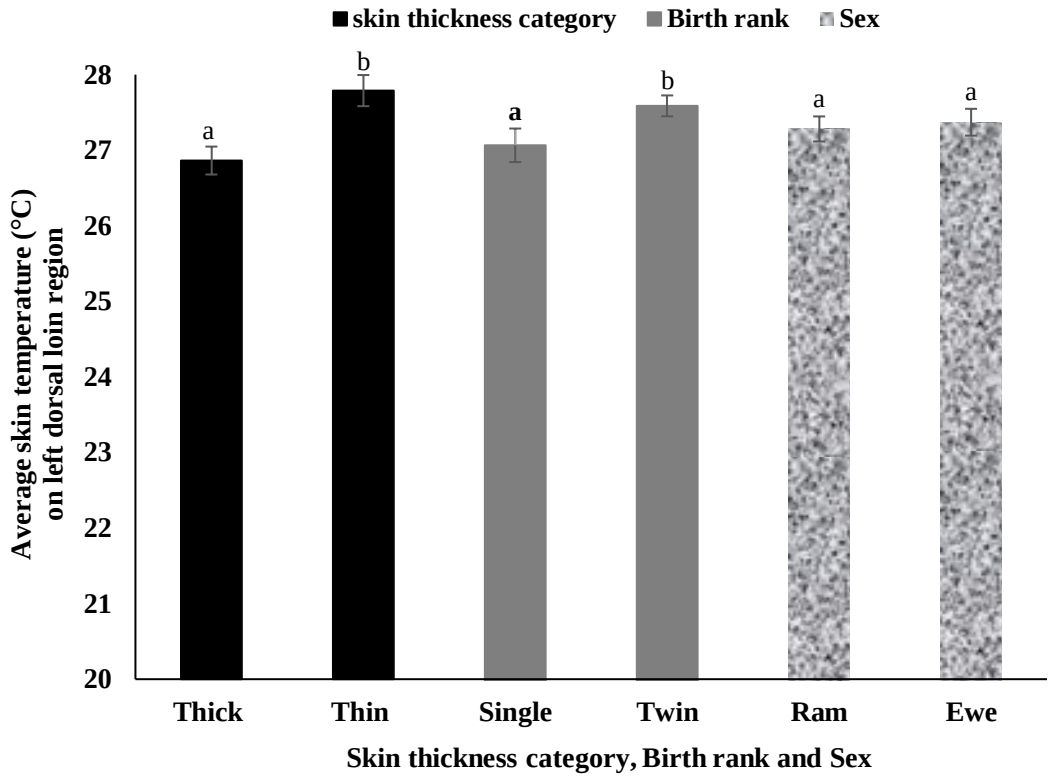


Figure 4.4. The effects of skin thickness category, birth rank and sex on overall skin surface temperature considering all the time-points (baseline, A, B, C, and D). All values are presented as Least Squares Means ± standard errors. Values with different letters, within each category, are significantly different (P<0.001 for skin thickness category and P<0.05 for birth rank). The mean skin thickness (mm) for lambs contributing to these values were respectively 3.20 and 2.13 for thick- and thin-skinned lambs, 2.64 and 2.74 for singles and twins, 2.66 and 2.76 for ewe and ram lambs.

As shown in Figure 4.4, the effect of sex on overall skin surface temperature considering all the time-points during the calorimetry was not significant (P>0.05). But, the results indicated a significant interaction (P<0.05) between skin thickness category and sex of lamb for skin temperature as an indicator of heat loss. As shown in Figure 4.5, within ram lambs, those with thick skins lost less heat from their skin surface compared to lambs with thinner skins (P<0.001). However, there was no significant difference between ewe lambs of different skin thickness categories (P>0.05). Furthermore, ram lambs of thick-skinned group showed lower skin temperatures than ewe lambs of the same skin category (P<0.05), whereas no significant difference was observed between ewe and ram lambs categorized as thin-skinned group (P>0.05). A previous study (McCutcheon, 1981) found female lambs to have greater skin

temperatures than males when they were exposed to ambient temperatures of 30, 10 and 5°C. At the two low ambient temperatures they observed the effect of sex only when lambs were dry, whereas this effect was evident for both wet and dry lambs in lambs exposed to 30°C.

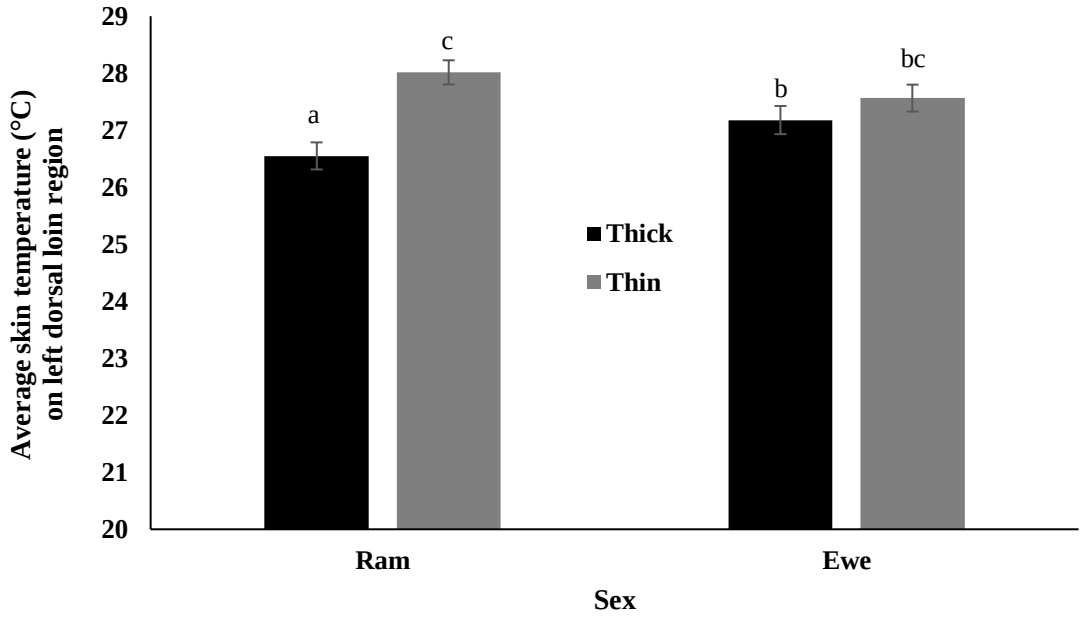


Figure 4.5. The effect of skin thickness category, within different sexes, on overall surface temperature considering all the time-points (baseline, A, B, C, and D). All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different ($P<0.05$). The mean skin thickness (mm) for lambs contributing to these values were respectively 3.20 and 2.16 for thick- and thin-skinned ram lambs, and 3.19 and 2.10 for thick- and thin-skinned ewe lambs.

Although the overall effect of skin thickness category on skin surface temperature was significant, this effect was not evident at all the time points. As shown in Figure 4.6, there were no significant differences between lambs of different skin categories at the first two time-points ($P>0.05$). However, at the three next time points, thick-skinned lambs showed significantly ($P<0.01$, $P<0.05$ and $P<0.05$, respectively) lower heat loss from their skin surface, compared to that in thin-skinned lambs.

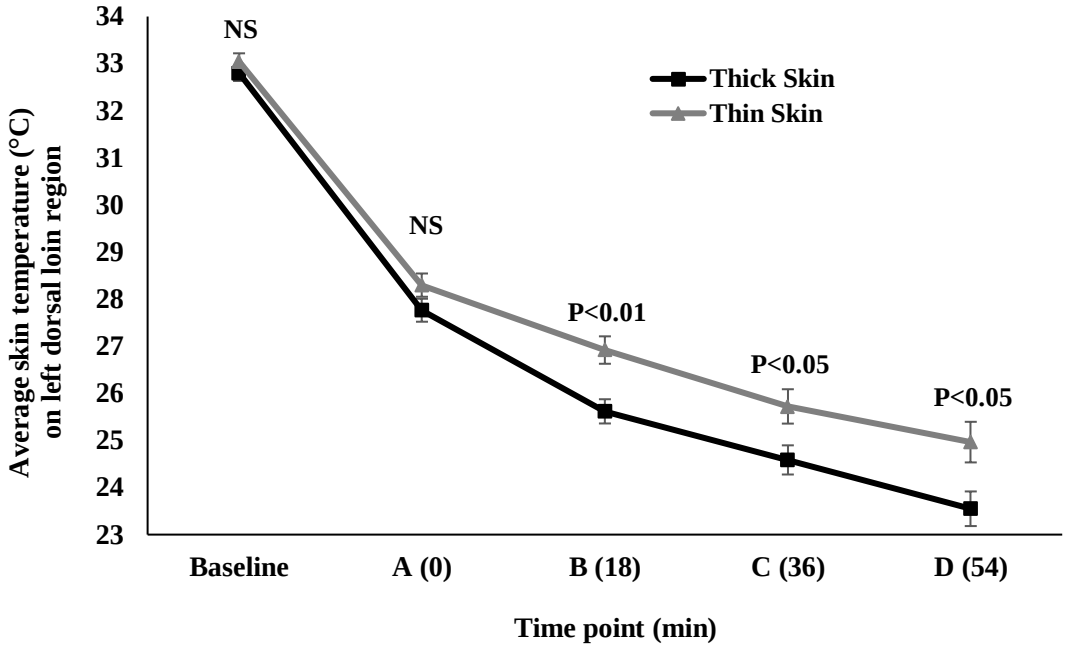


Figure 4.6. The effect of skin thickness category on skin surface temperature at different time points. Conditions applied were as follows. Baseline: 11-16°C, with no wind or rain; A-B: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s). All values are presented as Least Squares Means ± standard errors. The mean skin thickness (mm) of thick- and thin-skinned lambs contributing to these values were respectively 3.19 and 2.10 for baseline, 3.19 and 2.10 for point A, 3.20 and 2.16 for point B, 3.20 and 2.18 for point C, and 3.19 and 2.16 for point D.

The lack of an association between heat loss and skin thickness evident at the first two time points is unclear; however, one plausible reason could be variation in peripheral vasoconstriction each lamb employs as the first mechanism to maintain core body temperature in response to cold exposure. Although peripheral vasoconstriction has been widely considered as an all-or-none phenomenon in environments cooler than the lower critical temperature (Alexander, 1961, Alexander, 1962a), there are other reports suggesting that peripheral vasoconstriction might be controlled in a continuous manner (Gonzalez-Jimenez and Blaxter, 1962, Webster and Johnson, 1968). Some lambs might employ their abilities to a greater extent in restricting blood flow to peripheral blood vessels when they are exposed to severe cold conditions. Hence, there might be more homogeneity in peripheral vasoconstriction in more

severe cold conditions and consequently less variation in skin temperature arising from differences in the extent to which vasoconstriction has been employed. Consistent with this postulation, the significant effect of skin thickness on heat loss appeared after the lambs were exposed to cold, wet and windy conditions (time-points B, C and D), rather than when subjected to cold alone (Baseline and A). Therefore, it could be suggested that after peripheral vasoconstriction occurs to the maximum possible extent, skin thickness starts to play a significant role as an insulation against heat loss through skin surface. On the other hand, no subcutaneous fat was detected in the images taken by ultrasonography of the lambs, which is consistent with other studies (Alexander and Bell, 1975b, Alexander, 1978), reporting no subcutaneous fat in new-born lambs. This fact supports the hypothesis that skin thickness is most likely the main factor causing the difference observed in the skin temperatures in the lambs of different skin thickness categories in the present study.

Birth weight on the other hand, did not have any significant effect on skin surface temperature ($P>0.05$). Similar lack of association between birth weight and heat loss in new-born lambs has been observed in a recent study (Labeur *et al.,* 2017). On the contrary, a positive association has been reported between birth weight and skin temperature in wet Romney new-born lambs when exposed to wind at an ambient temperature of 10°C (McCutcheon, 1981). If the association was negative it could have been attributed to a positive correlation that might have existed between birth weight and skin thickness in their study, but it was not the case.

4.4.2. Heat production

Average heat production per kg of birth weight ($W\ Kg^{-1}$) was calculated for the five consecutive time-intervals into which cold stress period was divided. Calorimetry was performed only during the adjustment and severe cold stress periods. As shown in Figure 4.7, compared to thick-skinned lambs, thin-skinned lambs produced higher amounts of heat ($W\ Kg^{-1}$) during the first (adjustment), third (B-C) and fourth (C-D) time-periods with significance levels of

P<0.01, P<0.1 and P = 0.06, respectively. However, no significant difference (P>0.5) was evident for the heat (W Kg^{-1}) produced by lambs of different skin thickness categories during the second (A-B) and last (D-E) time-intervals.

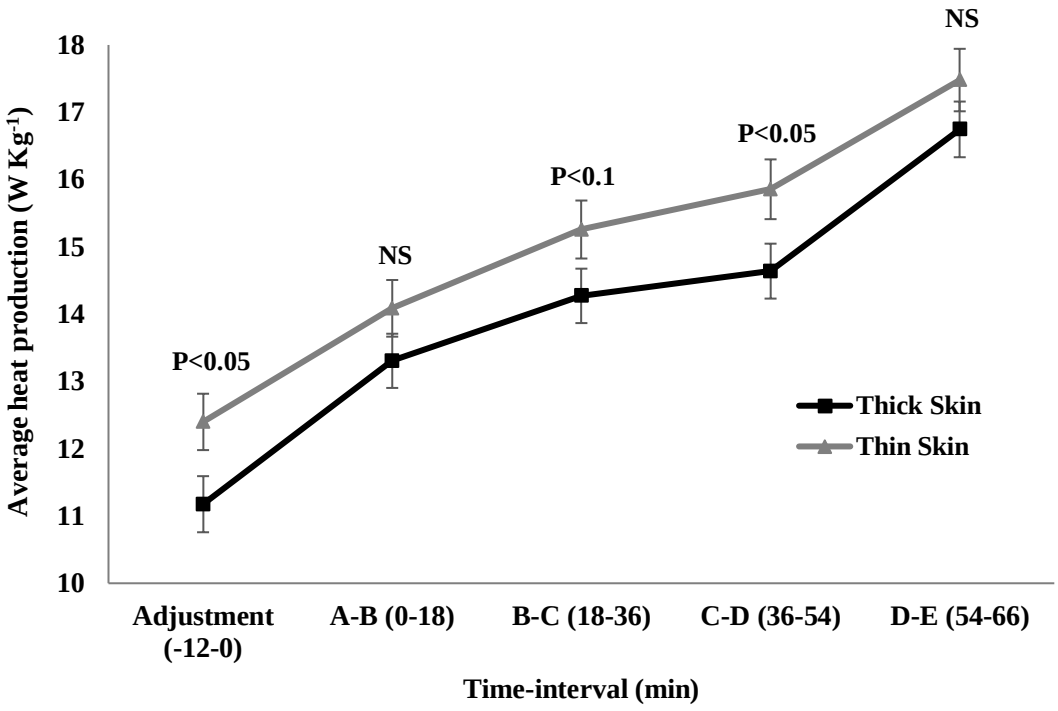


Figure 4.7. The effect of skin thickness category on average heat production (W Kg^{-1}**) during different time-intervals throughout calorimetry.** Conditions applied during each time-interval were as follows. Adjustment: 4°C, with no wind or rain; A-B: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D-E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors. The mean skin thickness (mm) of thick- and thin-skinned lambs contributing to these values were respectively 3.17 and 2.09 for adjustment, 3.19 and 2.12 for A-B, 3.19 and 2.13 for B-C, 3.18 and 2.14 for C-D, 3.19 and 2.13 for D-E periods.

Thin-skinned lambs producing higher amounts of heat during the time periods B-C (min 18-36) and C-D (min 36-54) is consistent with the fact that from the time-point B (min 18) onwards, they showed significantly higher skin surface temperatures compared to their thick-skinned peers (Figure 4.6). To maintain their core body temperature, lambs would normally produce more heat as they lose heat into the environment. However, it is not clear why there is a significant (P<0.01) difference in heat production in lambs of the two categories during the

adjustment cold period (min -12-0) while both groups had similar ($P > 0.05$) skin temperatures at the baseline time-point before commencement of calorimetry. On the contrary, while the thin-skinned lambs showed significantly ($P < 0.05$) higher skin temperatures at the time-points B (min 18), C (min 36) and D (54), there was no significant ($P>0.05$) difference in heat produced by the lambs in the two categories. This might be attributed to some extent to the fact that during the D-E time-interval (min 54-66), a considerable number of lambs (12 out of 18 and 12 out of 27 in thin and thick-skinned categories, respectively) reached their summit metabolism (Maximum heat production, MHP), while in the previous stages only few lambs attained MHP during each time period. Hence, the average heat production ($W\ Kg^{-1}$) calculated for this time period is expected to be a function of differences in MHP and consequently a poor predictor of average heat production itself. Those lambs that could not maintain their body temperature during each time-period, were excluded from the rest of experiment. This might have contributed to the difference between two skin categories in heat production being non-significant during the last time-interval.

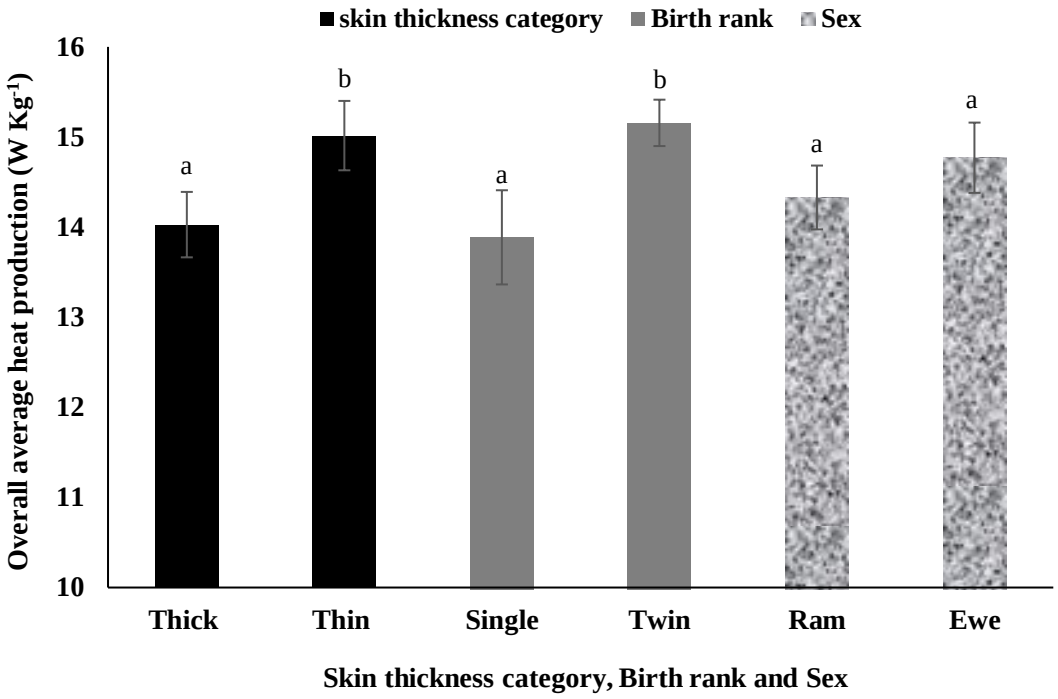


Figure 4.8. The effects of skin thickness category, birth rank and sex on overall average heat production (W Kg^{-1}**) considering all time-intervals (adjustment, A-B, B-C, C-D, and D-E respectively) during calorimetry.** All values are presented as Least Squares Means ± standard errors. Values with different letters, within each category, are significantly different (P<0.05 for both skin thickness category and birth rank). The mean skin thickness (mm) for lambs contributing to these values were respectively 3.19 and 2.12 for thick- and thin-skinned lambs, 2.61 and 2.7 for singles and twins, 2.62 and 2.75 for ewe and ram lambs.

While the differences in the amounts of heat (W Kg^{-1}) produced by lambs of different skin thickness categories were not significant during all the time periods, the overall effect of skin thickness category on heat production was significant at P = 0.05 (Figure 4.8). The thin-skinned lambs producing greater overall amount of heat during the experiment seems likely to be in response to their higher heat loss compared to the lambs with thick skin. This finding is consistent with the previously discussed fact that thin-skinned lambs showed an overall higher skin surface temperature (considering all time-points), compared to their thick-skinned peers (Figure 4.4).

Furthermore, considering all time-intervals, the effect of birth rank on overall heat production during the calorimetry was significant at P<0.05, so that compared to singles, twin-born lambs

produced more ($P<0.05$) heat on a per kg of body weight basis (Figure 4.8). This result is consistent with the finding that compared to singles, twin lambs had higher ($P<0.05$) overall skin surface temperatures taking into account all time-points (Figure 4.4). Hence, part of the difference in heat produced by singles and twins is likely to be due to a response twins showed to compensate higher heat loss through skin surface.

Although coat properties (depth, type and weight) were not measured in the present study, according to the literature (McCutcheon *et al.*, 1983b) the mean coat depth (middle and hip areas) of twin lambs were reported to be 1.1 mm less than that of singles, indicating that twins may be suffering from slightly inferior coat insulation. In their study (McCutcheon *et al.*, 1983b) shorter coat depth was related to higher heat production required to maintain rectal temperature under cold stress. However, it does not seem to be the case in our study, since wool was removed from the dorsal and lateral surfaces of all lambs and only a small amount of intact wool was remaining on the ventral surface, which is unlikely to have contributed to the variation in heat produced by singles and twins. A higher ratio of surface area to body weight in twins compared to singles (McCutcheon *et al.*, 1983b) might also impose more heat loss and consequently higher heat production in twins. Consistent with this assumption and to some extent with our results, in a previous study in new-born lambs (Kerslake *et al.*, 2000) heat production ($W\ Kg^{-1}$) calculated during a cold period comparable to the adjustment period in our study tended to be higher in triplets compared to twins. However, in another report by the same authors (Kerslake *et al.*, 2009), there was no difference between twins and triplets in terms of heat production. On the other hand, another plausible reason that could have contributed to the effect of birth rank on heat production in the present study is a possible bias that might have resulted from the lower number of single lambs compared to twins (12 vs 52).

As shown in Figure 4.8, the effect of sex on overall heat production considering all the time-intervals during the calorimetry was not significant ($P>0.05$). In a previous study (Kerslake *et*

al., 2000), no effect of sex was reported for heat production (W Kg^{-1}) in new-born lambs exposed to severe cold stress. On the contrary, in another study (McCutcheon *et al.*, 1983b), female lambs had significantly greater metabolic rate (W Kg^{-1}) than males when exposed to a cold stress. However, in a different study by the same authors (McCutcheon *et al.*, 1983a), sex of the lamb did not influence metabolic rate.

Rectal temperature at the start of calorimetry had a positive effect ($P<0.01$) on heat production during calorimetry, with a 1°C increase in rectal temperature being associated with 1.1 ± 0.4 W Kg^{-1} increase in heat production. Birth weight on the other hand, had no significant effect on heat production on a per kg of body weight basis. Consistent with this finding, a previous study (Kerslake *et al.*, 2000), which is comparable to the first stage of calorimetry in the present study, did not indicate any effect of birth weight on base heat production (W Kg^{-1}).

4.4.3. Maximum heat production (MHP)

The mean (± SE) maximum heat production of 52 lambs that attained summit metabolism in the present study was 18.55 ± 42 W Kg^{-1}, which is comparable to a mean of 18.03 ± 0.63 W Kg^{-1}reported in a previous study on new-born Romney lambs (McCutcheon *et al.*, 1983a). In contrast, other studies (Alexander, 1962b, Alexander and Bell, 1975a) on lambs of several breeds in Australia reported means that were 1.0-3.5 W Kg^{-1} lower than that obtained in our study. The observed difference is possibly due to the use of different breeds and differences in cold stress conditions used in the experiments.

Skin thickness category did not have any significant effect ($P>0.05$) on maximum heat production per unit body weight (Figure 4.9). Having the same capacity for maximum heat production as thin-skinned lambs while losing less heat through skin surface, would make the thick-skinned lambs superior over the other group in maintaining core body temperature.

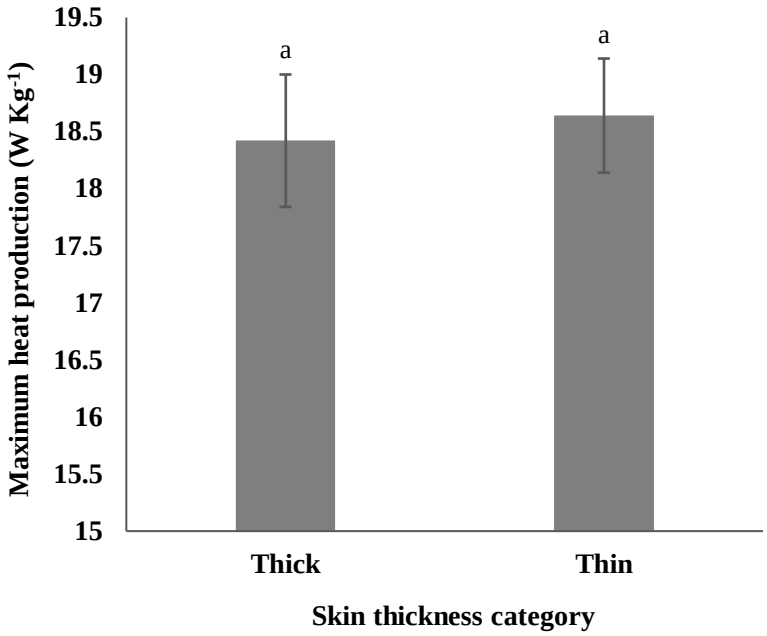


Figure 4.9. The effect of skin thickness category on maximum heat production (W Kg^{-1}**) during cold stress period.** The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05). The mean skin thickness (mm) of thick- and thin-skinned lambs contributing to these values were 3.19 and 2.11, respectively.

Rectal temperature at the start of adjustment period (min -12) had a positive effect (P<0.01) on maximum heat production (W Kg^{-1}), with a 1°C increase in rectal temperature being associated with a 3.29. ± 0.85 W Kg^{-1} increase in maximum heat production (Figure 4.10). This is consistent with a 1°C increase in rectal temperature during the adjustment period being associated with a 2 W Kg^{-1} increase in maximum heat production in a study on twin and triplet-born lambs of Romney type. (Kerslake *et al.*, 2009). On the contrary, in another study (Alexander, 1962b) on Merino and Border Leicester × Merino lambs, at body temperatures near normal, summit metabolism was not predictable from rectal temperature, while at temperatures below 36°C the metabolic rate increased with rising rectal temperature. Since the breed and experimental conditions used in the present study and the other study (Kerslake *et al.*, 2009) were the same, the lack of a relationship between maximum heat production and rectal temperature, at body temperatures near normal (Alexander, 1962b), is likely to be due to

the use of different breeds and differences in cold stress conditions used in the experiments. Therefore, this relationship needs to be further investigated in other breeds.

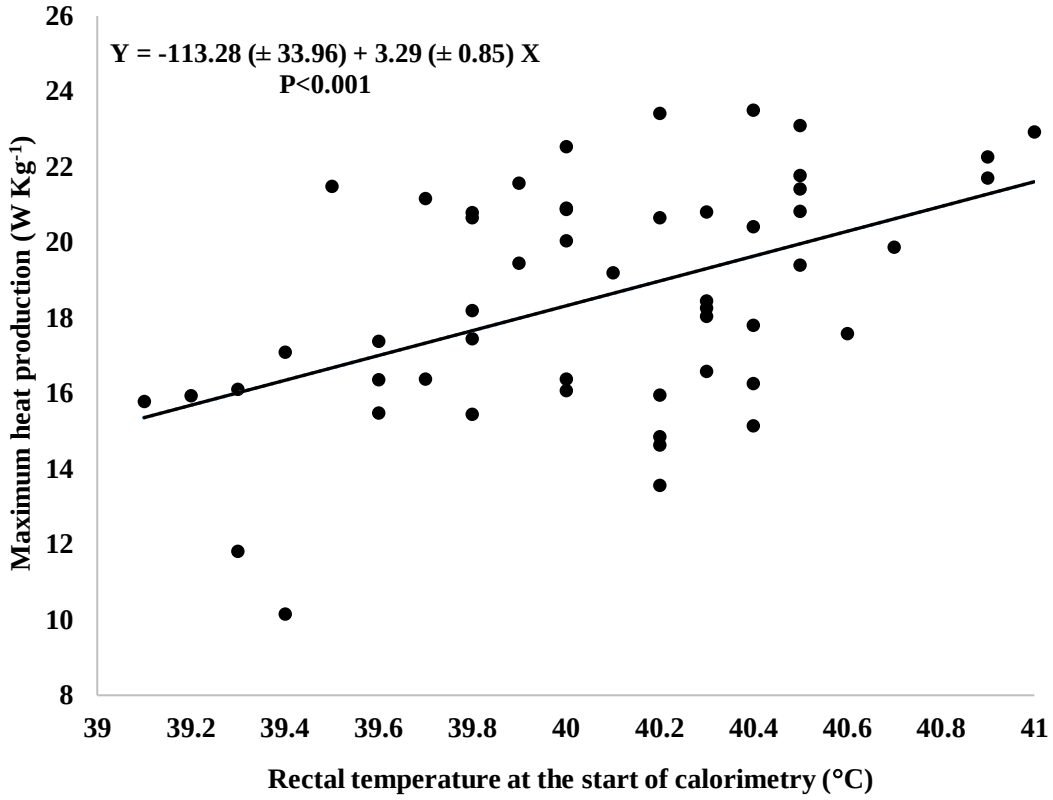


Figure 4.10. The association of rectal temperature at the start of calorimetry (°C) with maximum heat production (W Kg^{-1}**) during cold stress period.**

Body weight in the present study did not show any association (P>0.05) with maximum heat production on a per kg of body weight basis. Likewise, there were studies (Alexander, 1962b, Stott and Slee, 1987) reporting maximum heat production (W Kg^{-1}) to be independent of body weight in new-born lambs. However, in another study (Eales and Small, 1980a), summit metabolism per unit body weight decreased as body weight increased. Interestingly, another study (Kerslake *et al.*, 2009) found that maximum heat production on a per kg of body weight basis was positively associated with birth weight in triplet-born lambs, but independent of body weight in twins.

Birth rank could not demonstrate any significant effect (P>0.05) on maximum heat production (W Kg^{-1}) which is consistent with results from other studies (Alexander, 1962b, Alexander and Bell, 1975b, Kerslake *et al.*, 2000, Kerslake *et al.*, 2009). On the other hand, there is evidence suggesting that twin lambs have lower summit metabolic rate per kg body weight compared with singles (McCutcheon *et al.*, 1983b). Contrary to this report (McCutcheon *et al.*, 1983b), another study (Stott and Slee, 1987) showed single lambs to have higher summit metabolic rate per kg body weight compared with twins. Lambs of different sexes had the same amount of maximum heat production (W Kg^{-1}). In line with our result, no effect of sex was reported in other studies (Kerslake *et al.*, 2000, Kerslake *et al.*, 2009). Another study (Stott and Slee, 1987), however, demonstrated greater maximum heat production (on a per kg of body weight) in female lambs compared with males.

4.4.4. Time to reach maximum heat production

As presented in Figure 4.11a, before making adjustment for body weight, time taken to reach maximum heat production was significantly (P<0.05) longer in thick-skinned lambs compared with thin-skinned lambs. After including body weight in the model, the difference persisted but was not significant (P>0.05, Figure 4.11b). Based on the lower heat loss and consequently higher rectal temperature in thick-skinned lambs, they are expected to reach maximum heat production at a later time-point, compared to thin-skinned lambs. The difference being non-significant after making adjustment for body weight could be attributed to some extent to an inter-corralation between body weight and skin thickness (heavier lambs had thicker skin). Therfore including an effect into the model reduced the significance of the other one, and the significance of each effect was impossible to evaluate independently. On the other hand, during the cold stress period, 12 out of 64 lambs did not attain maximum heat production from which 10 were thick-skinned. Hence, these 12 lambs were not included in the model used for the analysis of the time to reach maximum heat production. This might have also contributed to

the difference between two skin categories in time to reach maximum heat production being non-significant, after adjusting for body weight. To compensate for heat loss through body surface, a lamb can increase its rate of heat production up to a point it reaches its maximum heat production (maximum heat production). It seems that under the conditions used in this study, those 12 lambs have been able to maintain their core body temperature without any need to achieve their maximum sustainable metabolic rate (maximum heat production). It is likely that if the cold stress period was allowed to be continued for a longer time, those 12 lambs would have finally attained their maximum heat production, and therefore could be included in the analysis. This could have further increased the mean time to reach maximum heat production in case of thick-skinned lambs, possibly resulting in a significant difference between the two skin categories. However, even in that case, the fact that those 12 lambs (from which 10 were thick-skinned) were heavier than the other 54 lambs (6.57 vs 5.57 kg), might make it impossible to determine which effect (skin thickness, body weight, or both) might have been responsible for a possible difference between two skin categories in time to reach maximum heat production.

To test this scenario, those 12 lambs were presumed to have attained their maximum heat production at the end of cold stress period (a time-point that was even sooner than the time at which they might have reached their summit metabolic rate if the cold stress period was allowed to be continued), and therfore were included in the analysis. Interestingly, time taken to reach maximum heat production was significantly ($P<0.05$) longer in thick-skinned lambs compared with thin-skinned lambs even after including body weight in the model.

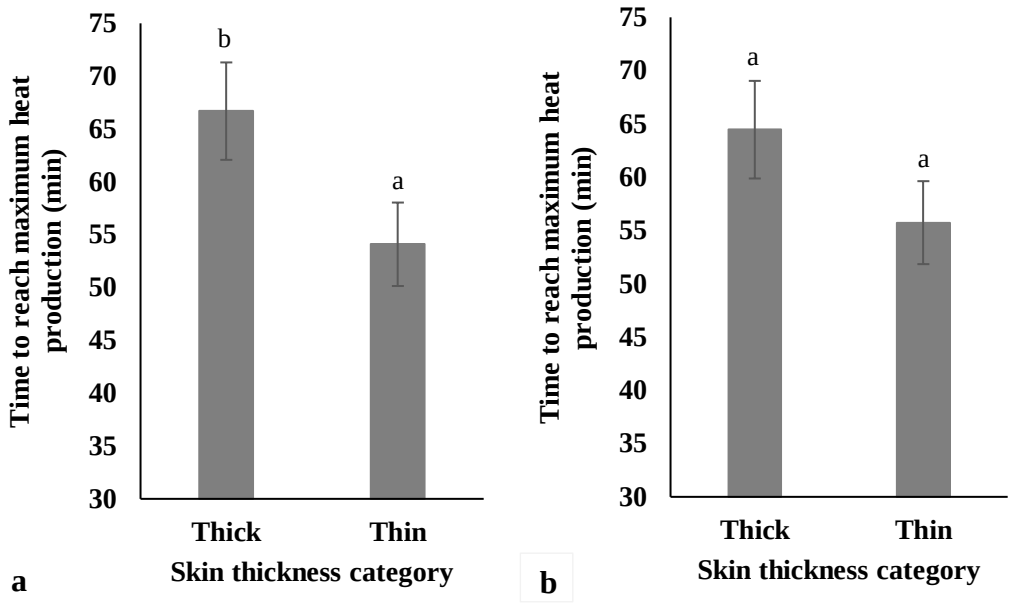


Figure 4.11. The effect of skin thickness category (a. before and b. after making adjustment for body weight) on time to reach maximum heat production (min) during the cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05). The mean skin thickness (mm) of thick- and thin-skinned lambs contributing to these values were 3.19 and 2.11, respectively.

Body weight showed a positive relationship (P<0.05) with time to reach maximum heat production (min) with a 1 kg increase in body weight leading to a 7.64 ± 3.60 minutes increase in time to reach maximum heat production. In a previous study (Stott and Slee, 1987), time to reach maximum heat production was independent of body weight. The association found in this study could be attributed to the fact that lambs with greater body weight have lower surface area to body mass ratio and possibly more brown adipose tissue (BAT) reserves in heavier lambs. The former implies better thermoregulation in heavier lambs, which achieve maximum sustainable metabolic rate later than lighter lambs. Furthermore, rectal temperature at the start of adjustment period (min -12) had a positive effect (P<0.05) on time to reach maximum heat production (min) with a 1°C

increase in rectal temperature being associated with a 16.38 ± 6.68 minutes increase in time to reach maximum heat production.

Birth rank and sex of lamb did not show any significant effect (P>0.05) on time to reach maximum heat production in the current study. In contrast, a previous study (Stott and Slee, 1987) indicated a longer time to reach maximum metabolic rate in female and single lambs compared to males and twins respectively. In this study (Stott and Slee, 1987), female lambs showed greater mean skin thickness and greater mean fleece depth than males, but had similar body weight. So, the longer time females took to reach maximum heat production in their study (Stott and Slee, 1987) was suggested to be a result of better insulation in females than males. Singles on the other hand, had the same skin thickness as twins, but greater body weight. Therefore, greater time to reach maximum heat production in single lambs could have been driven from a lower surface area to body mass ratio and consequently better thermoregulation in singles compare with twins.

In this study, males and females did not show any significant difference (P>0.05) in skin thickness or body weight. Males and females are expected to take the same time to reach maximum heat production. Likewise, no significant difference was observed between singles and twins in skin thicknes. However, compared to twins, single lambs were significantly heavier (P<0.05). But this difference was not reflected in singles taking significantly longer (P>0.05) time to reach maximum heat production compared with twins with or without making adjustment for body weight. The reason for this result is not clear, but it might have been due to a bias resulting from the lower number of single lambs compared to twins that achieved summit metabolism in this study (6 vs 46). It should be noted that 6 out of 12 lambs that did not attain MHP during the cold stress, were singles and 6 were twins. It means that half of the singles used in the experiment did not reach MHP while the proportion of twins not attaining MHP was much smaller (6 out of 52). Hence

including or excluding those 12 lambs in/from the analysis would probably affect singles more than twins in terms of mean time to reach MHP. In the scenario explained before, when those 12 lambs were included in the analysis, time taken to reach MHP was significantly ($P<0.05$) longer in single lambs compared with twins. This difference was not significant when body weight was included in the model. It indicates that the observed difference could probably be a result of singles having more BAT reserves and also being heavier and consequently having a smaller surface area to body mass ratio compared with twins.

4.4.5. Rectal temperature at the onset and during cold stress period

As presented in Figure 4.12, at the first three time-points (adjustment, A and B), there was no significant difference ($P>0.05$) between rectal temperatures of thick-skinned and thin-skinned lambs. At the next three time-points (C, D and E) however, thick-skinned lambs demonstrated higher rectal temperatures compared with their thin-skinned peers ($P = 0.08$, $P = 0.01$ and $P<0.01$ at the time-points C, D, and E, respectively). Consistent with the trend observed for heat loss (skin temperature) in thin- and thick-skinned lambs (Figure 4.6), from time-point C (min 36, the next time-point after the former group started to show a higher skin temperature than the latter category) onward, thin-skinned lambs showed a lower rectal temperature than thick-skinned lambs. Even the greater amounts of heat the thin-skinned lambs produced compared to thick-skinned lambs during the time-intervals B-C and C-D (Figure 4.7), could not compensate the higher heat loss observed in thin-skinned lambs. This resulted in a decrease in rectal temperature of thin-skinned to a higher magnitude compared to the corresponding decrease observed in thick-skinned lambs, and continued to the last time-point E (Figure 4.12). Rectal temperatures of the two groups declined and the difference between the two groups increased over time (Figure 4.12). There was a considerable drop in rectal temperature measured at time-point E, part of which could be due to a

higher rate of chilled water applied on the back of lambs after time-point D (min 54). Between time-points D and E (minutes 54 to 66), 9 out of 18 and 6 out of 27 in thin- and thick-skinned lambs, respectively experienced large drops in rectal temperature and could not maintain their core body temperature despite attaining maximum sustainable rate of heat production (summit metabolism) during this time.

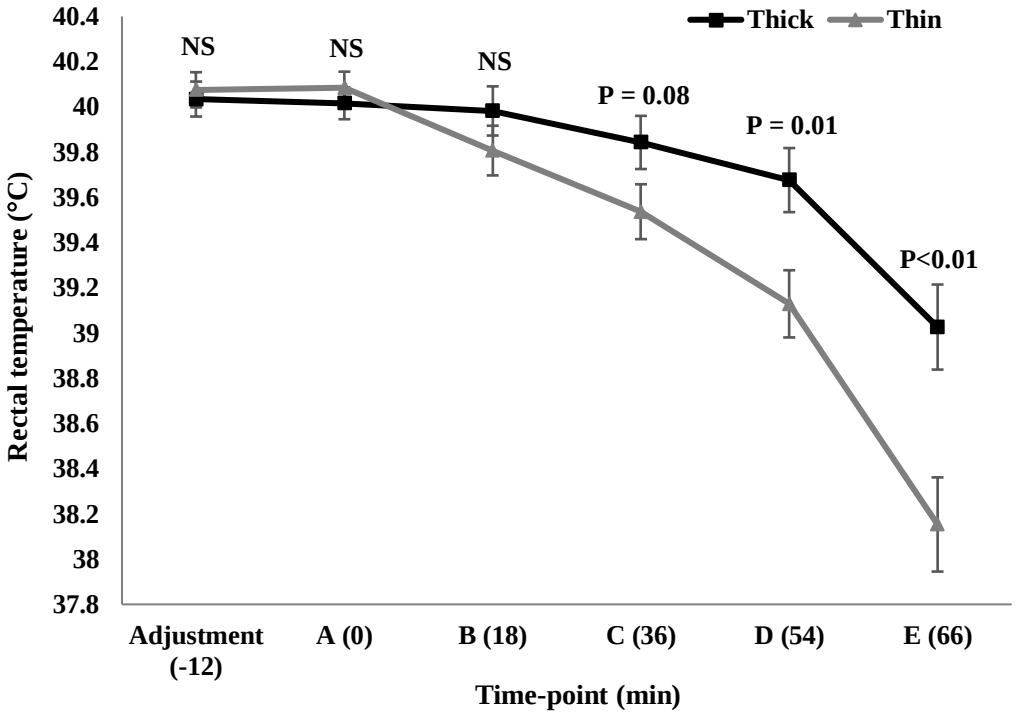


Figure 4.12. The effect of skin thickness category on rectal temperatures at different time-points during the cold stress period. Conditions applied at each time-point were as follows. Adjustment: 4°C, with no wind or rain; A: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s);, B: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s); E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors. The mean skin thickness (mm) of thick- and thin-skinned lambs contributing to these values were respectively 3.19 and 2.10 for adjustment, 3.19 and 2.10 for A, 3.19 and 2.12 for B, 3.19 and 2.13 for C, 3.18 and 2.14 for D, 3.19 and 2.13 for E time-points.

Skin thickness category had a significant effect ($P<0.05$) on the overall rectal temperature considering all the time-points during adjustment and severe cold stress periods (minutes -12, A, B, C, D and E), so that thick-skinned lambs had a higher overall temperature compared to their thin-skinned peers (Figure 4.13). According to this result, it seems that the higher ($P<0.05$) overall average heat (W Kg^{-1}) produced by thin-skinned lambs compared to thick-skinned lambs during the calorimetry (Figure 4.8) was not able to compensate the higher ($P<0.001$) overall heat they lost (indicated by higher skin temperature, Figure 4.4), considering all the time-points (baseline, A, B, C and D). This clearly shows the importance of heat retention to maintain core body temperature.

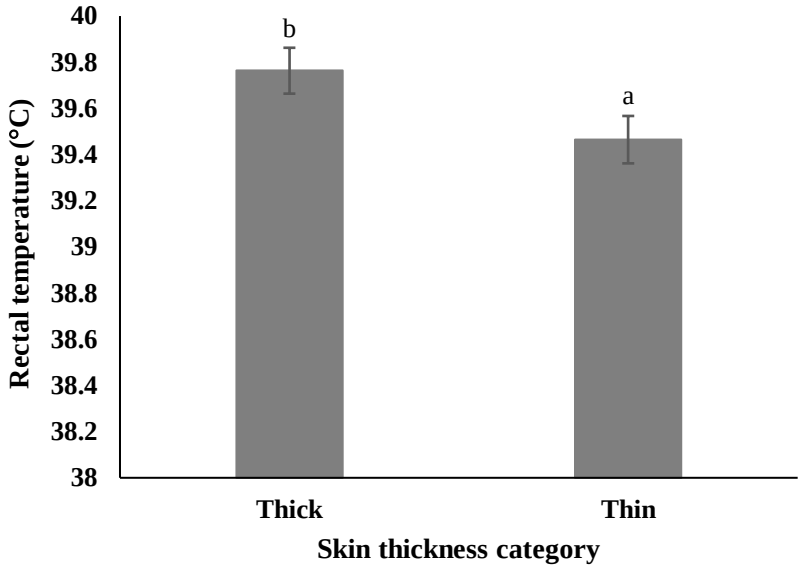


Figure 4.13. The effect of skin thickness category on overall rectal temperature considering all time-points during adjustment and severe cold stress periods. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different ($P<0.05$). The mean skin thickness (mm) of thick- and thin-skinned lambs contributing to these values were 3.19 and 2.12, respectively.

The average cold stress index (CSI) on the day of birth had a negative effect ($P<0.05$) on the rectal temperature of the lambs at all the time-points (minutes -12, 0, 18, 36, 54 and 66), with a 0.0012 $KJ/m^2/h$ increase in the cold stress index being associated with a 1°C decrease in rectal temperature. A previous report (Kerslake *et al.*, 2009) also demonstrated a negative influence of CSI in the first 24 h of life on rectal temperature before the onset of severe cold stress (adjustment period), although the effect was larger in their study than the corresponding effect observed in the present study (0.008 vs 0.0012).

4.4.6. Likelihood of maintaining the body temperature

As presented in Table 4.1, compared with thin-skinned lambs, thick-skinned lambs were 7.1 times more likely ($P<0.05$) to maintain their core body temperature during cold stress when adjustment was made for body weight. This result could be attributed to the fact that thick-skinned lambs lost less heat through skin surface compared with thin-skinned lambs while having the same capacity for attaining summit metabolism (indicated by maximum heat production ($W\ Kg^{-1}$)). This indicates a smaller ratio of heat loss to heat production and consequently better ability to maintain core body temperature by thick-skinned lambs compared with thin-skinned lambs. Previous studies (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991) reported a positive relationship between skinfold thickness and cold resistance, defined as the time taken for a lamb to become hypothermic (rectal temperature falls to 35°C) subsequent to progressive cooling in a water bath.

For every one kg increase in body weight, lambs were 6.9 times more likely ($P<0.01$) to maintain their core body temperature during the cold stress period (Table 4.1), which is likely to be a result of the smaller surface area to weight ratio and possibly higher BAT reserves in heavier lambs. As demonstrated in the current study and previous reports (Alexander, 1962b, Stott and Slee, 1987), maximum heat production per unit body weight is independent of body weight in new-born lambs.

Therefore, maximum heat production per unit area increases with increasing body weight (Alexander, 1962b, McDonald, 1962), because surface area to weight ratio decreases as body weight increases. As a result, heavier lambs will exhibit a smaller ratio of heat loss to heat production and are better at maintaining their core body temperature compared with lighter lambs. Consistent with this finding, body weight has been shown to be associated with cold resistance (as defined previously) in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991, Plush *et al.*, 2016).

Table 4.1. Odds ratios (OR) and 95% confidence intervals (CI) for variables effecting likelihood of maintaining rectal temperature during cold stress.

Variable	Maintained	Not maintained	P value	OR (95% CI)
	n	n		
Body weight	12	52	<0.01	6.9 (1.85-25.90)
Skin thickness category (Thick vs Thin)	10 vs 2	22 vs 30	<0.05	7.1 (1.11-45.67)

In a previous study (Samson and Slee, 1981), the relationship between skinfold thickness and cold resistance was attributed to an association between skin thickness and nutritional status and consequently greater energy reserves, or due to a direct effect of skin thickness on body insulation. The results in this study support the idea that the positive association of skin thickness with improved thermoregulation is most likely due to improved insulation rather than an association between skin thickness and nutritional status. New-born lambs do not possess subcutaneous fat (Alexander, 1978), so thicker skin might be associated with a deeper location of blood vessels and therefore with reduced heat flow (Stott and Slee, 1987) or with a different type of birth coat, which

itself could influence cold resistance (Slee, 1978, Slee *et al.*, 1991). Cold resistance may be associated with coat depth (Slee *et al.*, 1991, Stott and Slee, 1987), which itself could be correlated with skin thickness (Slee *et al.*, 1991). However, even if an effect of coat properties on thermoregulation is assumed, it is unlikely to have been able to exert an effect on the cold stress indices investigated in the present study since wool was removed from the dorsal and lateral surfaces of all lambs and only a small amount of wool was remaining on the ventral surface.

4.5. Conclusions

The following conclusions could be drawn from the results presented in this study:

1) Thick-skinned lambs showed significantly lower skin temperature than their thin-skinned peers during the cold stress period, implying less heat loss from the skin surface and better thermoregulation through increased skin thickness.
2) Thick-skinned lambs produced significantly less heat (per Kg body weight) than thin-skinned lambs as a response to less heat loss in the first group. This means there was less need to consume body reserves as a source of energy and consequently better conservation of body reserves in the thick-skinned lambs.
3) Thick-skinned lambs had higher rectal temperature and were more likely to maintain body temperature than thin-skinned lambs during cold stress period.
4) Thick-skinned lambs had the same capacity for maximum heat production as the thin-skinned lambs.

Taking these findings together, it can be concluded that increased skin thickness at birth can positively affect thermoregulation in new-born lams through decreased heat loss from skin surface, thereby minimizing the heat production required to maintain core body temperature. Brown adipose tissue (BAT) which is responsible for up to half the total cold-induced heat production in

new-born lambs (Alexander and Williams, 1968, Stott and Slee, 1987) and as a very important source of energy is depleted within a few days of life in new-born lambs (Alexander and Williams, 1968, Alexander and Bell, 1975a). Although increased capability of lamb in terms of heat production could be a satisfactory way of achieving high cold resistance in short-term laboratory tests, an improved insulation might be more beneficial for lamb survival in the field (Eales *et al.*, 1982).

Thick-skinned lambs seem more likely to be more resistant to hypothermia in the field which is responsible for a major proportion of lamb deaths within the first three days of life. Therefore, skin thickness could be considered as a potential alternative to selection criterion for cold resistance and consequently lamb survival. However, care must be taken to consider unfavorable genetic correlations with other economically important traits.

Different modeling and simulation methods have been used to predict heat loss through different ways of heat transfer (radiation, conduction, convection, and evaporation) in the food industry including carcass chilling (Mallikarjunan and Mittal, 1994), cooling of fruits and vegetables (Raval *et al.*, 2013), and processing of meat products (Hu and Sun, 2000, Marcotte *et al.*, 2008). Further study to predict heat loss from the body of lambs using appropriate modeling and simulation methods and to investigate how skin thickness would affect the rate of heat lost would be of interest.

Chapter 5

Is skin thickness measured at eight months of age a reliable indicator of the trait at birth?

Part of this chapter has been published in the conference proceedings cited below.

Soltani-Ghombavani, M., Dukkipati, R., & Blair, H. T. (2018). Correlations of skin thicknesses measured at monthly intervals from birth to eight months of age in New Zealand Romney sheep. In Proceedings of the 11th World Congress on Genetics Applied to Livestock Production, Auckland, New Zealand, 11-16 February 2018 (Vol. Electronic Poster Session - Biology - Growth and Development, pp. 333).

5.1. Abstract

Previous studies have shown increased skin thickness at birth to be associated with an increase in cold resistance in new-born lambs, which can lead to a decrease in lamb mortality arising from starvation/exposure. From practical and economic points of views, it would be more beneficial to measure skin thickness at a later age at the same time as recording of other traits of interest, instead of at birth. However, this would be possible only if skin thickness at later ages is a reliable indicator of the trait at birth. The current experiment aimed to explore whether skin thickness was a repeatable trait when measured on nine occasions between birth and eight months of age. Three hundred and twenty four single and twin lambs born to two batches of Romney-type ewes (3-4-year-old) that were estrus synchronized seven days apart, were scanned for skin thickness measurement within 24 hours of birth using ultrasonography. Out of 324 lambs, 100 were selected and categorized into three groups of significantly different skin thicknesses based on the measurement at birth (32, 36 and 32 lambs in thin-, thick- and medium-skinned categories, respectively), and were all maintained on the farm under identical conventional conditions until eight months of age, and their skin thickness was measured ultrasonically at monthly intervals. Ultrasound skin thickness recorded at birth, had a mean of 2.66 mm (ranging from 1.66 to 4.31 mm) and was positively associated with birth weight, with a 1 kg increase in body weight being associated with 0.1 ± 0.02 mm increase in skin thickness. Birth rank had an effect on skin thickness ($P<0.05$), with singles having thicker skin than twins, only before making adjustment for birth weight, while sex did not show any effect ($P>0.05$) on the trait neither before nor after adjustment for birth weight. Also, every one-day increase in gestation length in the ewes resulted in 0.06 ± 0.01 mm increase ($P<0.0001$) in skin thickness in the lambs. Lambs born to the ewes from the second batch had a greater skin thickness compared to lambs of the first batch ($P<0.0001$).

Significant ($P<0.05$) correlations were observed between skin thickness at birth and the measurements taken at 6, 7, and 8 months, with Pearson correlation coefficient values of 0.29, 0.33, and 0.34, respectively. There were no differences ($P>0.05$) in growth rates, or live weights of lambs from different skin thickness categories obtained from birth to any of the monthly measurements. Based on the results of this study, skin thickness measured during 6-8 months could be considered as a moderate indicator of skin thickness at birth.

5.2. Introduction

Starvation/exposure as a major cause of lamb mortality in the first three days of life is responsible for approximately 30% of lamb mortalities (Williams and Thornberry, 1992), especially in cold and wet outdoor conditions. A few studies have reported an increase in cold resistance with increased skin thickness in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991). Furthermore, a recent study (chapter four) demonstrated increased skin thickness measured ultrasonically at birth to be associated with improved thermoregulation in new-born lams due to decreased heat loss from the skin surface, thereby minimizing the heat production required to maintain core body temperature.

Skin thickness has a moderate to high heritability (Gregory, 1982a, Slee *et al.*, 1991, Soltani-Ghombavani *et al.*, 2017) and could be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000), unlike cold resistance, whose assessment needs laboratory-based techniques that are not feasible for breeders. Hence, skin thickness at birth can be considered as an alternative to cold resistance and consequently lamb survival. However, unlike fat depth and eye muscle depth that are commonly measured ultrasonically as traits of economic importance in the sheep industry (Gilmour *et al.*, 1994, Dewi *et al.*, 2002, Fischer *et al.*, 2006), measuring skin thickness is not a common practice. Even if a positive effect of skin thickness at birth on improved

thermoregulation in new-born lambs is considered as a reason for measuring skin thickness, from a practical point of view, it would be almost infeasible and costly for farmers to measure the trait in new-born lambs on farm. Therefore, it would be more practical and cost-effective if skin thickness could be measured at the same time with other traits of interest like fat depth and eye muscle depth, i.e. at a later age instead of at birth. For this to be implemented, skin thickness at later ages should be confirmed to be a reliable indicator of the trait at birth.

Changes in skin thickness have been studied from 10 days after birth to five months of age in sheep (Wodzicka, 1958a). That study did not measure skin thickness at birth when the trait is likely to reflect cold tolerance, nor at 6-8 months when breeders assess muscle and fat depth. Furthermore, the result from that study might not be highly reliable because of the small number of animals (12 lambs) they used. There is no published study reporting the association of skin thickness at birth with the measurements taken at later ages and its changes from birth onwards.

Therefore, the main objective of the current study was to examine the correlations (as an estimate of repeatability) among skin thickness measurements obtained at monthly intervals from birth to 8 month of age, in order to find out whether skin thickness at an older age is an appropriate indicator of the trait at birth. Furthermore, the effects of environmental factors on the trait, and also the effect of skin thickness at birth on growth rates and live weights from birth onwards were examined.

5.3. Material and methods

5.3.1. Ethics statement

The study protocol was approved by the Massey University Animal Ethics Committee (Protocol#16-21). Animals were all closely monitored throughout the experiment and no animal health or welfare issues were observed during or after the experiment.

5.3.2. Estrous synchronization

Four hundred and thirty Romney-type ewes (3-4-year-old) from the Massey University Keeble farm that were managed under commercial conditions were randomly selected for this experiment. In order to better manage the experiment in respect to skin thickness measurement and also because a number of lambs with the thickest and thinnest skin were needed for a separate experiment (chapter four), ewes were estrous synchronized to have their lambs born within a short period of time. The ewes were randomly divided into two batches of 215 and synchronized seven days apart using Eazi-BreedTM Controlled Intravaginal Drug Release (CIDR), containing 0.3 g progesterone (Zoetis, Auckland, New Zealand). CIDRs were inserted into vagina 12 days prior to mating. On the same day when CIDRs were removed (17 and 24 April 2016 for the first and second batches, respectively), eight rams equipped with harnesses and crayons were introduced and allowed to mate for seven consecutive days. All ewes marked with crayons were kept and the remaining ewes were excluded from the study. Ultrasound pregnancy testing of ewes was carried out 50 days after rams were introduced to the first batch and those ewes recognized as non-pregnant were removed. The second day after CIDR removal was considered as the conception day for all the ewes, so that a gestation length could be estimated for each ewe. Triplet-bearing ewes were excluded from the study. All ewes remaining in the study were managed under identical conditions throughout pregnancy.

5.3.3. Animal measurements

During the lambing period (9-24 September 2016), all lambs were weighed and tagged within 12 hours of age and their sex, birth rank, and date of birth recorded. Ultrasound scanning was performed within 24 hours of birth. To ensure lambs were within 24 hours of age, scanning was conducted every morning for lambs born over the previous 24 hours. A commercial operator did the scanning using an ultrasound scanning machine (Sonosite M Turbo, FUJIFILM Sonosite, Inc., Bothell, Washington, United States) with a 38 mm probe at 7.5 MHz set at a depth of 40 mm on the left dorsal loin region of the lambs around the 12th rib. Out of 324 lambs, 100 were selected and categorized into three groups of significantly different skin thicknesses based on ultrasound measurement at birth. The three categories included 64 lambs of the thickest and thinnest skin (32 lambs in each group) and 36 lambs of medium skin thickness. Within 24 to 48 hours of age, the 64 lambs (thin and thick-skinned categories) were used for a separate calorimetry experiment (chapter four) in addition to the current study. All 100 lambs were maintained on the farm under identical conventional New Zealand sheep farming conditions until eight months of age, and their skin thickness measured ultrasonically in the same region of body at monthly intervals by the same operator using the same scanning machine. Measurements were taken twice on the same day at the fifth month, to test the reliability of the measurements taken by the operator. Since some of the lambs died during the course of the study and a few were affected by dermatitis at the scanning region, the records for skin thickness at subsequent months after birth were less than 100. Live weights of the lambs were measured at the same time as skin thicknesses were taken at the monthly intervals and growth rates from birth to each measurement time were calculated accordingly.

5.3.4. Statistical analysis

The univariate procedure in SAS software (SAS, 2011) was used to check for normality and edit the data obtained for the 324 lambs (removing two outlier observations). Cleaned data for the 322 lambs were analyzed by the PROC MIXED of the SAS software (SAS, 2011) to identify significant effects influencing skin thickness at birth. The initial model contained the effects of sex (male and female), birth rank (single and twin), synchronization batch (first and second), and their two-way interactions as fixed effects and date of birth, body weight, gestation length, and average ambient temperature on the day of birth (obtained from a weather station at the AgResearch Limited, Grasslands Research Centre, located near the Massey University Keeble farm in Palmerston North, where the lambs were born) as covariates. All non-significant fixed effects ($P>0.05$), interactions ($P>0.05$) and covariates ($P>0.05$) were excluded from the final model. The final model included the effects of synchronization batch, birth weight, gestation length, and average temperature on the day of birth. The PROC CORR of the SAS software (SAS, 2011) was used to calculate Pearson correlation coefficients among skin thickness readings obtained at monthly intervals from birth to 8 months of age.

To test if the difference observed between skin thickness of the three categories at birth would continue as lambs age, the skin thicknesses of the three groups obtained at the subsequent measurements were compared using the PROC MIXED of the SAS software (SAS, 2011). For these analyses, in addition to the effect of skin thickness category, all the effects used in the initial analysis of skin thickness at birth were included in the models. However, after excluding non-significant effects ($P>0.05$), apart from the effect of category, the final models included the effects of synchronization batch for the first, third and seventh measurements, live weight for the first to fifth measurements, and age for the seventh measurement.

Any possible effect that skin thickness at birth could have on growth rate and live weight at different ages was examined. For this purpose, the means of these traits obtained from the three categories were compared after adjustment was made for other significant effects influencing growth rate and live weight. The initial models contained the effects of sex (male and female), birth rank (single and twin), synchronization batch (first and second), skin thickness category (thick, medium and thin), their two-way interactions as fixed effects and birth weight and age at measurement (for live weight) as covariates. All non-significant fixed effects ($P>0.05$), interactions ($P>0.05$) and covariates ($P>0.1$) were excluded from the final models. Therefore, the final models for live weight analysis included the effects of birth rank for the measurements taken from one to five months of age, sex for the third and last measurements, age for all except the first and eighth measurements, and birth weight for all measurements except the first one. Also, the final models for the analysis of growth rate included the effects of birth rank for the measurements taken from birth to five months of age, sex for the third and last measurements, and birth weight for all except the seventh and eighth measurements.

To test the reliability of the measurements taken by the operator, the two repeated measurements of each lamb (74 lambs) taken on the same day during the fifth month were used for calculation of within-day repeatability. For this purpose, repeatability was considered as intra-class correlation, and calculated as between-animal variance divided by total variance (McLaren *et al.*, 1991). Variance components were estimated by the PROC VARCOMP of the SAS software (SAS, 2011) using REML (restricted maximum likelihood, mixed models) method. For the analysis, a random model with animal and residual effects was assumed. Total variance therefore, was the sum of between-animal variance (differences among scan measurements associated with different animals) and residual variance. Also, the PROC CORR of the SAS software (SAS, 2015) was used

to calculate Pearson correlation coefficient between the two repeated ultrasound readings obtained on the same day.

5.4. Results and discussion

Table 5.1 presents the descriptive statistics for skin thicknesses measured at monthly intervals from birth to eight months of age. Ultrasound skin thickness in this study, recorded at birth, had a mean of 2.66 mm (Table 5.1), which was slightly lower than what was reported by (Jopson *et al.*, 2000) in new-born Coopworth lambs in New Zealand measured by skinfold callipers. The difference in skin thickness could be due to differences in breeds and/or techniques used for measuring skin thickness in these two studies.

Table 5.1. Descriptive statistics and number of records for the skin thicknesses from birth to 8 months of age measured at monthly intervals.

Measurement time (month)	No. of records	Mean (mm)	SD	Min.	Max.	CV (%)
Birth[1]	324	2.66	0.45	1.66	4.31	17.12
Birth[2]	100	2.59	0.52	1.66	4.31	20.08
1	92	3.36	0.38	2.46	4.39	11.43
2	91	3.61	0.44	2.39	4.78	12.08
3	91	4.11	0.50	2.85	5.48	12.21
4	86	3.99	0.41	3.16	5.16	10.29
5	75	3.47	0.44	2.46	4.58	12.64
6	77	3.78	0.45	2.54	4.70	11.90
7	76	3.57	0.42	2.54	4.62	11.70
8	76	3.83	0.43	2.39	5.08	11.08

[1 , 2]: Descriptive statistics for Birth[1] and Birth[2] are respectively based on all the 324 lambs scanned, and the 100 lambs that were selected from them.

Birth weight in the present study showed a positive association (P<0.0001) with skin thickness at birth with a 1 kg increase in body weight being associated with 0.1 ± 0.02 mm increase in skin thickness (Figure 5.1). In line with this result, Jopson *et al.* (2000) reported a positive association between skin thickness and body weight at birth in Coopworth lambs in New Zealand, with a 1 kg increase in body weight being associated with 0.37 ± 0.06 mm increase in skin thickness.

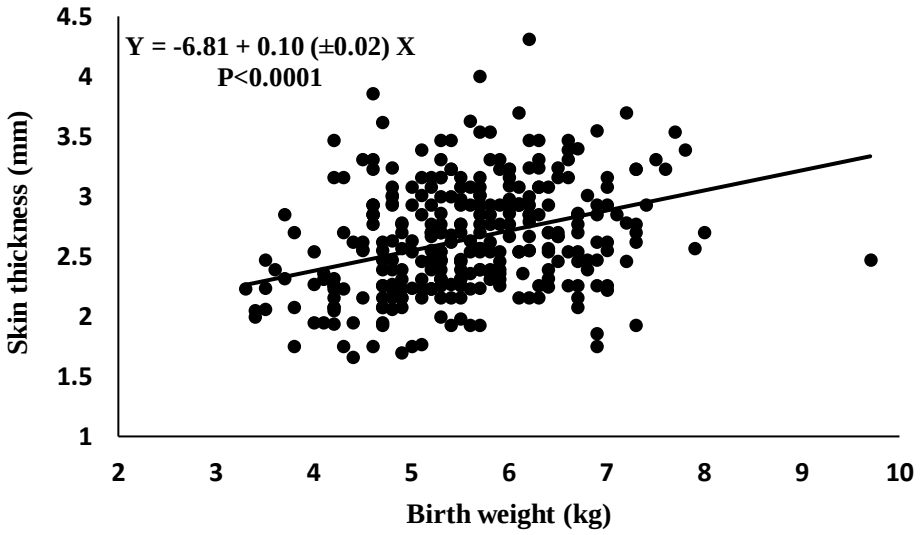


Figure 5.1. The association of birth weight (Kg) with skin thickness (mm) at birth after making adjustment for all other significant effects influencing skin thickness.

The effect of birth rank on skin thickness was significant before including birth weight as a covariate, so that single lambs had thicker (P<0.05) skin compared to twins (Figure 5.2). However, there was not any significant difference (P>0.05) between singles and twins when adjustment was made for birth weight (Figure 5.3). The difference between singles and twins in skin thickness before adjustment for body weight could be only due to singles being heavier than twins (6.32 kg vs 5.20 kg) implying that birth rank would not have any effect on skin thickness *per se*. Sex did

not show any effect on skin thickness neither before nor after adjustment for birth weight (Figures 5.2 and 5.3). Based on their higher birth weight, male lambs were expected to have thicker skin compared to females before making adjustment for birth weight. However, it seems that the difference in birth weight between males and females (5.78 kg vs 5.36 kg) was not large enough to result in a difference in skin thickness. Consistent with these results, in a study by Jopson *et al.* (2000), neither sex nor birth rank had an effect on skin thicknesses measured at birth when adjustment was made for birth weight.

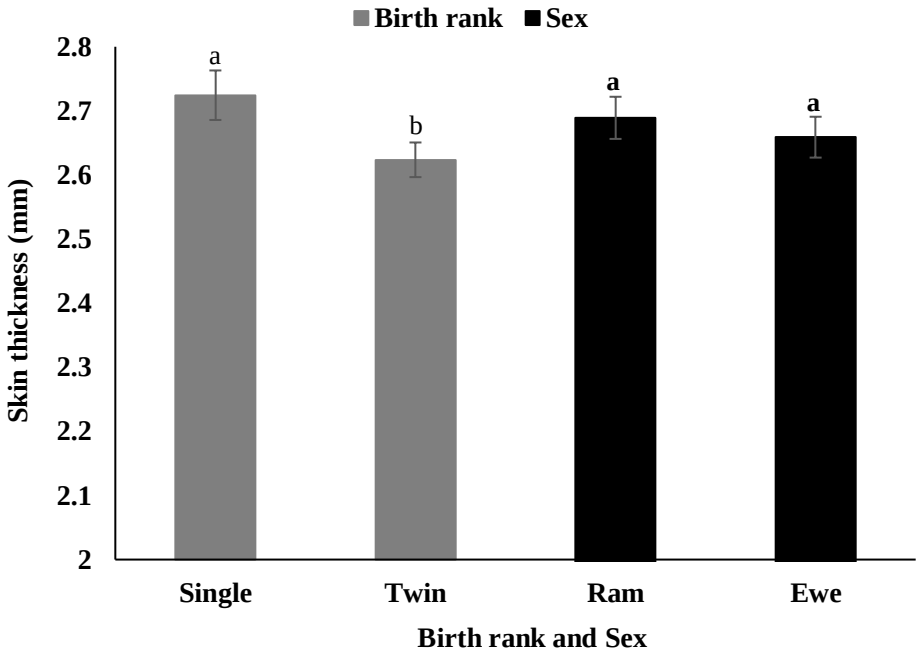


Figure 5.2. The effects of birth rank and sex on skin thickness (mm) measured at birth before making adjustment for birth weight. All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

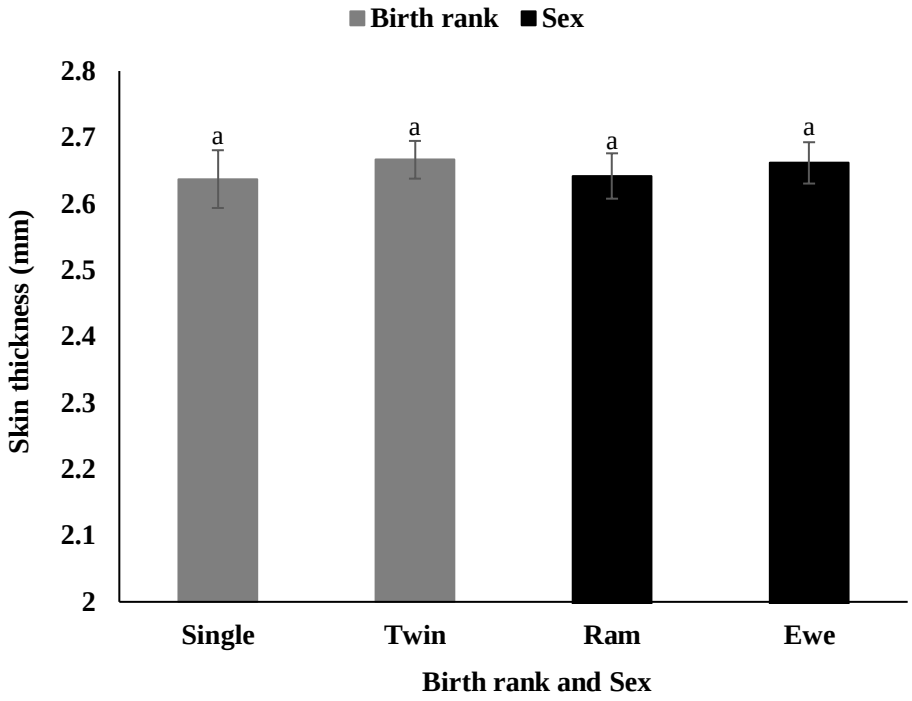


Figure 5.3. The effects of birth rank and sex on skin thickness (mm) measured at birth after making adjustment for birth weight. All values are presented as Least Squares Means ± standard errors. Values with the same letters are not significantly different (P>0.05).

Estimated gestation length had a positive significant effect (P<0.0001) on skin thickness in new-born lambs, so that a 1-day increase in gestation length resulted in 0.06 ± 0.01 mm increase in skin thickness at birth (Figure 5.4). There was also a positive association between birth weight and gestation length, with a 1-day increase in gestation length being associated with 0.10 ± 0.03 kg increase in body weight. After making adjustment for birth weight, the effect of gestation length on skin thickness was significant, though to a lesser extent compared with before making the adjustment (0.06 mm/day vs 0.07 ± 0.01 mm/day). Accordingly, the association between gestation length and skin thickness might be attributed to some extent, to the positive relationship that existed between gestation length and birth weight. Further assessment is required to investigate how gestation length can effect skin thickness in new-born lambs.

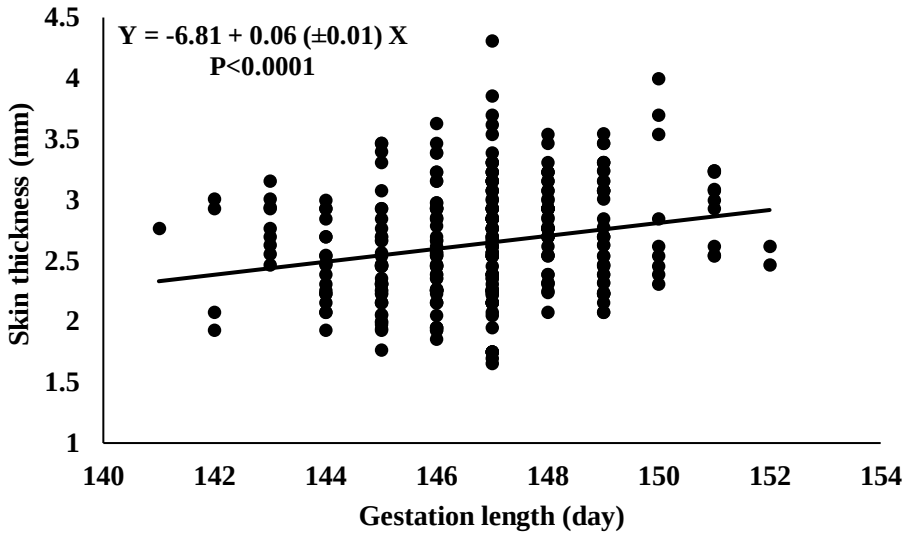


Figure 5.4. The association of gestation length (day) with skin thickness (mm) at birth after making adjustment for all other significant effects influencing skin thickness.

Estrous synchronization batch had a significant effect (P<0.0001) on skin thickness at birth, so that lambs born to ewes from the second batch had a greater skin thickness compared to their first-batch peers (Figure 5.5). Lambs of both batches had similar (P>0.05) birth weights (5.52 and 5.61 kg in the first and second batches, respectively) and almost the same proportions of singles to twins (55:107 and 52:110 in the first and second batches, respectively). So, the difference in skin thickness observed between two batches is unlikely to be due to the effect of birth weight and/or birth rank. Also, the greater proportion of males to females in the second batch (87:75) compared to the first one (73:89) is unlikely to be the reason for the greater skin thickness in the second batch since there was no effect of sex on skin thickness neither adjusted nor unadjusted for birth weight. Ewes of the first batch had longer (P<0.01) gestation period compared to the ewes of the second batch (147.15 d vs 146.48 d). Allowing for the positive effect of gestation length on skin thickness at birth, the difference in gestation length of the two batches was not large enough to cause the

difference in skin thickness between the batches. Even in that case, the first batch should have had thicker skin than the second batch due to longer gestation length in the first batch. All ewes were managed under the same conditions since the time of mating onwards and throughout pregnancy. So, the difference in skin thickness observed in lambs of different batches is unlikely to be a result of maternal nutritional differences. Difference in the time of mating and consequently the time of conception was the only difference exerted on the ewes of different batches. Ewes from the first and second batches were allowed to mate for seven consecutive days starting from the same day when CIDRs were removed (17 and 24 April for the first and second batches respectively). Since estrus synchronization was used for all the ewes, the second day after CIDR removal was considered as the conception day for all the ewes. Conception in the ewes of second batch happened 7 days after the first batch leading to shorter day length (photoperiod) exposure in the later compared to the former. So, a difference in photoperiod length to which dams of different batches were imposed before and on the day of conception, is possible to have influenced skin thickness in new-born lambs. Consistent with this, Vole pups born to dams exposed to shorter day length before gestation had thicker coats than those born to dams exposed to longer day length before gestation (Lee and Zucker, 1988). The thickening of skin observed in the present study might be an adaptive response that lambs born to the ewes of second batch showed to the shorter photoperiod length in dams transmitted to the developing fetus in uterus via melatonin signaling originating from the mother (Lee and Zucker, 1988, Lee *et al.*, 1989). It is a tautological prediction that reduced day length precedes winter (Broad *et al.*, 2016) and this adaptation enables the new-born to survive cold better than they would with thinner skin. Interestingly, the difference in skin thickness observed between the first and second batches was evident at two and eight months of

ages. Consistent with this, Lee and Zucker (1988) reported that pups born to dams exposed to shorter day length before gestation, had thicker pelage even at 49 days of age.

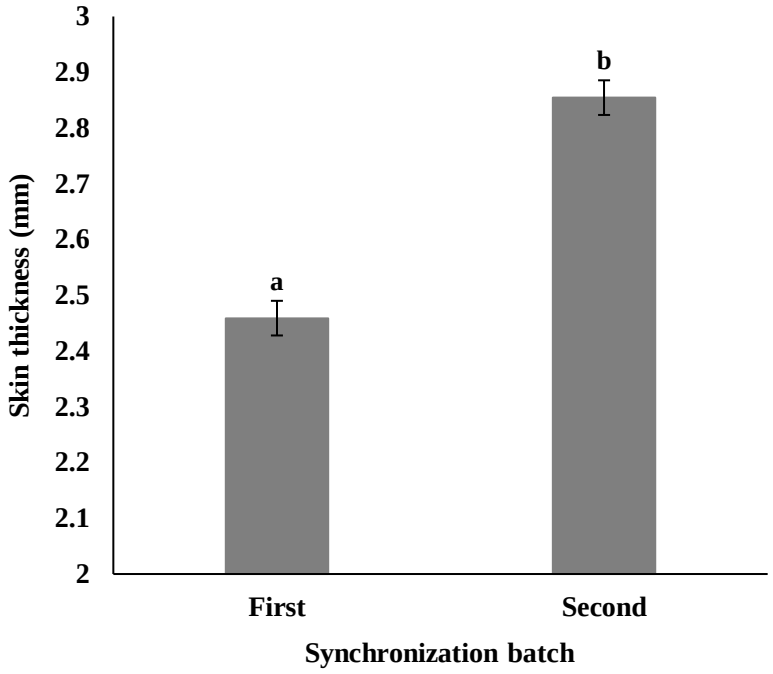


Figure 5.5. The effect of estrous synchronization batch on skin thickness (mm) measured at birth. All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.0001).

Average temperature on the day of birth had an effect (P<0.05) on skin thickness in the new-born lambs with a 1°C decrease in average temperature being associated with 0.02± 0.01 mm increase in skin thickness. The increase in skin thickness with decreasing temperature might be an adaptive response that lambs showed to better survive the cold environment they faced.

5.4.1. Repeatability of ultrasound scanning

The results of our study found a high within-day repeatability of 0.75 (the intra-class correlation of skin thickness measurements taken twice on 74 lambs on the same day at five months of age). Also as a measure of repeatability, a high Pearson correlation coefficient of 0.75 (P<0.0001) was indicated between the two repeated ultrasound measurements of skin thickness taken on the same day (Figure 5.6), which is in line with the repeatability calculated by intra-class correlation. These results demonstrate the reliability of the ultrasound skin thickness measurements taken by the operator. Although repeatability of ultrasound measurements has been widely evaluated in different livestock species for fat depth and muscle depth (McLaren *et al.*, 1991, Gilmour *et al.*, 1994, Herring *et al.*, 1994, Hassen *et al.*, 1998), the current experiment is the first repeated study reporting the repeatability of skin thickness measured by ultrasound.

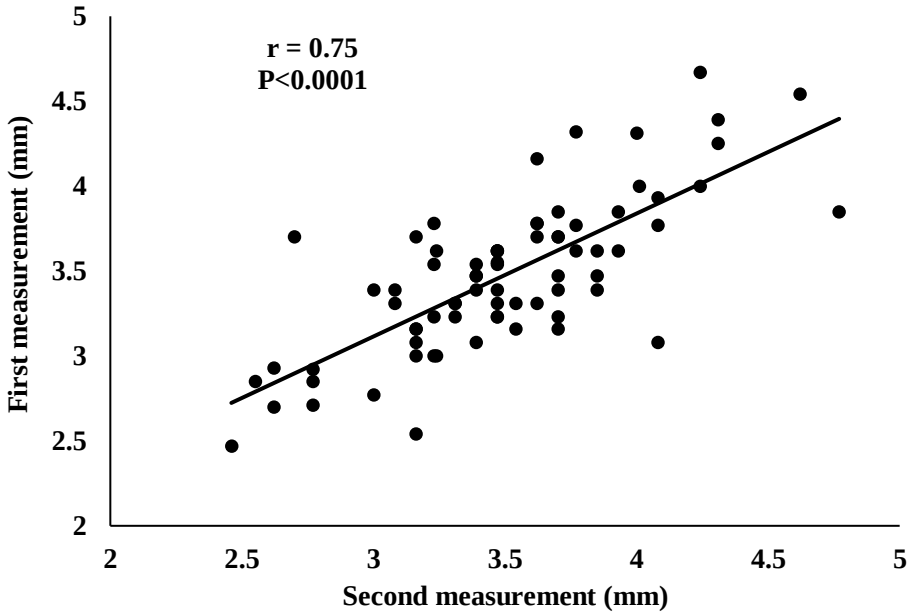


Figure 5.6. The correlation of the two repeated ultrasound measurements of skin thickness taken on the same day at five months of age. Pearson correlation coefficient has been indicated by r.

5.4.2. Changes of skin thickness from birth to 8 months of age

As presented in Table 5.1, from birth to three months of age, the mean skin thickness increased to 4.11 mm, followed by two consecutive decreases (3.99 mm and 3.47 mm at four and five months, respectively). Then, the mean skin thickness again showed an increase at six months of age (3.78 mm), followed by a decrease (3.57 mm), then another increase (3.83 mm) in the last two measurements. In a similar long-term study (Wodzicka, 1958a) that monitored skin thickness of 12 Romney wether lambs from 10 days of birth until five months of age at monthly intervals, skin thickness was found to increase by some 14% until the lambs were 5-10 weeks of age. However, contrary to our results, by 13 weeks skin thickness decreased to the original thickness at birth and remained at this value until five months of age.

As shown in Table 5.2, there was no significant correlation ($P>0.05$) between skin thickness at birth and any of the measurements taken between one and five months, while there were significant ($P<0.05$) correlations between skin thickness at birth and the measurements at six ($r = 0.29$), seven ($r = 0.33$), and eight ($r = 0.34$) months.

Table 5.2: Pearson correlation coefficients (below diagonal) among skin thicknesses measured at monthly intervals from birth to 8 months of age, and the number of records used for each pair (above diagonal).

Month	1	2	3	4	5	6	7	8	9
1		92	91	91	86	75	77	76	76
2	0.04		90	90	85	74	76	75	76
3	0.09	0.18		91	86	75	77	76	76
4	-0.13	0.02	0.29**		86	75	77	76	76
5	0.02	0.24*	0.35***	0.36***		71	74	73	73
6	0.22	0.05	0.20	0.26*	0.36**		71	69	69
7	0.29*	0.17	0.12	0.10	0.51***	0.42***		75	75
8	0.33*	0.05	0.14	0.22	0.48***	0.50***	0.61***		75
9	0.34*	0.08	0.23	0.18	0.40***	0.50***	0.50***	0.51***	

Significance: *P<0.05; **P<0.01; ***P<0.001.

Consistent with the correlation coefficients, when the lambs were categorized into three groups of significantly different skin thickness at birth (thin, medium, and thick-skinned), their mean skin thickness (thin-skinned vs thick-skinned) differed only during 5-8 months, but not during first five measurements either adjusted or unadjusted for other significant effects influencing skin thickness (Figures 5.7 and 5.8).

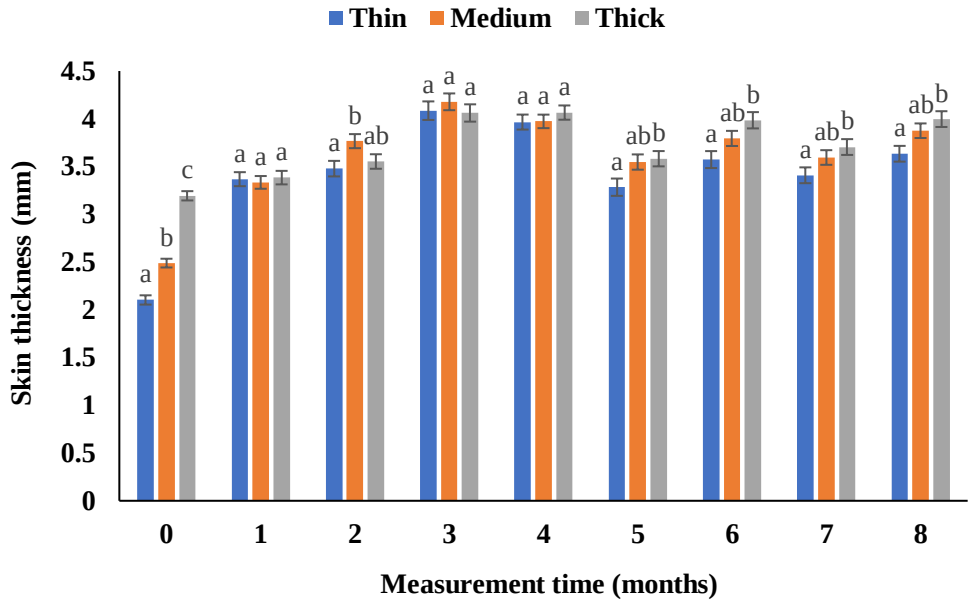


Figure 5.7. The average skin thickness in different skin thickness categories measured at monthly intervals from birth to 8 months of age before making adjustment for other significant effects. All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

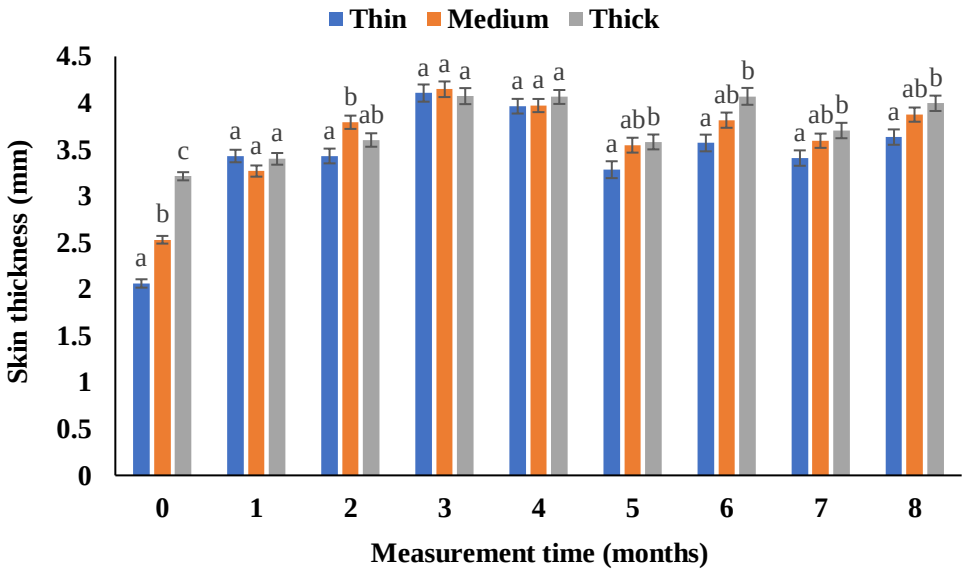


Figure 5.8. The average skin thickness in different skin thickness categories measured at monthly intervals from birth to 8 months of age after making adjustment for other significant effects. All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

The reason behind the trend observed in skin thickening is not clear. This could be attributed, to some extent, to changes in environmental factors like seasonal changes in climate and changes in nutrition that vary over the birth to eight months of age timeframe that might affect skin thickness. Lambs of different skin thickness might show different adaptation responses to survive cold environments they faced, based on their thermoregulatory capabilities during the early stages of life. Since thin-skinned lambs would generally lose more heat from skin surface, they need to thicken their skin more than thick-skinned ones. However, as body size and wool grow, skin might play a lesser role in thermoregulation, which could be the reason for the significant correlations appearing from the sixth month onwards. Similarly, in a study by Wodzicka-Tomaszewska (1960), skin thickness increased in sheep after a period of cold stress. Furthermore, Wodzicka (1958b) observed thickening of the skin, following shearing and this was attributed to cold stress.

On the contrary, shearing of the lambs two weeks before 4-month skin thickness measurement caused a reduction in skin thickness in our study (3.99 mm vs 4.11 mm, respectively for two weeks before and two weeks after shearing). Although this reduction could not be completely attributed to the effect of shearing, since skin thickness was not measured immediately before and after shearing. So, there might have been changes in skin thickness during the two-week period from 3-month skin thickness measurement to shearing that have not been seen. Also, skin thickness might possibly have increased as a result of cold stress due to shearing and then decreased as wool grew a few days after shearing. However, since skin thickness was measured two weeks after shearing, this increase could not be detected in the present study.

5.4.3. The effect of skin thickness category on growth rate and live weight

To test how skin thickness at birth might be associated with growth, live weights of the lambs in the three skin thickness categories measured at monthly intervals, were compared. Adjustment was

made for other significant effects influencing growth rate and live weight to ensure, as far as possible, that any difference in these traits observed between the three groups would be due to a difference in skin thickness not any other effect. There were no differences (P>0.05) between growth rates of lambs of different skin thickness categories calculated from birth to any of the monthly measurements (Figure 5.9).

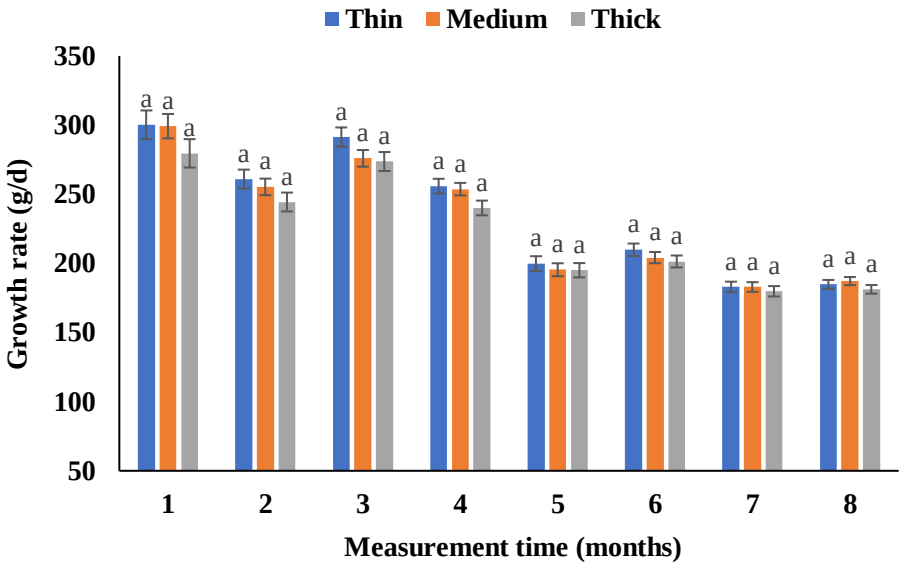


Figure 5.9. The effect of skin thickness category at birth on growth rates measured at monthly intervals from birth to 8 months of age after making adjustment for other significant effects. All values are presented as Least Squares Means ± standard errors. Values for each measurement are growth rates from birth to each corresponding point. Values with the same letters are not significantly different (P>0.05).

In terms of live weight, only weight at birth was associated with skin thickness category, so that lambs of thick-skinned group had greater (P<0.05) birth weight than their thin-skinned peers (Figure 5.10). However, there was no difference (P>0.05) between birth weight of medium-skinned group and the other two skin categories. Also, consistent with the finding that skin thickness at birth was not associated with growth rate from birth onwards, none of live weights

measured at one month of age to eight month of age were affected (P>0.05) by skin thickness category at birth (Figure 5.10). Therefore, based on the results of the present study, selection for increased skin thickness at birth for better thermoregulation would not affect growth from birth to at least eight months of age, which is favourable. However, it should be considered that this lack of association was only based on phenotypic data, not genetic data. So, further investigations are required to examine possible genetic association of skin thickness and weight at different ages.

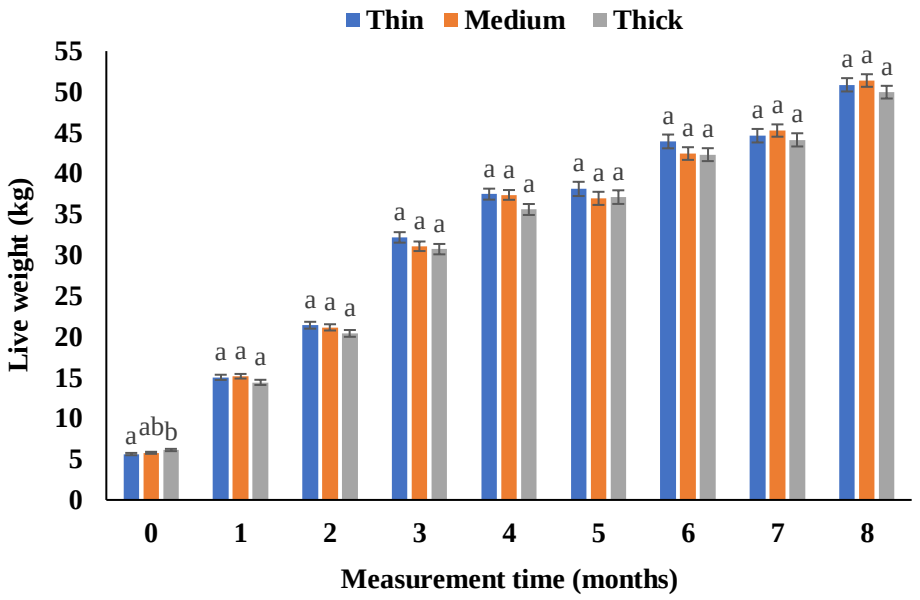


Figure 5.10. The effect of skin thickness category at birth on live weights measured at monthly intervals from birth to 8 months of age after making adjustment for other significant effects. All values are presented as Least Squares Means ± standard errors. Values with the same letters are not significantly different (P>0.05).

5.5. Conclusion

In conclusion, skin thickness measured at eight months of age is a moderately reliable indicator of skin thickness at birth. Further, the findings of this study support the idea that the positive genetic correlation of skin thickness at eight months of age with lamb survival found in chapter four might

be due to improved thermoregulation through less heat loss from skin surface in new-born lambs. The correlation found is of high importance from both practical and economic points of view, since measuring skin thickness at eight months of age is much easier than at birth. Furthermore, ultrasound measurement of skin thickness at eight months of age, instead of at birth, facilitates simultaneous recording of other traits of importance like fat depth and eye muscle depth, which are normally measured at this age, consequently saving money and time. Nevertheless, it should be noted that some breeders make selection decisions based on ultrasound measurements made at an earlier age like four to five months of age at which skin thickness was not a reliable indicator of the trait at birth based on our results. Therefore, doing the same experiment in larger populations to find if skin thickness measured at an early age of four to five months could be a reliable indicator of the trait at birth or not, would be promising. Also, since this study was performed on a small number of lambs of only one breed, this study could be repeated on a larger population of other breeds. It would also be of interest to do a similar experiment in different seasons and/or on housed lambs with controlled experimental conditions and see how skin thickness changes from birth to an older age, as well as skin thicknesses measured at different ages would be correlated. Finally, from a biological perspective, it would be interesting to investigate further to find out if at all and when the skin thickness in growing lambs would return back to birth-level.

Chapter 6

The effect of genetic variation in the uncoupling protein 1 (*UCP1*), prolactin (*PRL*), and prolactin receptor (*PRLR*) genes on cold resistance in new-born lambs

6.1. Abstract

This study aimed to explore the possible effect of genetic variation in three possible candidate genes (uncoupling protein 1 (*UCP1*), prolactin (*PRL*), and prolactin receptor (*PRLR*)) on cold resistance in new-born lambs exposed to cold-stress. Sixty four Romney-type lambs (32 lambs with the thickest skin (thick-skinned category) and 32 lambs with the thinnest skin (thin-skinned category)) were selected based on their skin thickness measured on the left dorsal loin region of the lambs around the 12th rib through ultrasound scanning performed within 24 hours of birth. Lambs were exposed to a period of cold stress and their heat production were calculated using oxygen consumption measured by indirect open-circuit calorimetry. Skin surface temperature (as an indicator of heat loss) on left dorsal loin region was obtained during the experiment using an infrared thermography (IRT) camera. Four different regions of ovine genome including 1) part of the promoter of the *UCP1* gene, 2) exon 5 of the *UCP1* gene, 3) part of intron 2, exon 3, as well as part of intron 3 of the *PRL* gene, and 4) part of intron 1 of the *PRLR* gene were chosen for analysis. Genetic variations were detected by polymerase chain reaction single-stranded conformational polymorphism (PCR-SSCP). Only one SSCP banding pattern was detected for the amplicon of the exon 5 of the *UCP1* gene. Therefore, no further association analysis was performed for that region. Lambs of genotype AA of the *UCP1* gene promotor had lower ($P<0.05$) skin temperature compared to those of genotype BB at the last two time-points of calorimetry, as well as lower ($P<0.05$) overall temperature. Lambs of genotype AA tended ($P<0.1$) to have lower skin temperature compared to those of genotype AC at the first and last time-points, and overall. Lambs of genotype AB tended ($P<0.1$) to have lower skin temperature compared to those of genotype BB at the last two time-points. Compared to genotype AA, lambs of genotype AB tended ($P<0.1$) to have higher amounts of heat production (W Kg^{-1}) during the second time-period.

Furthermore, lambs of genotype BB tended ($P<0.1$) to have lower rectal temperature compared to those of genotype AB at the second time-point. In case of the *PRL* gene, compared to AA genotype, lambs of genotype AB produced higher ($P<0.05$) amount of heat during the last time-period of calorimetry, while having lower rectal temperature at the first two time-points ($P<0.05$ and $P<0.06$, respectively). However, polymorphism in the amplified region of the *PRLR* gene did not show any effect on any of the cold resistance indices. Although genetic variations in the *UCP1*, *PRL*, and *PRLR* genes had effects on some of the cold resistance indices at/during some time-points/periods, those associations seem to be mostly due to biases resulting from low number of lambs. Therefore, further studies using larger populations and other breeds are needed to confirm these findings.

6.2. Introduction

Starvation/exposure is a major contributor to lamb mortality during the first few days of life (McCutcheon, 1981). Improved thermoregulation through decreased heat loss from skin surface can act to reduce the amount of heat production required to maintain body temperature (chapter four). Nevertheless, in order to maintain its core body temperature, it is vital for new-born lamb to produce enough heat to compensate for any heat lost from body.

Thermogenesis, therefore, can play an important role in lambs at risk of cold exposure. Non-shivering thermogenesis in brown adipose tissue (BAT) is a major source of heat production in the new-born lamb (Alexander and Williams, 1968). It accounts for approximately one-half of heat generated during summit metabolism in new-born lambs exposed to cold stress (Stott and Slee, 1985, Slee *et al.*, 1987). Brown adipose tissue is able to produce large amounts of heat due to the presence of a unique uncoupling protein 1 (UCP1) on the inner mitochondrial membrane (Cannon and Nedergaard, 2004) which uncouples oxidative phosphorylation from adenosine triphosphate (ATP) synthesis and releases energy as heat. Therefore, rapid activation of UCP1 is critical in

preventing hypothermia in the new-born (Clarke *et al.*, 1997b). UCP1 is first activated at birth following cold exposure to the extra-uterine environment and intense endocrine stimulation (Symonds, 2013, Symonds *et al.*, 2015). A failure to utilize non-shivering thermogenesis during neonatal development is associated with unexpected death (Symonds *et al.*, 1989).

In the ovine fetus BAT is mainly found in the perirenal region and accumulates rapidly relative to body weight between 70-120 days of pregnancy (Alexander, 1978). After this stage only a small amount of BAT accumulation is evident compared to total fetal growth. A key stage in the maturation of BAT is the gradual rise in the abundance of UCP1 (Symonds *et al.*, 2003b). Fetal UCP1 is present from the beginning of the third trimester and peaks around the time of birth (Clarke *et al.*, 1997a) and its activity also increases with gestational age and reaches a maximum just after birth (Clarke *et al.*, 1997b). During late gestation, when the amount and activity of UCP1 rises, an increase in the plasma concentrations of catecholamines, thyroid hormones, cortisol, leptin and prolactin (PRL) has been observed that may act individually, or in combination, to promote UCP1 expression (Symonds *et al.*, 2003b).

Prolactin administration throughout gestation to the mother, which results in substantial transfer of prolactin into the fetus (Yang *et al.*, 2002), has been shown to promote UCP1 abundance in the rat fetus (Budge *et al.*, 2002). Furthermore, it has been shown that administration of PRL can elicit a thermogenic effect in neonatal sheep through an increase in lipolysis occurring in conjunction with a rise in the thermogenic potential of UCP1, suggesting a potential role for PRL in the regulation of UCP1 expression and function (Pearce *et al.*, 2005a). Hence, PRL can act as a potential thermogenic hormone over the first few days of postnatal life.

Prolactin receptor (PRLR) through which PRL exerts its physiological roles, can also possibly have an effect on BAT activity. The stage of fetal development at which PRLR mRNA becomes

abundant coincides with the first appearance of UCP1 (Symonds *et al.*, 1998). Increase in UCP1 abundance in fetuses of well-fed sheep has also been reported to be closely associated with increased abundance of PRLR-1 (Budge *et al.*, 2000). Furthermore, the abundance of both UCP1 and PRLR peak at birth (Casteilla *et al.*, 1989, Clarke *et al.*, 1997a) coincides with maximum expression of other mitochondrial membrane proteins within adipose tissue, including voltage-dependent anion channel (VDAC) (Mostyn *et al.*, 2003), which has a role in regulating the supply of mitochondrial adenosine diphosphate (ADP) and ATP (Gottlieb, 2000). Also, the results of a study on new-born lambs has shown that the disappearance of UCP1 from BAT is very closely correlated with loss of the PRLR in the tissue (Pearce *et al.*, 2005a). Hence, in addition to *UCP1, PRLR* can also be considered as a marker of brown adipose tissue function (Pope *et al.*, 2014).

Due to their effects on BAT and consequently on thermogenesis in new-born lambs, any change in the sequence of genes translating UCP1, PRL, and PRLR that might result in a modification in either gene expression or structure of the translated protein, could possibly lead to a change in BAT and as a result, in thermoregulation. Although genetic variation and its effect on some economic traits have been studied for the ovine *UCP1* (Yang *et al.*, 2014, An *et al.*, 2018), *PRL* (Vincent and Rothschild, 1997, Chu *et al.*, 2009, Staiger *et al.*, 2010, Miltiadou *et al.*, 2017), and *PRLR* (Chu *et al.*, 2007, Ozmen *et al.*, 2011, Wang *et al.*, 2015, Dettori *et al.*, 2020) genes, to our knowledge, there is no published study reporting association of genetic variation in these genes with thermoregulation in new-born lambs.

Therefore, the objective of the present study was to identify genetic variations in the *UCP1*, *PRL*, and *PRLR* genes using polymerase chain reaction single-stranded conformational polymorphism

(PCR-SSCP) analysis and evaluate the effects of variations on a few indices of cold resistance in New Zealand (NZ) Romney-type new-born lambs exposed to cold stress.

6.3. Materials and methods

6.3.1. Experimental animals, calorimetry under cold stress and phenotype recording

This study utilized phenotype data from the new-born lamb thermoregulation study (described in chapter four and summarized as follows). The original study protocol was approved by the Massey University Animal Ethics Committee (Protocol#16-21). Sixty four Romney-type lambs (32 lambs with the thickest skin (thick-skinned category) and 32 lambs with the thinnest skin (thin-skinned category)), born to 215 ewes (estrous synchronized in two batches, a week apart) were selected based on their skin thickness measured on the left dorsal loin region of the lambs around the 12th rib through an ultrasound scanning as described in chapter four. The lambs were weighed, wool removed from the back and sides and baseline (room temperature ranged between 11 and 16°C) skin surface temperature recorded (as described in chapter four) for 10 min using an infrared thermography camera (Fluke TiX500, Fluke Corporation, Everett, WA, USA). The lambs were then subjected to calorimetry (in pairs) in a cold chamber (described in chapter four). In the cold chamber, the lambs were exposed to an initial ambient temperature of 4°C for 12 minutes (referred to as the adjustment period (min -12 to 0), after which artificial chilled rain (on the ventral surface) and moving air (from behind) were applied to induce summit metabolism (maximal heat production). This cold stress period was divided into four consecutive stages indicated as A-B (min 0 to 18), B-C (min 18 to 36), C-D (min 36 to 54), and D-E (min 54 to 90). Rectal temperature, dorsal skin surface temperature and oxygen consumption were recorded throughout the period of calorimetry. Calorimetry was continued until min 90 or until the lambs reached maximal heat production (MHP), whichever occurred first. Summit metabolism (MHP) was assumed to have

been reached when the rectal temperature of the lamb declined at the rate of 1°C/20 min and there was no further increase in the rate of oxygen consumption (Alexander, 1962b). Heat production, as an indicator of metabolic rate, was calculated from oxygen consumption (Revell *et al.*, 2002).

The following phenotypes (from the original study) were used for this study:

1) Skin surface temperature at different time-points
2) Rectal temperature at different time-points
3) Heat production during different time-periods during calorimetry
4) MHP
5) Time to reach MHP

In order to make an adjustment for different weather conditions each lamb experienced on the day of birth, an average cold stress index (CSI) was also calculated (Donnelly, 1984) and included in the models used for the statistical analysis.

6.3.2. Blood collection and DNA extraction

A 10 ml blood sample was taken from each lamb using jugular venipuncture with Lithium Heparin tube. All blood samples were placed on ice immediately after being taken and then stored at −20°C until DNA extraction. Genomic DNA was extracted from the whole blood samples using PureLink Genomic DNA Mini Kit (Invitrogen, CA, USA) according to the manufacturers' instructions. The quantity and quality of isolated genomic DNAs were assessed by a NanoDrop Spectrophotometer. The purified DNA samples were then stored at −20°C until PCR amplification.

6.3.3. PCR amplification

Four different regions of ovine genome including 1) part of the promoter of the *UCP1* gene, 2) exon 5 of the *UCP1* gene, 3) a segment spacing part of intron 2, exon 3, as well as part of intron 3

of the *PRL* gene, and 4) part of intron 1 of the *PRLR* gene were chosen for analysis. An effort was made to choose those regions of the genes that had been reported to be moderately polymorphic based on previous studies. Since a small number of animals were used in this experiment, choosing a highly polymorphic region would have resulted in a high number of genotypes for each region. This could lead to excluding some animals (genotypes) from the genetic association analyses due to considering a minimum sample size of five for each genotype to be included in the analyses. On the contrary, choosing a lowly polymorphic region could lead to finding no polymorphism in a region with no statistical analysis performable for that region. The sequence of the primers used for the amplification of the selected regions were based of previous studies as presented in Table 6.1.

Table 6.1. The sequence of primers used to amplify different regions of the *UCP1*, *PRL*, and *PRLR* genes.

Gene - region amplified	Primer sequence	Amplicon size (bp)	Reference
UCP1 - promoter	F: AGA TAC AAG CGG AAG AGA CAC R: TGA AGG GTT GGG TCT GTC A	352	(Yang *et al.*, 2014)
UCP1 – exon 5	F: TGT TGA CCT GTC TCA TCG R: TAG ATA CAG AAG AAC ACA TC	234	(Yang *et al.*, 2014)
PRL – partial intron 2-exon 3-partial intron 3	F: GCC CAA ACA ACC CTA ATG AA R: CGT GAA GCC AGG TAA CAT CA	219	(Miltiadou *et al.*, 2017)
PRLR – intron 1	F: TGT CAG TAA GCG TCA GAG GGC R: GGC TGG TGG AAG GTC ACT CTT	176	(Chu *et al.*, 2007)

PCR amplifications were carried out using a thermal cycler in a final volume of 50 µL containing 25 ng of genomic DNA, 90 nmol of $MgCl_2$, 20 nmol of each dNTP, 20 pmol of each primer, 1 U of Platinum®Taq DNA polymerase (Invitrogen, CA, USA), and 1X reaction buffer. The thermal cycling conditions for the four regions amplified were 5 min initial denaturation at 95°C, followed

by 35 cycles [denaturation for 30 s at 95°C, annealing for 30 s at an optimized annealing temperature for each region (*UCP1* - promoter at 56°C, *UCP1* - exon 5 at 54°C, *PRL* - partial intron 2-exon 3-partial intron 3 at 56°C, and *PRLR* - intron 1 at 56°C) and extension at 72°C for 30 s], with a final extension for 7 min at 72°C.

PCR products were then subjected to electrophoresis on 2.5% agarose gel in TBE (tris borate EDTA) buffer, containing 200 ng/ml of ethidium bromide. After completion of the electrophoresis, the gels were exposed to ultraviolet light and the images of the gels taken using a gel documentation system. The amplified PCR products were then stored at −20°C until SSCP analysis.

6.3.4. SSCP analysis

An aliquot of 3 μL (25 ng) of each amplicon was mixed with 9 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol). After denaturation at 95°C for 12 min, samples were cooled rapidly on wet ice and then loaded onto 16 × 20 cm acrylamide/bisacrylamide (37.5:1) (Bio-Rad Laboratories, CA, USA) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad Laboratories, CA, USA). Conditions used for each of the four amplicons have been presented in Table 6.2.

Table 6.2. Conditions used for single-stranded conformational polymorphism (SSCP) analysis of each amplicon.

Amplicon	Acrylamide gel (%)	Voltage (V)	Temperature (°C)	Time (h)
UCP1 - promoter	14	225	15	18
UCP1 – exon 5	14	250	18	18
PRL – Partial intron 2-exon 3-partial intron 3	12	250	15	18
PRLR – intron 1	11	250	15	18

After completion of the electrophoresis, gels were silver-stained according to a method described by Byun *et al.* (2009). In this method, gels were fixed and stained in a solution containing 10% ethanol, 0.5% acetic acid, and 0.2% silver nitrate (for anywhere between 3 and 20 min). The gels were then rinsed with distilled water once and then developed with a solution of 3% NaOH and 0.1% HCOH (pre-warmed to 55°C) until dark staining bands appeared on the yellow background of the PCR-SSCP gels (varied between 5 and 10 min). Development was then stopped with a solution containing 10% ethanol and 0.5% acetic acid. SSCP banding patterns could be then observed directly from the gels.

6.3.5. Sequencing and sequence analysis

PCR amplicons representative of different SSCP patterns (genotypes) for each gene were submitted to the Massey Genome Service (Palmerston North, NZ) for Sanger sequencing. In order to increase the accuracy of the sequencing, for each SSCP pattern, three PCR amplicons were sequenced, unless there was less than three samples for any given pattern. Sequencing was performed individually for each of those three PCR amplicons. The whole length was sequenced from both sides for each amplicon. To improve the quality of the sequencing, the PCR products selected for sequencing were first purified using PureLink PCR purification kit (Invitrogen, CA, USA) following the manufacturer protocol to remove primers, primer dimers, short spurious PCR products, dNTPs, enzymes, and salts from the PCR products. Sequence alignments, comparisons, and translations were performed using the geneious software, version 9.1.8 (https://www.geneious.com).

6.3.6. Statistical analysis

Skin surface temperatures on left dorsal loin region taken at the five selected time-points were analyzed by repeated measures ANOVA using the PROC MIXED of the SAS software (SAS,

2011). The time-points included baseline (last min of the 10-min period prior to the calorimetry at a room temperature of 11-16°C), point A (min 0, the end of cold adjustment period), point B (min 18), point C (min 36), and point D (min 54). The effect of genotype on CSI was assessed independently for each region using separate models. The initial models contained the effects of genotype, sex (male and female), birth rank (single and twin), oestrous synchronization batch (first and second), skin thickness (ST) category (thick and thin), time point (baseline, A, B, C, and D), and their two-way interactions as fixed effects and body weight and average CSI on the day of birth as covariates.

For statistical analysis, genotypes were coded as categorical (AA, AB, BB, …) not numerical (0, 1 and 2). Also, no linkage disequilibrium was considered between different regions, and statistical analyses were performed independently for each region using separate models for each region. Owing to having a low number of samples used in this study, considering linkage disequilibrium and generating different haplotypes from a combination of alleles from different regions could lead to excluding some animals from the analyses due to not having a minimum sample size of five for each haplotype to be included in the analyses. Since only one genotype (SSCP banding pattern) was detected for the exon 5 of the *UCP1* gene, no further statistical analysis was performed for this region. Furthermore, for any given region, a minimum sample size of five was considered for each genotype to be included in the analyses. As a result, the number of lambs included in the analysis for the promotor of the *UCP1* gene, *PRL* and *PRLR* genes were 62, 57, and 61, respectively.

Some of the fixed effects and covariates that were revealed to have a significant ($P<0.05$) influence on CSI in our previous study on the same lambs (chapter four), were only significant at $P<0.1$ (not at $P<0.05$) in the current study. One reason for this could be having less lambs used in the analyses

of the present study compared with 64 lambs used in the previous experiment (chapter four). Another reason could be the effect of genotype that were included in the current analyses. However, excluding these effects from the final models could possibly lead to unreliable results due to biases. Therefore, all the fixed effects and covariates that were revealed to have a significant ($P<0.05$) influence on CSI in the previous study, were included in the final models if they were significant at $P<0.1$ instead of $P<0.05$. The final models for all three regions included the effects of time-point, birth rank, ST category, genotype, time-point*genotype, time-point*ST category (except for the *PRLR* gene), and sex*ST category.

Similarly, for rectal temperature, measurements recorded at the five time-points (baseline, A, B, C, and D) were considered as repeated measures and therefore analysed by repeated measures ANOVA using the PROC MIXED of the SAS software (SAS, 2011). The fixed effects and covariates used in the initial models were the same as those used in the analysis of skin temperature. However, after excluding the non-significant fixed effects ($P>0.1$), interactions ($P>0.1$) and covariates ($P>0.1$), the final models for all three regions included the effects of time-point, ST category, genotype, time-point*genotype, time-point*ST category, and average CSI.

A repeated measures ANOVA using the PROC MIXED of the SAS software (SAS, 2011) was carried out also for the analysis of heat production as five averages of heat production calculated for each of the five time-intervals (adjustment (min -12 to 0), A-B (min 0 to 18), B-C (min 18 to 36), C-D (min 36-54), and D-E (min 54 to 66)) as the repeated measures of heat production. In addition to the fixed effects and covariates used in the analysis of skin temperature, rectal temperature at the onset of adjustment period was included as a covariate in the initial models. After removing the non-significant fixed effects ($P>0.1$), interactions ($P>0.1$) and covariates ($P>0.1$), the effects of time-point, birth rank (except for the *PRLR* gene), ST category, genotype,

time-point*genotype, rectal temperature at the start of adjustment period remained in the final models for all three regions. Due to a temporary fault in the calorimeter, oxygen consumption measured in the first and second readings were not correct for six lambs. Therefore, for those six lambs the averages of heat production calculated for the adjustment period (min -12 to 0) were excluded from the analysis.

For all the three above-mentioned analyses using repeated measures ANOVA, three commonly used covariance structures including unstructured (UN), autoregressive (AR(1)), and compound symmetric (CS), were applied. The type of covariance structure used in the final models was based on model fit that was determined by the Akaike information criterion (AIC), where smaller values indicate better fit. The unstructured (UN) type of covariance structure was used in the final models for the analysis of skin temperature and rectal temperature. However, for the analysis of heat production, autoregressive (AR(1)) type of covariance structure was used in the final model due to an error (Convergence criteria met but final hessian is not positive definite) after running the model with the unstructured (UN) type. Also, for the data analysed by repeated measures ANOVA, the random effect of animal was included in the initial models. But the effect was excluded from the final models since either it did not improve model fitness or the model with the random effect of animal resulted in an error after running the model (convergence criteria met but final hessian is not positive definite or stopped because of infinite likelihood).

The maximum heat production and time to reach maximum heat production were analyzed by the PROC MIXED of the SAS software (SAS, 2011) with genotype, sex, birth rank, synchronization batch, ST category, and their two-way interactions as fixed effects and body weight, rectal temperature at the start of adjustment period, and average CSI on the day of birth as covariates included in the models. After excluding the non-significant fixed effects ($P>0.1$), interactions

(P>0.1) and covariates (P>0.1), the effects of genotype and rectal temperature at the start of adjustment period remained in the final models for maximum heat production, and genotype, category (only for the *UCP1* gene), body weight and rectal temperature at the start of adjustment period in the final models for time to reach maximum heat production.

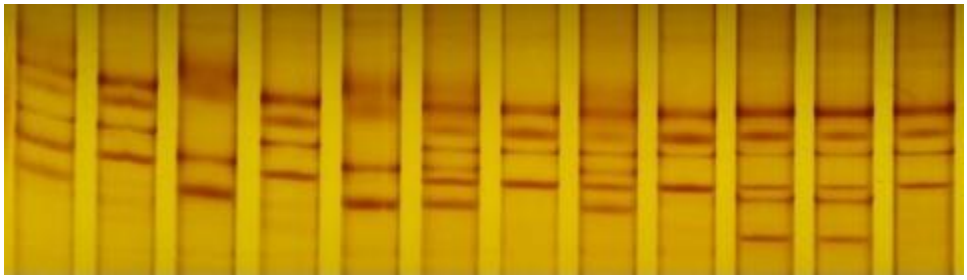
A lamb was considered as being not able to maintain its body temperature during the cold stress period if the lamb experienced four consecutive falls of at least 0.2°C in rectal temperature per each 3-minute period. Otherwise, it was considered as being able to maintain its body temperature during the cold stress period. A logistic regression model using PROC LOGISTIC of the SAS software (SAS, 2011) was used to identify factors affecting the likelihood of a lamb maintaining its body temperature during the cold stress period. The fixed effects and covariates used in the initial models were the same as those used in the analysis of maximum heat production. However, after excluding non-significant fixed effects (P>0.1), interactions (P>0.1) and covariates (P>0.1), only the effects of genotype, ST category and birth weight were retained in the final models.

6.4. Results and discussion

6.4.1. Variations detected in the *UCP1*, *PRL* and *PRLR* genes

The results of SSCP analysis for the 352-bp amplicon of the promoter region of the *UCP1* gene revealed five SSCP banding patterns, genotypes AA, BB, AB, AC, and AD (Figure 6.1). Sequencing of the amplicons corresponding to these banding patterns detected three SNPs in the promoter region (Figure 6.2). All of these three SNPs were previously reported by the International Sheep Genomics Consortium (ISGC) in the public database European Variation Archive (http://www.ebi.ac.uk/eva/). The two SNPs in the positions 1060 and 1095 were previously detected in the NZ Suffolk and NZ Romney sheep (Yang *et al.*, 2014) and a variety of sheep breeds

from NZ and China (An *et al.*, 2018), and have been deposited into the National Center for Biotechnology Information (NCBI) GenBank with accession numbers KC243136 - KC243138.



AD AA BB AA BB AB AA AB AA AC AC AA

Figure 6.1. The single-stranded conformational polymorphism (SSCP) patterns detected in the promoter region of the *UCP1* gene.

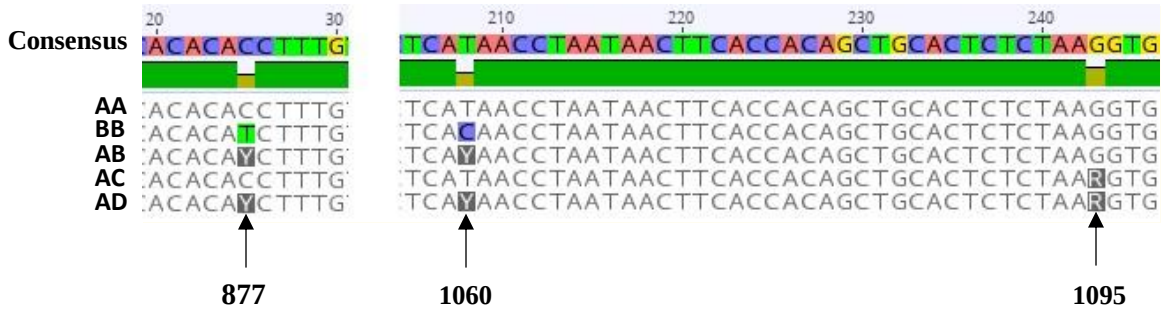


Figure 6.2. Sequence comparison of different genotypes of the promotor region of the ovine *UCP1* gene. The SNP positions shown by arrows are numbered relative to GenBank JN604985.1. Y:CT, R:AG.

As evident in Table 6.3, genotype AA of the promoter of the *UCP1* gene had the highest frequency followed by AB, AC, BB, and AD. Also, the A allele showed the highest frequency followed by alleles B, C, and D (Table 6.3).

Table 6.3. Allelic and genotypic frequencies of identified variants in the promoter of the *UCP1* gene.

Allele	Frequency	Genotype	Frequency	Number
A	0.66	AA	0.41	26
B	0.27	BB	0.09	6
C	0.06	AB	0.34	22
D	0.02	AC	0.13	8
		AD	0.03	2

Only one SSCP banding pattern was detected for the 233-pb amplicon of the exon 5 of the *UCP1* gene in all the lambs studied (Figure 6.3). Therefore, no further sequencing and association analysis was performed for this region. In line with this finding, no variation had been observed in the same region of the *UCP1* gene in a previous study on the NZ Suffolk and NZ Romney sheep (Yang *et al.*, 2014).

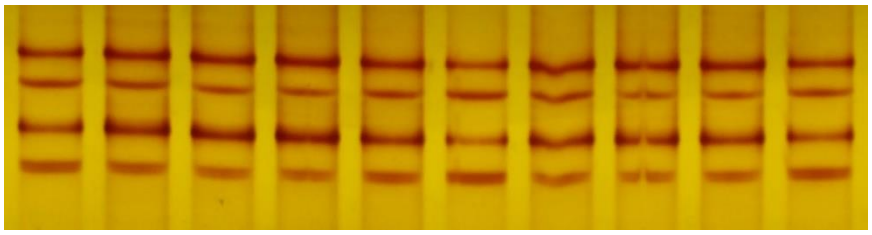


Figure 6.3. The single-stranded conformational polymorphism (SSCP) banding pattern in the exon 5 of the *UCP1* gene.

The results of SSCP analysis of the 219-bp amplicon of the *PRL* gene revealed six banding patterns, genotypes AA, BB, AB, AB, AC, AD, and CD (Figure 6.4). Sequencing of the amplicons corresponding to these banding patterns detected three SNPs in the amplified region of the *PRL* gene in our study (Figure 6.5). Two out of the three SNPs were located in the exon 3 of the gene (positions 2015 and 2101), while the other was in the intron 3 region (position 2131). All of these

three SNPs were previously reported by the ISGC in the public database European Variation Archive (http://www.ebi.ac.uk/eva/). The two SNPs in the positions 2015 and 2101 were previously detected in the Chios sheep from Cyprus (Miltiadou *et al.*, 2017).

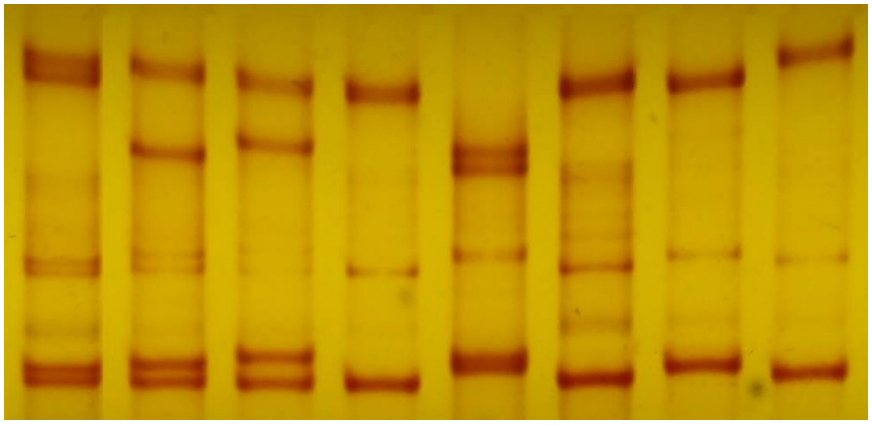


Figure 6.4. The single-stranded conformational polymorphism (SSCP) patterns detected in the amplified region of *PRL* gene.

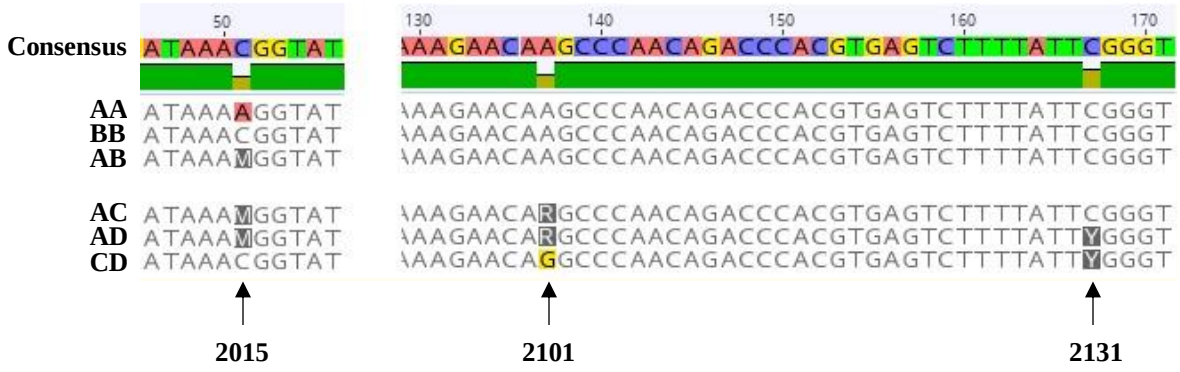


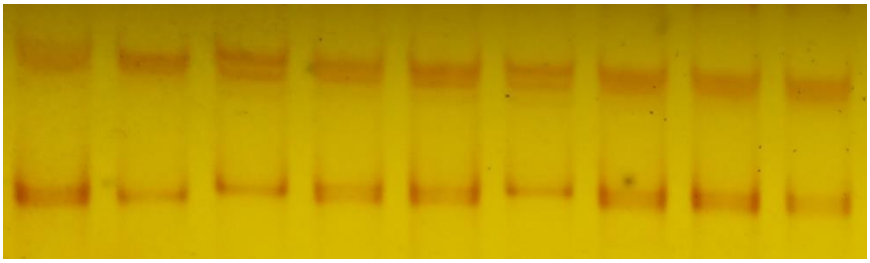
Figure 6.5. Sequence comparison of different genotypes of the exon 3 and intron 3 regions of the ovine *PRL* gene. The SNP positions shown by arrows are numbered relative to GenBank KC764410.1. M:CA, R:AG, Y:CT.

As presented in Table 6.4, genotype AA of the *PRL* gene had the highest frequency followed by AB, and AC, while genotypes BB, AD, and CD had the lowest frequency. Furthermore, allele A showed the highest frequency followed by alleles B, C, and D (Table 6.4).

Table 6.4. Allelic and genotypic frequencies of identified variants in the *PRL* gene.

Allele	Frequency	Genotype	Frequency	Number
A	0.87	AA	0.77	49
B	0.08	BB	0.02	1
C	0.04	AB	0.13	8
D	0.02	AC	0.06	4
		AD	0.02	1
		CD	0.02	1

The results of our SSCP analysis for the 176-pb amplicon of the intron 1 of the *PRLR* gene revealed three banding patterns, genotypes AA, BB, AB (Figure 6.6), resulting from only one SNP detected in the region (position 282, Figure 6.7). The existence of nucleotide C in position 282 of the 176-pb amplicon sequenced in our study is in accordance with the sequence of part of the ovine *PRLR* gene deposited into the NCBI GenBank with accession number AF042358.1. The presence of a T nucleotide observed in genotype AA in the corresponding position of the gene was reported in the Chios, White Karaman and Awassi sheep breeds (Ozmen *et al.*, 2011), and has been deposited into the NCBI GenBank with accession numbers HM437210- HM437214. The SNP found in the present study (C to T at position 282) was not reported by any other study, although similar C to T SNP was reported at an adjacent position (283) in the same region of the *PRL* gene in Small Tail Han ewes (Chu *et al.*, 2007). Also, they reported another polymorphism at position 373 (T to G) in the same population that was not observed in our study.



AB AA BB AB AB BB AB AB AB

Figure 6.6. The single-stranded conformational polymorphism (SSCP) patterns detected in intron 1 region of the *PRLR* gene.

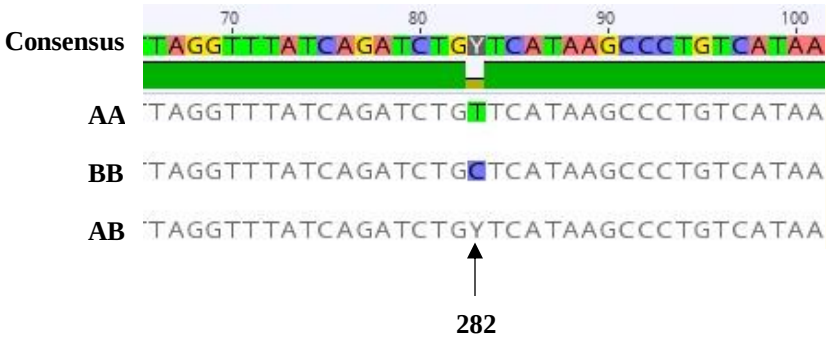


Figure 6.7. Sequence comparison of different genotypes of the intron 1 region of the ovine *PRLR* gene. The SNP position shown by arrow is numbered relative to GenBank AF042358.1. Y:CT.

As presented in Table 6.5, genotype AB of the *PRLR* gene had the highest frequency followed by AA, and BB, with the allele A showing higher frequency compared to allele B.

Table 6.5. Allelic and genotypic frequencies of identified variants in the *PRLR* gene.

Allele	Frequency	Genotype	Frequency	Number
A	0.68	AA	0.41	26
B	0.32	BB	0.05	3
		AB	0.55	35

6.4.2. Effect of variation in the *UCP1* gene on indices of cold resistance

To our knowledge, this is the first report describing relationship between variation in the *UCP1*, *PRL*, and *PRLR* genes and thermoregulation in new-born lambs. The impact of other effects like skin thickness, birth rank, sex, birth weight, cold stress index, and rectal temperature at the start of adjustment period on thermoregulation in the same lambs (that were subjects of the current study) has been previously described in detail (chapter four). So, in this chapter only the effect of genetic variation in the *UCP1*, *PRL*, and *PRLR* genes on indices of cold resistance will be discussed.

6.4.2.1. Skin surface temperature

Skin temperature in the loin region of lambs were recorded while the lambs were at room temperature (11 to 16°C) as well as in the cold chamber (2 to 4°C), during calorimetry.

Although five genotypes were detected for the promoter region of the *UCP1* gene (Figure 6.1 and 2), only four genotypes were included in the statistical analyses. Lambs of genotype AD of this region were not included only 2 out of the total 64 lambs had this genotype (Table 6.3).

As presented in Figure 6.9, lambs of genotype AA at the *UCP1* promotor locus had lower ($P<0.05$) overall average skin surface temperature compared to those of genotype BB. Nevertheless, when considering skin temperature at different time-points (Figure 6.8), this difference was only evident at the last two time-points C ($P<0.05$) and D ($P<0.01$). Regarding the lack of a difference between lambs of genotype AA and BB in terms of skin thickness, skin thickness does not seem to be the reason for the observed effect of genotype on heat loss in our experiment. Furthermore, although UCP1 has been shown to have a unique role in heat production in brown adipose tissue (Matthias *et al.*, 2000, Golozoubova *et al.*, 2001, Nedergaard *et al.*, 2001), there is no published literature reporting it to have an impact on heat dissipation from skin surface. Therefore, one possible

reason for this finding might be a bias resulting from the lower number of BB lambs compared to AA ones (6 vs 26 at the first and second time-points, and 5 vs 18, 4 vs 18 and 3 vs 19 at time-points B, C and D, respectively).

Furthermore, lambs of genotype AA tended ($P<0.1$) to have lower overall average skin surface temperature compared to those of genotype AC (Figure 6.9), though when considering skin temperature at different time-points (Figure 6.8), this tendency was only evident at the first and last time-points. For the same reasons as explained for the skin temperature difference observed between lambs of genotype AA and BB, one possible reason for this finding might be a bias resulting from the lower number of AC lambs compared to AA ones (8 vs 26 at the first and second time-points, and 7 vs 18, 6 vs 18 and 5 vs 19 at time-points B, C and D, respectively).

Also, compared to genotype BB, lambs of genotype AB tended ($P<0.1$) to have lower skin temperature at the two last time-points (Figure 6.8), though this tendency was not observed when the overall average skin temperature was considered (Figure 6.9). Similar to what was described for the other two differences (AA vs BB and AA vs AC), this difference could possibly be due to a bias resulting from the lower number of BB lambs compared to AB ones (8 vs 26 at the first and second time-points, and 7 vs 18, 6 vs 18 and 5 vs 19 at time-points B, C and D, respectively).

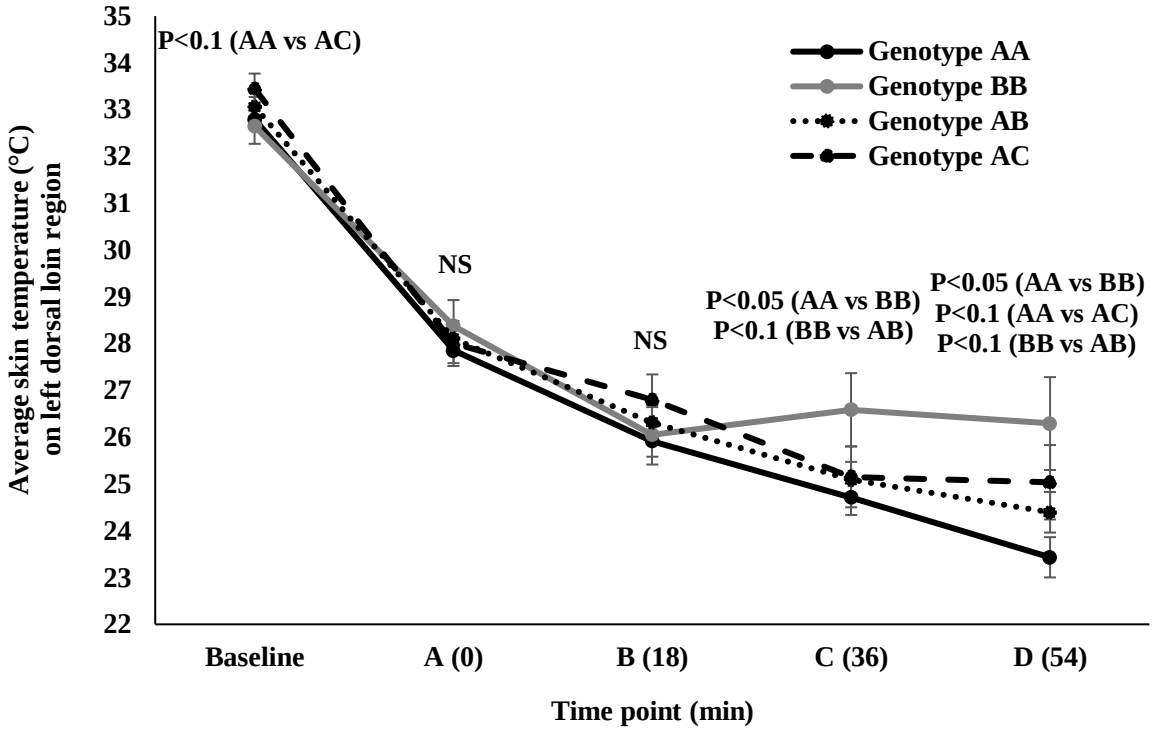


Figure 6.8. The effect of the *UCP1* genotype on skin surface temperature at different time points. Conditions applied were as follows. Baseline: 11-16°C, with no wind or rain; A-B: 4°C, rain (1°C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1°C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1°C) from below (1.08 l/min) and cold air (2.0 m/s). All values are presented as Least Squares Means ± standard errors.

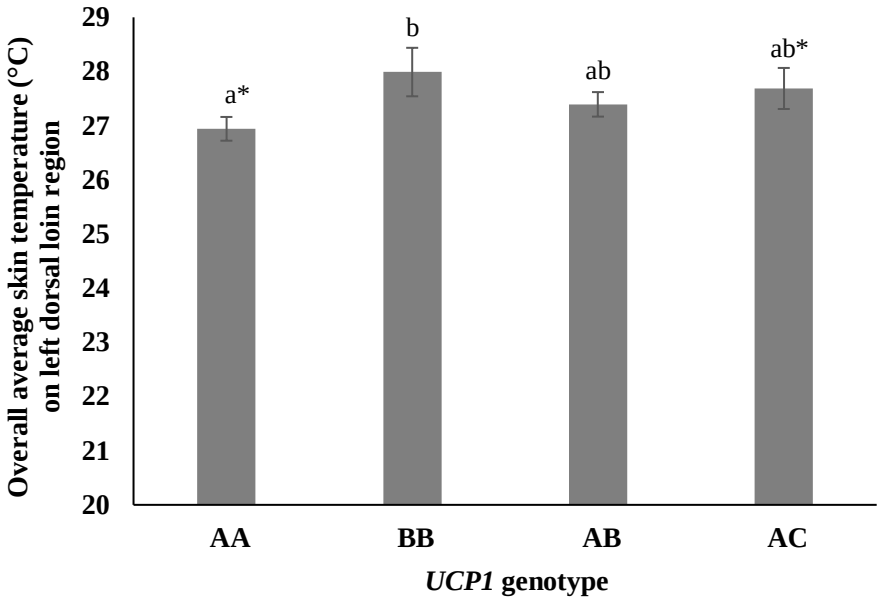


Figure 6.9. The effect of the *UCP1* genotype on overall surface temperature considering all the time-points (baseline, A, B, C, and D). All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05). * Genotypes AA and AC tended to be different at p=0.08.

6.4.2.2.Heat production

Average heat production per kg of birth weight (W Kg^{-1}) was calculated for the five consecutive time-intervals into which cold stress period was divided. Calorimetry was performed only during the adjustment and severe cold stress periods. As displayed in Figure 6.10, compared to genotype AA, lambs of genotype AB tended (P<0.1) to produced higher amounts of heat (W Kg^{-1}) during the second (A-B) time-period, though this difference was not observed for the other time-intervals (P>0.1, Figure 6.10) and overall heat production (P>0.1, Figure 6.11). Nevertheless, when considering the overall heat production and heat produced during these time-periods, AB lambs always produced higher heat compared to genotype AA though not significant either at P<0.05 or P<0.1. In the same way, compared to AA genotype, lambs of genotype AB had higher maximum heat production (summit metabolism) per kg of birth weight (W Kg^{-1}) though not significant

(P>0.05, Figure 6.12). To maintain their core body temperature, lambs would normally produce more heat as they lose heat into the environment. However, no difference was evident between heat lost from the skin of lambs of genotypes AA and AB (Figures 6.8 and 6.9). So, the higher heat produced by AB lambs compared to AA ones could not be simply attributed to the former losing greater heat from skin surface. On the other hand, with the reasonably reliable number of lambs having genotypes AA and AB (26 vs 22), the observed difference in heat production does not seem to be due to a bias resulting from low number of lambs with genotypes AA and/or AB. The main role of UCP1 in thermogenesis has been emphasized in previous studies (Matthias *et al.*, 2000, Golozoubova *et al.*, 2001, Nedergaard *et al.*, 2001). Therefore, the difference in heat production revealed in this study might be possibly due to the genetic variation observed between genotypes AA and AB (Figure 6.2) which could have led to an increased expression of the *UCP1* gene and consequently higher heat production in lambs of genotype AB compared to AA ones. Consistent with this, It has been shown that the sequence of the promoter region of the *UCP1* gene might influence the gene expression through its interaction with the CCAAT/enhancer binding-proteins α and β (Yubero *et al.*, 1994).

Although not significant (P>0.05), in comparison to genotype AA, lambs of genotype AC generally produced more heat throughout the calorimetry period (Figures 6.10 and 6.11). This finding could be attributed to some extent to the greater (though not significant (P>0.05)) heat lost from skin surface in lambs of genotype AC compared to AA ones (Figures 6.8 and 6.9). So, lambs of genotype AC might have produced more heat in an attempt to compensate the greater heat lost through skin to maintain their core body temperature. On the other hand this difference could also be due to a bias resulting from the lower number of AC lambs compared with AA ones (7 vs 23, 7 vs 25, 6 vs 22, 5 vs 21 and 5 vs 16 during the first to the last time-periods respectively). Consistent

with this postulation, lambs of both genotypes AA and AC had the same (P>0.05) amount of maximum heat production (W Kg^{-1}, Figure 6.12).

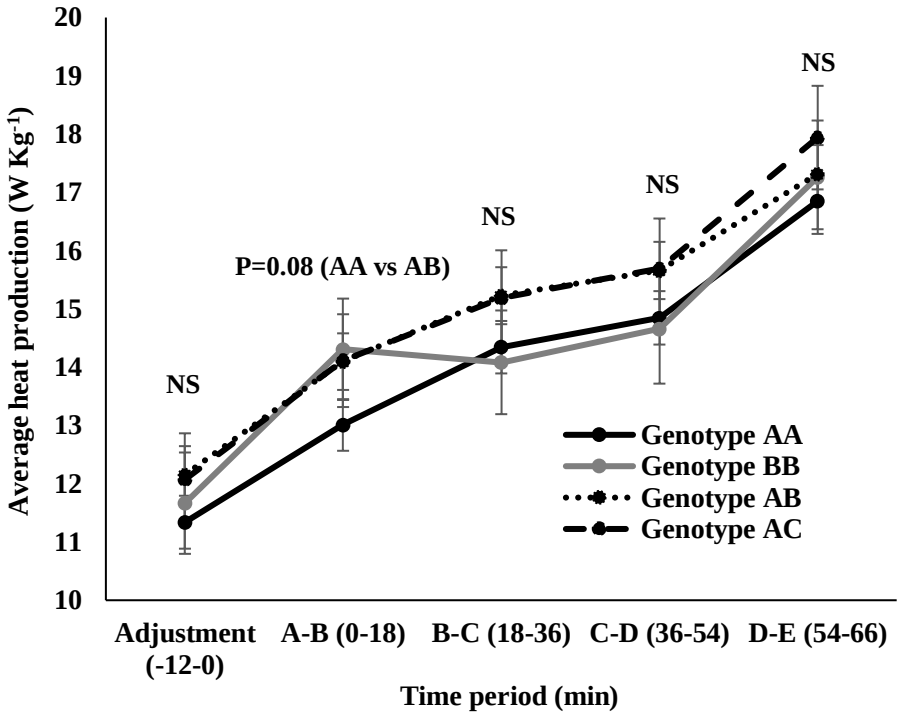


Figure 6.10. The effect of the *UCP1* genotype on average heat production (W Kg^{-1}) during different time-intervals throughout calorimetry. Conditions applied during each time-interval were as follows. Adjustment: 4°C, with no wind or rain; A-B: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D-E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors.

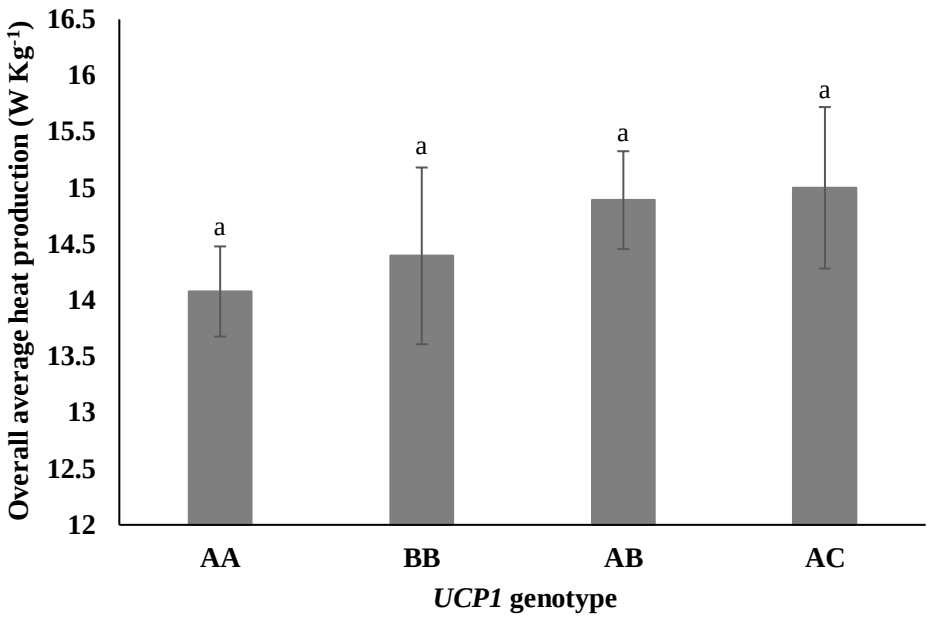


Figure 6.11. The effect of the *UCP1* genotype on overall average heat production (W Kg^{-1}) during cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

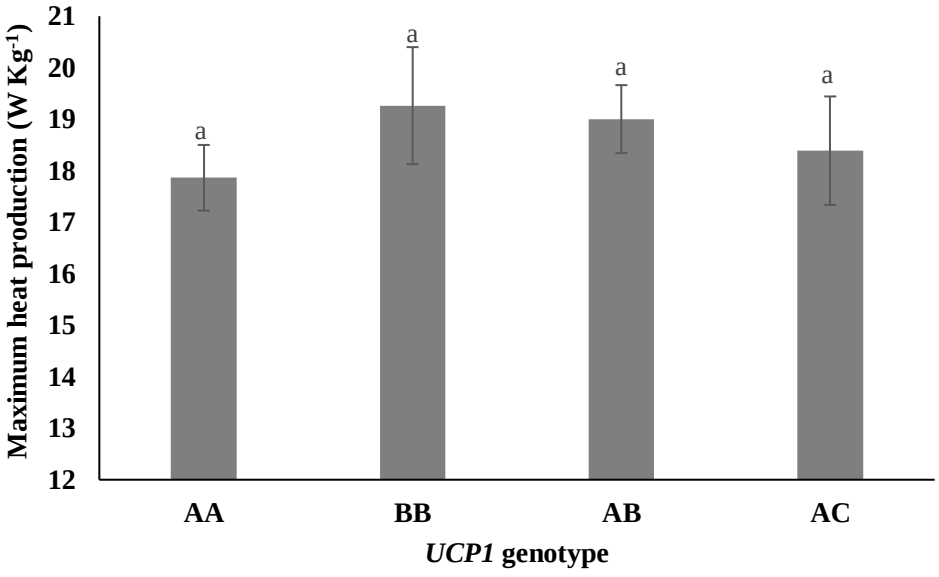


Figure 6.12. The effect of the *UCP1* genotype on maximum heat production (W Kg^{-1}) during cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.2.3.Time to reach MHP

As presented in Figure 6.13, time taken to reach MHP was the same (P>0.05) for lambs of all genotypes at the *UCP1* gene promotor locus. This is consistent with the fact that lambs of all four genotypes had generally the same skin heat loss and rectal temperature (apart from few exceptions that were attributed to a bias resulting from low number of lambs with genotypes BB and AC) during the experiment. To compensate for heat loss through body surface, a lamb can increase its rate of heat production up to a point it reaches its summit metabolism. So, when skin heat loss and rectal temperature, whose changes might induce summit metabolism, were the same in lambs of all genotypes, no difference was expected between lambs of different genotypes in time taken to reach MHP.

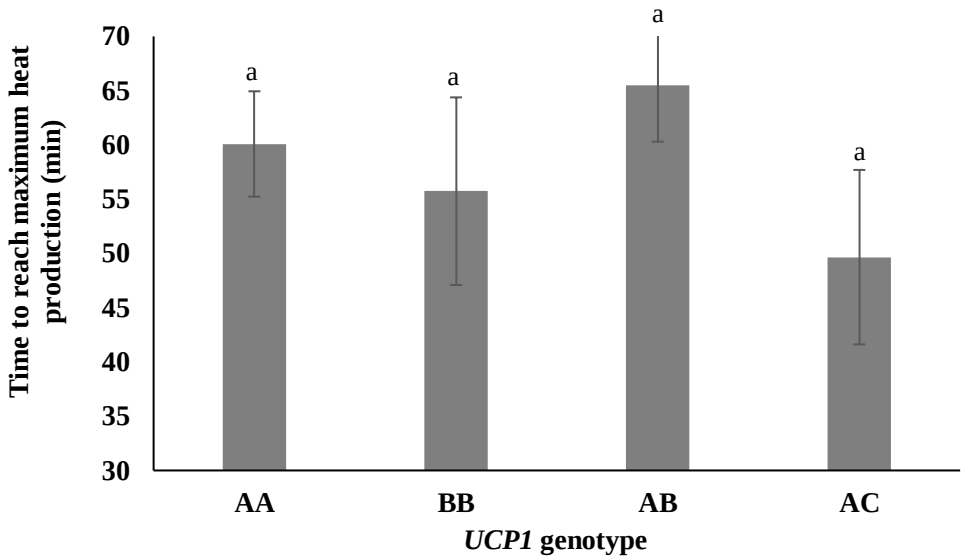


Figure 6.13. The effect of the *UCP1* genotype on time to reach maximum heat production (min) during the cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.2.4.Rectal temperature

As presented in Figure 6.14, lambs of genotype BB tended (P=0.06) to have lower rectal temperature compared to those of genotype AB at the second time-point. Also, this difference was evident at all the other time-points (Figure 6.14) and for the overall rectal temperature though it could not reach the significance level of P=0.05 (Figure 6.15). Similarly, compared with all the other genotypes, lambs of genotype BB had generally the lowest rectal temperature (though not significant (P>0.05)) at all the time-points (Figure 6.14). This general trend was also evident when the overall rectal temperature was considered (Figure 6.15). Although the number of lambs with genotypes AA and AB were reasonably reliable (26 and 22, respectively), the low number of individuals with genotypes BB and AC (6 and 8, respectively) makes it impossible to give a rational reason for these findings. However, these findings could be justified to some extent with the fact that compared to all other genotypes, lambs of BB genotype lost either the same or greater amount of heat through skin (Figures 6.8 and 6.9), while producing the same or lower amount of heat during the calorimetry (Figures 6.10 and 6.11). On the other hand, these observation could be attributed to some degree to a bias resulting from the lower number of lambs with genotypes BB and AC compared to the other two genotypes.

Furthermore, there were no significant differences between rectal temperatures of lambs of genotypes AA, AB, and AC neither at any of the time-points (Figure 6.14) nor when the overall rectal temperatures were considered (Figure 6.15). With the fact that compared to AA genotype, lambs of genotype AC tended (P<0.1) to lose more heat through skin (at the first (baseline) and last time-points (Figure 6.8) and overall (Figure 6.9)), the latter group was expected to have lower rectal temperature than the former one. However, this expectation was not observed in our results,

possibly due to generally higher (though not significant, P>0.05) amounts of heat produced by the latter one (Figures 6.10 and 6.11).

Lambs of genotype AB generally produced more (though not significant, P>0.05) heat than their AA peers (Figures 6.10 and 6.11), while they both lost the same (P>0.05) amounts of heat through their skin surface (Figures 6.8 and 6.9). Nevertheless, it seems that the difference in heat production was not so large to cause a difference in rectal temperatures of the lambs in the two groups.

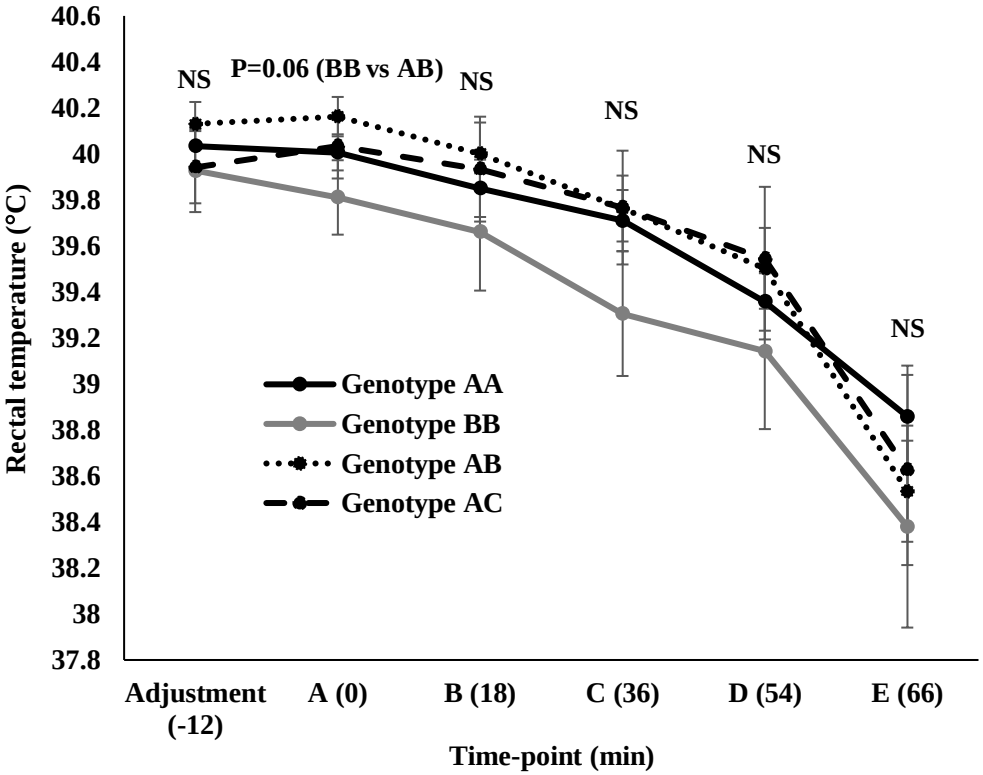


Figure 6.14. The effect of the *UCP1* genotype on rectal temperatures at different time-points during the cold stress period. Conditions applied at each time-point were as follows. Adjustment: 4°C, with no wind or rain; A: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s);, B: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s); E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors.

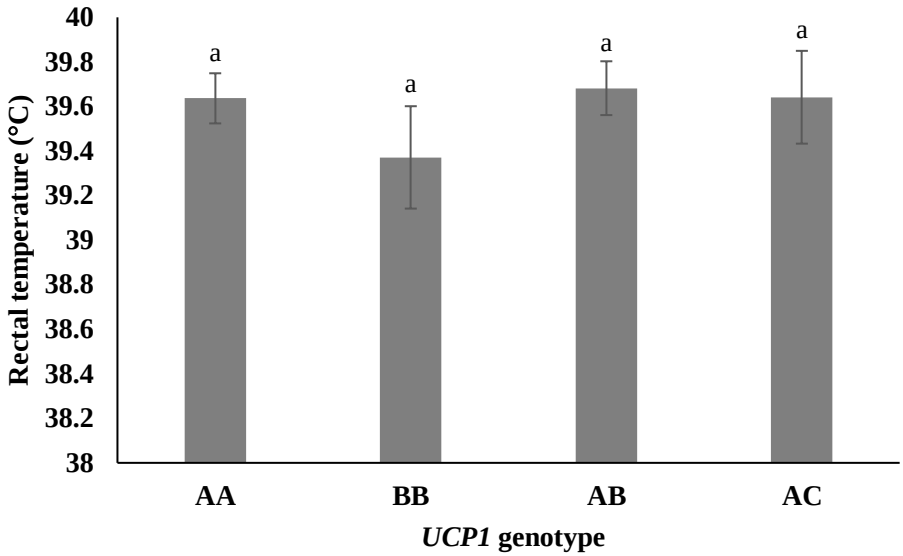


Figure 6.15. The effect of the *UCP1* genotype on overall rectal temperature considering all time-points during adjustment and severe cold stress periods. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.2.5.Likelihood of maintaining the body temperature

As presented in Table 6.6, the polymorphism in the promoter of the *UCP1* gene had no effect (P>0.05) on the likelihood of maintaining rectal temperature during cold stress period. The ability of a lamb to maintain its core body temperature during cold stress period is determined by a combination of other cold resistance indices like skin surface heat loss, heat production (average and maximum), time to reach maximum heat production, and rectal temperature at the onset and during cold stress period. As explained in detail in the previous study (chapter four), each of these indices might also be influenced by factors like skin thickness, birth weight, birth rank, and sex of lamb. As described in previous sections, apart from few exceptions where differences were observed between lambs of different genotypes at/during some time- period/points, lambs of these groups were the same (P>0.05) regarding the cold resistance indices investigated in this study. This is consistent with the fact that lambs of both genotypes AA and AB at the *UCP1* locus had

the same likelihood of maintaining rectal temperature during cold stress period. It seems that these differences were not so large to affect the likelihood of maintaining rectal temperature during cold stress period in lambs of different genotypes.

Table 6.6. Effects of body weight, Skin thickness category, and genetic variation in the *UCP1* gene on likelihood of maintaining rectal temperature during cold stress, presented as odds ratios (OR) and 95% confidence intervals (CI).

Variable	Maintained	Not maintained	P value	OR (95% CI)
	n	n		
Body weight	12	50	<0.01	7.12 (1.83-27.64)
Skin thickness category (Thick vs Thin)	10 vs 2	22 vs 28	<0.05	11.19 (1.23-101.94)
Genotype of the *UCP1* gene				
AA vs BB	7 vs 0	19 vs 6	>0.05	>999.999 (<0.001->999.999)
AA vs AB	7 vs 4	19 vs 28	>0.05	0.58 (0.89-3.85)
AA vs AC	7 vs 1	19 vs 4	>0.05	3.80 (0.27-53.92)
BB vs AB	0 vs 4	6 vs 28	>0.05	<0.001 (<0.001->999.999)
BB vs AC	0 vs 1	6 vs 4	>0.05	<0.001 (<0.001->999.999)
AB vs AC	4 vs 1	28 vs 4	>0.05	6.51 (0.33-127.50)

6.4.3. Effect of variation in the *PRL* gene on indices of cold resistance

6.4.3.1. Skin surface temperature

Although six genotypes were detected for the amplified region of the *PRL* gene (Figures 6.4 and 6.5), only two genotypes were included in the statistical analyses. Lambs of genotypes BB AC AD CD of this region were not included due to less than five lambs having each of these genotypes in the study (Table 6.4).

As presented in Figure 6.16, lambs of genotype AA of the *PRL* gene had the same (P>0.05) average skin surface temperature as those of genotype AB at all five time-points. Consistent with this, when the overall average skin surface temperature was considered, no difference (IP>0.05) was observed between these two genotypes (Figure 6.17). There are some reports indicating the role of prolactin in hair/wool growth and development (McCloghry *et al.*, 1993, Pearson *et al.*, 1996, Nixon, 2002). Therefore, prolactin might also have an effect on skin heat loss through its effect on wool properties of new-born lambs. However, based on the results of this study, variation in the amplified region of this gene had no effect (P>0.05) on skin heat loss. Although the number of lambs with genotypes AA was reasonably reliable (49 lambs), the low number of individuals with genotypes AB (8 lambs) makes it impossible to reach a rational conclusion. So, there might have been an effect of variation in the *PRL* gene on skin heat loss through a possible effect of prolactin hormone on wool properties of new-born lambs. However, this effect might have not been detected in this study possibly due to the low number of lambs. On the other hand, even if an effect of PRL on skin heat loss is assumed, it is unlikely to have been able to be exerted in the present study since wool was removed from the dorsal and lateral surfaces of all lambs and only a small amount of wool was remaining on the ventral surface.

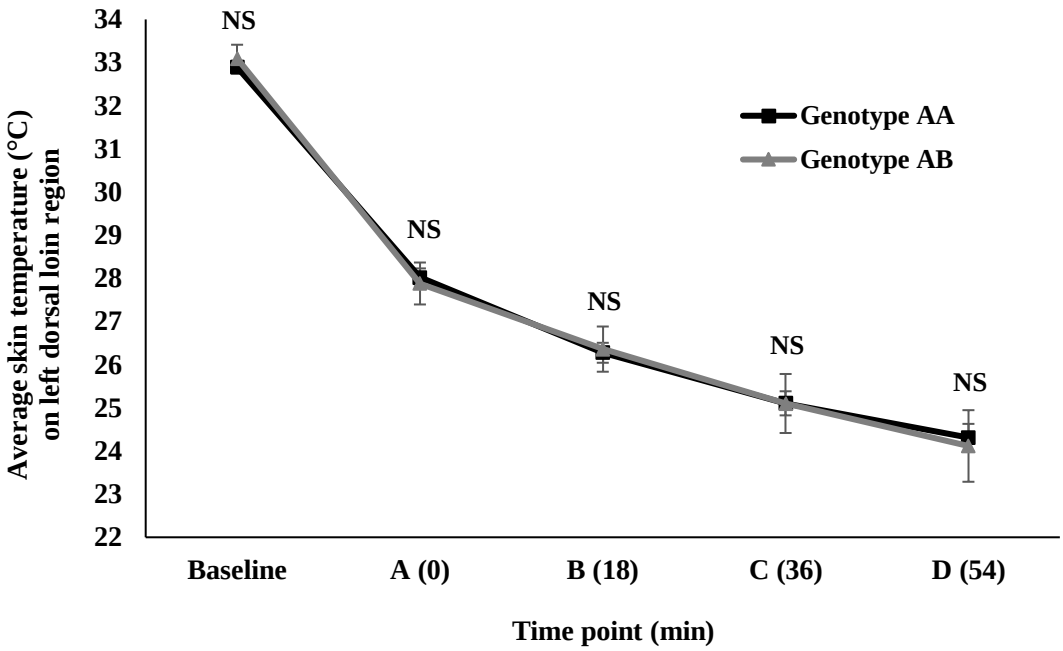


Figure 6.16. The effect of the *PRL* genotype on skin surface temperature at different time points. Conditions applied were as follows. Baseline: 11-16°C, with no wind or rain; A-B: 4°C, rain (1°C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1°C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1°C) from below (1.08 l/min) and cold air (2.0 m/s). All values are presented as Least Squares Means ± standard errors.

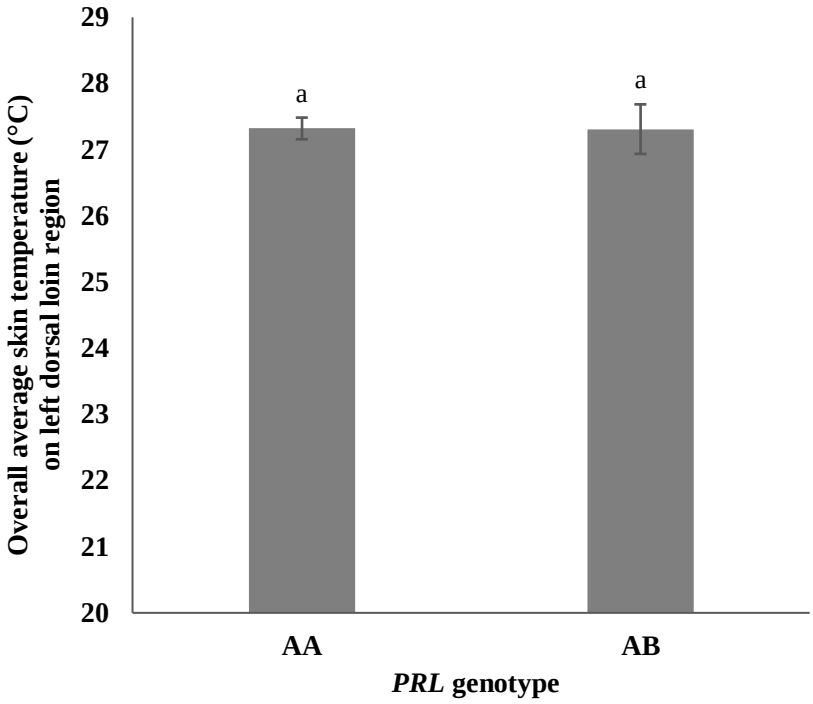


Figure 6.17. The effect of the *PRL* genotype on overall surface temperature considering all the time-points (baseline, A, B, C, and D). All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.3.2.Heat production

As shown in Figure 6.18, compared with AA genotype, lambs of genotype AB at the *PRL* locus produced more (P<0.05) heat (W Kg^{-1}) during the last time-point, though this difference was not observed for the other time-intervals (P>0.05). Furthermore, when the overall heat production was considered, lambs of both genotypes produced the same (P>0.05) amount of heat in the present study (Figure 6.19). In addition, no difference (P>0.05) was revealed between maximum heat production (summit metabolism) per kg of birth weight (W Kg^{-1}) of lambs of both genotypes AA and AB (Figure 6.20).

Due to a possible role of prolactin in the regulation of the UCP1 expression and function (Pearce *et al.*, 2005a), an effect of variation in the *PRL* gene on heat production can be assumed. Although the two SNPs detected in the exon 3 of the *PRL* gene (positions 2015 and 2101) were silent mutations that do not alter the functional conformation of the protein, there is strong evidence that even silent nucleotide substitutions in exon sequences may affect the speed and accuracy of translation (Drummond and Wilke, 2008). Furthermore, synonymous mutations predominantly located near intron-exon junctions, like the SNPs in positions 2015 and 2101, may affect how the pre-mRNA is processed and arranged (Parmley *et al.*, 2006). However, even if this effect exists in fact, it is possibly unlikely to be detected in the present study with low lumber of lambs (8 lambs of genotype AB vs 49 lambs of genotype AA for the average and 5 vs 42 for maximum heat production). Therefore, it is not clear if the higher amounts of heat produced by AB lambs compared to those of AA genotype during the last time-period is due to the variation in the *PRL* gene or not. With the lower number of AB lambs compared with AA ones especially during the last time-interval (4 vs 34), the difference in heat production is likely to be simply due to a bias resulting from the low number of lambs.

When the number of samples in a given group is low, which is the case for genotype AB, each individual lamb can sometimes have a huge effect on the group average for a trait. In our study, 2 out of 5 lambs present during the last time-point that had genotype AB were almost reaching their MHP during this time-interval, both with MHP greater than the average MHP of the population (almost 21 and 23.5 vs 18 ($W\ Kg^{-1}$)). This might possibly be the reason for the higher heat produced by AB lambs compared with those of AA genotype during the last time-point.

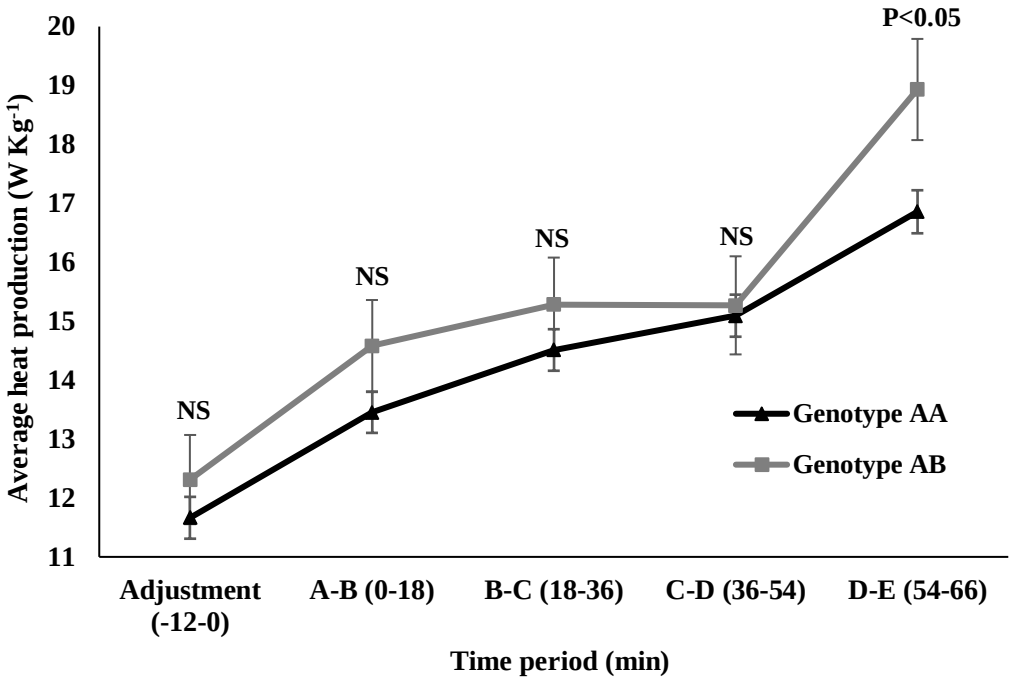


Figure 6.18. The effect of the *PRL* genotype on average heat production (W Kg^{-1}) during different time-intervals throughout calorimetry. Conditions applied during each time-interval were as follows. Adjustment: 4°C, with no wind or rain; A-B: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D-E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors.

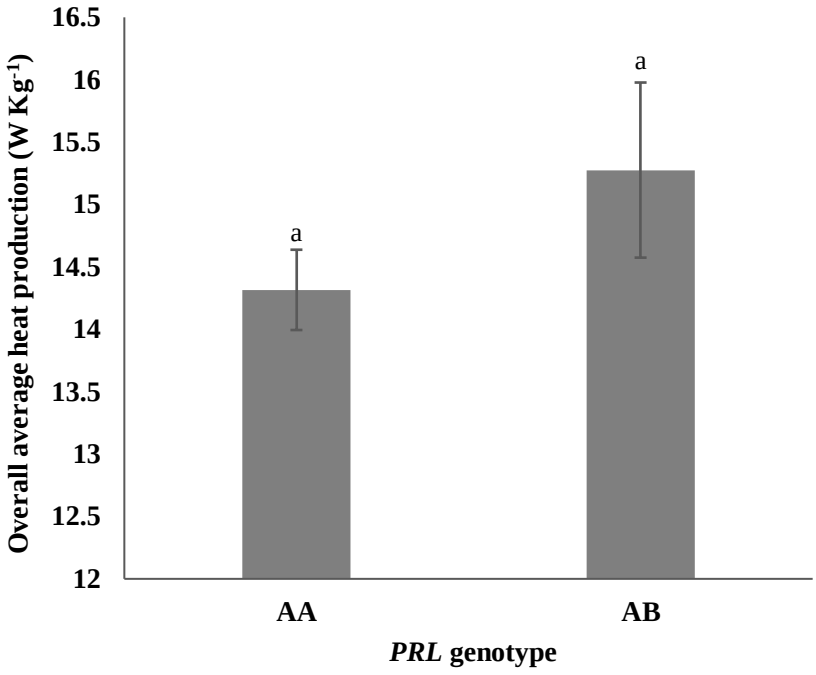


Figure 6.19. The effect of the *PRL* genotype on overall average heat production (W Kg^{-1}) during cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

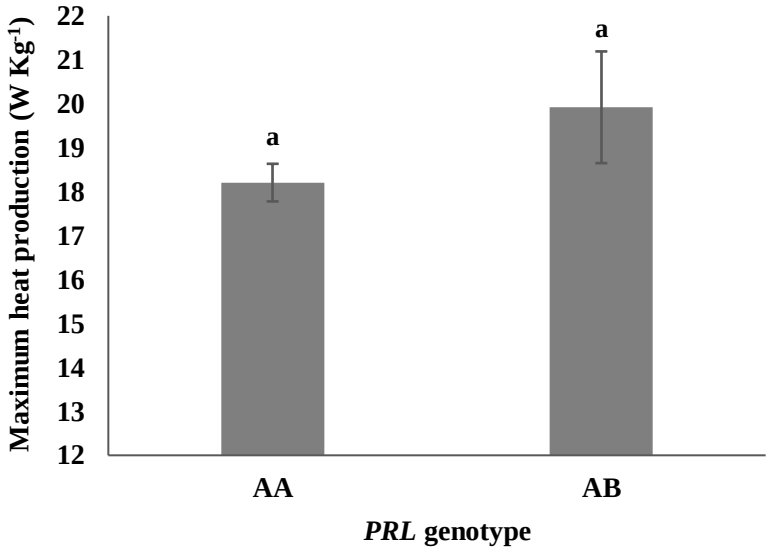


Figure 6.20. The effect of the *PRL* genotype on maximum heat production (W Kg^{-1}) during cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.3.3.Time to reach MHP

As presented in Figure 6.21, time taken to reach MHP was the same (P>0.05) for lambs of both genotypes AA and AB at the *PRL* gene locus. This is consistent with the fact that lambs of both genotypes had the same skin heat loss and generally the same rectal temperature (apart from the exceptions at two time-points that were attributed to a bias resulting from low number of lambs with genotype AB) during the experiment. To compensate for heat loss through body surface, a lamb can increase its rate of heat production up to a point it reaches its summit metabolism. So, when skin heat loss and rectal temperature, whose changes might induce summit metabolism, were the same in lambs of all genotypes, no difference was expected between lambs of different genotypes in time taken to reach MHP.

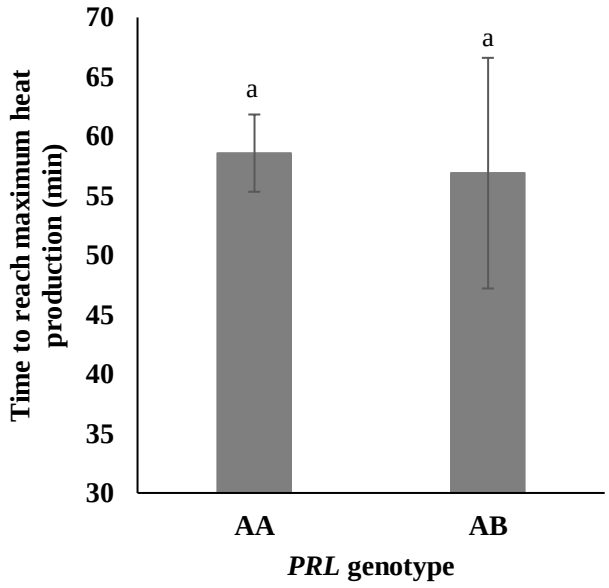


Figure 6.21. The effect of the *PRL* genotype on time to reach maximum heat production (min) during the cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.3.4.Rectal temperature

As presented in Figure 6.22, compared with genotype AB, lambs of genotype AA had significantly ($P<0.05$) higher rectal temperatures at the first time-point and tended ($P=0.06$) to demonstrate this difference at the second time-point. At the next four time-points (B, C, D and E) however, lambs of both genotypes had the same ($P>0.05$) rectal temperatures (Figure 6.22). Similarly, there was no significant difference ($P>0.05$) between rectal temperatures of lambs with these two genotypes when the overall rectal temperature was considered (Figure 6.23).

Although the number of lambs with genotypes AA was reasonably reliable (49 lambs), the low number of individuals with genotypes AB (8 lambs) makes it impossible to reach a rational conclusion. Nevertheless, the lack of a difference between rectal temperature in lambs of genotypes AA and AB (at time-points B, C, and D) is consistent with the fact that these two groups had the same skin heat loss (skin temperature) at the corresponding time-points (Figure 6.16) as well as the same amounts of heat produced during the first four time-intervals (Figure 6.18).

Having the same skin heat loss at all time-points (Figure 6.16) while producing higher ($P<0.05$) amounts of heat by lambs of genotype AB compared to AA during the last time-period (Figure 6.18, min 54-66) has been reflected in the fact that lambs of genotype AB showed higher (though not significant, $P>0.05$) rectal temperatures than AA lambs at the last time point (Figure 6.22).

The higher rectal temperatures of AA lambs at the first and second time-points could be attributed to a possible bias resulting from the lower number of AB lambs compared to AA ones (8 vs 49 at the first and second time-points). Consistent with this, 1 out of 8 lambs of genotype AB which was present only at the first two time-points had a large effect on the rectal temperature mean of the AB genotype. This lamb had a thin skin of 1.94 mm and low birth weight of 4.61 (both of which are against maintaining core body temperature) and low rectal temperature of 39.4°C and 39.5°C

at the first and second time-points, respectively. When this lamb was excluded from the analysis, the differences at the first and second time-points were not significant (P>0.05) anymore.

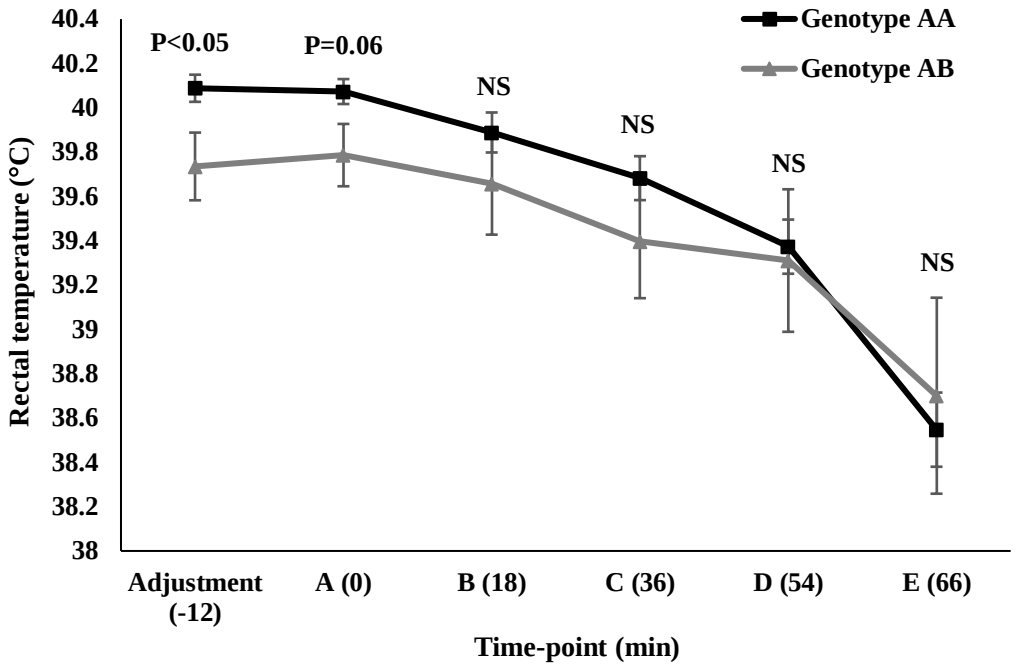


Figure 6.22. The effect of the *PRL* genotype on rectal temperatures at different time-points during the cold stress period. Conditions applied at each time-point were as follows. Adjustment: 4°C, with no wind or rain; A: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s);, B: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s); E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors.

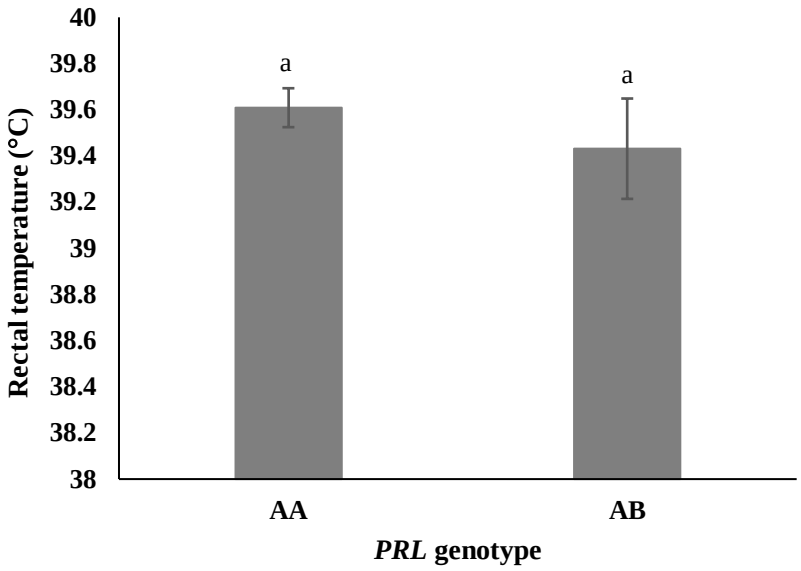


Figure 6.23. The effect of the *PRL* genotype on overall rectal temperature considering all time-points during adjustment and severe cold stress periods. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.3.5.Likelihood of maintaining the body temperature

As presented in Table 6.7, lambs of both genotypes, AA and AB, at the *PRL* gene locus had the same likelihood of maintaining rectal temperature during cold stress period. Apart from few exceptions observed for heat production and rectal temperature where differences were evident between lambs of genotypes AA and AB during/at some time- period/points (Figures 6.18 and 6.22), lambs of both groups were generally the same (P>0.05) regarding the cold resistance indices studied in this study, whose combination determines the ability of lamb to maintain its core body temperature. This is consistent with the fact that the polymorphism in the *PRL* gene had no effect (P>0.05) on the likelihood of maintaining rectal temperature during cold stress period.

Table 6.7. Effects of body weight, Skin thickness category, and genetic variation in the *PRL* gene on likelihood of maintaining rectal temperature during cold stress, presented as odds ratios (OR) and 95% confidence intervals (CI).

Variable	Maintained	Not maintained	P value	OR (95% CI)
	n	n		
Body weight	10	47	<0.01	33.24 (3.08-358.65)
Skin thickness category (Thick vs Thin)	8 vs 2	19 vs 28	<0.1	8.37 (0.72-96.82)
Genotype of the *PRL* gene AA vs AB	7 vs 3	42 vs 5	>0.05	0.53 (0.04-6.47)

6.4.4. Effect of variation in the *PRLR* gene on indices of cold resistance

6.4.4.1. Skin surface temperature

Although three genotypes were detected for the intron 1 of the *PRLR* gene amplified in this study (Figures 6.6 and 6.7), only two genotypes were included in the statistical analyses. Lambs of genotype BB were not included due to less than five lambs having this genotype in our experiment (Table 6.5).

As evident in Figure 6.24, lambs of genotype AA of the *PRLR* gene had the same (P>0.05) average skin surface temperature as those of genotype AB at all five time-points. Consistent with this, when the overall average skin surface temperature was considered, no difference (P>0.05) was revealed between these two genotypes (Figure 6.25).

Since prolactin exerts its physiological roles through its receptor, PRLR can possibly have an effect on lamb birth coat based on some studies reporting the role of prolactin in hair/wool growth and development (McCloghry *et al.*, 1993, Pearson *et al.*, 1996, Nixon, 2002).

Therefore, prolactin receptor might also have an effect on skin heat loss through its possible effect on wool properties of new-born lambs. However, based on the results of this study, variation in the amplified region of the *PRLR* gene had no effect ($P>0.05$) on skin heat loss. The SNP detected in the *PRLR* gene was located in an intron region, not in exon or regulatory regions of the gene. So, this variation have not been able to have any effect on the gene expression or the protein synthesized by this gene. This could be one reason for the lack of association between the variation in the intron 1 of the *PRLR* gene and skin heat loss in our study.

On the other hand, even if an association of this SNP with skin heat loss is assumed, through a possible genetic linkage between this SNP and a functional SNP that might influence wool properties, it is unlikely to be detected in the present study for two reasons. 1) The number of lambs in the study were low to show such an association. 2) Even if an effect of PRLR on skin heat loss is assumed, it is unlikely to have been able to be exerted in the present study since all lambs were the same in respect to coat at birth since wool was removed from the dorsal and lateral surfaces of all lambs and only a small amount of wool was remaining on the ventral surface.

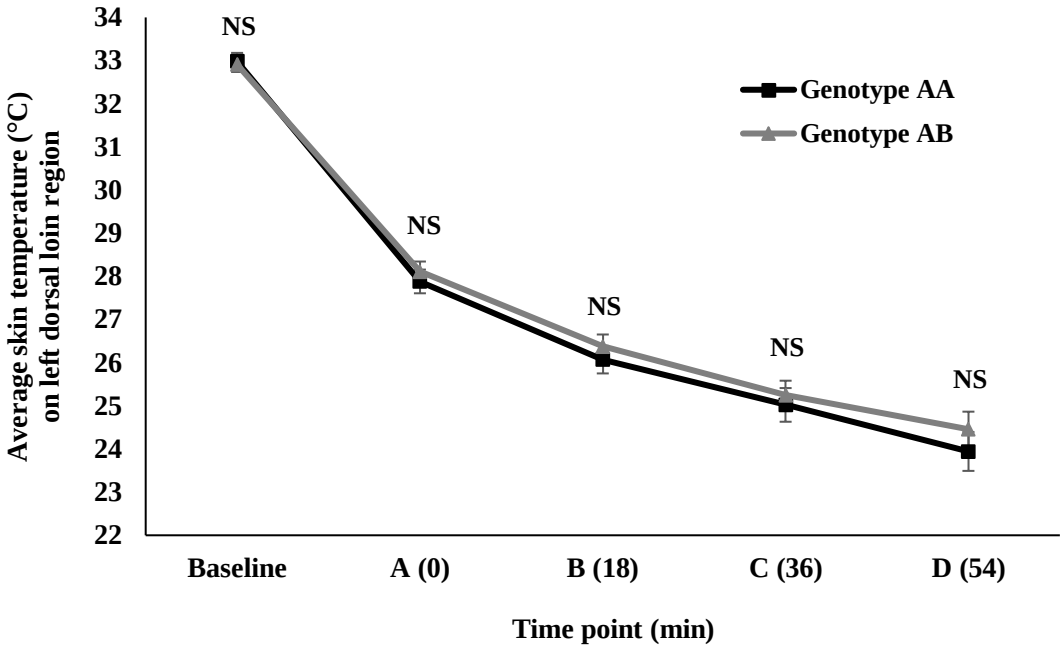


Figure 6.24. The effect of the *PRLR* genotype on skin surface temperature at different time points. Conditions applied were as follows. Baseline: 11-16°C, with no wind or rain; A-B: 4°C, rain (1°C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1°C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1°C) from below (1.08 l/min) and cold air (2.0 m/s). All values are presented as Least Squares Means ± standard errors.

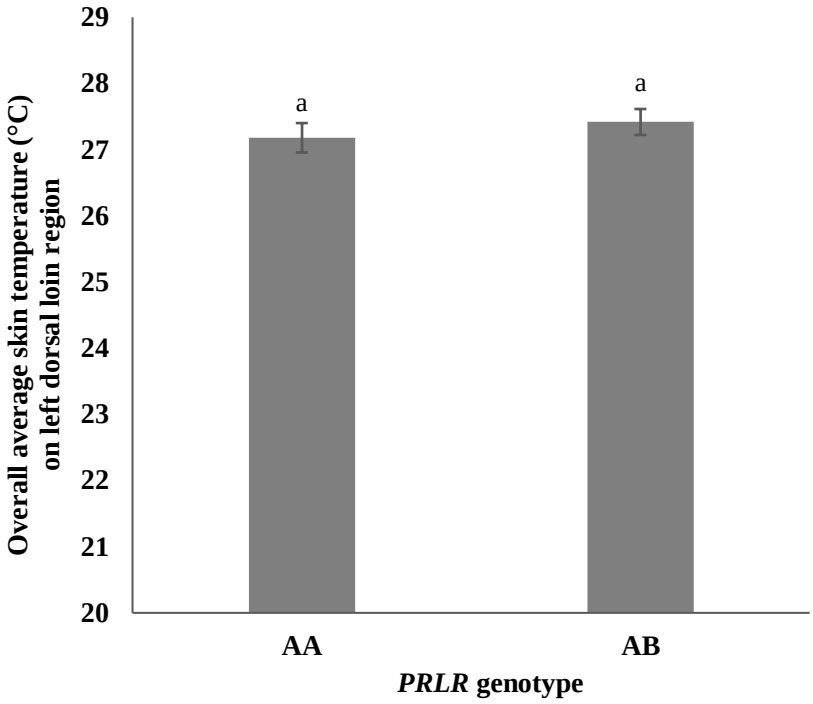


Figure 6.25. The effect of the *PRLR* genotype on overall surface temperature considering all the time-points (baseline, A, B, C, and D). All values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.4.2.Heat production

As shown in Figure 6.26, lambs of genotypes AA and AB produced the same (P>0.05) amounts of heat (W Kg^{-1}) during all five time-periods. Also, when the overall heat production was considered, lambs of both genotypes produced the same (P>0.05) amount of heat in the present study (Figure 6.27). Similarly, no difference (P>0.05) was revealed between MHP (summit metabolism) per kg of birth weight (W Kg^{-1}) of lambs of both genotypes AA and AB (Figure 6.28).

Prolactin receptor (PRLR) through which PRL exerts its physiological roles, can also have possibly an effect on BAT activity.

There is a close relationship between the abundance and disappearance of PRLR and UCP1 in the BAT of fetal and new-born lambs (Casteilla *et al.*, 1989, Clarke *et al.*, 1997a, Symonds *et al.*, 1998, Pearce *et al.*, 2005a). Therefore, any genetic variation in the *PRLR* gene that can alter the gene expression or the protein synthesized by this gene, might affect the abundance and disappearance of UCP1 in BAT and consequently the capacity of brown adipose tissue for thermogenesis.

However, based on the results of this study, variation in the amplified region of the *PRLR* gene had no effect ($P>0.05$) on heat production (neither average nor MHP). The SNP detected in the *PRLR* gene was located in an intron region of the gene that could not lead to any changes in the gene expression or the protein synthesized. This could be one reason why there was no association between the variation in the intron 1 of the *PRLR* gene and heat production in our study.

On the other hand, an association of this SNP with heat production could be assumed, through a possible genetic linkage between this SNP and a functional SNP that might have a real effect on thermogenesis. However, it is unlikely to be detected in the present study due to the low number of lambs in our study.

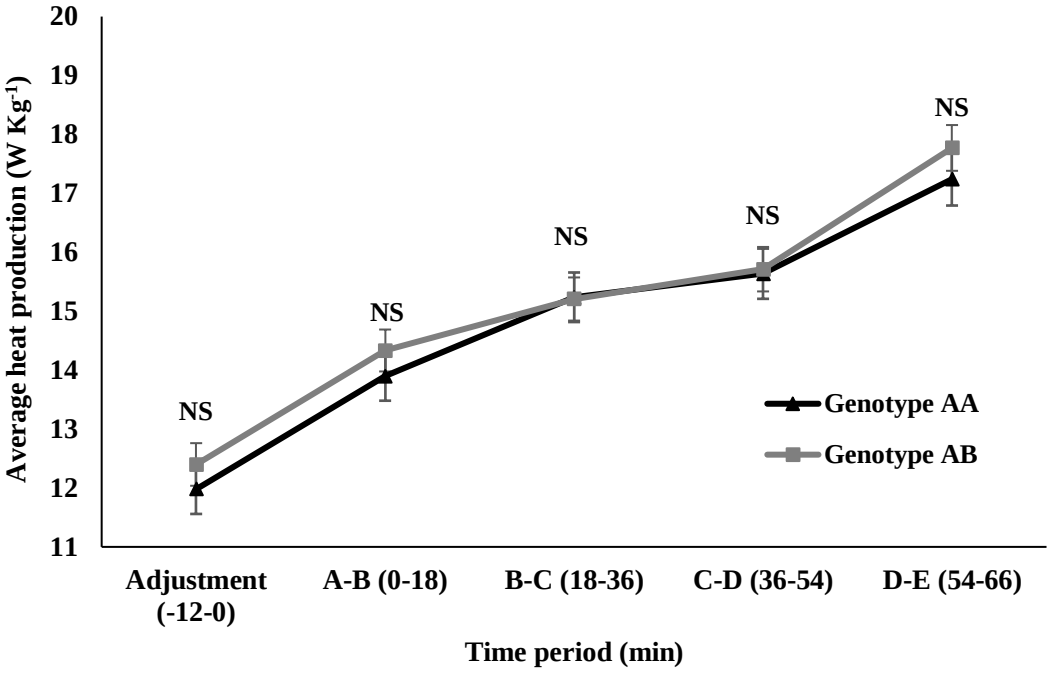


Figure 6.26. The effect of the *PRLR* genotype on average heat production (W Kg^{-1}) during different time-intervals throughout calorimetry. Conditions applied during each time-interval were as follows. Adjustment: 4°C, with no wind or rain; A-B: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s); B-C: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C-D: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D-E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors.

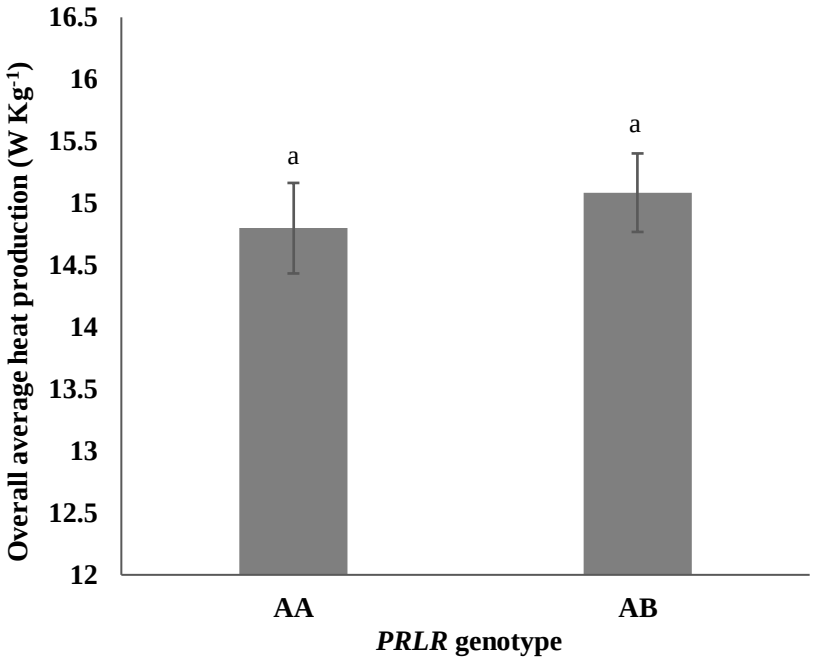


Figure 6.27. The effect of the *PRLR* genotype on overall average heat production (W Kg^{-1}) during cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

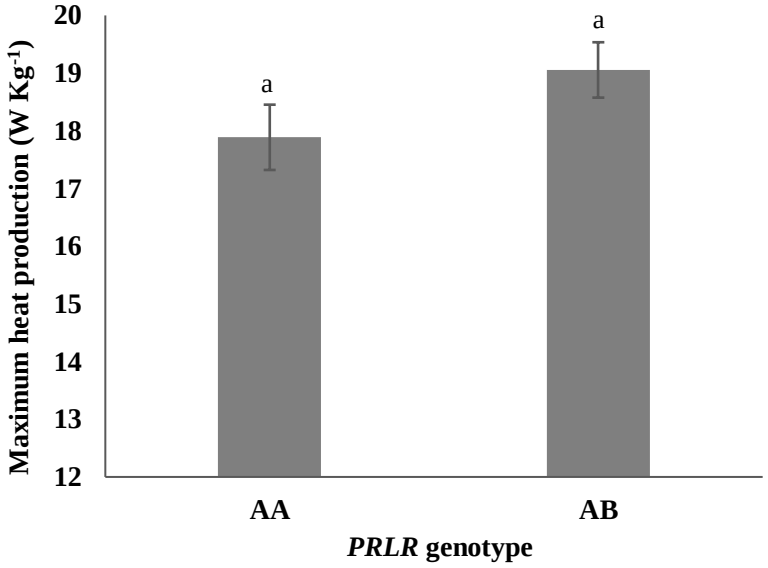


Figure 6.28. The effect of the *PRLR* genotype on maximum heat production (W Kg^{-1}) during cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.4.3.Time to reach MHP

As presented in Figure 6.29, time taken to reach maximum heat production was the same (P>0.05) for lambs of both genotypes AA and AB of the *PRLR* gene. This is consistent with the fact that lambs of both genotypes had the same skin heat loss and rectal temperature during the experiment. To compensate for heat loss through body surface, a lamb can increase its rate of heat production up to a point it reaches its summit metabolism. So, when skin heat loss and rectal temperature, whose changes might induce summit metabolism, were the same in lambs of all genotypes, no difference was expected between lambs of different genotypes in time taken to reach MHP.

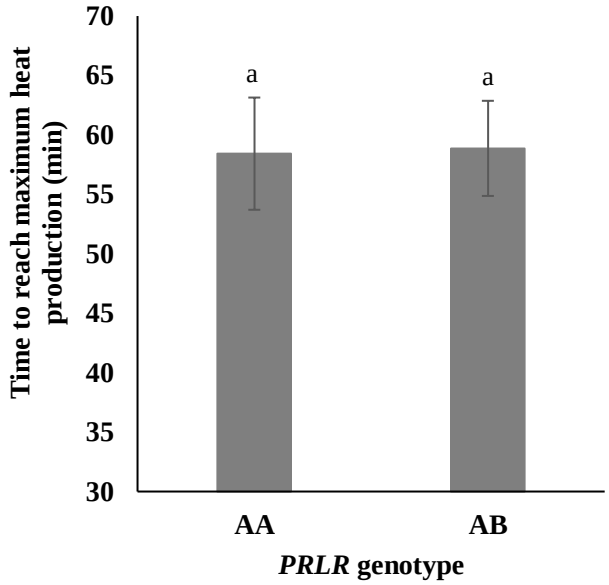


Figure 6.29. The effect of the *PRLR* genotype on time to reach maximum heat production (min) during the cold stress period. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.4.4.Rectal temperature

As evident in Figure 6.30, lambs of genotype AA of the *PRLR* gene had the same (P>0.05) rectal temperature as those of genotype AB at all six time-points. Consistent with this, when the overall rectal temperature was considered, no difference (P>0.05) was revealed between these two genotypes (Figure 6.31). This finding is consistent with the fact that these two groups had the same (P>0.05) skin heat loss (skin surface temperature) at all time-points (Figure 6.24) as well as the same (P>0.05) amounts of heat produced during all time-intervals (Figure 6.26).

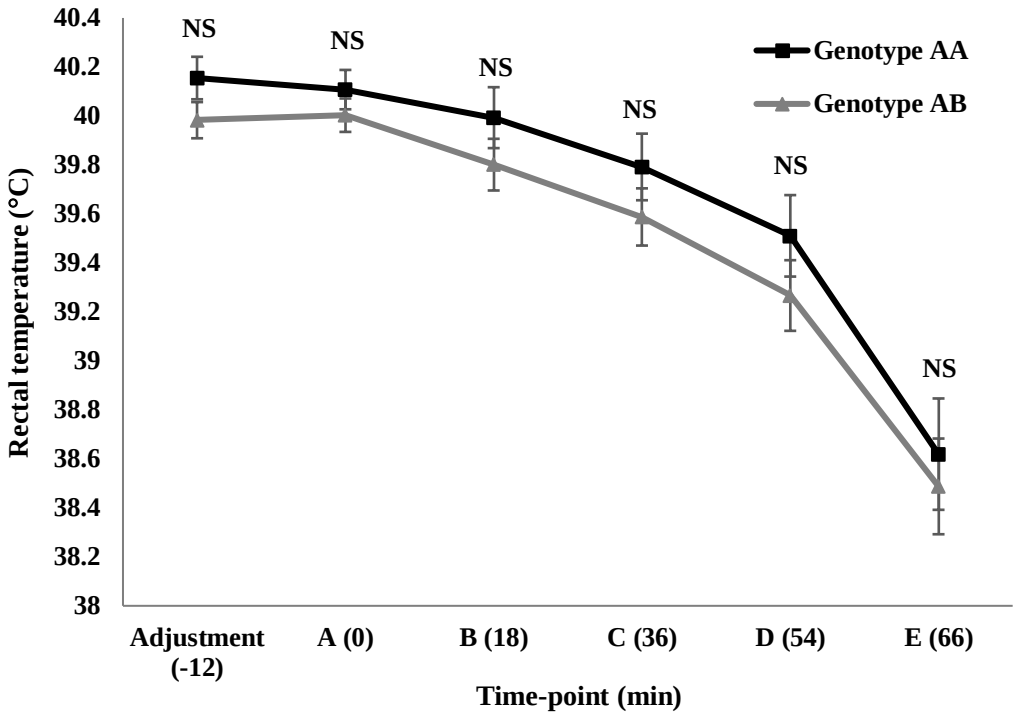


Figure 6.30. The effect of the *PRLR* genotype on rectal temperatures at different time-points during the cold stress period. Conditions applied at each time-point were as follows. Adjustment: 4°C, with no wind or rain; A: 4°C, rain (1 °C) from below (0.36 l/min) and cold air (1.0 m/s);, B: 3°C, rain (1 °C) from below (0.72 l/min) and cold air (1.5 m/s); C: 3°C, rain (1 °C) from below (1.08 l/min) and cold air (2.0 m/s); D: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s); E: 2°C, rain (1 °C) from below and top (1.08 l/min) and cold air (2.0 m/s). All values have been presented as Least Squares Means ± standard errors.

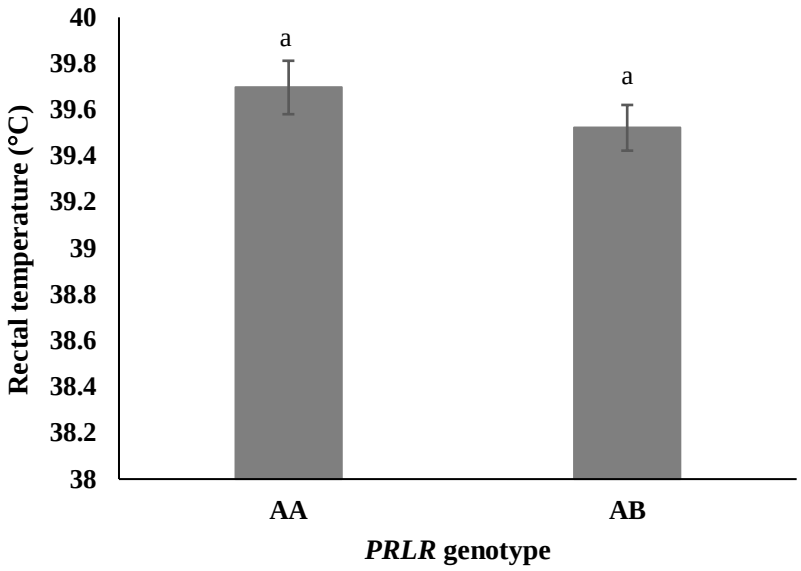


Figure 6.31. The effect of the *PRLR* genotype on overall rectal temperature considering all time-points during adjustment and severe cold stress periods. The values are presented as Least Squares Means ± standard errors. Values with different letters are significantly different (P<0.05).

6.4.4.5.Likelihood of maintaining the body temperature

As presented in Table 6.8, the polymorphism in intron 1 of the *PRLR* gene had no effect (P>0.05) on the likelihood of maintaining rectal temperature during cold stress period. As described in previous sections, the SNP detected in intron 1 of the *PRLR* gene could not show any significant effect (P>0.05) on any of the cold resistance indices in our study, whose combination determines the ability of lamb to maintain its core body temperature. This is consistent with the finding that lambs of both genotypes AA and AB of the *PRLR* gene had the same likelihood of maintaining rectal temperature during cold stress period.

Table 6.8. Effects of body weight, Skin thickness category, and genetic variation in the *PRLR* gene on likelihood of maintaining rectal temperature during cold stress, presented as odds ratios (OR) and 95% confidence intervals (CI).

Variable	Maintained	Not maintained	P value	OR (95% CI)
	n	n		
Body weight	11	50	<0.01	6.11 (1.65-22.62)
Skin thickness category (Thick vs Thin)	9 vs 2	21 vs 29	<0.1	6.06 (0.94-39.31)
Genotype of the *PRLR* gene AA vs AB	5 vs 6	22 vs 28	>0.05	0.95 (0.20-4.65)

6.5. Conclusions

Although the low number of lambs used in this study does not allow to reach reliable conclusion, the following conclusions could be generally drawn from the results presented in this study:

1) Lambs of genotype AA of the *UCP1* gene had significantly lower skin temperature compared to those of genotype BB at the last two time-points of calorimetry under cold stress, as well as low overall temperature. Lambs of genotype AA of the gene tended to have lower skin temperature compared to those of genotype AC at the first and last time-points, and overall. Lambs of genotype AB tended to have lower skin temperature compared to those of genotype BB at the two last time-points. These differences seem to be due to biases resulting from low number of lambs with genotypes AB, BB, and AC rather than being real differences.
2) Compared to genotype AA, lambs of genotype AB of the *UCP1* gene tended to produce higher amounts of heat (W Kg^{-1}) during the second time-period. Though not significant, this difference was also evident for all other time-periods and overall. With the reasonably

reliable number of lambs having genotypes AA and AB (26 vs 22), the observed difference in heat production does not seem to be due to a bias resulting from low number of lambs. Therefore, the difference might be possibly due to a possible effect of the genetic variation observed between genotypes AA and AB, which could have led to an increased expression of the *UCP1* gene and consequently higher heat production in lambs of genotype AB compared to AA ones. However, further study with more samples would be required to confirm this postulation.

3) Lambs of genotype BB tended to have lower rectal temperature compared to those of genotype AB of the *UCP1* gene only at the second time-point. This difference seems to be due to a bias resulting from low number of lambs with genotypes BB rather than being a real one. Otherwise, no difference was observed when lambs of all genotypes of this gene were compared to each other.
4) Lambs of all genotypes of the *UCP1* gene were the same regarding all other cold resistance indices investigated in this study.
5) Compared to AA genotype, lambs of genotype AB of the *PRL* gene produced higher amount of heat during the last time-period of calorimetry, while having lower rectal temperature at the first two time-points. These differences seem to be due to biases resulting from low number of lambs with genotype AB. Otherwise, the polymorphism in the amplified region of the *PRL* gene had no effect on the cold resistance indices and consequently thermoregulation of new-born lambs in this study.
6) The polymorphism in the amplified region of the *PRLR* gene did not show any effect on any of the cold resistance indices and consequently on thermoregulation of new-born lambs in our study.

Although the genetic variations in the *UCP1*, *PRL*, and *PRLR* genes had effects on some of the cold resistance indices at/during some time- points/periods, those associations seem to be mostly due to biases resulting from low number of lambs rather than being real. Therefore, further studies using larger populations and other breeds are needed to assess the association of these genetic variations with the cold resistance indices.

Chapter 7

General discussion

7.1. Summary of the experimental chapters and conclusions drawn

Skin thickness (ST), as a trait of moderate to high heritability (Gregory, 1982a, Slee *et al.*, 1991), has been revealed to be associated with a few other economically important traits including lamb survival (Jopson *et al.*, 2000), cold resistance in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991), wool traits (Gregory, 1982b, Williams and Thornberry, 1992, Ponzoni *et al.*, 1995, Hynd *et al.*, 1996), and carcass composition (Ripoll *et al.*, 2009). Therefore, skin thickness could be potentially used in indirect selection for these traits. However, ST first needs to be measured accurately using a feasible method. While many studies have used skin-fold calipers (plicometer) as a tool for measuring skin thickness (Gregory, 1982a, Gregory, 1982b, Slee *et al.*, 1991, Williams and Thornberry, 1992), it seems that differences in the subcutaneous fat layer and also pinching force during measurement could lead to inaccuracy in measurement (Alexander and Miller, 1979). Real time ultrasonography is another technique for measuring ST whose application has attracted increasing attention because of its advantages over skin-fold thickness measurement (Alexander and Miller, 1979, Brown *et al.*, 2000, Zanna *et al.*, 2012). However, prior to deciding on which ST measurement technique to use, the first step was to validate the accuracy of the ST measurements taken by ultrasound.

In the first experimental chapter of this thesis the accuracy of ultrasonically measured ST was assessed by plicometry and histometry in 35 New Zealand Romney lambs aged 9-11 months during August 2015 and July 2016 (15 and 20 lambs in the first and second years, respectively).

The results of this experiment indicated the ST measurements taken by ultrasound to be significantly ($P<0.01$) correlated with those measured by histometry, with a Pearson correlation coefficient of 0.52. This result is consistent with the result from a study by Zanna *et al.* (2012), who found a positive correlation between ST measured by ultrasonography and histometric

analysis in Shar-Peis and Beagle dogs. On the other hand, no significant (P>0.05) correlation was found between measurements made via ultrasonography and plicometery. Furthermore, no significant correlation was evident between ST measured by histometry and those obtained by plicometer. These two last findings could be attributed to some extent to differences in subcutaneous fat depth included as part of skinfold thickness measured by plicometery.

In conclusion, the results of the first experimental chapter of this thesis validated the reliability of ultrasonography as an accurate and non-invasive method for measurement of ST in lambs.

Lamb survival is a trait of high economic importance with a major effect on the overall productivity of ewes (Amer *et al.*, 1999, Conington *et al.*, 2004). However, low estimates of direct and maternal heritability reported for this trait (Lopez-Villalobos and Garrick, 1999, Morris *et al.*, 2000, Everett-Hincks *et al.*, 2005, Riggio *et al.*, 2008) implies that direct genetic selection is unlikely to be promising. Hence, indirect selection, based on selection for other traits of higher heritability that are genetically correlated with survival can be considered as a supplement to direct selection for the trait itself. Starvation/exposure has been reported to be responsible for approximately one third of lamb mortalities (Hartley and Boyes, 1964, McCutcheon *et al.*, 1981) especially in outdoor lambing, which is mostly common in countries like New Zealand, the UK and Australia. A few studies have reported a positive association between increased ST and increased cold resistance in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991). So, selection for ST might be a potential alternative to selection for lamb survival through its effect on cold resistance as a main component of lamb survival. However, prior to considering this trait in selection for lamb survival, it was prudent to first estimate its heritability and genetic association with other economic traits.

The heritability of ultrasonographically measured ST, fat depth (FD), and eye muscle depth (EMD) at an average age of about 9 months, lamb survival from birth to weaning (SBW), fleece weight at 12 months (FW12), and live weights at weaning (WWT), 8 months (LW8), scanning (LWS), and 12 months (LW12) were estimated in New Zealand Romney sheep in the second experimental chapter of this thesis. Furthermore, genetic, environmental, and phenotypic correlations of ST (as the proposed trait influencing lamb survival) with other traits of interest were also estimated in this chapter. Data were collected from four Terminal Romneys for Increased Genetic Gain (TRIGG) farms in the Manawatu region of New Zealand for lambing years 2010 to 2016. Appropriate animal and sire models were applied to estimate the genetic parameters using the ASREML software (Gilmour *et al.*, 2015).

The results of the second experimental chapter indicated that lamb survival from birth to weaning was lowly heritable, implying that the response of this trait to direct genetic selection would not be promising. Also, estimates of heritability for ST were 0.21 ± 0.03 and 0.20 ± 0.03, respectively from analyses with and without adjustment for LWS, implying that the trait would respond to genetic selection. Estimates of genetic correlation of ST with SBW from the analyses with LWS considered as a covariate for ST ranged from 0.16 to 0.35 depending on the number of progeny per sire for each trait, while the corresponding estimates from the analyses with LWS excluded ranged from 0.08 to 0.27. All the genetic correlations of ST with SBW had large standard errors which did not allow a clear interpretation. The results indicated that including LWS as a covariate for ST in analyses would result in higher genetic correlation estimates between ST and SBW, which is favorable and could be made use of in selection programs. The favorable genetic correlation of ST with SBW might be due to the effect of increased skin thickness on improved thermoregulation at birth as reported by previous studies (Samson and Slee, 1981, Stott and Slee,

1987, Slee *et al.*, 1991), and confirmed by the results from the third experimental chapter of this thesis. Interestingly, and unlike ST heritability estimates that were not affected by adjustment for LWS, the correlations (genetic, environmental, and phenotypic) of ST with other traits, particularly live weight traits at different ages, were highly influenced by the LWS covariate. When correction was made for LWS, ST showed genetic correlations of 0.21 ± 0.07, -0.13 ± 0.09, -0.32 ± 0.12, -0.23 ± 0.09, -0.10 ± 0.10, 0.02 ± 0.11, and 0.20 ± 0.11 with FD, EMD, WWT, LW8, LWS, LW12, and FW12, respectively. The corresponding estimates when no adjustment was made for LWS, were respectively 0.24 ± 0.08, -0.08 ± 0.10, -0.01 ± 0.12, 0.09 ± 0.09, 0.19 ± 0.09, 0.30 ± 0.10, and 0.20 ± 0.11.

In conclusion, the results of the second experimental chapter suggested the idea of considering this trait as a supplement to direct selection for survival in selection programs was feasible. Nevertheless, the large standard errors of the correlations of ST with SBW, and its unfavorable correlation with other traits should be taken into consideration.

The positive genetic correlation of ST measured ultrasonically at approximately nine months of age with lamb survival from birth to weaning was postulated to be due to the effect of ST on improved thermoregulation through its effect on heat loss at birth. Furthermore, a limited number of studies had revealed an association of ST with increased cold resistance in new-born lambs (Samson and Slee, 1981, Stott and Slee, 1987, Slee *et al.*, 1991). Nevertheless, the significance of each effect was impossible to evaluate independently in those studies, due to strong inter-correlations between ST and other traits affecting cold resistance like birth weight, coat depth and coat grade (Slee *et al.*, 1991) Also, the mechanism by which the skin might influence thermoregulation was not clear. Therefore, the third experimental chapter of this thesis aimed to explore the possible role of ST in thermoregulation through its effect on surface heat loss and a

few other indices of cold resistance in 64 new-born lambs (32 lambs with the thickest skin (thick-skinned category) and 32 lambs with the thinnest skin (thin-skinned category)) exposed to cold-stress.

The results of the third experimental chapter demonstrated that thick-skinned lambs had significantly lower skin temperature than their thin-skinned peers during the cold stress period, implying less heat loss from the skin surface and better thermoregulation through increased skin thickness. Also, thick-skinned lambs produced significantly less heat (per Kg body weight) than thin-skinned lambs as a response to less heat loss in the first group. These results indicate that there would be less need to consume body reserves as a source of energy and consequently better conservation of body reserves in the thick-skinned lambs. Furthermore, thick-skinned lambs had higher rectal temperature and were more likely to maintain body temperature than thin-skinned lambs during cold stress. In addition, thick-skinned lambs had the same capacity for maximum heat production as the thin-skinned lambs.

Overall, from the results of the third experimental chapter, it can be concluded that increased ST at birth can positively affect thermoregulation in new-born lambs through decreased heat loss from the skin surface, thereby minimizing the heat production required to maintain core body temperature.

Therefore, ST at birth can be considered as an alternative to cold resistance and potentially as a supplement to direct selection for lamb survival. However, unlike fat depth and eye muscle depth that are commonly measured ultrasonically as traits of economic importance in sheep industry (Gilmour *et al.*, 1994, Dewi *et al.*, 2002, Fischer *et al.*, 2006), measuring ST is not a common practice. Even if a positive effect of ST at birth on improved thermoregulation in new-born lambs is considered as a reason for measuring ST, from a practical point of view, it would be almost

infeasible and costly for farmers to measure the trait in new-born lambs on-farm. Therefore, it would be more practical and cost-effective if ST could be measured at the same time with other traits of interest like fat depth and eye muscle depth, which are commonly measured at six to eight months of age, not at birth. Therefore, the main objective of the fourth experimental chapter was to examine the correlations (as an estimate of repeatability) among ST measurements obtained at monthly intervals from birth to 8 month of age, in order to find out whether skin thickness at an older age is an appropriate indicator of the trait at birth. The results of this study revealed significant ($P<0.05$) correlations between ST measured at birth and the measurements taken at six ($r = 0.29$), seven ($r = 0.33$), and eight ($r = 0.34$) months of age. ST at birth was positively associated with birth weight in this study. Birth rank had an effect on ST ($P<0.05$), with singles having thicker skin than twins, only before making adjustment for birth weight, while sex did not show any effect ($P>0.05$) on the trait neither before nor after adjustment for birth weight. Interestingly, lambs born to the ewes from the second synchronization batch had a greater skin thickness compared to their first-batch peers in this experimental chapter. This finding was attributed to a difference in photoperiod length to which dams of different batches were exposed before and on the day of conception. Consistent with this, Vole pups born to dams exposed to shorter day length before gestation had thicker coats than those born to dams exposed to longer day length before gestation (Lee and Zucker, 1988). The thickening of skin observed in the present study might be an adaptive response that lambs born to the ewes of second batch showed to the shorter photoperiod length in dams transmitted to the developing fetus in uterus via melatonin signaling originating from the mother (Lee and Zucker, 1988, Lee *et al.*, 1989). It is a tautological prediction that reduced day length precedes winter (Broad *et al.*, 2016) and this adaptation enables the new-born to survive cold better than they would with thinner skin. Results of this chapter did not find any significant

effect of skin thickness at birth on growth rate and live weight at any of the monthly measurements from birth to eight months of age. This implies that selection for increased ST at birth for better thermoregulation would not affect growth from birth to at least eight month of age which is favorable. However, it should be considered that this lack of association was only based on phenotypic association not genetic.

In conclusion, based on the results of the fourth experimental chapter, ST measured at six to eight months of age could be considered as a moderately reliable indicator of ST at birth. Also, this finding supports the idea that the positive genetic correlation of ST at nine months of age with lamb survival, found in the second experimental chapter, might be due to improved thermoregulation through less heat loss from skin surface in new-born lambs.

Although improved thermoregulation through decreased heat loss from the skin surface was emphasized in this thesis, it is vital for new-born lamb to produce sufficient heat to compensate for any heat lost from body in order to maintain its core body temperature. Non-shivering thermogenesis in brown adipose tissue (BAT) is a major source of heat production in the new-born lamb (Alexander and Williams, 1968). It accounts for approximately one-half of heat generated during summit metabolism in new-born lambs exposed to cold stress (Stott and Slee, 1985, Slee *et al.*, 1987). Due to the reported effects of the *UCP1* (Clarke *et al.*, 1997a, Clarke *et al.*, 1997b, Symonds *et al.*, 2003a), *PRL* (Budge *et al.*, 2002, Yang *et al.*, 2002, Symonds *et al.*, 2003b, Pearce *et al.*, 2005a), and *PRLR* (Symonds *et al.*, 1998, Pearce *et al.*, 2005a, Pope *et al.*, 2014) genes on BAT, and consequently on thermogenesis in new-born lambs, any change in the sequence of these genes that might result in a modification of either gene expression or structure of the translated protein, could possibly lead to a change in BAT and as a result in thermoregulation. The final experimental chapter of the thesis, therefore, aimed to identify genetic variations in the *UCP1*,

PRL, and *PRLR* genes using polymerase chain reaction single-stranded conformational polymorphism (PCR-SSCP) analysis and to evaluate the effects of the variations on the indices of cold resistance in the same 64 new-born lambs which were used in the third experimental chapter.

Genetic variations in the *UCP1*, *PRL*, and *PRLR* genes had effects on some of the cold resistance indices at/during some time-points/periods. However, these associations seem to be mostly due to biases resulting from low number of lambs rather than being real.

7.2. Implementing the results of this study into practice

With the validation of the reliability of ST measurement using ultrasonography observed in the first experimental chapter, this method can be used in practice by sheep farmers/breeders as an accurate and non-invasive method for measurement of ST in lambs. Ultrasound scanning of ST could provide an objective, non-invasive and simple way for measuring skin thickness (Brown *et al.*, 2000). Another benefit of this method is that the wool generally does not need to be clipped before measurement, which might damage the skin and decrease the commercial value of the animal (Teixeira *et al.*, 2008). Clear images could be obtained simply by parting the wool and applying a small amount of vegetable oil (Brown *et al.*, 2000). Ultrasound also provides a real-time image of the skin, its structure and characteristics (Zanna *et al.*, 2012). There are imaging methods like computer tomography (CT) which is more precise and accurate compared to ultrasound. However, compared with CT, ultrasound is more accessible due to its lower cost and it is also noninvasive and inexpensive. Many countries around the world have incorporated ultrasound measurements of traits like fat depth and eye muscle depth and area into their genetic evaluation programs for the improvement of carcass quality due to the relatively low cost and portability of ultrasound equipment (Stanford *et al.*, 1998). Therefore, it would be also more practical and cost-effective if ST could be measured at the same time as these traits.

The estimates of heritability and positive genetic correlation of ST with lamb survival from birth to weaning, obtained in the second experimental chapter, suggests the idea of considering this trait as a supplement to direct selection for survival in selection programs. ST could be suggested as a likely trait in the indirect selection of lamb survival in selection programs. ST could be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000) at the same time when routinely measured ultrasound traits like fat depth and eye muscle depth are recorded. However, the large standard errors of the correlations of ST with lamb survival as well as its unfavorable correlation with other traits should also be considered. Otherwise, although selection of animals with thicker skin might result in lambs with improved survival as well as increased 12-month fleece weight and weights at scanning and 12 months of age, it could also lead to animals with more fat depth, and weighing less at weaning and 8 months of age. Also, increased ST in mature animals might negatively influence their performance in warm seasons due to a possible unfavorable effect of thicker skin on heat tolerance.

The positive effect of increased ST at birth on improved thermoregulation in new-born lams through decreased heat loss from skin surface is of great importance from a practical point of view. ST at birth can be considered as an alternative to cold resistance and consequently lamb survival. Unlike cold resistance, whose assessment needs laboratory-based techniques that are not feasible for sheep farmers/breeders, skin thickness could be easily measured in the field using objective techniques like ultrasonography (Brown *et al.*, 2000). Although increased capability of the lamb to generate heat (through increasing brown adipose tissue) could be a satisfactory way of achieving high cold resistance in short-term laboratory tests, an improved insulation might be more beneficial for lamb survival in the field (Eales *et al.*, 1982).

The finding that ST measured at six to eight months of age could be a moderately reliable indicator of skin thickness at birth, found in the fourth experimental chapter, is of high importance from both practical and economic points of views. Measuring ST at six to eight months of age is much easier than at birth for sheep farmers/breeders. Furthermore, ultrasound measurement of ST at six to eight months of age, instead of at birth, facilitates simultaneous recording of other traits of importance like fat depth and eye muscle depth, which are normally taken at these ages, consequently saving money and time. Some ram breeders in New Zealand are currently selecting for increased ST to improve lamb survival based on measurements taken at around 6 to 9 months of age, though this trait has still not been officially incorporated into genetic evaluation system run by the Sheep Improvement Limited (SIL).

7.3. Limitations of this thesis

In the dataset used in our analysis in the second experimental chapter, no data was available on lamb birth weight. This could have possibly influenced both the estimates of heritability for lamb survival and the genetic correlations of ST with lamb survival obtained in this study. Birth weight in relation to lamb survival is so important that some authors (Morris *et al.*, 2000, Gudex *et al.*, 2005) have suggested that it would be fruitless to study lamb mortality without considering lamb birth weight. For both single- and multiple-born lambs, birth weight has been reported to be the dominant factor affecting lamb survival (Hight and Jury, 1970, Hinch *et al.*, 1985).

The genetic correlations of ST with lamb survival from birth to weaning had large standard errors which do not allow a clear interpretation of the correlations. More data would be needed to confirm the correlations.

The positive genetic correlation of ST with lamb survival found in the second experimental chapter is believed to be due to the effect of ST on improved thermoregulation through less heat loss from skin surface in new-born lambs, as confirmed in the third experimental chapter of the thesis. This correlation was estimated using data from ST measured at an average age of 9 months, not at birth. If this correlation had been estimated using skin thickness measured at birth, it could have possibly resulted in a genetic correlation with a higher value than what was found in this study.

It should be noted that in the second experimental chapter, ST was measured at around 9 months of age not at birth. So, ST data was only available for those animals that were alive until ultrasound scanning, and no skin thickness data was available for those lambs that died from birth to weaning. This might have led to a bias in the resulting genetic correlation of skin thickness with lamb survival from birth to weaning. The solution to remove this kind of bias is to measure skin thickness at birth instead of at 9 months of age and estimate the genetic correlation using that data.

Due to a temporary fault in the calorimeter, oxygen consumption of six lambs could not be measured during the adjustment period of the cold stress period in the third experimental chapter. Therefore, for those six lambs the averages of heat production for the adjustment period were excluded from the analysis. This might have influenced the results of our analysis regarding average heat production.

In the analyses performed in the fourth experimental chapter, there was only data on a small number of lambs. So, no analysis could be done to find if there was any correlation (either genetically or phenotypically) between ST at birth and lamb survival. It would be ideal to have a large number of new-born lambs with their pedigrees known, so that the genetic association of ST at birth with lamb survival from birth to weaning could be estimated. With the positive effect of

increased ST at birth on improved thermoregulation in new-born lambs through decreased heat loss from skin surface, finding such a genetic correlation should be highly likely.

Small animal numbers also limited other features of the studies in this thesis. Firstly, the genetic association of ST at birth with growth rate and live weight could not be estimated at any of the monthly measurements from birth to eight months of age.

Secondly, the population used in the genetic association study were also small for this type of study, limiting the opportunity to discover useful relationships. While the low number of lambs available in several of the trials in this thesis did not allow firm conclusions from the results observed, sufficient evidence was gathered to suggest the line of enquiry that lamb survival, skin thickness and thermoregulation are all linked and that further investigations are warranted.

Although the results of the fourth experimental chapter showed that ST measured at six to eight months of age could be a moderately reliable indicator of ST at birth, they are not still perfect indicators. On the other hand, from a practical point of view, it would be almost infeasible and costly for farmers to measure ST in new-born lambs on farm. So, having genetic markers for this trait to be used in selection programs (marker-assisted selection) for improved thermoregulation would be of great interest. However, financial limitations did not allow for a genome-wide association study (GWAS) to look for possible markers or candidate genes associated with ST at birth as the main trait of interest in this thesis.

There are some reports indicating the role of prolactin in hair/wool growth and development (McCloghry *et al.*, 1993, Pearson *et al.*, 1996, Nixon, 2002). On the other hand, prolactin exerts its physiological roles through its receptor, PRLR. Therefore, both prolactin and the prolactin receptor might have an effect on skin heat loss through their effect on wool properties of new-born

lambs. However, as explained in the third and fifth experimental chapters, wool was removed from the dorsal and lateral surfaces of all lambs and only a small amount of wool was remaining on the ventral surface for two reasons. Firstly, to remove any possible bias in skin temperature readings (as an indicator of heat loss) obtained by infrared thermography, resulting from variation in coat properties (depth, type, weight). Secondly, to facilitate heat loss and consequently increase the chance of reaching the summit metabolic rate in lambs. Therefore, even if an effect of PRL and/or PRLR on skin heat loss is assumed, it is unlikely to have been exerted in the present study, since any possible variation that might have existed in birth coat properties of different lambs, were removed by the wool clipping. This could have contributed to the lack of an association between the genetic variations in *PRL*, and *PRLR* genes and heat produced by new-born lambs in the fifth experimental chapter.

All of the above limitations could be overcome with greater funding. When this thesis was initiated, there was very limited knowledge about the role of ST in lamb survival and thermoregulation. Given the positive outcomes generated by the research in this thesis, it should lead to future targeted research projects.

7.4. Suggestions for future work

Further experiments to test whether increased ST in mature animals would affect their performance in warm season is of interest due to a possible unfavorable effect of thicker skin on heat tolerance. A possible experiment would be to investigate thermoregulation in lambs with thick or thin skins, exposed to heat stress. Such an experiment might also be undertaken in mature ewes. Also, it would be of interest to estimate genetic parameters in other sheep breeds and flocks, especially those in the hill country areas of New Zealand where lamb survival can be critical. Estimation of genetic parameters was performed in a maternal breed of sheep while lamb survival is a trait of

economic importance in both maternal and terminal breeds. Therefore, further studies to estimate genetic parameters in terminal breeds is of great interest.

It would be of great interest to test if there is any association between the activity of BAT, as a major source of thermogenesis, and ST as a trait effecting heat loss, in new-born lambs. A possible experiment would be to expose groups of new-born lambs with different ST to different temperatures and to examine relevant gene expressions.

Based on the results of the fourth experimental chapter, the photoperiod length to which pregnant dams were exposed might have influenced ST in new-born lambs. This finding was also supported by a previous study (Lee and Zucker, 1988), in which Vole pups born to dams exposed to shorter day length before gestation had thicker coats than those born to dams exposed to longer day length before gestation. So, it would be of interest to investigate the effect of photoperiod length to which pregnant dams are exposed on ST in new-born lambs and to find the possible mechanism behind it. This might provide a way of manipulating ST in order to have a better thermoregulation in new-born lambs.

Doing a genome-wide association study (GWAS) to find possible candidate genes (markers) associated with lamb ST at birth would be promising. If useful associations could be found, this would obviate the need for the difficult and expensive measurement of ST at birth or thermoregulation. Similarly, it would be of interest to test the association of genetic variation in the *UCP1*, *PRL*, and *PRLR* genes with thermoregulation in larger populations and also in other breeds.

7.5. General conclusions

This thesis demonstrated that increased ST at birth improves thermoregulation through a reduction in heat lost from the skin surface. This finding was supported by the two other findings of this thesis, firstly, that ST measured at eight months of age is a moderately reliable indicator of ST at birth. Secondly, ST at nine months of age is positively genetically correlated with lamb survival, which itself can be greatly influenced by the effect of thermoregulatory capacity of new-born lamb. Also, ST measured at nine months of age was found to be heritable, implying that it would respond to selection. Finally, the reliability of ultrasonography as an accurate and non-invasive method for measurement of ST was validated in lambs. Considering all these findings, it could be generally concluded that ultrasonographically measured ST at about nine months of age could be considered as a supplement to direct selection for lamb survival in genetic improvement programs.

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Appendix

Statement of contribution to doctoral thesis containing publications

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Masoud Soltanighombavani
Name/title of Primary Supervisor:	Dr Rao Dukkipati
Name of Research Output and full reference:	
Soltani-Ghombavani, M., Dukkipati, V. S. R., & Blair, H. T. (2017). In Proceedings of the 22nd AAABG Conference, Townsville, Queensland, Australia, 589-592.	
In which Chapter is the Manuscript /Published work:	3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	75
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate did data sorting and analysis, interpreted the obtained results, and wrote the manuscript, all with guidance from supervisors.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	S.M.S. Digitally signed by Masoud Soltanighombavani Date: 2020.10.28 10:17:40 +03'30'
Date:	28/10/2020
Primary Supervisor's Signature:	Rao Dukkipati Digitally signed by Rao Dukkipati Date: 2020.10.29 00:23:46 +13'00'
Date:	29/10/2020

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MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Masoud Soltanighombavani
Name/title of Primary Supervisor:	Dr Rao Dukkipati
Name of Research Output and full reference:	
Soltani-Ghombavani, M., Dukkipati, R., & Blair, H. T. (2018). In Proceedings of the 11th WCGALP, Auckland, New Zealand, Electronic Poster Session - Biology - Growth and Development, pp. 333.	
In which Chapter is the Manuscript /Published work:	5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	75
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate designed and conducted the study, did data sorting and analysis, interpreted the obtained results, and wrote the manuscript, all with guidance from supervisors.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	S.M.S. Digitally signed by Masoud Soltanighombavani Date: 2020.10.28 10:27:01 +03'30'
Date:	28/10/2020
Primary Supervisor's Signature:	Rao Dukkipati Digitally signed by Rao Dukkipati Date: 2020.10.29 00:24:34 +13'00'
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