Exploring innovative ways, based on skin thickness and brown adipose tissue metabolism, for genetic improvement of new-born lamb survival

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Abstract

A major cause of new-born lamb mortality under pastoral conditions is starvation/exposure. Heritability for lamb survival is low and indirect selection for a trait that is both correlated with lamb survival and is heritable, may provide a more efficacious way to improve lamb survival. In addition to selecting for a lamb that can withstand cold environments, the ability to produce heat via non-shivering thermogenesis through brown adipose tissue (BAT) is essential. Consequently, the main aims of this thesis were to investigate the potential selection for skin thickness and temperature to indirectly improve lamb survival, to examine their interactions with other traits of importance, and to characterise the transcriptome and lipidome of BAT in new-born lambs exposed to a cold environment.

Heritability estimates were moderate for skin thickness and low for skin temperature when considering all sheep data from the FocusPrime, Texel, Romney and Highlander breeds. Positive and favourable correlations between skin thickness and other known insulation traits such as fat depth were found, as well as negative and favourable correlations between skin thickness and skin temperature. Therefore, selecting for thicker skinned lambs could enhance the lamb's insulation characteristics and survival.

A short-term cold challenge in new-born lambs was carried out to perform a ribonucleic acid sequencing (RNAseq) analysis to characterize the transcriptome of BAT and thyroid tissue. There was no evidence of thermogenic activity from any of the key thermogenic genes, such as *UCP1* (uncoupling protein 1) or any of the thyroid receptors. This may suggest that the heat production peak under cold exposure occurs swiftly and thus results in being undetectable in BAT by day three of life. These changes in expression

might give way to the whitening process of the adipose tissue, concluding the non-shivering thermogenesis period.

Within the same experimental short-term cold challenge, BAT and plasma samples were collected to identify lipidomic profiles. Profound changes were found after cold exposure, where significant increases in the lipid composition of glycerolipids, glycerophospholipids, sphingolipids and sterol lipids were observed. In some cases, an exponential increase of certain metabolites was recorded through the time of exposure, implying that their increase may be generated as a consequence of BAT activity. The overall differences in lipid types found could be associated with the improvement of lipid metabolism via BAT thermogenic activation, and adipocyte survival during cold adaptation.

In summary, this thesis suggests an alternative pathway to improve lamb survival and provides useful insights into molecular mechanisms and lipidomic composition of thermogenic tissues in new-born lambs.

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List of abbreviations for Chapters 3 and 4

Abbreviation	Full Description
h^2	Heritability
ST	Skin thickness
Stemp	Skin temperature
FD	Fat depth
EMD	Loin-eye muscle depth
EMW	Loin-eye muscle width
WWT	Weaning weight
LW6	6-month live weight
LW12	12-month live weight
FW12	12-month fleece weight
BirthYear_flock	Contemporary group
Birth_rearing type	Born as single, twin or triplet, and reared as single, twin or triplet
Lamb age*BirthYear_flock	Age of lamb according to its contemporary group

List of abbreviations for Chapter 5

Symbol	Full Name
UCP1	Uncoupling protein 1
ACTB	Beta-actin
ADRB1-3	Adrenoceptor beta 1-3
ADRA1A	Adrenoceptor Alpha 1A
BMP4	Bone morphogenetic protein 4
CYP1A1	Cytochrome P450 1A1
DIO2	Iodothyronine deiodinase 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HUWE1	HECT, UBA and WWE domain-containing 1
KI67	Monoclonal antibody KI67
LIPE	Lipase E
MGLL	Monoglyceride lipase
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PNPLA2	Patatin-like phospholipase domain-containing 2
PPARA	Peroxisome proliferator activated receptor alpha
PPARG	Peroxisome proliferator activated receptor gamma
RPL19	Ribosomal protein L19
THRA	Thyroid hormone receptor alpha
THRB	Thyroid hormone receptor beta
T3	Tri-iodothyronine
T4	Thyroxine
VEGFA	Vascular endothelium growth factor
ELOVL6	Fatty Acid Elongase 6
BMP7	Bone Morphogenetic Protein 7
BMP8B	Bone Morphogenetic Protein 8b
CIDEA	Cell Death-inducing DFFA-like Effector A
CKB	Creatine Kinase B
PDK4	Isozyme 4
TGM2	Transglutaminase 2
FNDC5	Fibronectin Type III Domain Containing 5
ACSL5	Acyl CoA synthetase 5
CPT1A	Carnitine Palmitoyltransferase 1A
FABP3	Fatty Acid-Binding Protein 3
PRKG1	Protein Kinase CGMP-Dependent 1
NOS3	Nitric Oxide Synthase 3
PDE3B	Phosphodiesterase 3B
VASP	Vasodilator Stimulated Phosphoprotein
LPL	Lipoprotein Lipase
PRDM16	PR domain-containing 16
EHMT1	Euchromatic Histone-Lysine N-Methyltransferase 1
GABPA	GA-Binding Protein alpha

List of abbreviations for Chapter 6

Category	Lipid Sub-Class	Abbreviation
Glycerolipids	Triacylglycerol	TG
	Ether-linked triacylglycerol	EtherTG
	Oxidised triglyceride	OxTG
	Diacylglycerol	DG
	Digalactosyldiacylglycerol	DGDG
	Diacylglyceryl-3-O-carboxyhydroxymethylcholine	DGCC
	Diacylglyceryl glucuronide	DGGA
Glycerophospholipids	Lysophophatidylcholine	LPC
	Phosphatidylcholine	PC
	Ether-linked phosphatidylcholine	EtherPC
	Lysophosphatidylethanolamine	LPE
	Phosphatidylethanolamine	PE
	Phosphatidylserine	PS
Sphingolipids	Sphingomyelin	SM
	Sulfonolipid	SL
	Phytosphingosine	SPB
	Ceramide	Cer
	Oxidised ceramide phosphoinositol	PI_Cer
	Ceramide hydroxy fatty acid-sphingosine	Cer_HS
	Ceramide hydroxy fatty acid-dihydrosphingosine	Cer_HDS
	Ceramide Esterified omega-hydroxy fatty acid-sphingosine	Cer_EOS
	Ceramide non-hydroxyfatty acid-sphingosine	Cer_NS
	Ceramide 1-phosphates	CerP
	Hexosylceramide hydroxyfatty acid-sphingosine	HexCer
	Acylhexosylceramide	AHexCer
	Hexosylceramide hydroxyfatty acid-sphingosine	HexCer_HS
	Phytosphingosine	PhytoSph
Sterol Lipids	Esterified glycodeoxycholic acid	GDCAE
	Esterified glycolithocholic acid	GLCAE

Chapter 1. General introduction

In New Zealand, lamb losses within the first three days of life range from 5% to 25% (Fisher & Mellor, 2002), therefore this first period of life is critical for survival. Starvation/exposure due to the cold, can account for up to 30% of the losses (Everett-Hincks et al., 2007; Hinch & Brien, 2014). These types of deaths are heavily influenced by the weather, whereby in outdoor pastoral systems, such as in New Zealand and Australia, new-born lambs can be subjected to wet and cold conditions at and around lambing (Mellor & Stafford, 2004; Tait et al., 2015). Hypothermia is the primary contributor to starvation/exposure mortality, causing a rapid decrease in the lambs' milk-suckling activity (Alexander & Williams, 1966), thereby accelerating its likelihood of death from starvation since its reserves are limited and are rapidly diminished (Dwyer & Lawrence, 2005). These losses have both an animal welfare implication (Dwyer, 2008a) and can have a great economic consequence for sheep farmers. Further, lamb losses lower the availability of animals for replacement selection (Forrest et al., 2006). Accordingly, improvement of lamb survival has been the objective of many research studies. However, direct selection for lamb survival itself is inefficient due to its low heritability (Lopez-Villalobos & Garrick, 1999; Safari et al., 2005; Cloete et al., 2009; Everett-Hincks & Cullen, 2009; Boujenane et al., 2013). A potential alternative way to genetically improve lamb survival is through indirect selection of a trait that is moderately to strongly correlated with survival and is more heritable. Skin thickness is moderately to highly heritable (Slee et al., 1991; Janssens & Vandepitte, 2004; Tait et al., 2015; Soltani-Ghombavani et al., 2017) and is positively correlated with lamb survival through its improvement of thermoregulation (Soltani-Ghombavani et al., 2021). Additionally, it can be measured via ultrasound at the same time as routinely-assessed production traits in New Zealand, such as muscle and fat depth at around five to 12 months of age. There is currently a lack of adequate information on skin thickness variation in different sheep breeds in New Zealand, with data only available for the Romney breed (Tait et al., 2015; Soltani-Ghombavani et al., 2017). To date, there appears to be no studies examining skin temperature as a complement of this skin trait in sheep.

In addition to the importance of reducing the heat losses from the skin surface to the environment, the thermoregulatory capacity of the new-born lambs plays a major role in survival by reducing sensitivity to cold exposure (Mellor & Stafford, 2004). Lambs have the ability to thermoregulate their body temperature within minutes after birth, by non-shivering thermogenesis through their brown adipose tissue (BAT) (Basse et al., 2015). The thermogenic activity of BAT involves a cascade of events and is aided through thyroid hormone receptors in the BAT adipocytes (Bianco & McAninch, 2013), which further induce heat production mechanisms. The interactions produced during adaptive thermogenesis result in a differential expression of many genes and in the abundance or type of lipids involved. Around the fourth day of life, the new-born lamb's heat production peak becomes nearly undetectable (Basse et al., 2015), and shivering thermogenesis will become the main source of heat (Symonds, 2013). The fat tissue will begin to whiten and act as the main fat storage for the body, losing the large mitochondrial phenotype of BAT and the expression and abundance of the network of factors involved in heat production (Liang & Ward, 2006; Ojha et al., 2013). These high-impact changes seem to occur fairly quickly in new-born lambs exposed to cold, however, knowledge of the molecular mechanisms of heat production and the signals exchanged between BAT and the thyroid glands is scarce.

Further, research regarding the lipidomic profile of thermogenic BAT in new-born lambs exposed to cold, and the reasoning behind the abundance and type of lipids present remains to be investigated.

This thesis sets out to determine if there could be an alternative pathway to improve new-born lamb survival through selection for skin traits, such as thickness and temperature, and how these are associated with other routinely assessed production traits in sheep. To cover these aims, heritabilities of skin thickness and temperature, plus the estimates of the production traits, such as fat and muscle depth, were calculated in Chapter 3. In

Chapter 4, correlations between skin and production traits were obtained and considering results from both Chapters 3 and 4, an indication of the potential for skin thickness-based selection to improve lamb survival was provided. In addition, an in-depth study of the molecular thermogenic network and lipidome composition of BAT during cold exposure in the new-born lambs was examined. To achieve this, blood, BAT, and thyroid tissue samples from 12-24hr old lambs induced to a short-term cold challenge were utilized for transcriptome analysis in Chapter 5 and further lipidome analysis in Chapter 6.

To accomplish these thesis aims, subsequent individual objectives were proposed:

- Estimation of the heritability and the genetic and phenotypic correlations of; skin thickness, skin temperature, subcutaneous fat depth, loin-eye muscle depth and width, 12-month fleece weight, and live weights at weaning, six months, and 12 months in the FocusPrime, Texel, Romney and Highlander sheep breeds of New Zealand (Chapters 3 and 4).

- Characterization of the BAT and thyroid tissue transcriptome through a ribonucleic acid sequencing (RNAseq) analysis in new-born Romney lambs exposed to either 20-22 °C or 4 °C for two days (Chapter 5).

- Identification of BAT and plasma lipid composition and exploration of potential biomarkers to predict BAT activity through liquid chromatography–mass spectrometry analysis (LC-MS) in new-born Romney lambs exposed to either 20-22 °C or 4 °C for two days (Chapter 6).

Chapter 2. Literature review

2.1 New-born lamb mortality

Most lamb deaths occur during the first three to five days after birth (Hight & Jury, 1970; Smith & Knight, 1998; Jacobson et al., 2020), and continues to be significant during the first year of life (Bingham & Hodge, 2022). Lamb losses in the range of 10 to 25% have been reported across many studies (Haughey, 1993; Quintela, 2007; Everett-Hincks et al., 2007b), while losses up to 40% have been recorded on some New Zealand farms (Fisher, 2004; Morris & Kenyon, 2004), and of up to 30% on some Australian farms (Hinch & Brien, 2014). In addition, Hatcher et al. (2009) reported that of all lamb deaths from a study in Australia (approximately 31% total deaths), 6% occurred either during the birth process itself or within the first 24 hours of life, 14% between days 1 and 7, 3% between days 7 and 30, and 8% between days 30 and 110.

For more income, farmers aim at increasing the total weight of lamb being sold by improving both the number of lambs weaned and their weight, since higher lambing percentage results in better efficiency of the farm in terms of kg of meat produced per kg of feed consumed or per ewe live weight (Morel & Kenyon, 2006; Mishra et al., 2007; Earle et al., 2017). On the other hand, a review on lamb survival rates to weaning by Kenyon et al. (2019), indicated that triplet lambs had a higher death rate (32.5%) than their single (10.5%) and twin (14.5%) counterparts. The increased proportion of multiples contribute to high losses worldwide (Kerslake et al., 2005; Everett-Hincks et al., 2005a; 2005b) and in New Zealand (Table 2.1).

Table 2.1. Overall mortality rates for new-born lambs reported in New Zealand through time.

Twin mortality rate (%)	Triplet mortality rate (%)	Overall mortality rate (%)	Source
.	.	10-25	Dalton et al. (1980); Fisher & Mellor (2002); Hight & Jury (1970)
.	.	5-25	Kelly (1982)
.	.	15	Hinch et al. (1985)
.	.	18-24	McQueen (1986)
15-32		.	Geenty (1997); McCorkindale (1999); Morris & Kenyon (2004); Sheath et al. (1999)
.	.	5-25	Fisher (2004)
20	40	.	Kenyon et al. (2006)
.	56 (light birthweight) 40 (medium birthweight) 28 (heavy birthweight)	.	Morel et al. (2008)
6	27	.	Kerslake et al. (2009)
15	33	11-33	Kenyon et al. (2019)

A dot is displayed where no data were available.

The sheep meat production sector is one of the most important revenue generators in New Zealand, where the exports contribute with around 47% to the global trade (Morris, 2009). According to Beef + Lamb New Zealand (2022) the total of lamb exports revenues were forecasted to be at $3.79 billion for the season 2021-2022, marking a 13% increase compared to the revenues for the 2020-21 period. Therefore, lamb losses that occur prior to the age at slaughter have a significant negative impact on the farmer's profits. A 2007 New Zealand study estimated that lamb losses costs New Zealand farmers over NZ$580 million annually (Everett-Hincks et al., 2007b), while an Australian study reported annual costs of approximately AU$250 million in 2002 (Walker et al., 2002). Moreover, these losses will decrease the selection potential by the reduced number of lambs to select from, adding further expenses (Forrest et al., 2006). These numbers indicate the importance of finding solutions to minimize the mortality of new-born lambs. As stated by Everett-Hincks (2007b), even the smallest improvement in lamb survival could increase the annual profit obtained from sheep, with just a 2% of improvement in lamb survival it would have an increase of individual farm returns of approximately NZ$3500

and boosted industry returns by NZ$47 million annually. Besides the economical perspective, decreasing lamb mortality rates would also improve animal welfare perceptions. For consumers and society in general, high lamb mortality is considered in addition to an animal welfare issue, an ethical one (Mellor & Stafford, 2004; Ferguson et al., 2014; Dwyer et al., 2016). Furthermore, the grand majority of New Zealand sheep farmers surveyed in a study believe that lamb survival is the most important of the research areas (Greer et al., 2015). Also, some Australian farmers showed concerns with the alarming lamb mortality rates and are worried about what consumers may perceive (Elliott et al., 2011). For these reasons, research regarding ways to reduce new-born lamb losses will bring production, animal welfare and economic benefits.

2.2 Causes of new-born lamb mortality

There are three complex interactions that compromise the survival of the neonate: climatic conditions around lambing, rearing ability of the mother and the lamb's viability (McDonald, 1962; Eales et al., 1983; Alexander, 1984, 1988). Moreover, other factors such as genetics, ewe nutrition during pregnancy, ewe and lamb behaviour, trauma and infection could further contribute to lamb mortality (Haughey, 1993). The majority of lamb losses revolve around starvation/exposure and dystocia (Kerslake et al., 2005; Everett-Hincks et al., 2007a), with each of them accounting for approximately 30% of all neonatal deaths. The remaining percentage can be attributed to other causes of death such as post-natal infection, abnormalities and misadventure (Hight & Jury, 1970; Dalton et al., 1980).

2.2.1 Starvation / Exposure

These types of death can be classified as simple starvation (exhaustion of all body reserves), simple exposure (death by hypothermia) or the mix between both (McCutcheon et al., 1981). Starvation occurs when the new-born lamb has an insufficient

or total lack of intake of colostrum and/or milk (Mellor & Stafford, 2004). It also happens when the new-born lamb fails to suck adequately due to weakness, poor mothering, deficient production of colostrum and/or milk or competition with litter mates (Alexander, 1984; Vermunt et al., 1995; Refshauge et al., 2016). This type of death has a negative relationship with birth weight and is frequently seen as the cause of death in multiple-born lambs due to lighter birth weight (Dalton et al., 1980; Le Floch et al., 2010). Heavier lambs are better prepared to survive harsh conditions as a result of them having more energy stored as brown fat and a better suckling drive for a greater duration than lighter lambs (Hight & Jury, 1970).

There is a very important link between lamb mortality and the weather when lambing occurs, as severe conditions are a major contributor to lamb deaths from starvation-exposure, especially in outdoors lambing (Slee, 1981; Mellor & Stafford, 2004; Tait et al., 2015). When a lamb is born, it passes from a warm uterine environment to a harsh and cold external one. It must immediately increase its rate of body heat production fifteen times more than what it was at the foetal level to compensate the heat loss (Alexander, 1962a), failing to achieve this can result in death. At birth, the body temperature of the lambs drops and subsequently increases to a normal temperature (between 39-40 ^{0}C) within a few hours of birth, but occasionally this decline continues and it ends in death for some lambs (Alexander & McCance, 1958). When a lamb goes through a high rate of body heat loss the requirement associated with a high metabolic rate concludes in a rapid consumption of the body reserves, and unless the lamb is suckling, an eventual depletion of the lamb's limited energy stored is expected (Alexander, 1962). Therefore, while the energy produced from the breakdown of body reserves is enough to allow the lamb to survive for three to five days in a warm place, survival time is much less than this in severe cold environments (McCutcheon et al., 1981). Starved lambs are likely to be more susceptible to hypothermia, and it has been recorded that the lamb's suckling reflex is considerably depressed when deep body temperature decreases below 37^{0}C

(Alexander & Williams, 1966). This explains, in part, why most starvation-exposure deaths occur within the first three days of life (McFarlane, 1961).

2.2.2 Dystocia

Dystocia, defined as a difficult birth brought on by an extended unassisted parturition or prolonged delivery that requires assistance (Arthur et al., 1982; Zaborski et al., 2009) is a significant cause of perinatal lamb mortality (Hight & Jury, 1970; Haughey, 1973; Holst et al., 2002). Between 5 and 50% of lamb losses within the first week of life are associated with difficult births (Everett-Hincks et al., 2007b), as a result of foeto-pelvic disproportions (Haughey, 1993; Refshauge et al., 2016; Noakes et al., 2018) or mal-presentation either due to the position of the lamb in the birth canal or entanglement with siblings (Alexander, 1984; Ennen et al., 2013; Mostefai et al., 2019). Moreover, the risk of death to dystocia increases as the birthweight of the individual lamb rises from the mean for its birth type (Everett-Hincks & Dodds, 2008; Geenty et al., 2014). As birth weight increases from an “ideal” weight, there is a rise in the rate of mortality resulting from dystocia (Scales et al., 1986; Fogarty et al., 1992). In accordance with this, several reports have attributed the deaths of heavy singles to dystocia as the primary cause of death (Arthur, 1967; Tarbotton & Webby, 1999; Kerslake et al., 2005). Due to the importance of this problem, breeding values of the trait “lambing ease” have been utilized for many years to avoid deterioration in ewe and lamb mortality as a result of problematic lambing (Brown, 2007). Essentially, lambing ease is scored based on requirements for assistance during lambing (Brown & Swan, 2015). Additionally, the breeding values for this trait include gestation length and the lamb’s birth weight, which are currently utilized in Australian breeding programs from Sheep Genetics Australia (Bunter et al., 2023).

2.2.3 Starvation-Exposure / Dystocia

If a lamb survives a problematic birth, it is more susceptible to starvation/exposure in comparison to other lambs that were born without any difficulties (Eales et al., 1982).

According to Dwyer and Morgan (2006), many new-born lamb deaths from starvation/exposure are in fact triggered by dystocia. Complicated and long labours impact negatively on the establishment of the ewe-lamb bond (Dutra et al., 2007) and increase in rejection behaviour of the dams (Dwyer et al., 2003). Difficult births also influence teat-seeking behaviour, suckling and maintenance of body temperature in lambs (Eales et al., 1982; Jacobson et al., 2020). In these cases, lambs have very low chances of survival (Murphy & Lindsay, 1996), and if they do survive, it will likely impact negatively on their later life. New-born lambs who suffered from hypoxia during a difficult birth tend to have their heat production system depressed (Stott & Slee, 1987), leaving the lamb more susceptible to hypothermia as a result of this impaired thermoregulation (Alexander et al., 1980). Ultimately, lambs that are impacted from the combination of dystocia and starvation/exposure would require human intervention for them to survive (Jacobson et al., 2020).

2.2.4 Post-natal infection

Infection accounts for a small proportion of neonatal losses (Eales & Small, 1986; Refshauge et al., 2016), where it has been associated with 4% of lamb deaths in an Australian study (Suter, 2010). New-born lambs absorb protective immunoglobins from ingesting the colostrum during the first 24 to 48 hours of life, before gut closure occurs, since they do not get immunoglobulins prenatally across the placenta (Ballabriga, 1980). If the new-born is inhibited by any way to ingest colostrum, it will be more susceptible to fatal infections (Collins et al., 1985; Hodgson et al., 1999). Infected animals will show signs such as fatigue, fever, listlessness, reduced social interaction, discomfort, inappetence, all signs of sickness behaviour linked to immunological responses to infection (Gregory, 1998). Lambs born indoors would be more prone to infections from being on used deep litter, however, lambs born outdoors are exposed to a more severe cold environment where starvation happens more frequently (Fisher & Mellor, 2002). Cold exposure could reduce the suckling activity of the lamb, and therefore, they would

be impaired to get the colostrum needed for immunization (Hinch & Brien, 2014), leaving the lamb unprotected from potential deadly infections.

2.3 Factors that make an impact on lamb survival

2.3.1 Breed

It is well established that lamb mortality is affected by breed, and lamb losses from crossbred ewes are generally less than those of pure breeds (Dalton et al., 1976; Fogarty et al., 2005). Non-domesticated sheep breeds (Boreray Blackface and Soay) and some mountain breeds (Cheviot, Scottish Blackface and Welsh) have been shown to have higher survival rates than many lowland breeds (Border Leicester, Southdown, Oxford, Finnish Landrace, Tasmanian and Merino) (Slee, 1981). Similarly, Slee (1981) found differences in the ability of different breeds to cope with a cold environment and Samson and Slee (1981) revealed that lambs of hill-based breeds exhibited an improved ability to resist hypothermia. Besides the differences between breeds and crosses, differences between strains of the same breed could make an impact on lamb survival, especially regarding maternal behaviour (Alexander, 1984). Adequate mothering, provision of colostrum and milk supply, and the capacity to give birth without complications are key for lamb survival (Brien et al., 2014). Merinos have been commonly recognized to have poor maternal characteristics, where in primiparous ewes this is often associated with lamb desertion (Alexander et al., 1993). On the other hand, differences among Merino bloodlines have been previously described, where variations in milking and rearing ability of the ewes have positively influenced lamb survival (Mortimer & Atkins, 1997). Moreover, evidence of improved maternal behaviour of Marshall Romney ewes was recorded when compared to Romney's, where the former showed the ability to lamb unassisted followed by a stronger maternal bond (Knight et al., 1988).

Besides maternal behaviour, some of the breed differences in respect to lamb mortality can be due to the diversity in ewe conformation, lamb traits and ewe milk supply

(Haughey et al., 1985). Other differences can come from maternal behaviour. Merinos have been commonly recognized to have poor maternal characteristics, where in primiparous ewes this is often associated with lamb desertion (Alexander et al., 1993). Breed differences in survival of lambs may also be linked to birthweight, where heavier breeds have higher survival rates (Hinch & Brien, 2014), however, a higher risk of dystocia may arise from having heavy lambs. Dalton (1980) stated that single lambs born from Dorset-Romney and Cheviot ewes had more incidence of dystocia than those born from Coopworth and Perendale ewes, but this finding was also linked to differences in birth weight, birth coat and cold resistance. Similarly, George (1976) stated that the incidence of dystocia ranged from 4.1% in Merino lambs to 34% in Dorset lambs.

2.3.2 Sex

It is well established that male lambs have a higher rate of mortality prior to weaning compared to female lambs (Dalton et al., 1980; Riggio et al., 2008; Matheson et al., 2012; McHugh et al., 2016), in fact, between 1-9% more (Hight & Jury, 1970; Johnson et al., 1982; Hatcher et al., 2009). According to a study by Southey et al. (2001) a male lamb has 23% greater chance of mortality within the first year of life than ewe lambs. The differences in survival between the sexes could be linked to body weight, such as greater size increasing risk of birthing difficulty (Alexander et al., 1955; Hight & Jury, 1970; Jopson et al., 2000). Also, another issue that marks a difference between sexes could be the litter size. Johnson et al. (1982) reported that survival variations to weaning of male and female lambs was more severe amongst singles (4.9% increased survival for females) lambs than multiples (2.3% increased survival for females). Alternatively it could be suggested that greater body weight of males at birth should have a positive effect on survival, as heavier lambs exhibited a greater resistance to cold stress (McCutcheon et al., 1983b). Stott and Slee (1987) reported that female lambs, regardless of their weight, showed higher metabolic rate and a greater total body insulation than males, concluding that the higher mortality rates for males are due to differences in thermoregulation.

2.3.3 Birth weight and body size

The weight of the lamb at birth is a dominant factor, which affects both singles and multiple lambs' survival (Hight & Jury, 1970; Hinch et al., 1985). It is influenced by many factors including litter size, sex, placental size, diseases and dam nutrition during pregnancy (Alexander, 1984). Lamb mortality has a curvilinear relationship, an inverted U (Figure 2.1), with the birth weight of the lamb, where lamb mortality is maximum at the birth weight extremes (Hight & Jury, 1970; Dalton et al., 1980; Holst et al., 2002; Geenty et al., 2014).

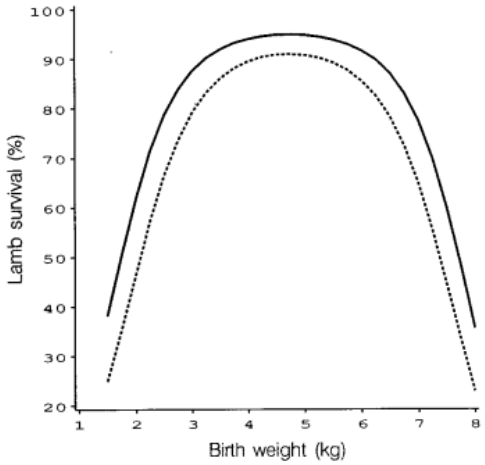

Figure 2.1. Lamb survival (%) versus birth weight (kg) in Marshall Romney (solid line) and Romney (dotted line) sheep. (From Knight et al. (1988))

Pettigrew et al. (2020) stated that the ideal birth weight for a large size breed was 6.5 kg, resulting in a survival rate between 86% and 91% being born to a mature ewe, whereas this birthweight resulted in only a 60% survival rate if born to a ewe lamb. On the other hand, the best birthweight for a smaller breed has been observed to be between 4.5 and 5.5 kg (Fogarty et al., 1992; Holst et al., 2002). Therefore, optimal birthweight would depend on the breed and the age of the ewe (Hinch & Brien, 2014). For several New

Zealand and Australian breeds, the greatest survival rate at birth have been recorded in lambs weighing between 3 and 5.5 kg (Dalton et al., 1980; Alexander, 1988; Morris et al., 2000; Sawalha et al., 2007). Previous observations show that there is a much bigger variation between heavier and lighter lambs in triplets than in twins (Kenyon et al., 2007). These deviations from the average weight, specially toward lower weights, increase lamb losses (Petersson & Danell, 1985; Burfening & Carpio, 1993), most of them by starvation/exposure due to insufficient body reserves (Scales et al., 1986; Yapi et al., 1990). Lighter lambs have a greater surface area to body weight ratio, which negatively affects their thermoregulatory capability and survival (Sykes et al., 1976). In addition, smaller lambs have poorer insulation and cold resistance, lower body reserves and reduced ability to recover from hypothermia, than bigger lambs (Alexander, 1984; Stott & Slee, 1987). On the other hand, a deviation toward a bigger birth weight can lead to a higher mortality rate by dystocia or birth injury (Dalton et al., 1980; Fogarty et al., 1992; Nowak & Poindron, 2006). However, if the lamb is able to survive the birth process, chances of survival increase for larger sized lambs (Morris et al., 2000).

2.3.4 Litter size / Litter mate survival

Several studies have shown that lamb mortality generally increases with litter size (Petersson & Danell, 1985; Kenyon et al., 2006; Riggio et al., 2008; Hatcher et al., 2009), and can be in excess of 30% (Mellor & Stafford, 2004; Hinch & Brien, 2014). Nicoll et al. (1999) indicated that mortality rates were lower for singles (9.9%) and increased with greater litter sizes (12.1% for twins and 26.4% for triplets). Similarly, Holst et al. (2002) observed losses of 11.1% for singles, 20.8% for twins and 46.2% for triplets, and Fowler (2007) reported losses of 16.5% for singles and 31.5% for twins. In triplet-born lambs, this increased mortality rate has been assigned to the lower birthweights that this birth type has, and it has been associated with much bigger risks for starvation and exposure (Dwyer & Morgan, 2006).

There seems to be an association between the survival of multiple-born lambs and the fate of their littermates. A study by Hatcher et al. (2009) observed that survival rates from birth to seven days of age was higher for twin-born lambs when both lambs survived the birth process (8% more), than for those who lost their littermates at birth. However, after seven days, the pattern of survival reversed and lambs that have lost their littermate had a higher survival rate to weaning that those with a surviving littermate. The authors of the study suggested that this reverse situation was likely to occur due to competition for milk, as the energy demand for growing lambs increases prior to their intake of hard feeds. Similar situations have been previously observed when the dam's milk supply starts to decrease. Also, a particular lamb of a multiple litter could often be severely handicapped in its milk consumption compared to its littermates, rising the risk of its pre-weaning death (Hinch, 1989).

2.3.5 Lamb vigour

In the first hour or so of a lamb's life there occurs the formation of an exclusive olfactory memory in the mother to her neonates by licking and grooming them. This will help them dry and restrict her maternal care only to them, rejecting the access of the udder to others (Keller et al., 2003; Nowak et al., 2007). Several reports have shown lamb vigour (lamb behaviour during the establishment of the ewe-lamb bond) to be correlated with maternal efforts of bonding and hence, ensuring that the lamb is not separated from its dam and this has a marked relevant influence on the lamb's survival (Lindsay et al., 1990; Murphy & Lindsay, 1996). It is known that a poor bond between the ewe and the lamb is a major cause of lamb mortality, as it causes hypothermia and starvation in the lamb which usually results in death on the day of birth (Dwyer et al., 2016). When the lamb's body temperature drops below 37^0C the sucking drive is considerably depressed, so in this case even a slight sign of hypothermia can pre-dispose lambs to starvation and death (Alexander & Williams, 1966). A good maternal behaviour, mostly by experienced ewes,

can prevent this and facilitate lamb-sucking responses; however, it is the behaviour of the lamb that will ultimately determine its survival (Dwyer et al., 2016).

2.3.6 Ewe nutrition and age

Maternal undernutrition can reduce lamb birthweight, negatively affect the capability of the lamb to thermoregulate (Dwyer et al., 2016), influence the mothering ability of the ewe and the amount and quality of the milk and colostrum produced (Dwyer et al., 2003). In addition, maternal undernutrition can cause the ewe to have metabolic disorders, a shortened gestation period, prolonged parturition, reduced lamb vigour, reduced lamb's body lipid concentration and a late onset of lactation (McDonald, 1962; Hight & Jury, 1970; Haughey, 1993). Several reviews and studies state that foetal growth, lamb birth and weaning weight are less likely to be affected by the nutrition of the ewe in early pregnancy than in the latter stages, except that the restriction is too severe (Symonds et al., 2007; Greenwood et al., 2010; Kenyon & Blair, 2014; Rooke et al., 2015; Dwyer et al., 2016). Over the last six weeks of gestation the unborn lamb gains 70% of the future birth weight and for that reason it is susceptible to low planes of the ewe's nutrition (Hight & Jury, 1970). If maternal undernutrition reaches a severely low level in these final days of gestation, it can reduce foetal growth by 30-70% or in some cases can cause a cessation of the foetus growth (Mellor, 1983).

Young ewes give birth to lambs with reduced lamb survival than older ewes (Atkins, 1980; Knight et al., 1988; Riggio et al., 2008; Everett-Hincks et al., 2014). Generally, lamb survival based on dam age is highest between the 4-6 year old range (Knight et al., 1988; Lopez-Villalobos & Garrick, 1999), while ewes that are 2 years old or older than 6 years have the lowest lamb survival rates (Hatcher et al., 2009). It is suggested that this survival response could be due to mature ewes being more experienced and giving birth to larger lambs, thus increasing lamb survival (Dalton et al., 1980; Afolayan et al., 2007). Moreover, younger ewes have a higher risk of dystocia, since they are often smaller and have a disproportion between their size and their lamb's size (McMillan, 1983).

Therefore, younger ewes have a longer parturition and are more prone to requiring birthing assistance than mature ewes (Matheson et al., 2012). According to Fowler (2007), lamb survival was observed to be 6.1% lower in single-bearing maiden ewes than in mature ewes.

2.4 Thermoregulation in new-born lambs

2.4.1 Thermoregulation / Heat production activation

When a lamb is born, it passes from a warm uterine environment to a harsh and cold external one. It must immediately increase its rate of body heat production fifteen times more than what it is at foetal level to compensate the heat loss (Dawes & Mott, 1959; Alexander, 1962a). As rectal temperature drops below 37 °C teat-seeking activity is reduced in the new-born and the metabolic rate capability declines (Alexander, 1962a; Alexander & Williams, 1968). When heat loss exceeds the lamb's highest sustainable metabolic rate, or the lamb fails to regulate the body temperature, the deep body temperature will decline, resulting in hypothermia and if it reaches a temperature below 30°C it can result in death (Alexander & McCance, 1958). Therefore, the ability to produce heat is crucial for survival. Like other mammals, lambs can thermoregulate due to the presence of functional brown adipose tissue (BAT) (Basse et al., 2015). Accounting for 60% of generated heat (Liang & Ward, 2006), this tissue is the principal source of non-shivering thermogenesis and is crucial for metabolic adaptation to low environment temperature in the new-born lamb (Alexander & Williams, 1968; Symonds, 2013). Under cold exposure, the hypothalamic–pituitary axis releases catecholamines, like norepinephrine, which then activate β-adrenergic receptors expressed on the surface of brown adipocytes (Contreras et al., 2015; Jiao et al., 2021). When active, these receptors can activate lipolysis and further release fatty acids in BAT (Forrest et al., 2007; Kurylowicz et al., 2015; Plush et al., 2016), which are the substrate for uncoupled oxidation and thus thermogenesis through *UCP1* (uncoupling protein 1)

(Cannon & Nedergaard, 2004). The key thermogenic factor *UCP1*, also known as Thermogenin, is a BAT-specific transport protein of the inner mitochondrial membrane which provides the thermogenic ability of BAT (Ridley et al., 1986; Zingaretti et al., 2009; Whittle et al., 2011). This protein can change the proton conductance mechanism in the mitochondria, allowing protons to run back along the gradient avoiding adenosine triphosphate (ATP) synthesis and instead, dissipate the excess of energy as heat (Garlid et al., 2000; Cannon & Nedergaard, 2004; Contreras et al., 2015; Plush et al., 2016). For this cascade of events to happen from the nervous system signalling up until heat production in BAT, the support by a complex network of genes and factors is needed. For instance, the expression of *PPARGC1A* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) has been attributed as indispensable for thermogenesis (Wu et al., 1999), since it is regarded as a master regulator of BAT differentiation and inductor of *UCP1* (Puigserver et al., 1998; Liang & Ward, 2006). Furthermore, thyroid hormone receptors in BAT adipocytes, like *THRA* and *THRB* (Thyroid hormone receptors A and B) can mediate between thyroid hormone signalling and the sympathetic nervous system in BAT (López et al., 2010), increasing the capacity of cells to respond to catecholamines (Rubio et al., 1995; Hellström et al., 1997). Consequently, these receptors increase the adrenergic sensitivity of the BAT cells (Ribeiro et al., 2001); hence, they can act as inductors of BAT thermogenic activity (Marrif et al., 2005; Bianco & McAninch, 2013). All these processes and inductions commence to decline within a matter of days after birth, where the β-adrenergic signalling starts to diminish (Finn et al., 1998; Shimizu et al., 2014).

Meanwhile, BAT starts its transformation into white adipose tissue (WAT), which will be predominant by day 30 in the lamb (Clarke et al., 1997; Ojha et al., 2013; Pope et al., 2014). The WAT will now act only as the main fat storage of the body, losing the large mitochondrial phenotype of BAT and the expression of its related genes involved in heat production (Liang & Ward, 2006; Ojha et al., 2013; Basse et al., 2015; Contreras et al.,

2015). At the end of this transition, shivering thermogenesis will become the main response to cold exposure (Symonds et al., 1989; Symonds, 2013).

2.4.2 Impact of brown fat reserves and body weight on thermoregulation

The BAT comprises 1-2% of the birth weight of the new-born lambs (Alexander & Bell, 1975) and is mainly contained in the perirenal-abdominal area (Alexander, 1978). Lambs can survive being exposed to cold conditions due to having adequate reserves of glycogen and sufficient BAT reserves (Merei et al., 1993), to produce about 60-70 kJ of heat per kg of body weight from both non-shivering and shivering thermogenesis (Alexander, 1979; Eales & Small, 1980; McCutcheon et al., 1983a). However, for new-born lambs the dominant metabolic fuel is fat rather than carbohydrate (Alexander, 1962b). The deposition of both energy stores seems to be determined partly by maternal nutrition and the metabolic environment (Gluckman et al., 1981; Lucas et al., 1990). According to McDonald (1962), fat body reserves provide the lamb's biggest source of energy and those reserves are greatly affected by prenatal nutrition, where lambs that are born from well fed ewes can have almost twice as much fat as those lambs born to poorly fed ewes. As maternal undernutrition during the mid-late gestation period results in a decrease in BAT deposition (Symonds et al., 1998), increasing maternal feed intake during this period could be promoting foetal BAT reserves (Gate et al., 1999). Moreover, several studies reported that lambs that were born to ewes that have lost weight during pregnancy cooled faster and tended to be slower in reaching the summit metabolic rate (SMR) than those born to ewes that gained weight in the late pregnancy period (Alexander & McCance, 1958; Alexander, 1962b). The SMR is used to measure the capability of the lamb to produce heat in response to cold, which is the maximum sustainable rate of heat production per unit body weight (McDonald, 1962; McCutcheon et al., 1981). Achieving SMR is associated with the development of metabolic acidosis, hyperlactatemia and increased plasma levels of glucose, glycerol and fatty acids (Alexander, 1962a; Eales & Small, 1980). For all lamb body weights, SMR is

approximately 17 kcal per kg body weight per hour, or between 200 and 300 kcal per m^2 of surface area per hour, thus heat production per unit area escalates with increasing body weight (McDonald, 1962; Alexander, 1962a; Kerslake et al., 2009). Therefore, heavier lambs will have a better chance to survive in the cold.

Body weight is related positively to the basal metabolic rate (Stott & Slee, 1987), negatively related in cold conditions to the rate of decline in rectal temperature (McCutcheon et al., 1983b), and proportional to heat production in the lamb (McCutcheon et al., 1983b). Further, heat loss is proportional to the surface area of the lamb (McDonald, 1962; Sykes et al., 1976) such that larger surface area in lambs results in losing more heat. Consequently, cold resistance is positively correlated to birth weight in lambs, probably as a result of a smaller surface area to weight ratio (Samson & Slee, 1981). Alexander (1962b), when examining the time to death from starvation in lambs, reported that the survival time raised with increasing lamb birth weight. Smaller lambs and lambs that have suffered intrauterine growth retardation have fewer fat reserves (Mellor & Murray, 1985a), reducing their margin of safety for maintaining homeothermy (Mellor & Cockburn, 1986). Furthermore, Stott and Slee (1987) noted that after adjusting for live weight, singles had a higher metabolic rate than twin lambs at both thermoneutral and cold conditions. They stated that single lambs had a proportionately greater cold resistance than twin lambs and this was due to higher metabolic rates, longer duration to reach and sustain summit metabolism and slower decrease in rectal temperature.

2.4.3 Lipidomic remodelling in activated brown fat

Cold exposure creates a flow of functions and inter-connections between many factors and tissues, as well as variance in gene expression towards BAT activity, as previously mentioned. As BAT is the central productor of non-shivering thermogenesis, deep changes within the abundance and type of lipids have been previously reported in humans (Boon et al., 2017; Lynes et al., 2018), mice (Marcher et al., 2015; Lu et al., 2017; Simcox et al., 2017), goats (Liu et al., 2022a) and pigs (Pan et al., 2019). One of

these changes is the release of long-chain fatty acids from triacylglycerol (TAG) lipolysis (Forrest et al., 2007; Kurylowicz et al., 2015; Plush et al., 2016), which is a crucial step, since fatty acids are the main substrate for *UCP1* thermogenic function (Cannon & Nedergaard, 2004; Schreiber et al., 2017). Moreover, glycerophospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can regulate the fluidity, homeostasis, and dynamics of the cellular membranes (Hishikawa et al., 2014), as well as adipocyte function and structure (Fuse et al., 2020). Therefore, these lipids could have an influence in the mitochondrial membrane, enhancing BAT uptake of the free fatty acids available through TAG lipolysis. Additionally, previous reports have linked heat-generating BAT with a greater abundance of phospholipids (Hoene et al., 2014), where these lipids may have a supportive role for mitochondrial biogenesis (Marcher et al., 2015; Lynes et al., 2018). Sterol lipids can also influence membrane fluidity and permeability (Ohvo-Rekilä et al., 2002). These types of lipids can further increase the lipolytic actions on TAGs (Schmid et al., 2019), as well as channeling the posterior free fatty acid uptake into BAT for consequent heat production (Broeders et al., 2015). However, fatty acids are known to be mild detergents (Alexaki et al., 2017), which can compromise the cell structure and function when being transported to and from adipocytes. For this reason, and to avoid deleterious effects when managing high concentrations of fatty acids, the sphingolipids from the adipocyte's plasma membrane protect the cells with their detergent-resistant functions (Meshulam et al., 2011). In this way, the adipocyte can keep its integrity, while still being able to utilise fatty acids as a means to produce heat when exposed to cold. Overall, exposure to cold can significantly remodel the amount or type of lipids in BAT, where these lipidome changes can influence and regulate adipocyte function towards thermogenesis (Lu et al., 2017).

2.5 Lamb body surface physiology and its relation to survival

2.5.1 Impact of coat type and depth on lamb survival

Several studies have demonstrated that there is a significant effect of birth-coat type on lamb survival (Purser & Karam, 1967; Obst & Evans, 1970; Hatcher et al., 2009; Brien et al., 2010). In severe cold environments, fine-coated lambs like Merinos are reported to have lower survival rates than other lambs with hairy coats (Cloete et al., 2009), however, these differences are reduced when lambs are born in less severe climates (McCutcheon et al., 1981). According to Allain et al. (2014), the main difference in lamb survival regarding the birth-coat type is attributed to the difference in coat surface temperature at birth. These authors observed that the coat surface temperature of long-haired new-born lambs was 5.1°C less than that for short-woolly coats, therefore, long-haired coats provide the new-born lamb with more insulation and less heat loss to the environment. Previous reports have researched the effect of birth-coat type on the capacity of the new-born lamb to regulate its body temperature. Alexander (1962a) estimated that for dry lambs in still air, the external insulation (coat and air) of fine coated Merino lambs was 60% that of hairy lambs, and if exposed to high windspeeds the estimated external insulation of each coat-type was halved. Likewise, Slee (1978) found an association between breed differences in mean birth-coat depth and the resistance to body cooling of lambs. These studies suggest that even though the insulative value of a unit depth of coat does not change if the coat is wet, deeper coats show a much higher reduction in depth when wet compared to finer coats. Therefore, having a wet coat reduces its insulative value and increases the rate of heat loss by surface evaporation (McCutcheon et al., 1981).

At birth, the lamb's fleece is wet with amniotic fluid, essentially making its thermal insulation capacity useless soon after birth (Hatcher et al., 2009). It has been recorded that the birth-coat of a new-born lamb can hold up to 400 mL of foetal fluids (McDonald, 1962). Although the dam can assist in drying off the lamb's coat by licking (Dwyer,

2008a), previous studies have demonstrated that more than half of the lambs were wet or damp 90 minutes post birth and most were not absolutely dry until at least three hours (Alexander & McCance, 1958). For the coat to be dry, evaporation needs to happen which will absorb the heat from the lamb as it occurs (Alexander, 1962c). It is estimated that approximately 100 kcal are necessary to evaporate 200 mL of fluid from the new-born lamb's coat (Moulk, 1954). Therefore, the coat-type can have an effect on thermoregulation in two ways; the amount of insulation it provides (e.g. hairier birth coated lambs conserve more energy than fine or medium coated) (Alexander, 1961, 1962b) and the amount of heat needed to dry the fleece (McDonald, 1962). This difference in fleece depth may contribute to lamb survival as an advantage in unfavourable weather conditions, as greater fleece depth could improve insulation (McCutcheon et al., 1981).

2.5.2 Skin physiology

The skin is the largest organ in the body (Mlosek et al., 2021). Comprising 16% of total body weight, its main function is to protect the body against the exposure to the elements and provides external sensory awareness (Venus et al., 2010; Aspinall & Cappello, 2020). In addition, it has an important role in thermoregulation and immunological defence (Samuelson, 2007). The skin is constituted by two main layers; the epidermis and dermis, accompanied by the hypodermis which is a subcutaneous tissue that lies internally to the dermis serving as an anchor for the skin to the adjacent structures (Samuelson, 2007). In addition, the skin is classified as thick or thin according to the thickness of the epidermis (Gartner & Hiatt, 2007).

The epidermis is the outermost layer of the skin, a keratinized stratified squamous epithelium that mainly acts as a seal that prevents the body from dehydration (Turton & Hooson, 1997). This layer is composed by two main cell populations: keratinocytes that form the epithelium and non-keratinocytes which are cells that have migrated into this layer, including macrophages, lymphocytes and melanocytes (Samuelson, 2007). In

response to injury, keratinocytes produce cytokines that are associated with proinflammatory properties (Wilmer et al., 1994). The epidermis and dermis are connected by the basement membrane and are thicker in regions that receive more abrasion (Samuelson, 2007).

The dermis layer is situated beneath the basement membrane and extends to the hypodermis, it consists of dense irregular connective tissue formed by elastic, collagen and reticular fibres embedded in an amorphous ground substance (Dellman, 1993). The dermis is divided into a superficial papillary layer and a deep reticular layer without a clear line of demarcation (Dellman, 1993). The papillary layer consists of loose connective tissue that may protrude into the epidermis forming the dermal papillae which make a strong attachment between the epidermis and dermis (Samuelson, 2007). Each of those dermal papillae has a capillary bed or loop that provides nutrition to the epidermis and has a role in regulating body temperature (Samuelson, 2007); by increasing or decreasing the blood flow, heat can be either dissipated or conserved (Melton & Swanson, 1996). The reticular layer of the dermis has larger and more compact fibres than the papillary layer; in thick skinned animals this layer appears to be very well-developed (Samuelson, 2007). Elements like glands (sebaceous and sweat), and hair follicles (fleece of fibre in the sheep) of the skin are mostly within this layer (Samuelson, 2007). Also, fat cells can be found forming scattered clusters, especially in the border of the reticular layer (Samuelson, 2007). These fat cells act as an energy reserve, and in the dermis of the adipose tissue, these cells increase the body insulation to reduce heat loss (Aspinall & Cappello, 2020). Also, in some places such as around the kidney, the adipose tissue acts as a protective layer (Aspinall & Cappello, 2020).

Lastly, the subcutaneous tissue or hypodermis, is located beneath the dermis and it consists of loose connective tissue and fat cells (Dellman, 1993). The thickness of the hypodermis can vary substantially with the physiological state of the animal, where this can be observed in animals that store subcutaneous fat for use in the winter (Turton & Hooson, 1997). Also, this is seen in animals that go through cyclical hair growth, where

the hypodermis can be five times thicker when the hair is growing (Turton & Hooson, 1997).

2.5.3 Impact of skin thickness on lamb survival

In countries where extensive outdoor production systems are applied, the thermoregulatory capacity of the lamb and its ability to conserve heat is critical for survival (Haughey, 1993; Mellor & Stafford, 2004). Therefore, to combat cold exposure, lambs would need to be equipped with better insulative properties, like a thicker skin (Tait et al., 2015). The skin has a major role on body insulation by reducing the heat losses from the body surface into the environment (Alexander, 1978). Numerous studies have shown a positive and significant correlation between skin thickness and cold resistance in new-born lambs, and also with increased insulation (Samson & Slee, 1981; Stott & Slee, 1987; Slee et al., 1991a). More recently, a study by calorimetry and infrared thermography in new-born Romney lambs subjected to cold and wet conditions concluded that thin-skinned lambs lost significantly more heat through the skin and consequently had to produce more heat to maintain body temperature (Soltani-Ghombavani et al., 2021). Hence the authors suggested that a thicker skin could potentially improve new-born lamb survival through its effects on cold tolerance, which is a major component of lamb survival.

A key benefit of skin thickness is that it is a moderately to highly heritable trait (Gregory, 1982a; Slee et al., 1991; Janssens & Vandepitte, 2004; Tait et al., 2015; Soltani-Ghombavani et al., 2017). This observation becomes crucial, since improvement of lamb survival by direct selection has a slow genetic change, linked to a low heritability estimate (Lopez-Villalobos & Garrick, 1999; Safari et al., 2005; Everett-Hincks & Cullen, 2009; Brien et al., 2010; Boujenane et al., 2013). Therefore, selection for a thicker skin that will potentially improve lamb survival becomes feasible. Furthermore, skin thickness can be measured via ultrasound at the farm, at the same time as routinely-measured economical traits, such as fat and muscle depth at around five to 12 months of age. Ultimately, skin

thickness could provide an alternative and cost-effective path to improve new-born lamb survival if selected for.

2.6 Methodologies for thermoregulation and lamb survival research

2.6.1 Ultrasonography: skin thickness

Ultrasonography is highly employed for skin examination under both clinical and research settings, as it is quick, easy to use, reliable and non-invasive (Schmid-Wendtner & Dill-Müller, 2008; Liu et al., 2017; Meikle et al., 2022). Moreover, high-frequency ultrasound has been utilized for skin diagnostic analysis, as this frequency offers sufficient resolution and depth to clearly identify the various skin structures (Alfageme, 2013). This methodology works by detecting the reflected sound waves through tissues that have inherently different acoustic characteristics (Zanna et al., 2012). For example, echoes in the dermis are formed by the reflection of the ultrasonography waves in the outer limits between dermal components like reticular and collagenous fibres, or sebaceous and sweat glands (Gniadecka & Quistorff, 1996). Therefore, the image that comes in the ultrasound screen consists of regions of different echogenicity that matches different histologic layers (Aspres et al., 2003).

Real time ultrasonography has shown much more advantages and accurate skin measurements than older methods in the sheep, such as skin-fold calipers (Gregory, 1982a; Slee et al., 1991a; Williams & Thornberry, 1992). One crucial advantage of the ultrasound over the calliper technique is that the wool does not needs to be clipped off before the measurement, which could injure the skin and diminish the commercial value of the animal (Brown et al., 2000; Teixeira et al., 2008). Further, due to the portability of the ultrasound equipment, several countries around the world have been including ultrasound measurements into genetic programs for lamb carcass quality improvement (Stanford et al., 1998; Tait, 2016). Therefore, including skin thickness measurements

into these routinely-measured economical traits appears to be feasible. This addition could provide key phenotype data for ram breeders to use for genetic improvement programmes towards cold tolerance and lamb survival.

2.6.2 Infrared thermography: skin surface temperature

Infrared thermography (IRT) is a fast, passive and non-contact technique that can be utilized to create a visual thermal profile (Vadlejch et al., 2010) and can monitor large areas simultaneously (Lahiri et al., 2012). It works through thermal cameras which collect the infrared radiation that is emitted by the surface of study and converts them into electrical signals, thus creating a thermal image that shows the distribution of the body superficial temperature (Speakman & Ward, 1998) (Figure 2.2). One of the best advantages of this technique is that it is completely non-invasive, thus it enables a remote reading of the temperature distribution (Speakman & Ward, 1998). This way, the skin temperature measurement is not influenced by the presence of any probes or human manipulation that might alter the temperature variation of the surface (Ludwig et al., 2014; Loyau et al., 2016). For these reasons, IRT has been a very valuable tool in veterinary and animal research to detect fluctuations in the body surface (Stewart et al., 2005; McCafferty, 2007; Ferreira et al., 2011; Loyau et al., 2016). In fact, IRT has been widely used for thermoregulation studies (Jones, 1998; Bouzida et al., 2009). Previous research has tested this technique in new-born lambs, where the radiated heat loss of the skin surface was measured while the lambs were under cold exposure (McCoard et al., 2014; Soltani-Ghombavani et al., 2021). The study by McCoard et al. (2014) stated that a rapid decrease in the skin surface temperature was observed (from 35^0C to 20^0C), and that IRT had proved to be a useful method to measure heat loss in new-born lambs. Therefore, it is possible to take advantage of this methodology by measuring the skin surface temperature of lambs to create a sheep trait that can be utilized to indicate the heat loss into the environment. If future studies validate the fact that lesser heat

measured could equal a better insulation, this trait could potentially be used as an indirect trait for lamb survival due to its cold tolerance significance.

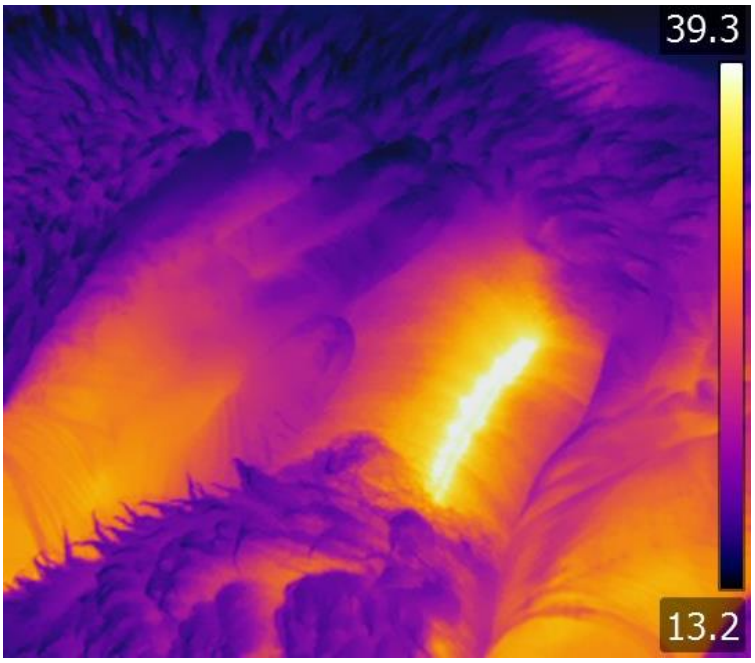

Figure 2.2. Thermal image of a lamb's skin from the experimental animals utilised in Chapters 3 and 4. Temperature values are in Celsius degrees.

2.6.3 Genetic parameters: skin selection for breeding programs

In order to utilize skin measurements for breeding selection as its thickness through ultrasound or its temperature through infrared thermography, these phenotypes need to be heritable. Heritability is defined as the proportion of phenotypic variation attributable to additive genetic variation (Falconer & Mackay, 1996). Heritability estimates tells the farmer and/or breeder how much trust to put in an animal's phenotypic performance when selecting parents for the following generation (Cassell, 2009). Heritability estimates that are lower than 0.20 are considered as low, estimates of 0.21 to 0.40 are considered as moderate and those above 0.40 as high (Bailey, 2014). Throughout the years, many studies have worked towards enhancing sheep's traits that have an important economic

value, as weight, muscle, fat and fleece traits. In fact, some of these studies have marked that there is room for genetic improvement of many of these traits, as they appear to be heritable (Table 2.2). Regardless, many of these estimates are not up to date and are lacking for New Zealand breeds, such as Highlander and FocusPrime. Meanwhile, only a handful have studied skin traits, such as skin thickness, and far less have studied this trait to enhance lamb survival through its effects on cold tolerance (Tait et al., 2015; Soltani-Ghombavani et al., 2017). On the other hand, research that studied skin temperature as a potential trait to be selected for lamb survival are lacking. This research gap provides the opportunity to explore these skin traits, as they could provide a potential pathway for farmers and/or breeders to use in future breeding programs for targeted cold survival. However, prior to including these skin traits into breeding programs, an in-depth exploration on how selection will affect other production traits is warranted, so that no unfavourable effects are unintentionally selected for.

Table 2.2. Means and heritability estimates for different sheep traits from previous research.

Trait	Mean	Heritability	Source
Skin thickness (mm)	4.28	0.35	Slee et al. (1991)
	3.00	0.21	Tait et al. (2015)
	3.05	.	Jopson et al. (2000)
	2.92	0.26	Soltani-Ghombavani et al. (2017)
	.	0.22	Janssens & Vandepitte (2004)
	.	0.25 - 0.60	Gregory (1982a)
Skin temperature (°C)	39.13	0.19	Morris et al. (1989)
	39.00	0.17	Dikmen et al. (2012)
Fat depth (mm)	2.86	0.36	Soltani-Ghombavani et al. (2017)
	6.31	0.35	Cameron & Bracken (1992)
	3.00	0.25	Tait et al. (2015)
Loin-eye muscle depth (mm)	21.40	0.28	Maximini et al. (2012)
	24.84	0.37	Brito et al. (2017)
	26.70	0.50	Pinares-Patiño et al. (2013)
Loin-eye muscle width (mm)	64.19	0.27	Brito et al. (2017)
	62.30	0.15	Fogarty et al. (2003)
	.	0.63	Waldron et al. (1992)
Weaning weight (kg)	21.90	0.29	Safari et al. (2007)
	.	0.26	Duguma et al. (2002)
	28.10	0.45	Brash et al. (1994)
6-month live weight (kg)	31.73	0.26	Riggio et al. (2008)
	.	0.44	Wuliji et al. (2001)
	40.10	0.41	Fossceco & Notter (1995)
12-month live weight (kg)	58.00	0.25	Janssens et al. (2000)
	65.20	0.45	Cloete et al. (2001)
	46.65	0.40	Huisman & Brown (2008)
12-month fleece weight (kg)	3.97	0.57	Mortimer et al. (2017)
	3.00	0.45	Johnson et al. (1995)
	3.78	0.46	Huisman et al. (2008)

A dot is displayed where no records were available for the trait.

2.6.4 Liquid chromatography–mass spectrometry (LC-MS): BAT biomarker identification

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that couples high resolution chromatographic separation with sensitive and specific mass spectrometric detection (Lim & Lord, 2002). The sample molecules are converted into ions and moved within a time-of-flight tube or through an electromagnetic field before they are measured by a detector (Zhou et al., 2012). As global characterization of lipids in biological samples, as lipidomics, appears to be a challenging task (Nygren et al., 2011), this technique has been widely chosen for lipidome analysis due to its sensitivity and selectivity (Jenkins et al., 2020). In addition, it has the potential to detect and quantify hundreds of intact lipid molecular species in parallel (Wenk, 2005; Orešič et al., 2008), as well as detecting and identifying novel lipids (Nygren et al., 2011).

All these particularities of the methodology make it possible to resolve complex lipidomes and to identify different lipid species, their biological activities, abundance, subcellular localization and tissue distribution (Han & Gross, 2003). Previous LC-MS-based lipidomic studies have utilised this technique in order to improve the understanding of the physiological role of lipids that were previously unknown or did not have a corresponding function (Fuse et al., 2020; Leiria & Tseng, 2020). More specifically, some studies have used this technique to study lipid metabolism through BAT and WAT profiles (Baker et al., 2014; Tumas et al., 2016; May et al., 2017; Grzybek et al., 2019). Under cold exposure, BAT metabolism has been observed to be considerably altered to maintain body temperature, triggering its thermogenic capacity, and inducing non-shivering thermogenesis (Marcher et al., 2015; Simcox et al., 2017). Under these conditions BAT vascularization is increased (Xue et al., 2009), further ensuring that a continuous supply of metabolic substrates needed for thermogenesis is secured (Cannon & Nedergaard, 2004). Consequently, lipid metabolites that are generated or consumed by BAT can be observed by the abundance or type of metabolites in plasma, hence, these lipid variations could be utilized as predictors for BAT activity. Evidence of

BAT lipidome changes in active BAT have been previously recorded in many mammal species, as stated above in this chapter (Marcher et al., 2015; Boon et al., 2017; Lu et al., 2017; Simcox et al., 2017; Lynes et al., 2018; Pan et al., 2019; Liu et al., 2022a). On the other hand, studies surrounding BAT activity in new-born lambs are lacking. Consequently, there is still room for research where lipid biomarkers identification could potentially be utilised as BAT activity predictors and contribute to the improvement of new-born lamb survival under cold conditions.

2.6.5 Ribonucleic acid sequencing (RNAseq): Identification and expression level of genes involved in thermogenesis

High-throughput sequencing, such as ribonucleic acid sequencing (RNAseq), has become the top option to measure differential gene expression levels (Mortazavi et al., 2008; Zhang et al., 2014). Moreover, this methodology can be performed without prior knowledge of the reference or sequence of interest (Costa-Silva et al., 2017), which is a crucial advantage when trying to understand different genomic functions (Stark et al., 2019). Ribonucleic acid sequencing is a multi-step process (Wang et al., 2009; Oshlack et al., 2010). Firstly, fragmented RNA is reverse transcribed into complementary DNA and sequencing adaptors are attached. These fragments are then sequenced in the high throughput platform. Resulting sequence reads are mapped to a reference genome or transcriptome and the expression levels for each gene are estimated. Finally, the mapped data is normalized using statistical methods and the differentially expressed genes are recognized, which will then be decoded within its biological context (Li et al., 2015). In summary, the key aim of this methodology is to quantify the changing expression levels of each transcript that take place under specific conditions (Wang et al., 2009). Therefore, RNAseq can be a useful tool to shed light on the molecular aspects that take place during for example, BAT thermogenesis under cold conditions. In fact, many studies have utilized this methodology, where differential expression of a wide variety of genes and pathways appeared to be actively enhancing heat production were

detected in BAT (Marcher et al., 2015; Liu et al., 2022a). Furthermore, another use of this methodology has been reported by Basse et al. (2015), for studying the molecular changes occurring during the transformation of thermogenic BAT into non-thermogenic WAT. All things considered, RNAseq has the potential to be an exploratory tool of the molecular components of heat production under cold exposure in new-born lambs, where little is known. Data produced by this kind of exploration could be key to understand the underlying regulatory mechanisms that control BAT thermogenesis.

2.7 Purpose and scope of the investigation

Between five and 25% of lambs are lost within the first few days of life, where a considerable part of these deaths is due to starvation/exposure. These high mortality rates cause not only an animal welfare concern, but also have economic implications for farmers and result in reduced number of animals for replacement selection. As the skin has a major role on body insulation, several studies have found a positive correlation between skin thickness and cold resistance in new-born lambs. Skin thickness has been proven to be moderately heritable in the Romney breed, making it a potential tool to be added in genetic programs to improve lamb survival. Regardless, studies that explore the heritability of this trait in other breeds and venture into its correlations with production traits are lacking. Therefore, there is scope for research regarding genetic parameters for skin traits selection and their potential for improving lamb survival. Within this thesis, heritability estimates of skin traits, such as skin thickness and skin temperature (as an indicator of heat loss into the environment), were calculated in FocusPrime, Texel, Romney and Highlander sheep breeds. Additionally, genetic estimates for production traits, such as fat and loin-eye muscle depth were calculated in all breeds mentioned. To provide more information for farmers on the potential outcomes of selecting for skin thickness, correlations of the skin traits with several production traits of economic importance were calculated. Besides the importance of selection for skin traits to help

reduce the heat loss into the environment, understanding the thermoregulatory capacity of the new-born lamb is crucial when targeting to improve lamb survival. During the first few days of the lamb's life, heat is produced from non-shivering thermogenesis through its reserves of BAT. This process is linked to a complex network of genes that work towards heat production, and at the same time, can change the abundance and type of lipids within BAT adipocytes and the circulating blood. This thesis studied the differential expression of genes proven to have a role in thermogenesis via an RNAseq approach of thermogenic tissues, BAT and thyroid glands, during a short cold challenge in new-born lambs. Moreover, a lipidomic profile through LC-MS was studied in this thesis, to understand the physiological role of lipids that appear within a thermogenic BAT. Overall, these methodological approaches provide further insight into the molecular thermogenic network and lipidome composition that occur in the new-born lamb when exposed to cold.

Chapter 3. Heritability estimates of skin thickness, skin temperature and production traits in FocusPrime, Texel, Romney and Highlander sheep

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References and supplementary data of this chapter, as submitted to the journal, have been moved to the "References" and "Appendices" sections, respectively, at the end of the thesis. Moreover, formatting has been changed from the submitted version to have a consistent style throughout the thesis.

Abstract

Lamb survival has significant influence on sheep farm profitability, however, direct selection has poor efficacy due to low heritability. Therefore, examination of alternative phenotypes to predict lamb survival breeding values is warranted. Skin thickness is considered a potential, non-invasive, cost-effective and heritable phenotype to indirectly improve lamb survival through its effect on cold tolerance. The aims of this study were to estimate the heritability for ultrasonic measured skin thickness and infrared measured skin temperature, plus several production traits in FocusPrime, Texel, Romney and Highlander sheep breeds. The analysis used records from 5564 lambs from the Focus Genetics breeding flocks in New Zealand. Heritability estimates of all breeds considered as one were 0.28 ± 0.04 for skin thickness, 0.15 ± 0.04 for skin temperature, 0.30 ± 0.05 for fat depth, 0.39 ± 0.05 for loin-eye muscle depth, 0.25 ± 0.04 for loin-eye muscle width, 0.43 ± 0.04 for weaning weight, 0.58 ± 0.05 for 6-month live weight, 0.38 ± 0.05 for 12-month live weight and 0.64 ± 0.08 for 12-month fleece weight. Fat depth, loin-eye muscle depth and width presented sufficient variation for genetic improvement if selected for production purposes. In addition, FocusPrime and Highlander showed high heritability for weights at weaning, six and 12 months, and Romney and Highlander showed high heritability for the fleece weight at 12 months. Skin thickness showed a wide phenotypic variation with moderate heritability, suggesting that it could be utilized for selection. In addition, skin temperature as an indicator of heat loss to the environment, was found to be lowly heritable, although the individual breed heritability for FocusPrime was moderate to high. In conclusion, skin thickness and skin temperature could be utilized as indirect selection phenotypes to improve lamb survival, providing their genetic correlations with lamb survival are suitable.

3.1 Introduction

Lamb mortality is a significant financial and welfare issue for sheep farmers internationally. Reports from many countries suggest lamb loses in the range of 10 to 25% (Hinch et al., 1983; Alexander, 1984; Hinch et al., 1985; Haughey, 1993), but higher rates of up to 30% were observed on some Australian farms (Hinch & Brien, 2014), or even up to 40% on some New Zealand farms (Fisher, 2004; Morris & Kenyon, 2004), especially with adverse weather conditions. Consequently, lamb survival has a high economic importance (Amer et al., 1999; Lopez-Villalobos & Garrick, 1999; Conington et al., 2004). For example, according to a study on hill sheep in Scotland, lamb survival is one of the main influences on overall sheep productivity (Conington et al., 2004). In addition, a decrease in selection potential brought on by fewer lambs to select from adds foregone benefit (Forrest et al., 2006). Lamb losses prior to the age at slaughter were estimated to cost over NZ$580 million annually to New Zealand farmers in 2007 (Everett-Hincks et al., 2007), and A$56 million to the Australian sheep industry in 2006 (Sackett et al., 2006). Besides the economic impact, these losses imply an animal welfare concern (Dwyer, 2008a), providing additional motivation to create efficient strategies to increase lamb survival.

Improving lamb survival by direct selection results in slow genetic change, mainly on account of its low heritability (h^2) (Safari et al., 2005), of between 0.002 and 0.05 (Lopez-Villalobos & Garrick, 1999; Kerslake et al., 2005; Safari et al., 2005; Cloete et al., 2009; Everett-Hincks & Cullen, 2009; Brien et al., 2010; Boujenane et al., 2013). An alternative way to improve lamb survival is through indirect selection of a trait of higher h^2, that is cost-effective to measure and has a strong genetic correlation with lamb survival. In countries such as in New Zealand and Australia, where lambing mostly occurs outdoors, the thermoregulatory capacity of the lamb and its ability to conserve heat is critical for survival since exposure to cold and/or starvation are the main causes of lamb deaths (Haughey, 1993; Mellor & Stafford, 2004). For that reason, lambs might be better suited

to combat cold exposure if they had better insulative properties, such as greater skin and/or fat thickness, and muscle depth (Tait et al., 2015). It is known that the skin plays a vital role in increasing body insulation and reducing heat loss from the body surface (Alexander, 1978), and some studies have observed that new-born lambs with thicker skin were more resistant to cold stress (Samson & Slee, 1981; Stott & Slee, 1987; Slee et al., 1991). As skin thickness is also a moderate to highly heritable trait (Gregory, 1982a; Slee et al., 1991; Janssens & Vandepitte, 2004; Tait et al., 2015; Soltani-Ghombavani et al., 2017), it should be possible to improve lamb survival by indirect selection for skin thickness through its effect on cold tolerance (Soltani-Ghombavani et al., 2017). Skin thickness can be ultrasonically measured, simultaneously with other routinely-measured ultrasound traits such as fat depth and muscle depth at around five to 12 months of age. Adding skin thickness ultrasound measurement to those already stated, would be a low cost and non-invasive phenotype for ram breeders to use in a genetic improvement programme.

Although many studies have been conducted on lamb survival, there are only a few studies that have simultaneously assessed ultrasound measurements of skin thickness (Tait et al., 2015; Soltani-Ghombavani et al., 2017). Besides from the survival perspective, there are only a few estimates of genetic parameters regarding sheep skin traits (McRae et al., 2022) and productive traits for New Zealand breeds such as Highlander and FocusPrime. Therefore, the aims of this study were to estimate the h^2 for skin thickness, skin temperature, subcutaneous fat depth, loin-eye muscle depth and width, 12-month fleece weight, and live weights at weaning, six months, and 12 months in FocusPrime, Texel, Romney and Highlander sheep breeds.

3.2 Materials and methods

3.2.1 Animals and data collection

The present study was undertaken on 2017- and 2019-born lambs, with data collected until 2020 from three Focus Genetics breeding farms in New Zealand: Waikite (Reporoa, 38°18'17.3"S 176°18'24.9"E), Goudies (Reporoa, 38°31'07.4"S 176°28'46.1"E) and Waipuna (Wanganui, 39°49'23.9"S 175°16'38.7"E). The study was composed of 1785 lambs born in 2019 and 3779 born in 2017, a total of 5564 lambs from four breeds: FocusPrime (2017-born = 1236 and 2019-born = 877, from Waikite), Texel (2017-born = 510 and 2019-born = 257, from Waikite), Romney (2017-born = 844 from Goudies) and Highlander (2017-born = 1189 and 2019-born = 651, from Waipuna). FocusPrime and Texel are terminal breeds selected for meat and growth production traits, while Highlander and Romney are maternal breeds with selection based on a self-replacement system (Pickering et al., 2018). The study protocol was approved by Massey University Animal Ethics Committee (MUAEC), protocol number 18/63.

Skin thickness (ST) of each lamb was measured ultrasonically during August 2018 in 2017-born lambs (i.e., approximately 11 months old), and around January/February 2020 in 2019-born lambs (i.e., approximately five months old). A commercial scanning operator undertook these measurements in both years, using a Mindray DP 50 ultrasound system (BCF Ultrasound Australasia, Auckland, New Zealand), with a 40 mm probe at 3.5 MHz set on the left dorsal loin region of the lambs around the 12^{th} rib. It was set up such that one mm of skin depth measured one cm on screen, and measurements were to a tenth of a mm. In case of the 2017-born lambs, on the same day of skin thickness measurement (i.e., when the lambs were approximately 11 months old), skin temperature measurements (Stemp) were also obtained through infrared thermography using an infrared camera (FLIR T650sc; Teledyne FLIR, Wilsonville, OR, USA), mounted on a tripod at a fixed distance (1.2 m at an angle of 50°). Prior to obtaining the infrared

image, the wool at the site was parted to either side using a metal rod so as to capture an accurate recording of the exposed skin. Using FLIR Research IR Max software (Teledyne FLIR, Wilsonville, OR, USA), the exposed skin area on the infrared image for each lamb was delimited and utilized to provide an average of the lamb's skin temperature. Subcutaneous fat depth (FD), loin-eye muscle depth (EMD) and width (EMW) around the 12th rib region were measured ultrasonically at around five months of age in all lambs by another commercial operator, utilizing SONOACE R3 ultrasound machine (Samsung Medison co. Ltd., Seoul, South Korea), with a probe at 3.5 to 4.5MHz set to a depth of 220 mm.

Measurements and records of all traits regarding the Romney breed came only from ram lambs born in 2017. In 2017, ST and Stemp were measured on both ewe and ram lambs, except for the Romney flock, where only ram lambs were measured (Table S3.1). In 2019 ST was measured on ram lambs only. Ultrasound traits, FD, EMD and EMW of all breeds were only measured on ram lambs for both years. Pedigree data and other phenotypes were obtained from the Sheep Improvement Limited (http://www.sil.co.nz) and Focus Genetics databases (http://www.focusgenetics.com), and they included: date of birth, sex (ewe/ram), flock, recording mob for each trait at the time of measurement, birth rank, rearing rank, dam age, dam and sire identities, weaning weight (WWT; at approximately three months of age), 6-month live weight (LW6), 12-month live weight (LW12) and 12-month fleece weight (FW12).

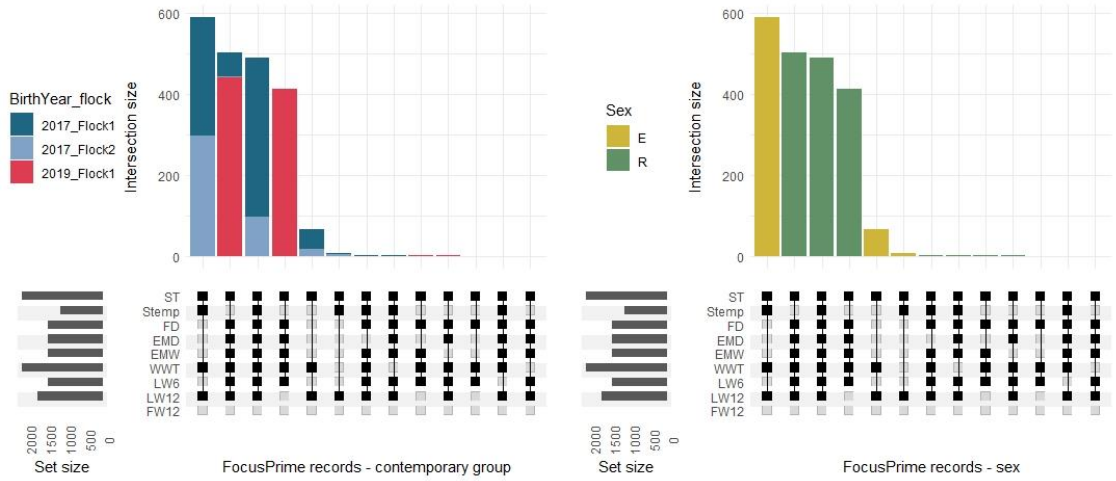
3.2.2 Data editing and plotting

Lambs of unknown parentage in the pedigree dataset (n = 83), and lambs that had insufficient record of birth rank-rearing rank (n = 35) were excluded from the final analysis. Dam ages of 6-years-old or more were all considered as five-years-old (n = 254), due to relative low numbers. Similarly, lambs with a birth rank of four (n = 36) were

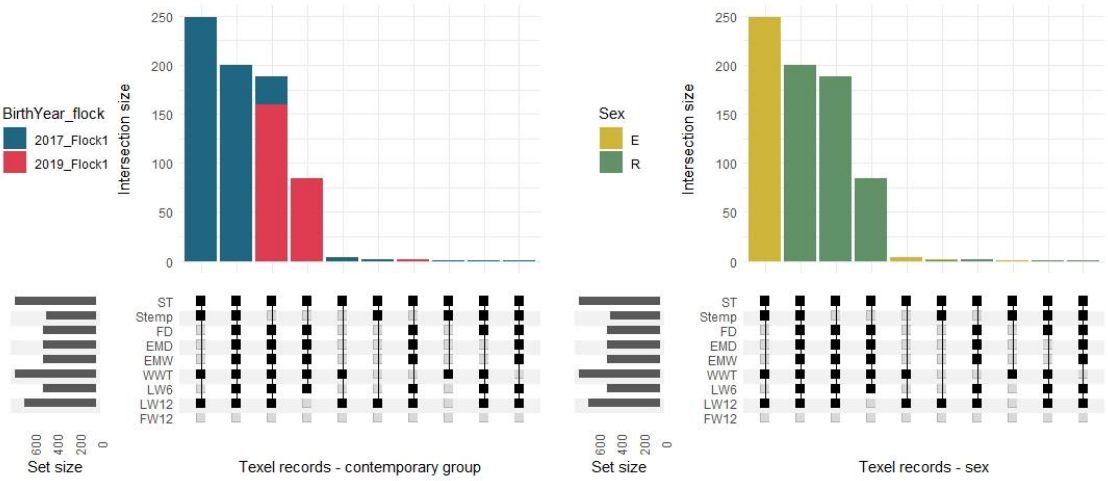
considered as triplets and those with a rearing rank of four (n = 28) were classified as lambs with rearing rank of three, due to relative low numbers. Outlier observations (i.e., values that were outside of the mean ± 4 SD range) were removed so all traits according to each breed followed a normal distribution. After editing, the final dataset had 5446 lambs that included 2088 FocusPrime lambs (born to 68 sires and 1501 dams), 732 Texel lambs (born to 37 sires and 505 dams), 825 Romney lambs (born to 58 sires and 762 dams) and 1801 Highlander lambs (born to 56 sires and 1302 dams). The number of records available for each trait per breed and sex are available in the supplementary section (Table S3.1).

UpSet plots (Figure 3.1) depicting the intersection of records available for each trait based on breed, the contemporary group (BirthYear_flock) and sex, were constructed in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the "tidyverse" package (version 1.3.1) (Wickham et al., 2019) and the "ComplexUpset" package (version 1.3.3) (Krassowski, 2020). In addition, Raincloud plots showing the lamb's measurement ranges for each trait according to breed (Figure 3.2) were plotted in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the "ggplot2" package (version 3.3.5) (Wickham, 2016).

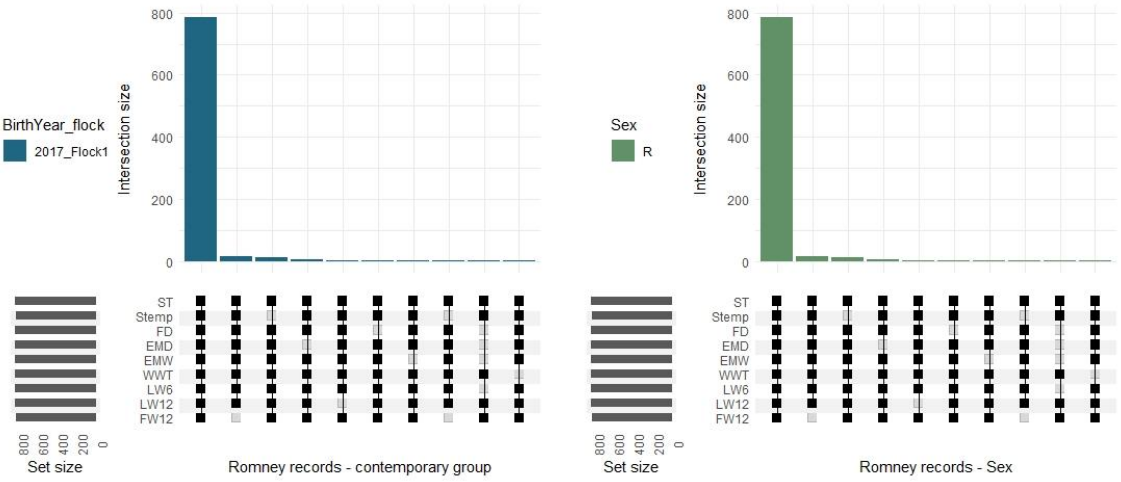
(**A**)



(**B**)


BirthYear_flock
2017_Flock1
2019_Flock1
Intersection size
250
200
150
100
50
0
ST
Stemp
FD
EMD
EMW
WWT
LW6
LW12
FW12
600
400
200
0
Set size
Texel records - contemporary group
Sex
E
R
Intersection size
250
200
150
100
50
0
ST
Stemp
FD
EMD
EMW
WWT
LW6
LW12
FW12
600
400
200
0
Set size
Texel records - sex

(**C**)


BirthYear_flock
2017_Flock1
Intersection size
800
600
400
200
0
ST
Stemp
FD
EMD
EMW
WWT
LW6
LW12
FW12
800
600
400
200
0
Set size
Romney records - contemporary group
Sex
R
Intersection size
800
600
400
200
0
ST
Stemp
FD
EMD
EMW
WWT
LW6
LW12
FW12
800
600
400
200
0
Set size
Romney records - Sex

(**D**)

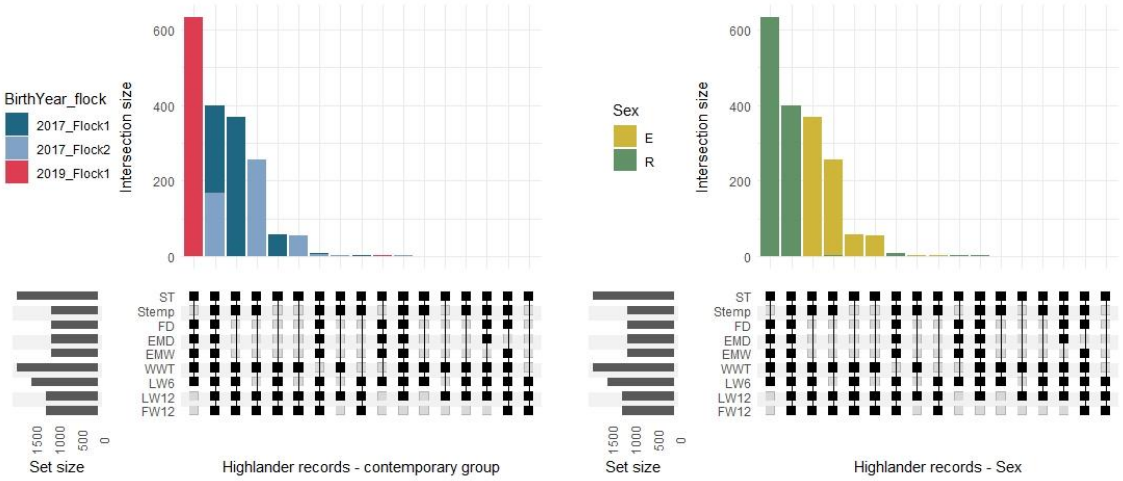


Figure 3.1. UpSet plots showing the intersection of records available for all traits based on breed, FocusPrime (**A**), Texel (**B**), Romney (**C**) and Highlander (**D**)**,** and the contemporary group (BirthYear_flock) (on left), and sex (ewe/ram) (on right). The UpSet plots visualize the intersection between sets, which are the rows in the matrix, and the columns correspond to the intersections between these sets. For the breed and contemporary group plot, it shows colour divided between the year 2017 in blue and light blue for the different flocks within the year, and in pink for the re-utilization of the Flock1 again in the 2019 measurements. For the breed and sex plot, it was divided between ewes (E, in yellow) and rams (R, in green). ST: skin thickness, Stemp: skin temperature, FD: fat depth, EMD: loin-eye muscle depth, EMW: loin-eye muscle width, WWT: weaning weight, LW6: 6-month live weight, LW12: 12-month live weight, FW12: 12-month fleece weight. The Romney dataset, in addition to FD, EMD and EMW measurements in all breeds, did not include ewe lambs' data.

3.2.3 Statistical analysis

Following data editing, the mean and standard deviation for each trait by breed were obtained using the MEANS procedure of SAS 9.4 (SAS Institute Inc. 2013, Cary, NC, USA). Using the same software, analyses of variance for each dependent trait was performed using the GLM procedure. The linear model included the fixed effects of the

contemporary group, which was defined as the group of lambs that were born in the same flock and year (BirthYear_flock, 1-3 levels depending on the trait/breed), sex (ewe/ram), Birth_rearing type (born as single, twin or triplet, and reared as single, twin or triplet, 6-8 levels depending on the trait/breed), the age of the dam (one to five years old, 4-5 levels depending on the trait/breed) and the recording mob for each trait at measurement (1-17 levels depending on the trait/breed). For ST analysis, the effect of the age at scanning (around five or 11 months of age) according to its contemporary group (Lamb age*BirthYear_flock) was included as a covariate. For FD, EMD and EMW analysis, the weight at six months (LW6) was included as a covariate. These fixed effects and covariates were included in the model for each breed according to the availability of the records per trait. Least squares means and standard errors were estimated for each class of the fixed effects and were used for multiple mean comparisons using Fisher's least significant differences. Means were considered significantly different when $P < 0.05$.

3.2.4 Estimation of variance components and heritability

A single-trait animal model was used for the estimation of the variance components in order to calculate the h^2 of each trait within each breed, and for all breeds together (considered as one). These calculations were undertaken using the statistical package ASReml version 4.2 (Gilmour et al., 2015). The model included the fixed effects and covariates previously described and the random effect of the animal.

In matrix notation, the single-trait model can be represented as:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{e}$$

where **y** is the vector of the phenotypic measure for the trait under analysis; **X** and **Z** constitute the design matrices pertaining to the fixed and random effects, respectively, on the phenotype, **b** is the vector of the fixed effects of the contemporary group, sex, birth-rearing type, the age of the dam and the recording mob; **a** is the vector of the

random effect of the animal for the trait; and **e** is the vector of random residual error for the trait. The distributional properties of the model with E and V indicating the expectation and variance were as follows:

$$E(\mathbf{y}) = \mathbf{Xb}$$

and

$$\text{Var}\begin{bmatrix}\mathbf{a}\\ \mathbf{e}\end{bmatrix} = \begin{bmatrix}\mathbf{A}\sigma_a^2 & 0\\ 0 & \mathbf{I}\sigma_e^2\end{bmatrix}$$

where **A** is the numerator relationship matrix of order equal to the total number of animals in the pedigree file, according to one breed being analyzed or all breeds together (considered as one); σ^2_a is the animals' (co)variance component for the trait considered; I is an identity matrix of order equal to the number of records; and σ^2_e is the residual (co)variance component for the trait.

The h^2 for each trait was calculated as:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_p^2}$$

where σ^2_g and σ^2_p are the animal additive genetic and residual variances, respectively, and their sum is the phenotypical variance.

The pedigree file containing all breeds had; 211 sires with 15 paternal grandsires and 19 paternal granddams, 4070 dams with 84 maternal grandsires and 517 maternal granddams. Additionally, the whole pedigree file had a range of 2-5 generations on the sire side and a range of 2-4 generations on the dam side.

Furthermore, a t-test for all heritability estimates was calculated to demonstrate that the variance was significantly different from zero (Table S3.2).

3.3 Results

3.3.1 Descriptive statistics

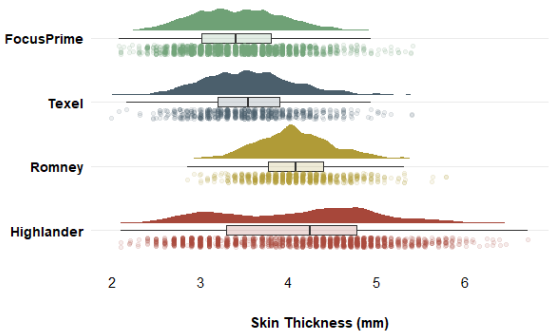
The number of records, mean and standard deviation (SD) for each trait by breed are presented in Table 3.1, with trait distributions shown in Figure 3.2. Mean ST was significantly higher ($P < 0.05$) in Romney and Highlander, followed by Texel and FocusPrime. In respect to the Stemp, mean values were significantly different ($P < 0.05$) between all breeds, where the highest mean value was recorded in Romney, followed in decreasing order by FocusPrime, Texel and Highlander. Ultrasound measured FD was observed as significantly higher ($P < 0.05$) in Focus Prime and Highlander**,** followed by Texel and Romney. In the case of EMD and EMW, mean values were significantly different ($P < 0.05$) between all breeds, where the highest mean value for both traits was recorded in FocusPrime, followed in decreasing order by Texel, Highlander and Romney. Weaning weight was heaviest in FocusPrime, followed by Texel, where both these values were significantly greater ($P < 0.05$) than in Romney and Highlander. Mean values for LW6 were significantly different ($P < 0.05$) between all breeds, where the heaviest mean value was recorded in FocusPrime, followed in decreasing order by Texel, Highlander and Romney. Mean values for LW12 were significantly different ($P < 0.05$) between all breeds, where the heaviest mean value was recorded in Romney, followed in decreasing order by FocusPrime, Texel and Highlander. Additionally, FW12 mean was significantly higher ($P < 0.05$) in Romney, compared to that in Highlander. There were no fleece records available for FocusPrime or Texel.

Table 3.1. Descriptive statistics (Mean ± SD) and number of records for each trait in FocusPrime, Texel, Romney and Highlander sheep.

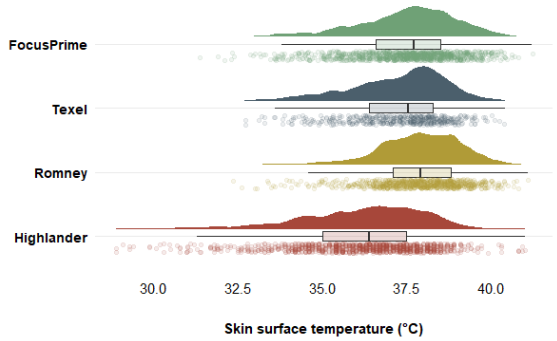
Trait	FocusPrime			Texel			Romney			Highlander		
	No. of records	Mean	SD	No. of records	Mean	SD	No. of records	Mean	SD	No. of records	Mean	SD
ST (mm)	2088	3.45^{a}	0.54	732	3.56^{b}	0.53	825	4.08^{c}	0.45	1801	4.12^{c}	0.88
Stemp (°C)	1099	37.47^{a}	1.53	454	37.24^{b}	1.44	813	37.84^{c}	1.32	1049	36.14^{d}	1.97
FD (mm)	1422	3.78^{a}	1.00	476	3.64^{b}	1.06	822	3.17^{c}	0.97	1050	3.83^{a}	1.19
EMD (mm)	1414	30.42^{a}	3.03	475	29.08^{b}	3.36	820	22.84^{c}	2.30	1049	26.58^{d}	3.03
EMW (mm)	1419	73.24^{a}	6.05	475	69.75^{b}	6.74	822	62.00^{c}	5.28	1049	64.46^{d}	5.61
WWT (kg)	2074	38.21^{a}	7.10	727	33.86^{b}	6.18	824	28.71^{c}	5.35	1785	28.50^{c}	4.78
LW6 (kg)	1421	47.38^{a}	7.35	476	42.76^{b}	6.85	824	35.98^{c}	4.93	1485	36.62^{d}	6.25
LW12 (kg)	1672	64.32^{a}	11.98	647	59.80^{b}	9.04	823	67.57^{c}	6.30	1163	52.75^{d}	8.50
FW12 (kg)	0	.	.	0	.	.	809	3.14^{a}	0.38	1153	2.32^{b}	0.61

ST: skin thickness, Stemp: skin temperature, FD: fat depth, EMD: loin-eye muscle depth, EMW: loin-eye muscle width, WWT: weaning weight, LW6: 6-month live weight, LW12: 12-month live weight, FW12: 12-month fleece weight. For each trait, mean values across breeds with different superscripts differ significantly ($P < 0.05$). A dot is displayed where no records were available. The Romney dataset, in addition to FD, EMD and EMW measurements in all breeds, did not include ewe lambs' data.

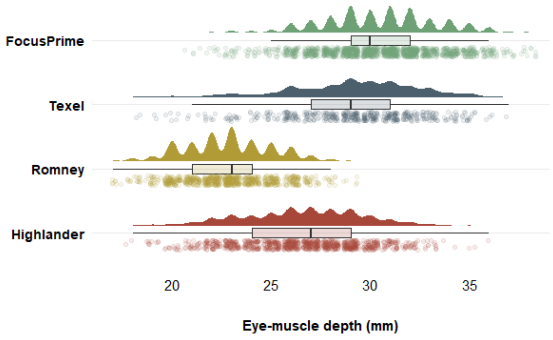
(A)


FocusPrime
Texel
Romney
Highlander
2
3
4
5
6
Skin Thickness (mm)

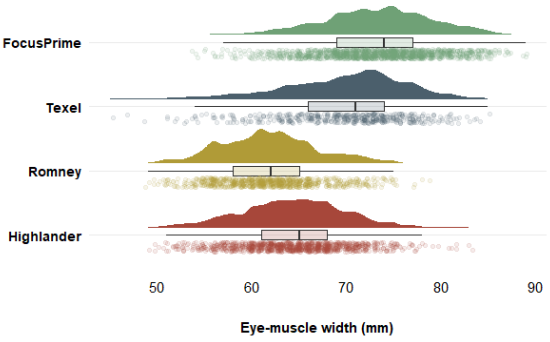
(B)


FocusPrime
Texel
Romney
Highlander
30.0
32.5
35.0
37.5
40.0
Skin surface temperature (°C)

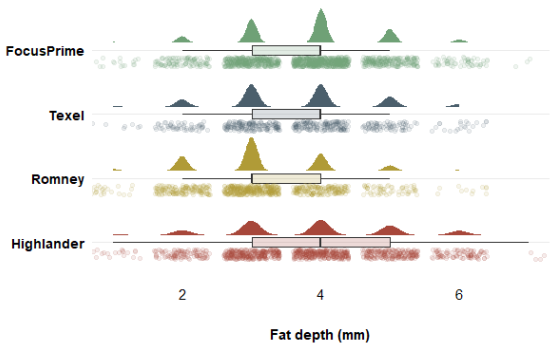
(C)


FocusPrime
Texel
Romney
Highlander
20
25
30
35
Eye-muscle depth (mm)

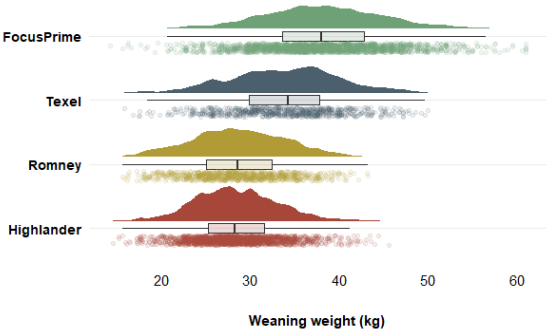
(D)


FocusPrime
Texel
Romney
Highlander
50
60
70
80
90
Eye-muscle width (mm)

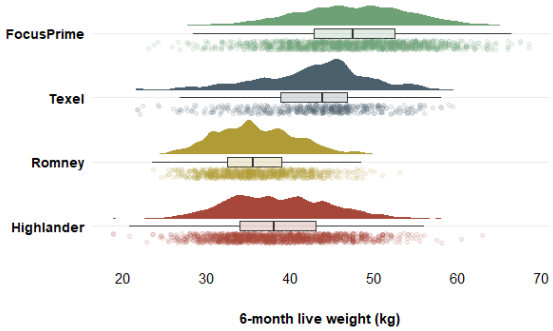
(E)


FocusPrime
Texel
Romney
Highlander
2
4
6
Fat depth (mm)

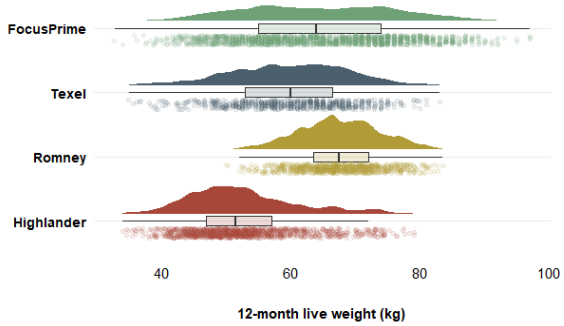
(F)


FocusPrime
Texel
Romney
Highlander
20
30
40
50
60
Weaning weight (kg)

(**G**)



(**H**)



(**I**)

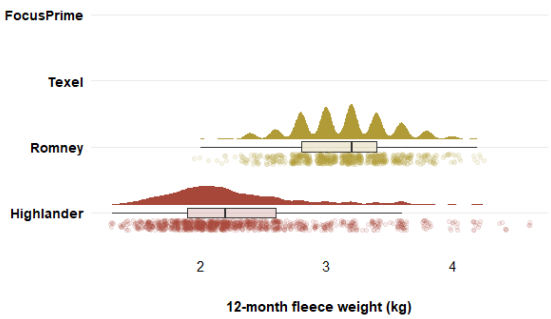


Figure 3.2. Raincloud plots showing measurement ranges for each sheep trait according to breed. (**A**) skin thickness, (**B**) skin temperature, (**C**) loin-eye muscle depth, (**D**) loin-eye muscle width, (**E**) fat depth, (**F**) weaning weight, (**G**) 6-month live weight, (**H**) 12-month live weight and (**I**) 12-month fleece weight. The raincloud plots visualize the data distribution (the “cloud”), with the jittered raw data (the “rain”), where in between a boxplot (showing the minimum to maximum towards the right, with the interquartile range depicted in between) was added as supplementary standard visualization.

3.3.2 Factors affecting ST

Analysis of variance results for different fixed and covariate factors on ST in each breed are presented in Table 3.2.

Table 3.2. F-values for fixed and covariate factors affecting skin thickness in FocusPrime, Texel, Romney and Highlander sheep.

Factor	FocusPrime	Texel	Romney	Highlander
BirthYear_flock	5.22**	0.15	.	1.67
Sex	33.40***	63.30***	.	38.52***
Birth_rearing type	1.50	1.68	1.08	0.99
Age of dam	2.72*	3.02*	1.22	0.72
Recording mob	38.65***	12.01***	1.28	87.72***
Lamb age*BirthYear_flock	11.63***	1.76	.	1.84

*P < 0.05, ** P < 0.01, *** P < 0.001

A dot is displayed where no records were available for effects calculation.

The contemporary group BirthYear_flock had a significant effect (P < 0.05) on ST only in the FocusPrime breed. The effect of sex on ST was significant (P < 0.05) in FocusPrime, Texel and Highlander. FocusPrime and Texel ewe lambs had 0.41 ± 0.07 and 0.48 ± 0.06 mm lower ST than respective ram lambs, whereas Highlander ewe lambs had 0.30 ± 0.06 mm higher ST than respective ram lambs. Birth_rearing type did not have a significant effect on ST in any of the breeds studied. The effect of age of the dam on the ST was significant (P < 0.05) in FocusPrime and Texel lambs. In FocusPrime, two to four-year-old dams were associated with 0.08 ± 0.03, 0.07 ± 0.03 and 0.11 ± 0.04 mm less thickness, respectively, when compared to a 5-year-old dam. However, 1-year-old Texel dams were associated with 0.33 ± 0.13 mm greater thickness, when compared to 5-year-old dams. The effect of the recording mob was significant in FocusPrime, Texel and Highlander. Lastly, the age at ultrasound scanning (Lamb age*BirthYear_flock) had a significant effect on ST (P < 0.05) only in FocusPrime.

3.3.3 Factors affecting Stemp

Analysis of variance results for different fixed and covariate factors on Stemp in each breed are presented in Table 3.3.

Table 3.3. F-values for fixed and covariate factors affecting skin temperature in FocusPrime, Texel, Romney and Highlander sheep.

Factor	FocusPrime	Texel	Romney	Highlander
BirthYear_flock	0.03	.	.	0.54
Sex	149.5***	108.47***	.	1.99
Birth_rearing type	1.54	1.31	1.00	0.78
Age of dam	0.61	2.15	0.38	0.46

*P < 0.05, ** P < 0.01, *** P < 0.001

A dot is displayed where no records were available for effects calculation.

The contemporary group (BirthYear_flock) did not have a significant effect on Stemp in FocusPrime nor Highlander. Sex had a significant effect (P < 0.05) on Stemp in FocusPrime and Texel, where ewe lambs in the two breeds had 1.12 ± 0.09 and 1.22 ± 0.12 °C higher Stemp, respectively, than the respective ram lambs for each breed. Birth_rearing type did not have a significant effect on Stemp in any of the breeds studied. The effect of age of the dam on the lamb's Stemp was non-significant in all of the breeds studied. Recording mob was not fitted in this analysis, as there was only one per sex and would have confounded with sex in the model.

3.3.4 Heritabilities

Estimates of variance components (genetic, residual and phenotypic) and h^2 for each trait per breed, and for all breeds considered as one, are presented in Table 3.4. Moreover, the variance for these heritability estimates has proven to be significantly different than zero via a t-test (Table S3.2). Heritability estimates can be categorized according to Bailey et al. (2014), where a value less than 0.20 is considered as low,

values of 0.21 to 0.40 as moderate and those above 0.40 as high. The h^2 estimates for ST were mostly moderate, with a lower value in Highlander. Estimates for Stemp were mostly low, reaching a disparity of almost-zero in Romney and a moderate to high value in FocusPrime. Estimates for ultrasonic FD were consistently moderate in all breeds. For both muscle traits, moderate estimates were found in FocusPrime and Romney, whereas a low estimate was found in Texel. Highlander showed a moderate estimate for EMD and a lower one for EMW. In the case of WWT, LW6 and LW12 the h^2 estimate was highest in FocusPrime and Highlander, compared to the other two breeds which were mostly moderate. For FW12, a much greater h^2 estimate was recorded for Highlander, compared to Romney.

Table 3.4. Estimation of variance components (additive genetic, residual and phenotypic) and heritabilities (± SE) for each trait in FocusPrime, Texel, Romney, Highlander, and all breeds considered as one.

		Variance			
Trait	**Breed**	**Genetic**	**Residual**	**Phenotypic**	**Heritability**
ST	**Focus Prime**	0.08 ± 0.02	0.15 ± 0.01	0.23 ± 0.01	0.36 ± 0.06
	Texel	0.06 ± 0.02	0.19 ± 0.02	0.25 ± 0.01	0.23 ± 0.10
	Romney	0.06 ± 0.02	0.14 ± 0.02	0.20 ± 0.01	0.28 ± 0.10
	Highlander	0.04 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.17 ± 0.05
	All breeds	0.06 ± 0.01	0.17 ± 0.01	0.23 ± 0.00	0.28 ± 0.04
Stemp	**Focus Prime**	0.82 ± 0.26	1.24 ± 0.21	2.06 ± 0.10	0.40 ± 0.11
	Texel	0.27 ± 0.18	1.39 ± 0.18	1.66 ± 0.12	0.16 ± 0.11
	Romney	0.07 ± 0.05	1.82 ± 0.10	1.76 ± 0.09	0.04 ± 0.03
	Highlander	0.48 ± 0.23	3.41 ± 0.24	3.88 ± 0.17	0.12 ± 0.06
	All breeds	0.37 ± 0.09	2.19 ± 0.09	2.56 ± 0.06	0.15 ± 0.04
FD	**Focus Prime**	0.20 ± 0.06	0.44 ± 0.05	0.64 ± 0.03	0.32 ± 0.08
	Texel	0.20 ± 0.09	0.42 ± 0.08	0.62 ± 0.04	0.33 ± 0.13
	Romney	0.14 ± 0.06	0.46 ± 0.06	0.60 ± 0.03	0.24 ± 0.10
	Highlander	0.17 ± 0.07	0.66 ± 0.06	0.83 ± 0.04	0.21 ± 0.08
	All breeds	0.21 ± 0.04	0.49 ± 0.03	0.70 ± 0.02	0.30 ± 0.05
EMD	**Focus Prime**	1.63 ± 0.42	2.51 ± 0.33	4.14 ± 0.18	0.39 ± 0.09
	Texel	0.69 ± 0.39	2.87 ± 0.38	3.56 ± 0.24	0.19 ± 0.11
	Romney	1.06 ± 0.35	1.57 ± 0.30	2.63 ± 0.14	0.40 ± 0.12
	Highlander	1.08 ± 0.38	2.24 ± 0.32	3.33 ± 0.17	0.33 ± 0.11
	All breeds	1.45 ± 0.22	2.30 ± 0.18	3.75 ± 0.10	0.39 ± 0.05
EMW	**Focus Prime**	3.63 ± 1.16	12.08 ± 1.04	15.71 ± 0.63	0.23 ± 0.07
	Texel	2.55 ± 1.62	12.45 ± 1.62	15.01 ± 1.01	0.17 ± 0.11
	Romney	3.64 ± 1.62	13.03 ± 1.53	16.67 ± 0.86	0.22 ± 0.09
	Highlander	1.51 ± 0.87	11.43 ± 0.90	12.93 ± 0.58	0.12 ± 0.07
	All breeds	4.04 ± 0.72	12.05 ± 0.65	16.09 ± 0.39	0.25 ± 0.04
WWT	**Focus Prime**	14.54 ± 2.10	15.29 ± 1.57	29.83 ± 1.08	0.49 ± 0.06
	Texel	5.23 ± 2.09	14.60 ± 1.83	19.83 ± 1.13	0.26 ± 0.10
	Romney	4.14 ± 1.77	10.90 ± 1.59	15.04 ± 0.79	0.28 ± 0.11
	Highlander	6.46 ± 1.38	8.93 ± 1.06	15.39 ± 0.62	0.42 ± 0.08
	All breeds	9.43 ± 1.00	12.49 ± 0.78	21.92 ± 0.49	0.43 ± 0.04
LW6	**Focus Prime**	22.76 ± 3.84	13.41 ± 2.84	36.17 ± 1.66	0.63 ± 0.09
	Texel	5.03 ± 3.42	20.57 ± 3.22	25.60 ± 1.75	0.20 ± 0.13
	Romney	7.43 ± 2.48	11.33 ± 2.10	18.76 ± 1.02	0.40 ± 0.12
	Highlander	10.56 ± 2.25	12.08 ± 1.71	22.64 ± 1.01	0.47 ± 0.09
	All breeds	16.63 ± 1.78	12.13 ± 1.34	28.76 ± 0.76	0.58 ± 0.05

Trait	Breed	Variance			
		Genetic	Residual	Phenotypic	Heritability
LW12	**Focus Prime**	20.15 ± 4.23	26.91 ± 3.32	47.06 ± 1.91	0.43 ± 0.08
	Texel	8.58 ± 3.75	24.22 ± 3.30	32.8 ± 1.98	0.26 ± 0.11
	Romney	8.89 ± 3.11	21.43 ± 2.80	30.32 ± 1.58	0.29 ± 0.10
	Highlander	13.53 ± 3.41	15.12 ± 2.67	28.65 ± 1.42	0.47 ± 0.10
	All breeds	14.81 ± 2.07	23.99 ± 1.69	38.8 ± 0.95	0.38 ± 0.05
FW12	**Romney**	0.09 ± 0.02	0.06 ± 0.02	0.14 ± 0.01	0.61 ± 0.14
	Highlander	0.09 ± 0.02	0.04 ± 0.01	0.13 ± 0.01	0.67 ± 0.10
	All breeds	0.09 ± 0.01	0.05 ± 0.01	0.14 ± 0.01	0.64 ± 0.08

ST: skin thickness, Stemp: skin temperature, FD: fat depth, EMD: loin-eye muscle depth, EMW: loin-eye muscle width, WWT: weaning weight, LW6: 6-month live weight, LW12: 12-month live weight, FW12: 12-month fleece weight. No records were available for FW12 in FocusPrime or Texel.

3.4 Discussion

Alternative strategies are needed to genetically improve lamb survival, given that its h^2 is near-zero (Everett-Hincks et al., 2005; Cloete et al., 2009). Indirect selection for a trait that has a favourable genetic correlation with lamb survival, like ST, would enable faster genetic progress, thereby tackling lamb mortality issues for farmers in an achievable and cost-effective way. The present study reports summary statistics and estimates of heritabilities for ST and Stemp, in addition to a few production traits in FocusPrime, Texel, Romney and Highlander sheep breeds.

In the present study, ST was lowest in FocusPrime, while it was highest in the Romney and Highlander breeds. Variation in sheep ST are not well documented (Brown et al., 2000), nevertheless, thickness means reported here are within the range reported in previous studies (Slee et al., 1991; Jopson et al., 2000; Tait et al., 2015). The slight differences in ST between studies might be attributed to differences in breed and age at the time of measurement. There is also variation in the techniques employed; some studies used skinfold callipers (Slee et al., 1991; Jopson et al., 2000), while a few other

studies employed an ultrasound machine (Tait et al., 2015; Soltani-Ghombavani et al., 2017). In addition, accuracy of the ultrasound method varies. Brown et al. (2000) stated that there was a consistent difference in the results whether the skin was measured with skinfold callipers or by ultrasound, where for instance, the skin of a 2-year-old Merino in summer could measure 1.74 mm by callipers versus 2.56 mm by ultrasound. In addition, the time of the year can influence the skin measurement itself, since ST can vary with the seasons or even through cyclical hair growth (Turton & Hooson, 1997; Brown et al., 2000).

Skin thickness had moderate h^2 for all breeds except the Highlander in the present study. These estimates were found to be comparable with those compiled from Janssens & Vandepitte (2004), Gregory (1982a), Tait et al. (2015), Soltani-Ghombavani et al. (2017) and Slee et al. (1991). Slight variations between these estimates may come from differences in breeds, age, the techniques utilized, the time of the year when the measurements were made and statistical models utilized, which would likely affect the h^2 estimates.

Skin temperature was highest in Romney sheep and lowest in Highlander sheep. Moderate heritability was found in FocusPrime, and low estimates for the other breeds. However, estimates in Texel and Romney were linked to high standard errors. No published reports were found for the h^2 of Stemp in lambs of 11 months of age. Nevertheless, the results obtained here for most breeds were similar to the estimates for rectal temperature in cattle, as reported by Morris et al. (1989) or Dikmen et al. (2012), or for Stemp in chickens (Loyau et al., 2016). The estimates of Stemp obtained require verification by addition studies, and if confirmed to be moderate, the trait has great potential for selection.

In the present study, Stemp was measured alongside ST as an indicator of heat loss into the environment. In this context, the lowest Stemp value was recorded in the Highlander

breed which has the thickest skin among the breeds of this study. On the other hand, a relatively high temperature was recorded in the thinnest skin breed, FocusPrime. Considering these observations, it could be implied that sheep having better insulation through a thicker skin would lose less heat into the environment. In support of this notion, Soltani-Ghombavani (2021) observed that during an induced cold stress period, thick-skinned, new-born, Romney lambs had lower Stemp than thin-skinned lambs. Consequently, they proposed that having a thicker skin could positively affect thermoregulation in lambs by minimizing heat loss through the skin surface, while reducing the heat production required to maintain body temperature. Ram lambs of FocusPrime and Texel breeds in the present study showed thicker skin and lower Stemp than the ewe lambs. Data collected by Cloete et al. (2004) and Van Der Merwe et al. (2021) somewhat supports this, where ST appeared to be affected by sex, with rams having heavier skin weights or producing thicker leathers, compared to ewes. However, contradictory data was found by Soltani-Ghombavani et al. (2017), where ewe lambs of approximately eight months of age had thicker skin when compared to ram lambs, which would be in agreement of the results in the current study regarding the Highlander breed. Meanwhile, Jopson et al. (2000) found no difference between sexes.

In addition to the sex effect, ST of the lambs were affected by the age of the dam. Specifically, 2-year-old to 4-year-old FocusPrime dams had lambs with less ST, and 1-year-old Texel dams had lambs with greater ST, when compared to a 5-year-old dam within each breed. The effect of the dam age on lamb survival has been reported; older ewes have lambs with higher survival rates (Knight et al., 1988; Riggio et al., 2008), where the highest survival rate is observed in lambs born to ewes of four to six years of age (Lopez-Villalobos & Garrick, 1999). These observations are based on the concept that mature ewes are more experienced dams and give birth to heavier/larger lambs, thus increasing new-born lamb survival (Dalton et al., 1980). Unfortunately, there is a lack of studies reporting a link between the age of a dam with its lambs' ST. This is an

area that might require further research to allow for the non-genetic effect of dam age to be removed in the calculation of breeding values.

Ultrasound-measured FD was lowest in Romney, and highest in FocusPrime and Highlander, and a moderate h^2 estimate was observed in all breeds. Overall means and estimates observed for this trait in the Highlander and Romney breeds were within the range reported by Tait et al. (2015) and Brito et al. (2017). Meanwhile, slightly higher estimates from ultrasonic measurements as the ones by Soltani-Ghombavani et al. (2017) (0.36) and Cameron & Bracken (1992) (0.35) were comparable to the ones reported here in FocusPrime and Texel. Nonetheless, FD is still observed as a moderately heritable trait in all studies.

Other ultrasound measured traits such as EMD and EMW were lowest in Romney, and highest in FocusPrime. Heritability estimates for these traits across breeds were mostly moderate, although in Texel and Highlander the estimates for EMW were linked to high standard errors. Means and h^2 estimates for EMD were in the range reported by Maximini et al. (2012), Huisman & Brown (2008) and Brito et al. (2017), whereas reports by Pinares-Patiño et al. (2013) (0.50) observed a much higher estimate than the current study. In respect to EMW, means and h^2 estimates were similar to those reported by Brito et al. (2017) and Fogarty et al. (2003), and appeared to be much in the lower end if compared to Waldron et al. (1992) (0.63). These differences in estimates may come from different models for analysis, breeds, and ages at ultrasonic measurement, but overall, estimates were similar to those already reported.

Mean measurements for WWT were lowest in Romney and Highlander, and highest in FocusPrime. The h^2 estimate for this trait was high in Highlander and even greater in FocusPrime, while a moderate estimate was found in the other breeds. Heritability estimates in the Texel and Romney breeds were comparable to those reported by Safari et al. (2007) (0.29) and Duguma et al. (2002) (0.26). Meanwhile higher estimates as the

ones observed by Brash et al. (1994) (0.45) were comparable to the ones estimated here in FocusPrime and Highlander.

Regarding other weight traits, LW6 was lowest in Romney and highest in FocusPrime, whereas LW12 was lowest in Highlander and highest in Romney. Both traits showed similar h^2 estimates, which were high in FocusPrime and Highlander**.** It appears that in the span of time between LW6 and LW12, the Romney breed was able to increase its body weight more significantly than the other breeds and become the heaviest at 12 months of age. While some culling based on physical appearance, breeding values or selection indexes occurred before LW6, this process should have biased LW12 similarly for all breeds. Heritability estimates reported for LW6 in Texel were comparable to that reported by Riggio et al. (2008) (0.26), while estimates for this trait in Romney and Highlander were in the range of those reported by Wuliji et al. (2001) (0.44) and Fossceco & Notter (1995) (0.41). Regarding LW12, means and h^2 estimates in Texel and Romney were in agreement with the ones reported by Janssens et al. (2000), while reports by Cloete et al. (2001) (0.45) and Huisman & Brown (2008) (0.40) where in the range reported here in FocusPrime and Highlander.

Twelve-month fleece weight was only recorded in the two maternal breeds, where the lowest mean value was registered in Highlander, and the highest in Romney. For this trait, a high h^2 estimate was recorded in Romney and an even greater one was in Highlander. A somewhat comparable mean and h^2 estimate to the one observed in the present study in Romney was reported by Mortimer et al. (2017) (0.57). Other reports such as by Huisman et al. (2008) (0.46) or Johnson et al. (1995) (0.45), have comparable means but much lower estimates than the ones observed in the current study. It may be the case that the smaller sample size utilized in this study could have inflated the heritability estimates, if compared with the above-mentioned studies in far more animals. Regardless of the differences observed between these studies which might also imply

variation between breeds or in the time that the measurements were taken, this trait still appears to be highly heritable.

In summary, the h^2 estimates presented here could contribute to future breeding programs, since most traits described possess an adequate amount of variation that is needed for genetic gain through selection. Especially for ST, there would be sufficient phenotypic variation and moderate genetic control, implying that indirect selection for lamb survival through this trait could be achieved, providing the genetic correlations with lamb survival is favourable. It is suggested that for the FocusPrime breed, it would be particularly beneficial to also include Stemp as a trait itself, together with ST when selecting for lamb survival, since a moderate-high h^2 estimate was recorded for Stemp uniquely in this breed. All things considered, ST as the main trait of interest here, in addition to Stemp as an indicator of heat loss from the body, have the potential to be valuable traits to consider in future breeding programs to improve lamb survival. Nevertheless, it is important that before these skin traits are utilised in a selection index, more studies would be needed to explore relationships with production traits in young lambs, and as they age. Studies regarding how thicker-skinned animals would endure hot conditions, and how those conditions might affect their performance would also be needed, so that no unfavourable effects were unintentionally selected for.

3.5 Conclusions

Skin thickness was shown to have a moderate h^2, and in combination with its wide phenotypic variation, it shows promise for the sheep industry for improving lamb survival by indirect selection. In addition, this trait can be measured cost-effectively, and can be assessed simultaneously with routine ultrasound-measurements for production traits. Furthermore, skin temperature, as an indicator of heat loss to the environment, could be a beneficial addition to the selection for skin thickness, especially in FocusPrime due to its moderate to high heritability. Traits such as WWT, LW6 and LW12 in FocusPrime and

Highlander, and FW12 in Romney and Highlander, showed a considerable amount of variation needed for genetic improvement through selection. Additional research is required to estimate the magnitude of genetic covariation between skin thickness, skin temperature and important production traits and lamb survival to ensure that indirect selection is efficacious.

Chapter 4. Genetic and phenotypic correlations of skin thickness and skin temperature with key production traits in FocusPrime, Texel, Romney and Highlander sheep

Chapter submitted to the *Journal of Animal Science* as: **Graña-Baumgartner, A., Dukkipati, V. S., Biggs, P. J., Kenyon, P. R., Blair, H. T., Pickering, N. K., Van der Linden D. S. and López-Villalobos, N.** Genetic and phenotypic correlations of skin thickness and skin temperature with key production traits in FocusPrime, Texel, Romney and Highlander sheep.

References of this chapter, as submitted to the journal, have been moved to the "References" section at the end of the thesis. Moreover, formatting has been changed from the submitted version to have a consistent style throughout the thesis.

Abstract

A major contributor to new-born lamb mortality under pastoral conditions is starvation/exposure, which can be a welfare concern and cause production/economic losses. Hypothermia is a major contributor to starvation/exposure mortality rates, therefore, being able to select a lamb with enhanced ability to withstand cold environments would be of benefit. In this regard, skin thickness is a moderately heritable trait which is positively correlated with lamb survival, through its effects on thermoregulation and cold tolerance. However, prior to including this trait into breeding programs to improve lamb survival, it is essential to explore how selection based on the skin trait might affect other production traits such as live weight, fat, and loin-eye muscle depth, which are often included in breeding schemes in New Zealand. The aim of this study was to estimate genetic and phenotypic correlations of ultrasound-measured skin thickness and infrared-measured skin temperature with key production traits in the FocusPrime, Texel, Romney and Highlander breeds in New Zealand. The bivariate-trait analyses utilized a dataset of 5564 lambs from the Focus Genetics breeding flocks in New Zealand. Positive correlations were found between skin thickness and other known insulation traits, such as fleece weight, fat depth and body weight. In contrast, a negative correlation was found between skin thickness and skin temperature. An overall positive association of fat depth measurements with loin-eye muscle depth and width, and live weight at six months, was found. In addition, weight measurements at weaning, six and 12 months had a positive correlation with fleece weight at 12 months in Romney and Highlander. The present study suggests selecting for thicker skin should help improve lamb survival and it could be integrated into selection programs where hypothermia is a major cause of lamb losses.

4.1 Introduction

New-born lamb mortality can be a serious issue, causing worldwide economic and production losses in addition and also being a welfare issue (Mellor & Stafford, 2004). It is heavily influenced by the weather conditions at lambing in outdoor pastoral systems (Tait et al., 2015). Under cold and wet weather conditions, approximately 30% of lamb mortalities can be due to starvation/exposure within the first three days of life (Williams & Thornberry, 1992). Hypothermia is the major contributor to starvation/exposure mortality rates as it can cause a rapid decrease in lamb milk-suckling activity (Alexander & Williams, 1966). In this scenario, death from starvation becomes accelerated, as the neonate reserves are limited and can be swiftly diminished (Dwyer & Lawrence, 2005). In less extreme weather conditions, a cold environment can still be deadly if combined with undernourished small lambs, such as those from litters of two or more lambs, as they may lose heat more quickly due to their small size and are therefore more prone to be at risk from cold exposure (Slee et al., 1991). Accordingly, increased lamb survival has been the objective of many research studies, especially in extensive outdoor production systems (Riggio et al., 2008; Brien et al., 2010).

Lamb survival has a very low heritability of between 0.002 and 0.05 (Lopez-Villalobos & Garrick, 1999; Cloete et al., 2009; Everett-Hincks & Cullen, 2009; Boujenane et al., 2013), therefore selecting for the trait itself will only result in slow genetic progress. Consequently, utilizing indirect selection criteria, such as selecting for the individual components of lamb survival, for example heat loss/production or skin thickness, could provide alternate pathways to improve lamb survival. Previous studies have reported the existence of a positive correlation between skin thickness and cold tolerance in new-born lambs (Samson & Slee, 1981; Stott & Slee, 1987; Slee et al., 1991). A more recent study by Soltani-Ghombavani et al. (2021) found that skin thickness at around eight months of age in the Romney breed had a positive genetic correlation with lamb survival from birth to weaning. They reported that thick-skinned lambs had a greater ability to

maintain body temperature when exposed to cold, as opposed to thin-skinned paired contemporaries. In addition, skin thickness was observed as a moderately heritable trait in Chapter 3 and in previous reports (Slee et al., 1991; Janssens & Vandepitte, 2004; Tait et al., 2015; Soltani-Ghombavani et al., 2017). Hence, it is of interest to explore the potential of selection for skin thickness to improve lamb survival, for the enhancement of cold tolerance.

Skin thickness can be measured in the field by ultrasonography (Brown et al., 2000), at the same time as some of the routinely assessed production traits such as muscle depth at five to 12 months of age. It is already common practice in New Zealand sheep production systems to incorporate these ultrasound measurements into breeding programs, due to these traits being economically important. Therefore, it could be possible to extend those datasets and include skin measurements for lamb survival improvement, since skin thickness at birth has been correlated to skin thickness at seven to nine months of age (Soltani-Ghombavani et al., 2021). Nevertheless, it is important to first understand how selection based on skin traits, such as ultrasonic skin thickness and infrared-measured temperature, would be associated with other thermoregulation and production traits. To date this has not been undertaken. Therefore, the aims of this study were to estimate the genetic and phenotypic correlations of ultrasonic measured skin thickness and infrared measured skin temperature, fat depth, loin-eye muscle depth and width, live weights at weaning, six, and 12 months, and 12-month fleece weight, in the FocusPrime, Texel, Romney and Highlander breeds.

4.2 Materials and methods

The overall experimental design, data collection and editing, and pre-statistical analysis were previously described in depth in Chapter 3. In brief, the study was conducted on three Focus Genetics breeding farms in New Zealand, between 2017 and 2020 and involved 5564 lambs, born in 2019 (n = 1785) and 2017 (n = 3779), respectively,

comprising four breeds: FocusPrime, Texel, Romney and Highlander. While Highlander and Romney are maternal breeds used to generate flock replacements, FocusPrime and Texel are terminal breeds with all lambs sold for meat production (Pickering et al., 2018). The experimental protocol was approved by Massey University Animal Ethics Committee (MUAEC), protocol number 18/63.

At approximately five and 11 months of age skin thickness (ST) of each lamb was measured ultrasonically. At around 11 months of age, skin temperature (Stemp) was measured by infrared thermography. Loin-eye muscle depth (EMD) and width (EMW), and subcutaneous fat (FD) of each lamb were measured ultrasonically at approximately five months of age. Phenotypic and pedigree data available from the Focus Genetics database (http://www.focusgenetics.com) and Sheep Improvement Limited (http://www.sil.co.nz) included: date of birth, sex (ewe/ram), birth flock, recording mob for each trait at the time of measurement, birth rank, rearing rank, dam age, dam and sire identities, weaning weight (WWT; at approximately three months of age), 6-month live weight (LW6), 12-month live weight (LW12) and 12-month fleece weight (FW12). No ewe data was available for all Romney records, and for FD, EMD and EMW measurements in all breeds.

4.2.1 Data editing

When compiling and editing the final dataset, all lambs that had missing data on birth rank and rearing rank (n = 35) and had unknown parents in the pedigree dataset (n = 83), were excluded from the final analysis. Also, due to small numbers in each category, records of lambs with birth rank and rearing rank of four were considered as triplets (n = 64), and dam ages of six years or more were all considered as five-year-old (n = 254). Further, any outlier observations (i.e., value ranges that were outside of the mean ± 4 SD) were removed from each breed's dataset. After these editing steps, the final dataset

had 5446 lambs which included 2088 FocusPrime, 732 Texel lambs, 825 Romney lambs and 1801 Highlander lambs.

4.2.2 Estimation of genetic and phenotypic correlations

A bivariate-trait animal model was utilized for the estimation of (co)variance components required for the estimation of genetic and phenotypic correlations between the traits by breed, using the statistical package ASReml, version 4.2 (Gilmour et al., 2015). The model included the fixed effects of the contemporary group (BirthYear_flock), sex (ewe/ram), Birth_rearing type (born as single, twin or triplet, and reared as single, twin or triplet), the age of the dam and the recording mob for each trait at measurement. In addition, the effect of the actual age at scanning (around five or 11 months of age) according to its contemporary group (Lamb age*BirthYear_flock) was utilized as a covariate for ST only. Similarly, uniquely for FD, EMD and EMW, the trait LW6 was included as a covariate. All these fixed effects and covariates were included in each correlation model for each breed, based on the availability of the records per trait.

In matrix notation, the bivariate model can be represented as:

$$\begin{bmatrix}\mathbf{y_1}\\ \mathbf{y_2}\end{bmatrix} = \begin{bmatrix}\mathbf{X_1} & 0\\ 0 & \mathbf{X_2}\end{bmatrix}\begin{bmatrix}\mathbf{b_1}\\ \mathbf{b_2}\end{bmatrix} + \begin{bmatrix}\mathbf{Z_1} & 0\\ 0 & \mathbf{Z_2}\end{bmatrix}\begin{bmatrix}\mathbf{a_1}\\ \mathbf{a_2}\end{bmatrix} + \begin{bmatrix}\mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix}$$

where $\mathbf{y_1}$ and $\mathbf{y_2}$ is the vector of phenotypic measures for two traits under analysis; $\mathbf{X_1}$ and $\mathbf{X_2}$, and $\mathbf{Z_1}$ and $\mathbf{Z_2}$ constitute the design matrices pertaining to the fixed and random effects, respectively, on the phenotypes; $\mathbf{b_1}$ and $\mathbf{b_2}$ is the vector of the fixed effects of the contemporary group, sex, birth-rearing type, the age of the dam and the recording mob; $\mathbf{a_1}$ and $\mathbf{a_2}$ is the vector of random effects of animal for each trait; and $\mathbf{e_1}$ and $\mathbf{e_2}$ is the vector of random residual errors for each trait. The distributional properties of the model with E and V indicating the expectation and variance were as follows:

$$\mathrm{E}\begin{bmatrix}\mathbf{y_1}\\ \mathbf{y_2}\end{bmatrix} = \begin{bmatrix}\mathbf{X_1} & 0\\ 0 & \mathbf{X_2}\end{bmatrix}\begin{bmatrix}\mathbf{b_1}\\ \mathbf{b_2}\end{bmatrix}$$

and

$$V\begin{bmatrix}\mathbf{a_1}\\ \mathbf{a_2}\\ \mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix} = \begin{bmatrix}\mathbf{A}\sigma^2_{a1} & \mathbf{A}\sigma_{a1a2} & 0 & 0\\ \mathbf{A}\sigma_{a1a2} & \mathbf{A}\sigma^2_{a2} & 0 & 0\\ 0 & 0 & \mathbf{I}_1\sigma^2_{e1} & \mathbf{I}_1\sigma_{e1e2}\\ 0 & 0 & \mathbf{I}_1\sigma_{e1e2} & \mathbf{I}_1\sigma^2_{e2}\end{bmatrix}$$

where **A** is the numerator relationship matrix of order equal to the total number of animals in the pedigree file, according to the breed being analyzed; σ^2_{a1}, σ^2_{a2} and σ_{a1a2} are the animals' (co)variance components for the two traits considered; I_1 is an identity matrix of order equal to the number of records; and σ^2_{e1}, σ^2_{e2} and σ_{e1e2} are the residual (co)variance components for the traits. Genetic (r_G) and phenotypic (r_p) correlations were calculated as:

$$r_G = \frac{\sigma_{a1a2}}{\sigma_{a1} \times \sigma_{a2}}$$

and

$$r_p = \frac{\sigma_{p1p2}}{\sigma_{p1} \times \sigma_{p2}}$$

where σ_{p1p2} is the phenotypic covariance between trait 1 and 2, which is equivalent to $\sigma_{a1a2} + \sigma_{e1e2}$; σ_{p1} is the phenotypic standard deviation for trait 1, equivalent to $\sqrt{\sigma^2_{a1} + \sigma^2_{e1}}$, and σ_{p2} is the phenotypic standard deviation for trait 2, equivalent to $\sqrt{\sigma^2_{a2} + \sigma^2_{e2}}$.

4.3 Results

4.3.1 Genetic and phenotypic correlations

Mean values (± SD) and heritabilities for each trait considered in this study within the FocusPrime, Texel, Romney and Highlander breeds, have been described in Chapter 3. Here, the genetic and phenotypic correlations between the traits were estimated for each breed and are presented in Table 4.1 from the pairwise ASReml analysis.

4.3.1.1 Skin thickness

Genetic and phenotypic correlations of ST with Stemp were negative for all breeds, with strong genetic correlations in Texel (-0.60 ± 0.33) and Romney (-0.53 ± 0.81), but linked to high standard errors. Genetic correlations of ST with FD, EMD, EMW, LW6, LW12 and FW12 were generally positive for all breeds and especially strong in Texel with EMW (0.81 ± 0.31) and with LW12 (0.57 ± 0.26). Contrarily, genetic correlations of ST with EMD and EMW were negative in Highlander (-0.51 ± 0.19 and -0.22 ± 0.29, respectively), but linked to high standard errors. In addition, genetic correlations of ST with FW12 were especially strong in Highlander (0.56 ± 0.18). Overall phenotypic correlations of ST with all other traits were mostly positive and low.

4.3.1.2 Skin temperature

Genetic and phenotypic correlations of Stemp with FD were generally negative for all breeds, where genetic correlations were especially strong in Texel (-0.63 ± 0.28). Genetic correlations of Stemp with muscle traits were generally negative, except for the moderate positive correlation with EMD and EMW in Highlander (0.48 ± 0.27 and 0.39 ± 0.42, respectively), but linked to high standard errors. Genetic correlations of Stemp with the weight traits were mostly negative for all breeds and strong in Romney with WWT (-0.57 ± 1.18) and with LW12 (-0.57 ± 1.15), but linked to high standard errors. Phenotypic correlations of Stemp with EMD, EMW, WWT, LW6 and LW12 were generally negative and low. A low and negative genetic and phenotypic correlation of Stemp with FW12 was found in Highlander, whereas no correlation could be determined in the other three breeds. Genetic and phenotypic correlations of Stemp with EMD, EMW, LW6 and FW12 were not calculable in the Romney breed with the available dataset.

4.3.1.3 Fat depth and muscle traits

Correlations of FD with the muscle traits were positive overall, where a positive strong genetic association of FD with EMD was found in Highlander (0.65 ± 0.19). Phenotypic correlations of FD with the muscle traits tended to be weakly positive. Genetic and phenotypic correlations were mostly negative for FD with WWT, positive for FD with LW6 and mixed for FD with LW12. Strong genetic and phenotypic correlations of FD with LW6 were found in FocusPrime (0.60 ± 0.10 and 0.53 ± 0.02, respectively), Texel (0.76 ± 0.17 and 0.54 ± 0.05, respectively), Romney (0.87 ± 0.08 and 0.58 ± 0.03, respectively), and Highlander (0.57 ± 0.16 and 0.46 ± 0.03, respectively). Genetic correlations of FD with FW12 were moderately positive in Romneys and low negative in Highlander, and both phenotypic correlations were almost zero.

A similar trend of association was observed for FD with the weight traits as for EMD and EMW with the weight traits, where both were positive with LW6 and mixed with LW12. On the other hand, WWT was mostly negatively correlated with EMD, whereas it was correlated positively with EMW. Strong genetic and phenotypic correlations of EMD with LW6 were found in Romney (0.70 ± 0.12 and 0.63 ± 0.02, respectively) and in Highlander (0.52 ± 0.15 and 0.59 ± 0.02, respectively), and within its genetic correlation only in FocusPrime (0.51 ± 0.12) and within the phenotypic correlation for Texel (0.70 ± 0.02). In addition, a strong negative genetic correlation of EMD with LW12 was found in Highlander (-0.56 ± 0.17).

All genetic and phenotypic correlations of EMW with LW6 were strong and positive. Genetic and phenotypic correlations of EMD with FW12 were mostly negative, whereas on the other hand, genetic correlations of EMW with FW12 were moderate positive in Romney and low negative in Highlander, and both phenotypic correlations were low positive.

4.3.1.4 Weight traits

Genetic and phenotypic correlations of WWT with LW6, LW12 and FW12 were positive in all breeds. Particularly, all correlations of WWT with LW6 and the genetic correlations with LW12 were strong in all breeds, meanwhile, all correlations with FW12 were low. Similarly, genetic and phenotypic correlations of LW6 with LW12 were mostly strong in all breeds. Genetic and phenotypic correlations of FW12 with LW6 and LW12 were mostly low to moderate, except for the Highlander breed which had a strong genetic correlation between FW12 and LW12 (0.72 ± 0.10).

Table 4.1. Phenotypic (above the diagonal) and genetic (below the diagonal) correlations (± SE) between skin thickness (ST), skin temperature (Stemp), subcutaneous fat depth (FD), loin-eye muscle depth (EMD) and width (EMW), 12-month fleece weight (FW12), and live weights at weaning (WWT), 6 months (LW6), and 12 months (LW12) in (**A**) FocusPrime, (**B**) Texel, (**C**) Romney and (**D**) Highlander breeds.

(**A**)

	ST	**Stemp**	**FD**	**EMD**	**EMW**	**WWT**	**LW6**	**LW12**
ST		-0.23 ± 0.03	0.08 ± 0.03	0.01 ± 0.03	-0.02 ± 0.03	0.05 ± 0.03	0.11 ± 0.03	0.10 ± 0.03
Stemp	-0.44 ± 0.15		-0.05 ± 0.04	-0.02 ± 0.05	-0.01 ± 0.05	-0.08 ± 0.04	-0.14 ± 0.05	-0.04 ± 0.04
FD	0.19 ± 0.18	-0.25 ± 0.19		0.15 ± 0.03	0.20 ± 0.03	-0.05 ± 0.03	0.53 ± 0.02	-0.03 ± 0.03
EMD	0.08 ± 0.17	0.14 ± 0.18	0.20 ± 0.18		0.45 ± 0.02	0.10 ± 0.03	0.29 ± 0.04	0.02 ± 0.03
EMW	0.07 ± 0.12	0.06 ± 0.21	0.25 ± 0.19	0.63 ± 0.12		0.29 ± 0.03	0.66 ± 0.02	0.08 ± 0.03
WWT	0.07 ± 0.12	-0.19 ± 0.15	-0.13 ± 0.14	0.10 ± 0.03	0.54 ± 0.12		0.79 ± 0.01	0.46 ± 0.02
LW6	0.01 ± 0.13	-0.19 ± 0.14	0.60 ± 0.10	0.51 ± 0.12	0.78 ± 0.06	0.91 ± 0.03		0.45 ± 0.03
LW12	0.00 ± 0.15	0.11 ± 0.19	0.20 ± 0.16	0.18 ± 0.16	0.26 ± 0.17	0.70 ± 0.08	0.73 ± 0.09	

(**B**)

	ST	Stemp	FD	EMD	EMW	WWT	LW6	LW12
ST		-0.24 ± 0.04	0.10 ± 0.05	0.08 ± 0.05	-0.05 ± 0.05	0.06 ± 0.04	0.18 ± 0.05	0.09 ± 0.04
Stemp	-0.60 ± 0.33		-0.08 ± 0.07	0.04 ± 0.07	0.08 ± 0.07	-0.09 ± 0.05	-0.10 ± 0.07	-0.13 ± 0.05
FD	0.11 ± 0.31	-0.63 ± 0.28		0.17 ± 0.05	0.21 ± 0.05	-0.22 ± 0.06	0.54 ± 0.05	-0.11 ± 0.05
EMD	0.34 ± 0.34	-0.27 ± 0.50	-0.24 ± 0.41		0.45 ± 0.04	-0.17 ± 0.06	0.70 ± 0.02	-0.12 ± 0.05
EMW	0.81 ± 0.31	-0.40 ± 0.44	0.30 ± 0.33	0.35 ± 0.36		0.04 ± 0.06	0.72 ± 0.02	-0.05 ± 0.05
WWT	0.09 ± 0.30	0.02 ± 0.41	-0.08 ± 0.28	-0.48 ± 0.29	-0.17 ± 0.39		0.83 ± 0.01	0.46 ± 0.03
LW6	0.09 ± 0.36	0.15 ± 0.55	0.76 ± 0.17	0.49 ± 0.42	0.65 ± 0.30	0.98 ± 0.06		0.47 ± 0.04
LW12	0.57 ± 0.26	-0.33 ± 0.35	-0.29 ± 0.30	0.21 ± 0.35	0.08 ± 0.36	0.71 ± 0.16	0.55 ± 0.26	

(**C**)

	ST	Stemp	FD	EMD	EMW	WWT	LW6	LW12	FW12
ST		-0.05 ± 0.04	0.07 ± 0.04	0.09 ± 0.04	0.06 ± 0.04	0.01 ± 0.04	0.00 ± 0.04	0.13 ± 0.04	0.21 ± 0.04
Stemp	-0.53 ± 0.81		-0.12 ± 0.05	-	-	-0.04 ± 0.04	-	-0.09 ± 0.04	-
FD	0.32 ± 0.26	-0.28 ± 1.45		0.26 ± 0.04	0.33 ± 0.03	-0.06 ± 0.04	0.58 ± 0.03	0.03 ± 0.04	0.04 ± 0.04
EMD	0.44 ± 0.22	-	0.25 ± 0.26		0.37 ± 0.03	-0.11 ± 0.04	0.63 ± 0.02	-0.03 ± 0.04	-0.02 ± 0.04
EMW	0.07 ± 0.31	-	0.47 ± 0.27	0.77 ± 0.19		0.22 ± 0.04	0.53 ± 0.03	0.06 ± 0.04	0.10 ± 0.04
WWT	0.04 ± 0.28	-0.57 ± 1.18	0.44 ± 0.29	-0.24 ± 0.26	0.37 ± 0.29		0.77 ± 0.02	0.44 ± 0.03	0.12 ± 0.04
LW6	0.09 ± 0.25	-	0.87 ± 0.08	0.70 ± 0.12	0.77 ± 0.13	0.79 ± 0.10		0.52 ± 0.03	0.12 ± 0.04
LW12	0.10 ± 0.24	-0.57 ± 1.15	0.23 ± 0.26	0.07 ± 0.24	0.07 ± 0.29	0.58 ± 0.21	0.67 ± 0.15		0.36 ± 0.04
FW12	0.20 ± 0.22	-	0.47 ± 0.21	0.10 ± 0.21	0.37 ± 0.27	0.22 ± 0.23	0.14 ± 0.21	0.17 ± 0.20	

(**D**)

	ST	Stemp	FD	EMD	EMW	WWT	LW6	LW12	FW12
ST		-0.08 ± 0.03	0.13 ± 0.03	0.01 ± 0.03	0.09 ± 0.03	0.14 ± 0.03	0.25 ± 0.03	0.02 ± 0.03	-0.04 ± 0.03
Stemp	-0.33 ± 0.27		0.00 ± 0.06	-0.06 ± 0.05	-0.08 ± 0.05	-0.04 ± 0.03	-0.13 ± 0.04	-0.14 ± 0.03	-0.08 ± 0.03
FD	0.22 ± 0.25	-0.40 ± 0.35		0.22 ± 0.03	0.22 ± 0.03	-0.20 ± 0.04	0.46 ± 0.03	-0.15 ± 0.05	0.03 ± 0.05
EMD	-0.51 ± 0.19	0.48 ± 0.27	0.65 ± 0.19		0.47 ± 0.03	-0.08 ± 0.04	0.59 ± 0.02	-0.18 ± 0.05	-0.02 ± 0.05
EMW	-0.22 ± 0.29	0.39 ± 0.42	0.02 ± 0.33	0.47 ± 0.24		0.11 ± 0.04	0.61 ± 0.02	-0.12 ± 0.05	0.06 ± 0.05
WWT	0.42 ± 0.17	0.16 ± 0.26	-0.40 ± 0.19	-0.37 ± 0.18	0.02 ± 0.28		0.72 ± 0.01	0.44 ± 0.03	0.24 ± 0.03
LW6	0.43 ± 0.17	-0.13 ± 0.25	0.57 ± 0.16	0.52 ± 0.15	0.84 ± 0.08	0.87 ± 0.05		0.55 ± 0.03	0.33 ± 0.03
LW12	0.38 ± 0.20	0.33 ± 0.25	-0.40 ± 0.23	-0.56 ± 0.17	-0.44 ± 0.31	0.56 ± 0.14	0.77 ± 0.10		0.47 ± 0.03
FW12	0.56 ± 0.18	-0.09 ± 0.23	-0.18 ± 0.20	-0.42 ± 0.15	-0.18 ± 0.24	0.20 ± 0.13	0.50 ± 0.10	0.72 ± 0.10	

A hyphen is displayed where correlations could not be determined with the dataset available.

4.4 Discussion

Selection for skin thickness may be a potential tool to indirectly select for lamb survival, as this trait is heritable and correlated with survival (Tait et al., 2015; Soltani-Ghombavani et al., 2017). In addition, skin temperature could also be combined with skin thickness selection, since it acts as an indicator of heat loss to the environment and is also moderately heritable as observed in Chapter 3. Scanning measurements of skin thickness at around eight months of age is a reliable indicator of the skin measurements at birth (Soltani-Ghombavani et al., 2021). Accordingly, selecting for skin thickness from the measurements of older lambs should have a positive effect on new-born lambs thermoregulation and survivability, since having a thicker skin would improve heat insulation thereby losing less heat from the skin surface (Soltani-Ghombavani et al., 2021). In addition, undertaking ultrasound scanning measurements at five to 12 months of age is already a routine practice on many New Zealand sheep breeding farms for traits such as muscle depth. Therefore, measuring skin thickness and temperature could be done with little extra cost and labour required. Nonetheless, prior to considering skin thickness and/or temperature as new additions to ultrasound measurements and into breeding programs, knowledge of how selection for these traits will affect production traits is crucial.

4.4.1 Selection for skin thickness (ST) and temperature (Stemp) and its potential impact on new-born lamb survival

As noted in Chapter 3, FocusPrime and Texel lambs had thinner skins, and were positioned in the middle to high range of Stemp when compared to the other breeds. In addition, Highlander lambs had thicker skin that correlated with lower Stemp. All genetic correlations of ST with Stemp were moderately negative in all breeds, and specifically strong in Texel and Romney. A lower skin temperature would indicate that more endogenous heat would remain within the body with less loss to the environment,

thereby maintaining homeothermy in cold conditions (Dwyer & Lawrence, 2005). Results from this study suggest that selecting for a thicker skin should decrease the level of heat escaping from the body surface, which would presumably have a positive effect on lamb survival. However, there is a lack of data directly examining this association on lambs at birth.

Selection to increase cold tolerance is known to be positively associated genetically with traits that provide insulation, such as skin thickness, fat depth, body weight and wool (Stott & Slee, 1987). In other words, animals that have thicker skin, larger body weight, deeper wool coat and that possess a great amount of fat reserves would be more suitable to endure cold conditions, and therefore have greater chances of survival under adverse conditions. Within the present study, genetic and phenotypic correlations of ST with FD were positive in all breeds. These results agree with Jopson et al. (2000), where sheep selected for increased backfat had thicker skin. Previous studies have observed a high correlation between ultrasonic fat depth measurements of lambs at different ages, from post-weaning (around five months old) to hoggets (around 14 months old) (McEwan et al., 1993; Huisman & Brown, 2009). A greater fat deposition that is associated with a thicker skin would provide the lambs with an increased tolerance to cooling (Plush et al., 2016), as well as more available energy stored for further thermogenic metabolism when nutrition does not meet the metabolic demand. If the fat depth at birth was found to be genetically related to FD at older ages, then a positive survival response would be expected in adverse weather conditions. Further research is required to confirm this.

In addition to fat being an important insulation trait, the conjoined effects of a larger body weight, especially if at birth with thicker skin, could exert a positive effect on cold tolerance (Plush et al., 2016). In the current study, body weight traits had a positive association with ST across all breeds. Previous studies have observed a positive correlation between birth weight and skin thickness at birth, where for a one kilogram increase in body weight, a 0.10 ± 0.02 mm (Soltani-Ghombavani et al., 2021), or 0.37 ±

0.06 mm (Jopson et al., 2000) increase in skin thickness was recorded. For this reason, it would be likely to observe heavier body weights linked to a thicker skin in new-born lambs after selecting for skin thickness, enhancing further body insulation and therefore potential lamb survival in adverse weather conditions during lambing.

Another important insulative trait, FW12 had a mainly positive association with ST. Previous research has shown that a high genetic and phenotypic correlation exists between measuring the fleece weight at ten months with its measurement at 16 months and older (Hickson et al., 1994; Brash et al., 1997). There appears to be little information at younger ages, although Di et al. (2011) observed a positive correlation between pre-weaning staple length with its measurement at 15 months. Since fleece weight is correlated with staple length (Safari & Fogarty, 2003), it could be the case that pre-weaning fleece weight is correlated to later fleece weights. Therefore, the positive association of thicker skin with heavier fleece as observed in the current study, could indicate a potentially similar relationship at birth. Further studies to support this notion are needed. Given a deeper coat provides greater insulation (Stott & Slee, 1987; Slee et al., 1991; Plush et al., 2016), this partnership with thicker skin would become essential for survival in cold environments if apparent at birth.

4.4.2 Selection for skin thickness and temperature and its impact on sheep performance

A positive association of ST at scanning with FD was recorded in all breeds, which might be regarded as unfavourable for the meat market (Soltani-Ghombavani et al., 2017). Nevertheless, in the case of extensively managed animals that live in colder environments, such as hill sheep, more layers of insulation would increase survival (Dwyer & Lawrence, 2005) and increase numbers available for sale. For this reason, it might be more desirable to have a fatter lamb, despite the market needs (Greeff et al., 2008; Mortimer et al., 2017). Besides, the fat depth measurements observed within the

dataset analysed, which varied between 2.20 to 4.96 mm on average, seem to be considered within the “low fat content” fat class by the New Zealand Meat Classification Authority (2004), therefore, a modest increase in FD is unlikely to incur a market penalty. An overall negative genetic and phenotypic association was found between Stemp and FD. It may be that a greater fat depth would mean more insulation, therefore less heat would be lost through the skin. Given the absence of studies examining the correlation of the skin temperature with production traits in the sheep, this is a prime topic for future research.

Skin thickness and the muscle traits at scanning showed generally a low to moderate positive association in all breeds, besides the negative genetic correlations for both muscle traits in Highlander. Previously, an unfavourable genetic correlation was observed between ST and EMD in Romney lambs (Soltani-Ghombavani et al., 2017). That study included ewe lamb data and utilized a much bigger dataset exclusively from Romney lambs, as opposed to the smaller sample size of the current study. This considered, and given there is limited literature exploring the relationship between skin thickness and muscle traits, further research into how muscle traits are associated with ST is required. Furthermore, the correlations of Stemp with the muscle traits were generally negative. Nevertheless, contrasting positive results were found regarding the genetic correlations of Stemp with the muscle traits in Highlander. Hence, it seems that selecting for Stemp might impact the muscle traits in different directions according to the breed.

In the present study, body weight traits were positively associated with ST across all breeds. This observation is in accordance with previous reports, where skin growth is positively correlated with the increase in body weight (Passman & Sumner, 1987), therefore heavier lambs would have heavier skins (Van der Merwe et al., 2021). Moreover, correlations of Stemp with weight traits were observed to be mostly negative,

therefore heavier animals would potentially have more insulation and lose less heat to the environment.

Genetic and phenotypic correlations of FW12 were mainly positive with ST measurements at scanning in Romney and Highlander. These results were in agreement with previous reports, where thicker skin was associated with greater fleece weight at around eight months (Soltani-Ghombavani et al., 2017) and 15 months (Gregory, 1982b). Accordingly, sheep breeders have highlighted the importance of considering ST in making selection decisions towards wool improvement (Gifford et al., 1995). This would be especially important in the Highlander breed, due to its high genetic correlation between ST and FW12, where selecting for skin thickness would favourably affect the genetic factors that increase the quantity of wool.

Correlations of Stemp with FW12 were inconclusive due to the undetermined results for Romney with the available dataset, and the very low and negative correlation in the Highlander breed, in addition to the lack of data from Texel and FocusPrime breeds. Consequently, insights on how Stemp might influence the fleece are still unresolved. Supplemental studies on the correlation between these traits are clearly needed.

4.4.3 Correlations between production traits

Correlations of FD with the muscle traits were mostly positive. Positive genetic associations have been previously recorded for muscle and fat (Brito et al., 2017; Mortimer et al., 2017; Fitzmaurice et al., 2020; Medrado et al., 2021), which agrees with the results of most breeds observed here. Moreover, an overall positive trend of association of the live weight at six months with FD and with the muscle traits was observed in the current study and agreed with previous research (Huisman et al., 2008; Mortimer et al., 2017; Fitzmaurice et al., 2020). Mixed and inconclusive results between FD and the muscle traits were observed when associated with the live weight at weaning and at 12 months. Further, FW12 was observed to have an overall positive genetic and

phenotypic correlation with the weight traits in Romney and Highlander. Accordingly, the correlation between fleece weight and body weight has been regarded as positive through several studies (Hanford et al., 2006; Gowane et al., 2010), while a recent study by Medrado et al. (2021) recorded a strong genetic correlation of greasy fleece weight and weaning weight. However, the association between fleece weight with fat or muscle is not as clear as with body weight. Results from the association between FW12 and both fat and muscle traits have been mixed in the current study. Correlations of fleece weight with FD have been previously reported to range between low positive values (Mortimer et al., 2017) to negative ones (Safari et al., 2005; Greeff et al., 2008). In the same manner, correlations of fleece weight with muscle traits have been observed as positive (Safari et al., 2005), as well as negative (Greeff et al., 2008). These differences in correlation values between fleece weight with fat and muscle traits may come from different breeds or even from different models for analysis, therefore further analysis is needed to confirm these associations.

In summary, skin thickness was shown here as an enhancer of lamb insulation, positively correlated to several other insulative characteristics and with decreased heat loss to the environment. As skin thickness and skin temperature as an indicator of heat loss, have proven to be heritable, they could potentially be added into breeding programs to improve thermoregulation and ultimately lamb survival. Still, more research would be needed to utilize skin thickness for selection, since negative genetic correlations were observed with muscle traits at five months of age in the Highlander breed. Further studies with much larger datasets would provide more reliable estimates of correlations for the combination of these traits. In addition, it would be wise to perform future studies to investigate how a thicker skin would impact on animal performance, as it is known that thermal stress can affect production indices in hot areas (Silanikove, 2000).

4.5 Conclusions

Previous studies have revealed that skin thickness is correlated with lamb survival, exhibits a moderate heritability, and is cost-effective when measured ultrasonically with other routinely recorded production traits. This trait has been shown here to be positively correlated with other insulation traits, such as fleece weight, fat depth and body weight. In addition, an overall favourable negative correlation was found here between skin thickness and infrared-measured skin temperature. The accumulation of insulative layers will allow the conservation of heat in lambs, which becomes crucial in extensive production systems where lambing occurs outside. For this reason, skin thickness and skin temperature could potentially be included in sheep programmes aimed at producing better insulated lambs that can conserve energy for improved thermoregulation, and therefore have better chances of survival.

Chapter 5. RNAseq analysis of brown adipose tissue and thyroid of new-born lambs subjected to short-term cold exposure reveals signs of early whitening of adipose tissue

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References and supplementary data of this chapter, as a published paper, have been moved to the "References" and "Appendices" sections, respectively, at the end of the thesis. Moreover, formatting has been changed from the published version to have a consistent style throughout the thesis. In addition, methodological information was included for this thesis version after the recommended PhD examiners' emendations. The dataset supporting the conclusions of this article is available in the NCBI Gene Expression Omnibus (GEO accession number: GSE210259, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE210259).

Abstract

During the early postnatal period, lambs have the ability to thermoregulate body temperature via non-shivering thermogenesis through brown adipose tissue (BAT), which soon after birth begins to transform into white adipose tissue. An RNAseq approach was used to characterize the transcriptome of BAT and thyroid tissue in new-born lambs exposed to cold conditions. Fifteen new-born Romney lambs were selected and divided into three groups: group 1 (n = 3) was a control, and groups 2 and 3 (n = 6 each) were kept indoors for two days at an ambient temperature (20–22 °C) or at a cold temperature (4 °C), respectively. Sequencing was performed using a paired-end strategy through the BGISEQ-500 platform, followed by the identification of differentially expressed genes using DESeq2 and an enrichment analysis by g:Profiler. This study provides an in-depth expression network of the main characters involved in the thermogenesis and fat-whitening mechanisms that take place in the new-born lamb. Data revealed no significant differential expression of key thermogenic factors such as uncoupling protein 1, suggesting that the heat production peak under cold exposure might occur so rapidly and in such an immediate way that it may seem undetectable in BAT by day three of life. Moreover, these changes in expression might indicate the start of the whitening process of the adipose tissue, concluding the non-shivering thermogenesis period.

5.1 Introduction

In temperate countries such as New Zealand, where lambing occurs outdoors, new-born lambs transit rapidly from a warm uterine environment to a potentially harsh and cold external one. Under these conditions, lambs must immediately increase the rate of body heat production to fifteen times more than the fetal level to compensate for the increased heat loss (Dawes & Mott, 1959; Alexander, 1962a). As the ability to produce heat is essential for survival, lambs can thermoregulate their body temperature within minutes after birth, due to the presence of functional brown adipose tissue (BAT) (Basse et al., 2015). This tissue, predominantly found in the peri-renal and pericardial areas, is the principal source of non-shivering thermogenesis in the new-born lamb (Alexander & Williams, 1968; Symonds, 2013), accounting for 60% of the generated heat (Liang & Ward, 2006). The thermogenic activity of BAT is mainly regulated by catecholamines such as norepinephrine that are released from the sympathetic nervous system during cold exposure (Villarroya et al., 2007), which then activate β-adrenergic receptors expressed in the brown adipocytes (Contreras et al., 2015). This interaction results in a cascade of metabolic events that eventuate in the release of free fatty acids, which are the substrate for uncoupled oxidation and subsequently thermogenesis (Cannon & Nedergaard, 2004).

A BAT-specific transport protein of the inner mitochondrial membrane, uncoupling protein 1 (*UCP1*) or Thermogenin, supplies the thermogenic ability of BAT, and is activated by long-chain fatty acids produced by lipolysis upon adrenergic stimulation (Ridley et al., 1986; Zingaretti et al., 2009; Whittle et al., 2011). *UCP1* has the ability to increase proton permeability and form a proton-motive force through the mitochondrial matrix, where the energy produced from this force is then released as heat rather than stored as adenosine triphosphate (ATP) (Garlid et al., 2000; Cannon & Nedergaard, 2004; Plush et al., 2016). In addition, the thyroid hormone T3 (tri-iodothyronine) can act through its roughly 8000 receptors per brown adipocyte cell (Bianco & Silva, 1987),

stimulating the actions of *UCP1*, hence enhancing BAT thermogenesis (Marrif et al., 2005; Bianco & McAninch, 2013). In addition to this influence, thyroid hormone receptors act as a central inducer of BAT by mediating the synergism between thyroid hormone signalling and the sympathetic nervous system in BAT (López et al., 2010), increasing the capacity of cells to respond to catecholamines (Rubio et al., 1995; Hellström et al., 1997), and thereby increasing adrenergic sensitivity (Ribeiro et al., 2001). These findings demonstrate the multifaceted contribution to thermogenesis involving the crucial relationship between thyroid hormones and BAT, and the importance of their joint contribution to energy homoeostasis (Bianco & McAninch, 2013).

The secretion and plasma concentrations of many of these factors decline over the first few days and weeks of a lamb's life (Symonds et al., 1995; Bispham et al., 2002), as shivering thermogenesis becomes the main response to cold exposure, replacing the non-shivering mechanisms of heat production (Symonds et al., 1989; Symonds, 2013). This replacement has an evolutionary implication, since although BAT is an efficient heat producer, it is small and localised, so the heat it produces needs to be circularised through the body to maintain the core body temperature (Bal et al., 2017). As opposed to this, skeletal muscle is dispersed throughout the body, making it possible to produce heat *in situ* in many parts of the body at the same time (Bal et al., 2017). Consequently, heat produced from skeletal muscle shivering is more cost-effective than BAT; thus, it reduces the demands on diet intake, giving the possibility to invade and explore new geographic areas that are cooler and where food availability is low (Rowland et al., 2015).

At birth, *UCP1* is at its maximum expression but this decreases rapidly, and by day four of life it seems to become nearly undetectable, as observed by Basse et al. (2015). Consequently, brown adipocyte formation and BAT's thermogenic activity also decrease, as BAT begins its transition into white adipose tissue (WAT) which becomes predominant by day 30 (Clarke et al., 1997; Ojha et al., 2013; Pope et al., 2014). WAT then only acts as the main fat storage of the body, losing the large mitochondrial

phenotype of BAT and the expression of its related genes involved in heat production (Liang & Ward, 2006; Ojha et al., 2013; Basse et al., 2015; Contreras et al., 2015). Studying the molecular components of heat production, and the signals exchanged between BAT and the thyroid glands is crucial to help the understanding of the underlying regulatory mechanisms that control thermogenesis in new-born lambs. No published reports appear to have used a ribonucleic acid sequencing (RNAseq) approach in BAT and thyroid gland tissue, to explore the new-born lamb metabolic activity in response to cold exposure. For that reason, the objective of this study was to characterise the transcriptome of brown adipose tissue and thyroid tissue through RNAseq in new-born Romney lambs exposed to either 20-22 °C or 4 °C for 2 days.

5.2 Materials and methods

This study was undertaken in spring 2018 at the Massey University Animal Physiology Unit (APU) with new-born Romney-type lambs born on Keebles Farm (40°24′ S, 175°36′ E), Palmerston North, New Zealand.

5.2.1 Animals and sampling

During the lambing period, which was outdoors under pastoral conditions, fifteen new-born Romney lambs (9 males and 6 females) were randomly procured. Lambs were born as twins, but only one lamb per dam was used (the heaviest of the set). Within 12–24 h after birth, the selected lamb was tagged and the ewe together with both of its lambs were brought indoors to the APU. The selected lamb within each twin set was then weighed and allocated randomly to one of the three treatment groups: group 1 (n = 3; two males weighing 4.8 kg and 5.2 kg, respectively, and a female weighing 4.4 kg) as a control; group 2 (n = 6; two females weighing 4.9 and 4.5 kg, respectively, and four males weighing 4.6, 6.0, 4.6 and 5.3 kg, respectively), which were kept indoors for two days at an ambient temperature (20–22 °C); and group 3 (n = 6; two females weighing 5.5 and

6.1 kg, respectively, and four males weighing 5.4, 4.5, 5.4 and 5.2 kg, respectively), which were kept indoors for two days at a cold temperature (4 °C). This followed the previously described method of Marcher et al. (2015) to induce a cold challenge. Soon after being brought to the APU, the three lambs in group 1 were euthanised via captive bolt to provide a baseline transcriptome profile. Samples of brown adipose tissue (BAT) from around the kidneys and the thyroid tissue were collected and stored at −80 °C. Lambs in groups 2 and 3, together with their dams and siblings were moved into indoor pens (2 m by 1 m) for two days. Siblings of the lambs in group 3, which were not subjects of the current study, were wrapped with wool covers (Woolover Limited, Christchurch, New Zealand) to enhance their comfort and to minimise impact of the cold. During the two days of indoor retention, the ewes were fed commercial roughage (FiberEzy, Fiber Fresh Feeds Ltd., Reporoa, New Zealand) and commercial grain-based pellets (10%) (NRM Sheep Nuts, Northern Roller Mills, Christchurch, New Zealand) and had unrestricted access to water. The ewes and lambs were monitored at least 3 times per day during this period, to ensure successful ewe/lamb bonding and that the lambs were successfully suckling the ewe. On day 2, after 48 h exposure at respective temperatures, the 12 lambs of these two groups (i.e., 6 per group) were euthanised by captive bolt. Samples of brown adipose tissue from around the kidneys and the thyroid tissue were collected and stored at −80 °C. In all groups, after the lambs had been euthanised, their dams and remaining siblings were returned to Keebles Farm to commercial farming conditions.

5.2.2 RNA isolation and transcriptome sequencing

Brown adipose tissue from around the kidneys and thyroid tissue samples were submitted to BGI Genomics (Hong Kong) for RNA extraction and subsequent sequencing. Approximately 60 mg of tissue per sample was ground with liquid nitrogen into powder, then transferred into a 2 mL tube containing 1.5 mL of TRIzol (Invitrogen,

Carlsbad, CA, USA). The samples were homogenised with the TissueLyser II (Qiagen, Hilden, Germany), and then incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes, before being centrifuged at 12,000 × *g* for 5 min at 4 °C (Centrifuge 5427R, Eppendorf, Hamburg, Germany). The resulting supernatant was transferred into a new 2 mL tube, 0.3 mL of chloroform/isoamyl alcohol (24:1) was added, and then mixed by vigorously shaking the tubes for 15 s. The samples were then centrifuged at 12,000 × *g* for 10 min at 4 °C, to separate the mixture into three layers; the lower phenol-chloroform phase, an interphase and the upper aqueous phase containing the RNA. The aqueous phase was transferred into a new 1.5 mL tube and an equal volume of isopropyl alcohol was added; this was mixed and incubated at −20 °C for 2 h for precipitation. After this time, the samples were centrifuged at 13,600 × *g* for 20 min at 4 °C. Following this centrifugation, the supernatant was removed, and the RNA pellet was washed with 1 mL of 75% ethanol then resuspended and centrifuged at 13,600 × *g* for 3 min at 4 °C. This step was repeated, and at the end the ethanol was removed without disturbing the pellet, which was let to air-dry in a biosafety cabinet (Esco Airstream, Esco Technologies, Horsham, PA, USA). Afterwards, 25–100 µL of diethyl pyrocarbonate-treated water was added to solubilise the RNA pellet.

The quality check of the extracted RNA was measured by RNA integrity number (RIN), where on average, a RIN of 7.8 for thyroid tissue and 6.8 for brown adipose tissue was observed (Table S5.1). Following the RNA isolation and quality checks, mRNA was purified from total RNA using oligo (dT)-attached magnetic beads. The resulting mRNA molecules were fragmented and reverse-transcribed into double-stranded cDNA by using random hexamer primers. The synthesised cDNA was subjected to an end repair and then 3′ adenylated, where adapters were ligated to the ends of these fragments. The cDNA fragments were amplified by PCR and product purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Afterwards, a quality check was conducted on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The PCR product was then heat-denatured and the single-strand DNA was

circularised by splint oligo and DNA ligase. Finally, all cDNA libraries were sequenced using paired-end strategy of 150 bp read length, through the BGISEQ-500 platform.

5.2.3 Quality control, mapping and reads quantification

All processes described in this section were carried out through the use of high-performance computing resources of the New Zealand eScience Infrastructure (NeSI, https://www.nesi.org.nz/). Several pipelines of analysis were tested in order to utilize the best one available for the present studies' samples, this process is attached in Appendix B.

The quality of the raw 150-base paired-end sequence files was examined using FastQC (version 0.11.9) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (as shown in figure S5.3) and then summarised by MultiQC (version 1.9) as described by Ewels et al. (2016). Next, the paired-end reads were trimmed to remove low-quality bases and adapter sequences with Trimmomatic (version 0.39) (Bolger et al., 2014), using the options: SLIDINGWINDOW:4:20, MINLEN:50, ILLUMINACLIP:TruSeq3-PE.fa:2:30:10. The read quality was re-assessed with FastQC and MultiQC to confirm that improvements had been made by trimming. Clean reads were mapped to a reference genome (Oar_rambouillet_v1.0) from the RefSeq database (https://www.ncbi.nlm.nih.gov/assembly/GCF_002742125.1/ accessed on 22 April 2021) using STAR (version 2.7.7a) (Dobin et al., 2013). After mapping, featureCounts (Subread package, version 2.0.0) was used to quantify the aligned reads (Liao et al., 2014), allowing multimapping reads to be counted using the option -M.

Principal component analysis (PCA) plots of normalised sequence reads were constructed in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the "ggplot2" package (version 3.3.5) (Wickham, 2016), to visualise the differences between control, cold and ambient temperature samples for BAT and thyroid tissue, according to the top 1000 genes per tissue selected by highest

row variance (sample variance). Heatmaps of the normalised counts per gene, for the 38 analysed genes, from the DESeq2 analysis for each group of samples (control, cold, and ambient) were made in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the “heatmap” package (version 1.0.12) (Kolde, 2018), and the“ RColorBrewer” package (version 1.1-2) (Neuwirth & Neuwirth, 2014). Scaling was set as default (i.e., by rows) utilising the Euclidian distances method. After scaling, the heatmaps were ordered according to the different treatment groups: controls, cold exposed and ambient exposed. The genes *ADRB3* and *THRB* were excluded from the thyroid tissue heatmap due to insufficient counts. Criteria for insufficient counts is detailed below.

5.2.4 Differential expression analysis

Differentially expressed genes (DEGs) were identified using the DESeq2 package (version 1.32.0) (Love et al., 2014), in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021). Low read counts were prefiltered before running DESeq2, by removing rows that had fewer than 10 reads in at least 10 samples. Each tissue (BAT and thyroid) was analysed separately and the DEGs were examined by treatment vs. control (ambient temperature vs. control, and cold temperature vs. control), in order to compare the differences in the transcriptome after being exposed to different temperatures for two days vs. not being exposed to them. Within DESeq2, the model design was computed as: design =~ control + treatmentCold, and design =~ control + treatmentAmbient. For normalisation, three selected reference genes were utilised: *ACTB*, *RPL19* and *HUWE1,* as they were previously tested (Taube et al., 2015; Sahu et al., 2018; de Brun et al., 2020). For both tissues, one control sample was removed as it had outlier counts overall, compared to all the other samples. Finally, genes with an absolute value of |log2Fold Change| ≥ 1 and an adjusted $P < 0.05$ were considered differentially expressed and were used in subsequent analyses (Table S5.2).

The adjusted *P*-value was calculated using the Benjamini–Hochberg False Discovery Rate (FDR) concept (Benjamini & Hochberg, 1995). In addition to the treatment vs. control analysis, an analysis of cold temperature vs. ambient temperature for each tissue was also performed in a unique set of 38 genes that, according to the literature, are involved in thermogenesis or fat whitening. Within DESeq2, the model design was computed as: design =~ treatmentCold + treatmentAmbient. These were: *UCP1*, *ADRB1*, *ADRB2*, *ADRB3*, *ADRA1A*, *PPARGC1A*, *PPARGC1B*, *PPARA*, *PPARG*, *ELOVL6*, *BMP4*, *BMP7*, *BMP8B*, *CIDEA*, *CKB*, *PDK4*, *TGM2*, *FNDC5*, *ACSL5*, *CPT1A*, *FABP3*, *PRKG1*, *NOS3*, *PDE3B*, *VASP*, *LPL*, *PRDM16*, *EHMT1*, *GABPA*, *VEGFA*, *CYP1A1*, *THRA*, *THRB*, *DIO2*, *PNPLA2*, *LIPE*, *MGLL* and *MKI67* (Table S5.3).

Plots depicting the intersection of the number of genes between experimental groups were constructed in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the “tidyverse” package (version 1.3.1) (Wickham et al., 2019) and the “UpSetR” package (version 1.4.0) (Conway et al., 2017). Volcano plots of the results from each tissue (showing treatment vs. control) were created using the“ EnhancedVolcano” package (version 1.14.0) (Blighe et al., 2019) in R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021).

5.2.5 Functional analysis of DEGs

Gene ontology (GO) enrichment analysis, Reactome pathway database and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed in g:Profiler (https://biit.cs.ut.ee/gprofiler/gost (accessed on 6 September 2021)) (Raudvere et al., 2019; Reimand et al., 2019; Jassal et al., 2020), as it has previously been utilised in RNAseq analysis (Singh et al., 2017; Al-Dalahmah et al., 2020; Varela-Martinez et al., 2021). Analysis was conducted through an ordered query with a significance threshold by the Benjamini–Hochberg FDR method (adjusted $P < 0.05$) (Table S5.4). A human database was set for all analyses, due to the scarcity of sheep GO data (Chopra-

Dewasthaly et al., 2017). Additional information regarding selected genes of interest was gathered through the NCBI database (https://www.ncbi.nlm.nih.gov/gene/).

5.2.6 Validation by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from BAT and thyroid tissue of all 15 lambs (3 of group 1, 6 of group 2 and 6 of group 3) was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Approximately 50–60 mg of tissue was homogenised with 1 mL of TRIzol and around 50 mg of a mixture of 1.0:2.3 zirconia/silica beads (dnature, Gisborne, New Zealand), in a TissueLyser II (Qiagen, Hilden, Germany) with 30 Hz frequency. This homogenisation was performed for 2 min for the BAT samples and 5 min for the thyroid samples, all conducted in intervals of 30 s with 1 min of in-ice storage in between. After homogenisation, the samples were incubated at room temperature for 5 min. Subsequently, a centrifugation step at 12,000× *g* at 4 °C for 10 min was performed and the supernatant was pipetted carefully into a clean 2 mL tube. To the supernatant, 200 µL of chloroform was then added and mixed by vortexing; after this, the sample was incubated for 5 min at room temperature. The sample was then centrifuged at 12,000× *g* at 4 °C for 20 min and the upper aqueous RNA phase was transferred into a new 2 mL tube without disturbing the interphase layer. The volume of the sample was estimated, and 1.5 volumes of 100% ethanol were added then mixed by inverting the tube several times. RNA was extracted from the ethanol-precipitated samples using RNeasy Mini Kit (Qiagen, Hilden, Germany), following the kit protocol. Final RNA was resuspended in 33 µL of RNAse-free water. The concentration of RNA was determined by measuring the absorbance at 260 nm and its purity evaluated at an absorption ratio of 260/280 nm, using a NanoDrop spectrophotometer (Thermo Scientific, MA, USA). The RNA was stored at −80 °C until RT-qPCR reactions were performed.

The expression levels of six selected DEGs were determined relative to *ACTB* and *GAPDH* endogenous control genes, which remained unchanged among samples in this study. Specific primers (Table S5.5) for *ACTB*, *KI67*, *BMP4*, *DIO2* and *PPARGC1A* were designed using Primer3, with gene sequences extracted from the reference genome described previously, through Geneious software (version 10.2.6). The primer sequences for *GAPDH*, *VEGFA* and *ADRB3* were based on previous sheep-based studies (Yu et al., 2009; Pope et al., 2014; Bedir et al., 2022). Single-step RT-qPCRs were performed using Verso 1-step RT-qPCR Kit (Thermo Fisher Scientific, MA, USA). Each 20 µL reaction mix included: 10 µL Verso 1 Step qPCR SYBR Mix, 1 µL Verso RT Enhancer, 0.2 µL Verso Enzyme Mix, 5 µL RNase/DNase-free water, 1.4 µL each of the forward and reverse primers (final concentration 700nM) (Integrated DNA Technologies, Coralville, USA) and 1 µL (containing approximately 2.20 ng) of diluted RNA. Reactions were set up in triplicate in a 36-disk Rotor-Gene Q RT-PCR cycler (Qiagen). The standard amplification conditions included: cDNA synthesis at 50 °C for 15 min, an initial denaturation of the cDNA and enzyme activation at 95 °C for 15 min, followed by 40 cycles of 30 s at 95 °C, 30 s at the appropriate annealing temperature (60 °C for *ACTB* and *GAPDH*, 55 °C for all other primer combinations), and 30 s at 72 °C with fluorescence capture. At the end of each run, dissociation curves (from 72 °C to 95 °C with a fluorescent absorbance reading after each 0.3 °C increment) were analysed to ensure that the desired amplicon was being detected and to discard contaminating DNA or primer dimers. Expression data were generated using the mathematical model of $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001) and normalised to the geometric mean expression of the endogenous control genes.

5.3 Results

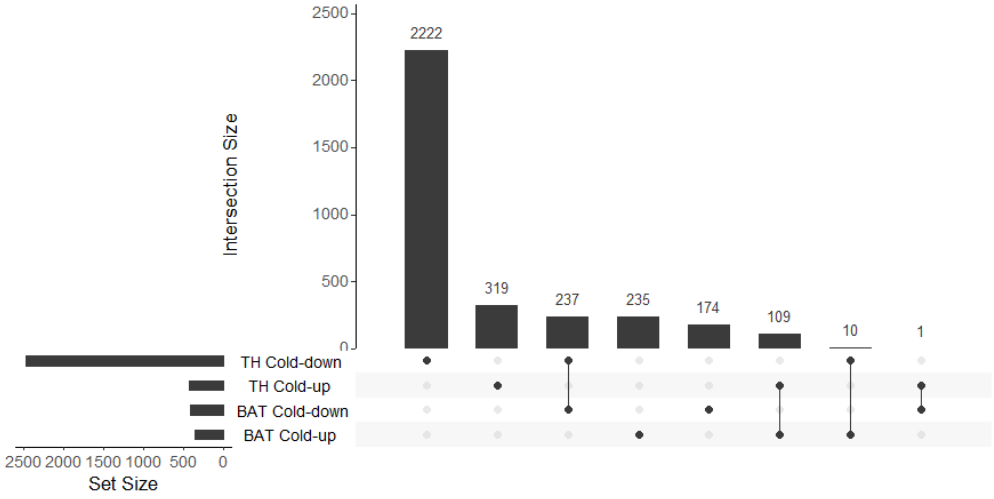
5.3.1 Summary of sequencing reads

On average from BGI sequencing, each BAT and TH sample had 73,597,270 and 73,552,149 reads, respectively. After quality control analysis, on average, 55,337,991 (75.28%) and 37,777,815 (51.36%) reads in BAT and TH, respectively, were mapped to the reference genome (Oar_rambouillet_v1.0) through the STAR software. From those, 33,953,923 (61.49%), and 27,886.474 (73.98%) reads, respectively, in BAT and TH were assigned to features through the featureCounts software. In summary, an average of 46.13% of reads in BAT and 37.91% in TH were assigned to features from the original sequencing reads. A detailed version on reads per sample throughout the analysis is presented in Supplementary Table S5.1. The PCA of normalised sequence reads revealed a separation of the three groups in BAT as well as thyroid (Figure S5.1).

5.3.2 Summary of DEGs

All DEGs with a cutoff of log2Fold Change ≥ |1| and an adjusted $P < 0.05$ (Table S5.2) were selected for comparison analysis against controls (Figure 5.1). Under cold conditions, 354 genes were found to be upregulated and 412 downregulated in BAT (Figure 5.2A), whereas 709 upregulated and 995 downregulated genes were identified under ambient conditions (Figure 5.3A). Under cold conditions, 429 upregulated and 2469 downregulated genes were detected in thyroid tissue (Figure 5.4A), whereas 441 upregulated and 2673 downregulated genes were found under ambient conditions (Figure 5.5A).

(**A**)



(**B**)

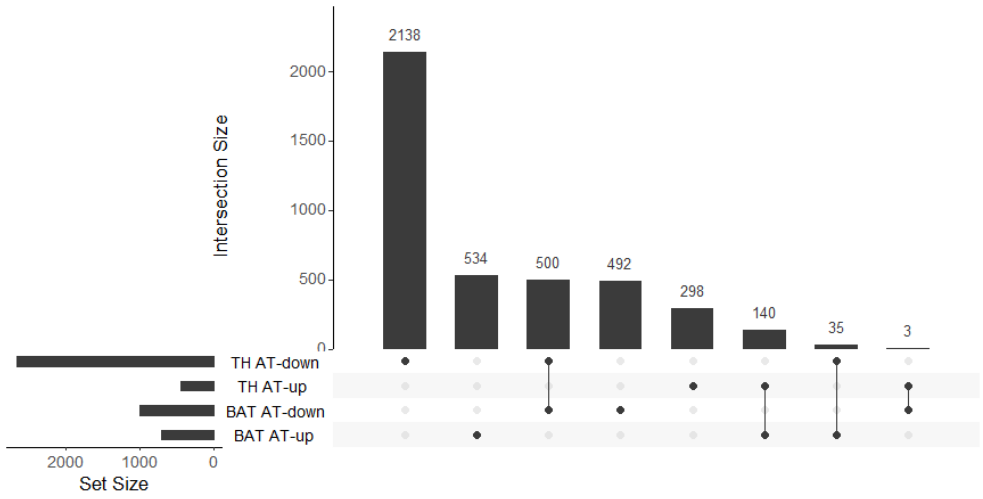


Figure 5.1. UpSet plots showing the intersection of the number of genes between experimental groups. (**A**) Intersection of genes expressed in BAT and thyroid tissue (TH) of lambs under cold temperature (4 °C). (**B**) Intersection of genes expressed in BAT and thyroid tissue of lambs under ambient temperature (AT, 20–22 °C). All genes depicted here had a cutoff of log2Fold Change ≥ |1| and an adjusted $P < 0.05$. The UpSet plots visualise the intersection between sets, which are the rows in the matrix, and the columns correspond to the intersections between these sets.

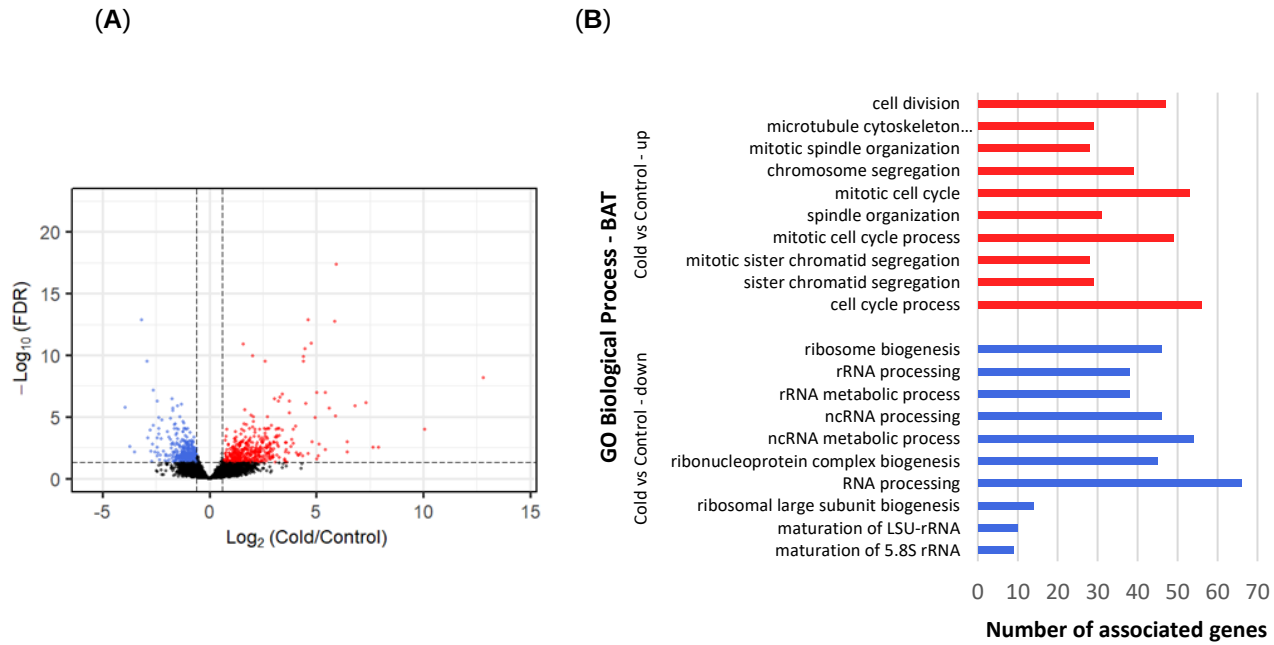


Figure 5.2. Differential gene expression and enrichment in BAT of lambs under cold temperature (4 °C). (**A**) Volcano plot comparing downregulated (blue dots) and upregulated (red dots) gene expression that occurs after cold exposure. Dotted lines indicate cutoffs, adjusted P < 0.05 and log2Fold Change (Cold/Control) ≥ |1|. Black dots represent genes that are not significantly different. (**B**) The most significant (those with lowest *P*-value) up (red lines) and downregulated (blue lines) DEGs enriched Biological Process GO terms, as determined by g:Profiler (adjusted P < 0.05), in brown fat tissue exposed to cold. Names of GO terms are indicated on the Y-axis, and the number of enriched genes for each term is represented on the X-axis.

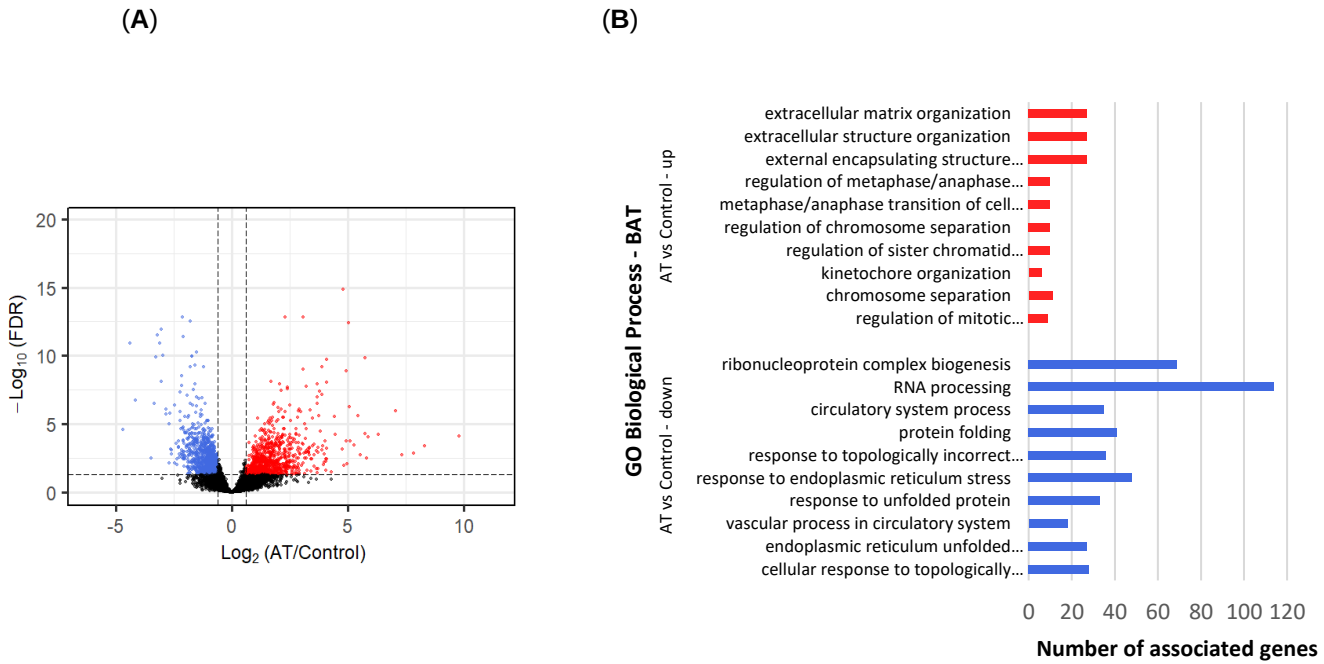


Figure 5.3. Differential gene expression and enrichment in BAT of lambs under ambient temperature (20–22 °C). (**A**) Volcano plot comparing downregulated (blue dots) and upregulated (red dots) gene expression that occurs at AT exposure. Dotted lines indicate cutoffs, adjusted $P < 0.05$ and log2Fold Change (AT/Control) ≥ |1|. Black dots represent genes that are not significantly different. (**B**) The most significant (those with lowest P-value) up (red lines) and downregulated (blue lines) DEGs enriched Biological Process GO terms, as determined by g:Profiler (adjusted $P < 0.05$), in brown fat tissue at AT. Names of GO terms are indicated on the Y-axis, and the number of enriched genes for each term is represented on the X-axis.

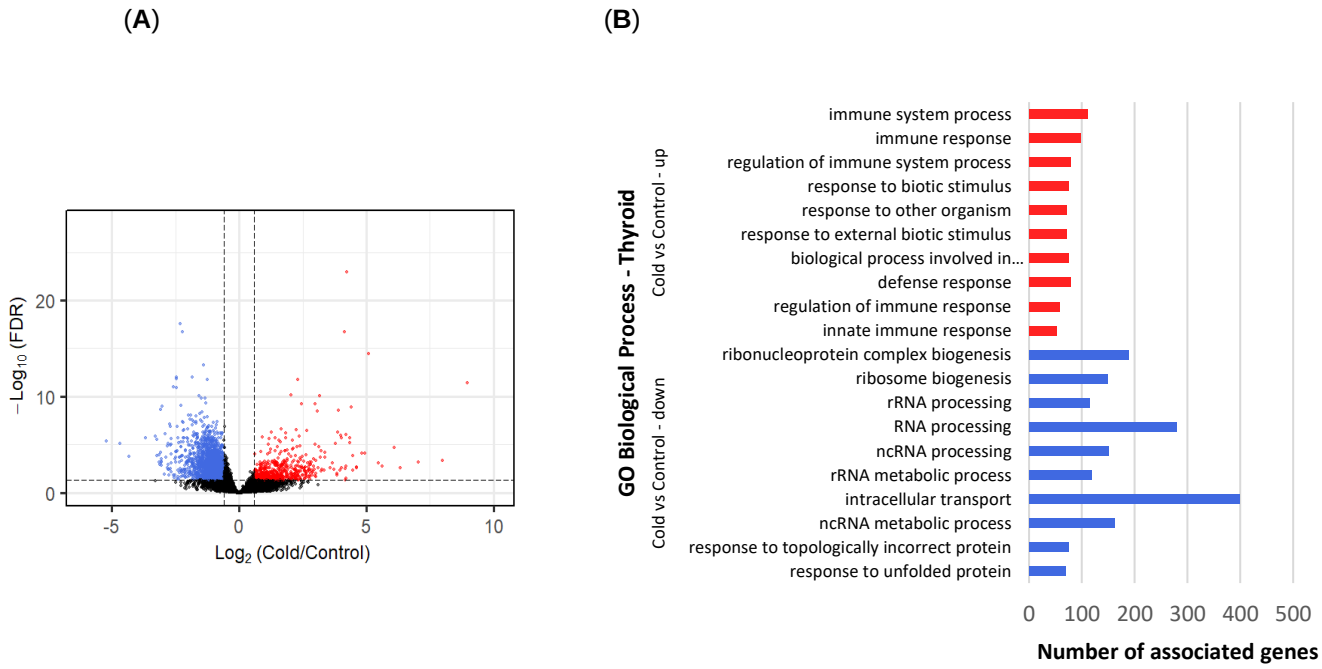


Figure 5.4. Differential gene expression and enrichment in thyroid tissue of lambs under cold temperature (4 °C). (**A**) Volcano plot comparing downregulated (blue dots) and upregulated (red dots) gene expression that occurs after cold exposure. Dotted lines indicate cutoffs, adjusted P < 0.05 and log2Fold Change (cold/control) ≥ |1|. Black dots represent genes that are not significantly different. (**B**) The most significant (those with lowest P-value) up (red lines) and downregulated (blue lines) DEGs enriched Biological Process GO terms, as determined by g:Profiler (adjusted P < 0.05), in thyroid tissue exposed to cold. Names of GO terms are indicated on the Y-axis, and the number of enriched genes for each term is represented on the X-axis.

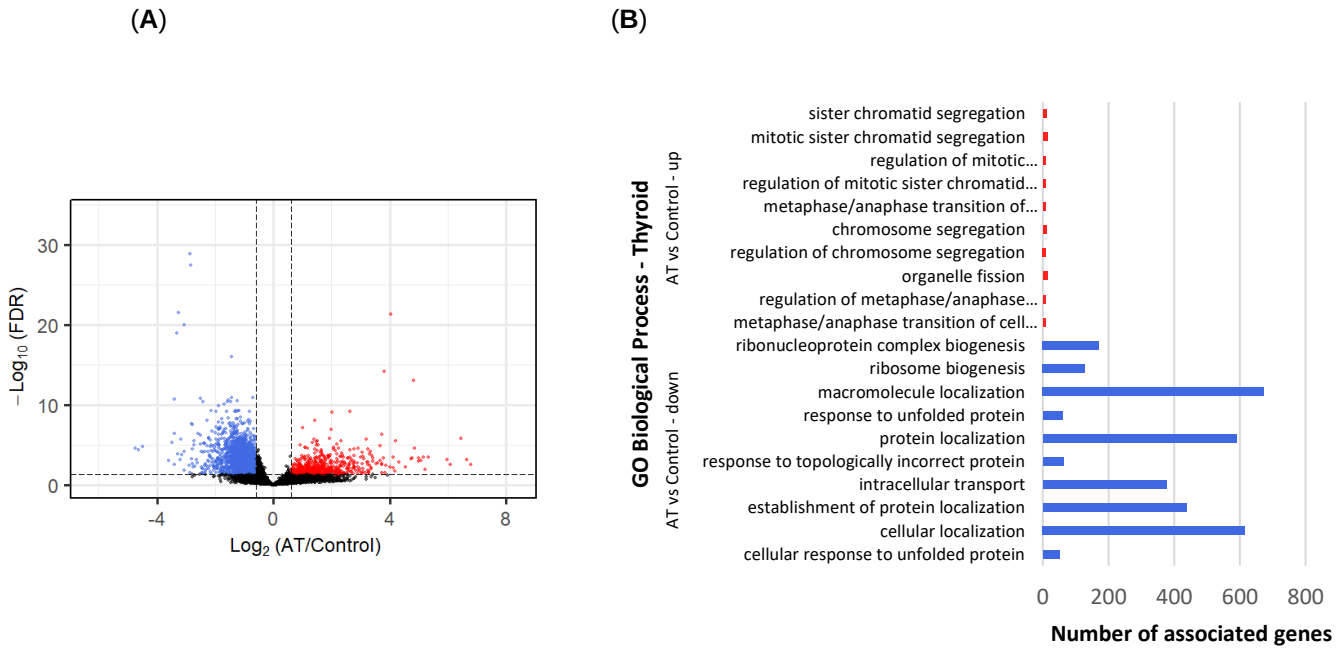


Figure 5.5. Differential gene expression and enrichment in thyroid tissue of lambs under ambient temperature (20–22 °C). (**A**) Volcano plot comparing downregulated (blue dots) and upregulated (red dots) gene expression that occurs at AT exposure. Dotted lines indicate cutoffs, adjusted P < 0.05 and log2Fold Change (AT/Control) ≥ |1|. Black dots represent genes that are not significantly different. (**B**) The most significant (those with lowest P-value) up (red lines) and downregulated (blue lines) DEGs enriched Biological Process GO terms, as determined by g:Profiler (adjusted P < 0.05), in thyroid tissue at AT. Names of GO terms are indicated on the Y-axis, and the number of enriched genes for each term is represented on the X-axis.

5.3.3 Differential gene expression and enrichment in BAT under cold conditions

A total of 638 upregulated Biological Process (BP) terms were found (Table S5.4). The most significant ones (those with the lowest P-value) were involved in the cell cycle with terms such as “cell division”, “chromosome segregation” and “mitotic cell cycle” (Figure 5.2B). Moreover, there were a considerable number of terms regarding immune defence, such as “response to virus” (26 genes), “immune system process” (33 genes) and “defence response” (24 genes). Stress-related BP terms were also found: “response to stress” (35 genes) and “regulation of response to stress” (17 genes). In addition, “response to stimulus” terms appear significant, such as “regulation of response to external stimulus” (34 genes) and “cellular response to stimulus” (55 genes). Further, cell communication and signalling terms appeared, such as “positive regulation of signaling” (31 genes), “cell surface receptor signaling pathway” (17 genes) and “regulation of signaling” (55 genes). For the Molecular Function (MF) category, “microtubule binding” (20 genes), “extracellular matrix structural constituent” (13 genes) and “ATPase” (19 genes) were found as being the most significant. Within the Cellular Component category (CC) “chromosome-centromeric region” (24 genes), “extracellular matrix” (31 genes) and “cell periphery” (97 genes) were part of the most significant terms. The top Reactome pathways were “arachidonic acid metabolism” (3 genes) and “fatty acid metabolism” (4 genes).

A total of 544 downregulated BP terms were found (Table S5.4). These terms were focused on ribosome synthesis and maturation, with “ribosome biogenesis”, “rRNA processing” and “RNA processing” as the most significant ones (Figure 5.2B). In addition, many terms associated with protein synthesis and maturation were found: “protein folding” (21 genes), “positive regulation of cellular protein localization” (18 genes) and “protein maturation” (16 genes). In the MF category, the most enriched terms were “RNA binding” (101 genes), “unfolded protein binding” (13 genes) and “ribonucleoprotein complex binding” (12 genes). The most enriched terms for CC

included “preribosome” (23 genes), “nucleolus” (53 genes) and “ribonucleoprotein complex” (47 genes). Further, the most significant pathway for KEGG analysis was “Ribosome biogenesis in eukaryotes” (15 genes) and for Reactome analysis it was “rRNA processing” (26 genes).

5.3.4 Differential gene expression and enrichment in BAT under ambient-temperature conditions

A total of 40 upregulated BP terms were found (Table S5.4). The most significant ones were focused on cell cycle and the extracellular space, with terms such as “extracellular matrix organization”, “regulation of metaphase/anaphase transition of cell cycle” and “regulation of chromosome separation” (Figure 5.3B). These were followed closely by immune system terms, such as “antiviral innate immune response” (3 genes), “type I interferon signaling pathway” (9 genes) and “response to virus” (5 genes). Within the MF category, “aldehyde dehydrogenase (NAD+) activity” (7 genes), “extracellular matrix structural constituent” (16 genes) and “integrin binding” (13 genes) were found as most significant. The most significant CC terms were “extracellular matrix” (39 genes), “cell periphery” (159 genes) and “chromosome, centromeric region” (16 genes). The term “interferon alpha/beta signaling” (4 genes) was seen as the most significant in Reactome pathways.

A total of 187 downregulated BP terms were found (Table S5.4). The most significant terms were linked to RNA and protein processing, such as “ribonucleoprotein complex biogenesis”, “RNA processing” and “protein folding” (Figure 5.3B). Further, stress-related BP terms appeared as well, such as: “cellular response to stress” (168 genes) and “regulation of cellular response to stress” (69 genes). The most significant MF terms were “RNA binding” (180 genes), “enzyme binding” (166 genes) and “protein binding” (846 genes). Intracellular-related terms were found to be the most significant type of CC

terms, including: “cytoplasm” (739 genes), “intracellular membrane-bounded organelle” (742 genes) and “intracellular anatomical structure” (867 genes). Within Reactome pathways, “metabolism of RNA” (93 genes) was the most significant term.

5.3.5 Differential gene expression and enrichment in thyroid tissue under cold conditions

A total of 1149 upregulated BP terms were found (Table S5.4). Immune defence terms were seen as most significant, such as “immune response”, “regulation of immune system process” and “defence response” (Figure 5.4B). Those were followed by terms regarding response to external changes, including: “response to external stimulus” (88 genes), “positive regulation of response to stimulus” (83 genes), and “response to stimulus” (186 genes). A good number of BP terms surrounding cell communication and signalling have been found, with terms such as “cell surface receptor signaling pathway” (98 genes), “signaling” (160 genes) and “signal transduction” (147 genes). Moreover, cell cycle and differentiation terms were significant, such as: “sister chromatid segregation” (13 genes), “regulation of cell activation” (36 genes) and “metaphase/anaphase transition of cell cycle” (11 genes). In addition, stress-related terms were present: “response to stress” (42 genes), “regulation of response to stress” (45 genes) and “cellular response to stress” (4 genes). The most significant MF terms were “extracellular matrix structural constituent” (20 genes), “immune receptor activity” (11 genes) and “signalling receptor activity” (44 genes). The most significant CC terms consisted of “extracellular matrix” (45 genes), “cell periphery” (180 genes) and “extracellular region” (124 genes). The most significant Reactome pathways found were “arachidonic acid metabolism” (6 genes) and “extracellular matrix organization” (19 genes).

A total of 1982 downregulated BP terms were found (Table S5.4). The most significant terms were involved in RNA synthesis and maturation, such as: “ribonucleoprotein complex biogenesis”, “ribosome biogenesis” and “RNA processing” (Figure 5.4B). These were closely followed by protein maturation-related terms, such as “response to unfolded protein” (70 genes), “protein localization” (534 genes) and “protein transport” (376 genes). The most significant terms for the MF category were “RNA binding” (449 genes), “protein binding” (2112 genes) and “ribonucleoprotein complex binding” (61 genes). The most significant terms for the CC group included “intracellular membrane-bounded organelle” (1916 genes), “cytoplasm” (1849 genes) and “intracellular anatomical structure” (2188 genes). Pathways related to proteins and ribosome processing were found through KEGG analysis: “protein processing in endoplasmic reticulum” (70 genes) and “ribosome biogenesis in eukaryotes” (29 genes), as well as through Reactome “rRNA modification in the nucleus and cytosol” (44 genes) and “metabolism of RNA” (231 genes).

5.3.6 Differential gene expression and enrichment in thyroid tissue under ambient-temperature conditions

A total of 968 upregulated BP terms were found (Table S5.4). Most significant terms regarded the cell cycle, such as “sister chromatid segregation”, “regulation of mitotic metaphase/anaphase transition” and “regulation of chromosome segregation” (Figure 5.5B). In addition, many terms involved in the immune defence appeared significant, such as “regulation of immune system process” (32 genes), “antiviral innate immune response” (6 genes) and “immune response” (76 genes). For the MF category, these terms were regarded as most significant: “extracellular matrix structural constituent” (24 genes), “microtubule binding” (20 genes) and “ATPase” (24 genes). Within the most significant terms of the CC group, “extracellular matrix” (40 genes), “cell periphery” (182 genes) and “chromosome, centromeric region” (9 genes) were annotated. The most

significant Reactome pathways were “mitotic spindle checkpoint” (11 genes) and “interferon alpha/beta signalling” (4 genes).

A total of 1860 downregulated BP terms were found (Table S5.4). The most significant terms were involved in ribosome and protein synthesis and metabolism, with terms such as “ribonucleoprotein complex biogenesis”, “ribosome biogenesis” and “protein localization” (Figure 5.5B). These were followed by catabolism terms such as “regulation of catabolic process” (265 genes), “catabolic process” (558 genes) and “cellular catabolic process” (490 genes). Further, a couple of stress-related terms appeared significant: “cellular response to stress” (438 genes) and “response to stress” (179 genes). The most significant terms in the MF category were “RNA binding” (440 genes), “protein binding” (2280 genes) and “enzyme binding” (427 genes). For the CC group, “intracellular membrane-bounded organelle” (2065 genes), “intracellular anatomical structure” (2362 genes) and “cytoplasm” (1982 genes), were the most significant terms. The most significant KEGG pathways were “protein processing in endoplasmic reticulum” (52 genes) and “protein export” (12 genes), accompanied by “rRNA modification in the nucleus and cytosol” (40 genes) and “metabolism of RNA” (214 genes) by Reactome pathways.

5.3.7 RT-qPCR validation of RNAseq results, and expression of selected genes involved in thermogenesis and BAT whitening

The trend and magnitude of the fold change of expression (log2FC ± SE) detected in the RT-qPCR analysis for all the tested genes, was similar to that detected in RNAseq results. Therefore, the results from the validation through RT-qPCR (Figure S5.2) from selected genes confirmed the accuracy of the RNASeq data to quantify gene expression in BAT and thyroid tissue. Fold change values (RT-qPCR vs. RNAseq analysis) were analysed in BAT (cold/control) for *VEGFA (*−3.20 ± 0.22 vs. −1.79 ± 0.42), *PPARGC1A (*−0.47 ± 0.15 vs. −0.52 ± 0.53), *KI67* (1.36 ± 0.25 vs. 2.39 ± 0.58), *BMP4* (1.28 ± 0.15

vs. 0.97 ± 0.40), *ADRB3* (−1.22 ± 0.28 vs. −1.23 ± 0.58) and *DIO2* (−0.82 ± 0.38 vs. −0.83 ± 1.15). Fold change values (RT-qPCR vs. RNAseq analysis) were analysed in BAT (AT/control) for *VEGFA (*−3.02 ± 0.24 vs. −1.53 ± 0.42), *PPARGC1A (*−1.47 ± 0.18 vs. −1.30 ± 0.53), *KI67* (0.64 ± 0.22 vs. 1.45 ± 0.58), *BMP4* (0.86 ± 0.11 vs. 1.08 ± 0.40), *ADRB3* (−1.20 ± 0.28 vs. −0.87 ± 0.58) and *DIO2* (−1.97 ± 0.78 vs. −1.12 ± 1.15). Fold change values (RT-qPCR vs. RNAseq analysis) were analysed in thyroid tissue (cold/control) for *VEGFA (*−1.78 ± 0.56 vs. −1.32 ± 0.32), *PPARGC1A (*−1.61 ± 0.07 vs. −1.23 ± 0.27), *KI67* (2.34 ± 0.17 vs. 3.01 ± 0.70), *BMP4* (0.73 ± 0.35 vs. 0.43 ± 0.52), *ADRB3* (−0.05 ± 0.56 vs. NA) and *DIO2* (−0.45 ± 0.12 vs. −1.23 ± 0.63). Fold change values (RT-qPCR vs. RNAseq analysis) were analysed in thyroid tissue (AT/control) for *VEGFA (*−1.51 ± 0.34 vs. −1.16 ± 0.32), *PPARGC1A (*−1.40 ± 0.25 vs. −1.20 ± 0.27), *KI67* (1.59 ± 0.23 vs. 3.33 ± 0.70), *BMP4* (0.52 ± 0.15 vs. 1.23 ± 0.52), *ADRB3* (−0.53 ± 0.51 vs. NA) and *DIO2* (−0.75 ± 0.35 vs. −1.03 ± 0.63). There were not enough counts for the calculation of fold change values for *ADRB3* from RNAseq analysis in thyroid tissue, for neither the cold nor ambient-temperature groups.

A set of 38 genes (Table S5.3) which are known to have an active role directly or indirectly on thermogenesis and BAT whitening (Figure 5.6) were selected and analysed in both tissues as cold temperature vs. control, ambient temperature vs. control and cold temperature vs. ambient temperature (Table 5.1). In the analysis of cold temperature vs. control, *FABP3*, *NOS3* and *VEGFA* were downregulated in BAT, whereas *CYP1A1* and *MKI67* where upregulated; meanwhile, in thyroid tissue *PDK4*, *TGM2*, *ASCL5*, *CTP1A*, *FABP3*, *NOS3*, *VASP* and *VEGFA* were downregulated, while *CYP1A1A* and *MKI67* were upregulated. In the case of ambient temperature vs. control, *CPT1A*, *FABP3*, *NOS3*, *VASP* and *VEGFA* were downregulated in BAT, whereas *FNDC5*, *PRDM16* and *CYP1A1A* where upregulated. In thyroid tissue, *TGM2*, *ACSL5*, *CPT1A*, *FABP3*, *NOS3*, *VASP* and *VEGFA* were downregulated, while *CYP1A1A* and *MKI67* were upregulated. In the cold temperature vs. ambient temperature comparison, only *CYP1A1A* was observed as upregulated in BAT. There were no significant differences recorded in any

tissue/treatment for *UCP1*, *ADRB1-3*, *ADRA1A*, *PPARGC1B*, *PPARA*, *PPARG*, *ELOVL6*, *BMP7*, *BMP8B*, *CIDEA*, *CKB*, *PRKG1*, *PDE3B*, *LPL*, *EHMT1*, *GABPA*, *THRA*, *THRB*, *DIO2*, *PNPLA2*, *LIPE* and *MGLL*.

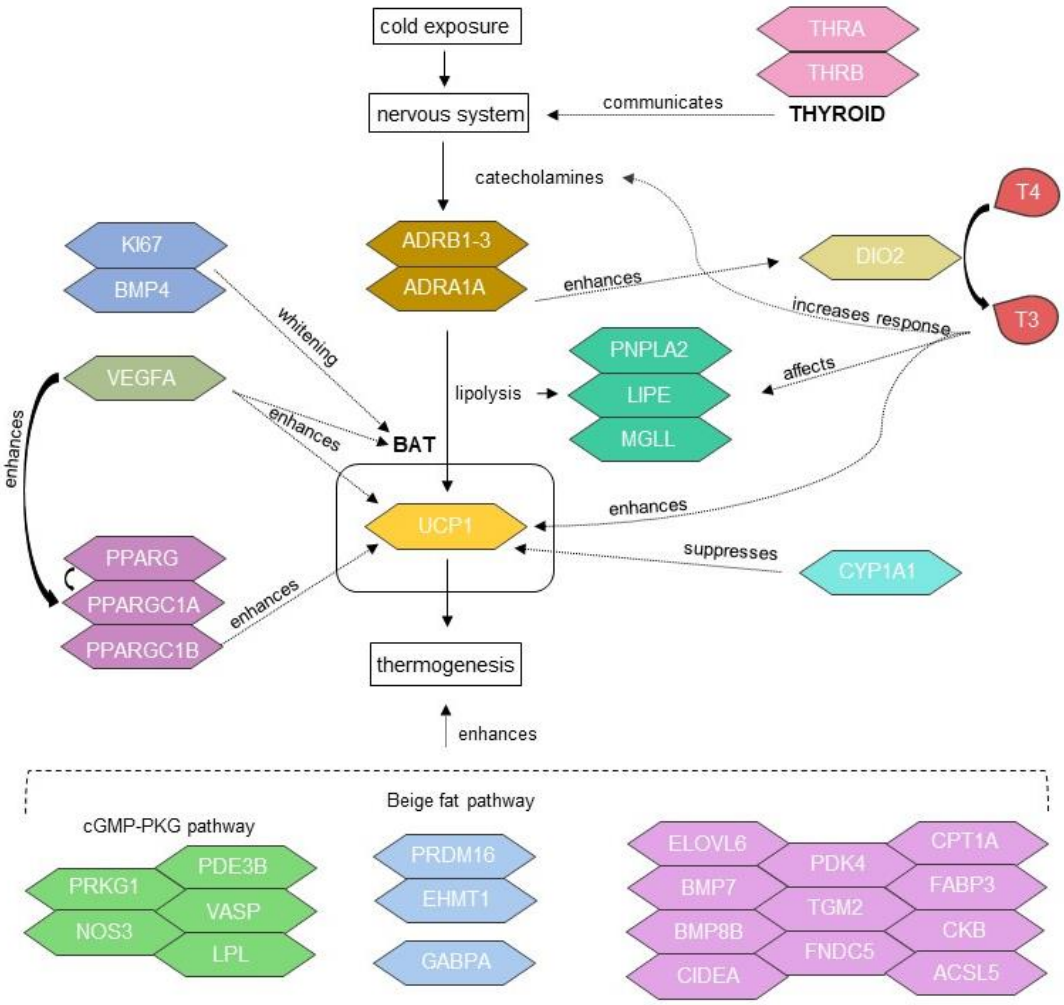
Table 5.1. Selected genes with a role in thermoregulation or BAT whitening within each group.

Gene	Tissue	Cold/Ctrl log2FoldChange	Cold/Ctrl *P*-adj	AT/Ctrl log2FoldChange	AT/Ctrl *P*-adj	Cold/AT log2FoldChange	Cold/AT P-adj
PPARGC1A	BAT	−0.52	0.64	−1.3	0.08	0.78	0.32
	Thyroid	−1.23	<0.05	−1.2	<0.05	−0.03	1.00
BMP4	BAT	0.97	0.13	1.08	<0.05	−0.11	0.89
	Thyroid	0.43	0.59	1.23	0.06	−0.80	0.96
PDK4	BAT	0.76	0.48	−0.29	0.79	1.04	0.17
	Thyroid	−2.40	<0.05	−1.83	0.09	−0.57	1.00
TGM2	BAT	0.14	0.85	−0.05	0.94	0.19	0.72
	Thyroid	−1.36	<0.05	−1.31	<0.05	−0.05	1.00
FNDC5	BAT	1.27	0.06	1.95	<0.05	−0.69	0.27
	Thyroid	0.33	0.65	0.97	0.11	−0.64	1.00
ACSL5	BAT	0.28	0.73	0.28	0.65	−0.01	0.99
	Thyroid	−0.88	<0.05	−0.81	<0.05	−0.07	1.00
CPT1A	BAT	−0.95	0.06	−1.59	<0.05	0.63	0.17
	Thyroid	−0.94	<0.05	−0.93	<0.05	0.00	1.00
FABP3	BAT	−1.74	<0.05	−2.01	<0.05	0.27	0.78
	Thyroid	−2.20	<0.05	−2.29	<0.05	0.09	1.00
NOS3	BAT	−1.40	<0.05	−1.87	<0.05	0.47	0.35
	Thyroid	−1.53	<0.05	−1.46	<0.05	−0.07	1.00
VASP	BAT	−0.38	0.33	−0.75	<0.05	0.37	0.23
	Thyroid	−0.94	<0.05	−0.92	<0.05	−0.02	1.00

Gene	Tissue	Cold/Ctrl log2FoldChange	Cold/Ctrl *P*-adj	AT/Ctrl log2FoldChange	AT/Ctrl *P*-adj	Cold/AT log2FoldChange	Cold/AT P-adj
PRDM16	BAT	0.98	0.15	1.16	<0.05	−0.18	0.81
	Thyroid	−0.12	0.87	0.72	0.21	−0.85	0.66
VEGFA	BAT	−1.79	<0.05	−1.53	<0.05	−0.26	0.73
	Thyroid	−1.32	<0.05	−1.16	<0.05	−0.16	1.00
CYP1A1	BAT	12.80	<0.05	7.33	<0.05	5.47	<0.05
	Thyroid	8.98	<0.05	6.44	<0.05	2.54	0.22
MKI67	BAT	2.39	<0.05	1.45	0.07	0.94	0.25
	Thyroid	3.01	<0.05	3.33	<0.05	−0.33	1.00

UCP1, *ADRB1-3*, *ADRA1A*, *PPARGC1B*, *PPARA*, *PPARG*, *ELOVL6*, *BMP7*, *BMP8B*, *CIDEA*, *CKB*, *PRKG1*, *PDE3B*, *LPL*, *EHMT1*, *GABPA*, *THRA*, *THRB*, *DIO2*, *PNPLA2*, *LIPE* and *MGLL* were not included in this table as they were not significant in any tissue/treatment (adjusted P < 0.05). Cold/Ctrl: analysis of cold temperature vs. control; AT/Ctrl: analysis of ambient temperature vs. control; Cold/AT: analysis of cold temperature vs. ambient temperature.

(**A**)


cold exposure
nervous system
communicates
THRA
THRB
THYROID
catecholamines
T4
T3
KI67
BMP4
ADRB1-3
ADRA1A
enhances
DIO2
increases response
whitening
VEGFA
lipolysis
PNPLA2
LIPE
MGLL
affects
enhances
BAT
UCP1
enhances
suppresses
CYP1A1
PPARG
PPARGC1A
PPARGC1B
enhances
thermogenesis
enhances
Beige fat pathway
cGMP-PKG pathway
PRDM16
EHMT1
GABPA
PRKG1
PDE3B
VASP
NOS3
LPL
ELOVL6
PDK4
CPT1A
BMP7
TGM2
FABP3
BMP8B
FNDC5
CKB
CIDEA
ACSL5

(**B**)

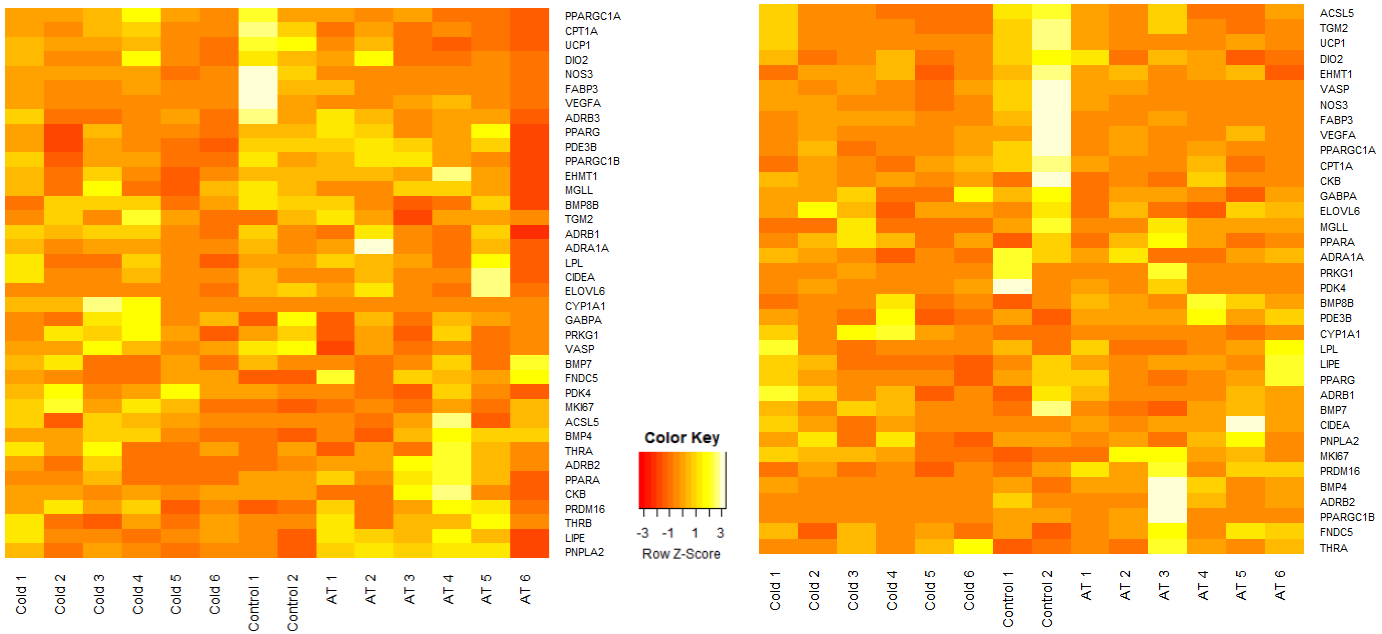


Figure 5.6. Characters involved in thermogenesis and BAT whitening. (**A**) Diagram of the thermogenic/whitening network, composed by the 38 analysed genes. (**B**) Heatmaps of the 38 uniquely analysed genes in BAT (left) and thyroid tissue (right), clustered from the normalised counts per gene matrix from the DESeq2 analysis for each group of samples; cold, control and ambient. The genes *ADRB3* and *THRB* were excluded from the thyroid heatmap due to insufficient counts. Cold 1 to Cold 6 represent animals kept at 4 °C (cold) for two days, AT 1 to AT 6 represent animals kept at 20–22 °C (ambient temperature) for two days, and Control 1 and Control 2 represent the control lambs that were euthanised within 12–24 h after birth.

5.4 Discussion

The thermogenic capacity of BAT under cold exposure is a multistep process, regulated by a complex network of genes and metabolic pathways (Marcher et al., 2015). Over the first few days of life these factors decline (Bispham et al., 2002), as BAT transforms into WAT and shivering becomes the main response to cold conditions (Symonds, 2013). In this context, transcriptome profiles from a comparative RNAseq analysis of new-born lambs under cold and ambient conditions provide insight into the molecular changes that occur over this period. As there is minimal information available to date regarding the molecular aspects of these transitions (Basse et al., 2015; Marcher et al., 2015), this approach adds an in-depth view of some of the major characters that take part directly or indirectly in thermoregulation and the fat-whitening transformation.

5.4.1 Analysis on the main factors that regulate thermogenesis

Exposure to cold leads to an expected cascade of events (Figure 5.6), starting from an increase in catecholamines such as norepinephrine that stimulate the various subtypes of β-adrenergic receptors (ADRBs) normally found on the surface of brown adipocytes in BAT (Strosberg, 1997; Leaver & Pappone, 2002; Henry et al., 2017). More importantly, it stimulates the adrenergic-B3-receptor, which can activate lipolysis and release fatty acids in BAT (Forrest et al., 2007; Kurylowicz et al., 2015; Plush et al., 2016). The key thermogenic factor *UCP1* (uncoupling protein 1), then utilises fatty acids to produce heat instead of ATP in cold conditions. All lambs under cold and ambient temperature conditions in the current study displayed no difference in the expression of the *UCP1* gene or any of the ADRBs (*ADRB1-3* and *ADRA1A*), at day three of age. This observation may support previous results that were found around this age by both Basse et al. (2015) and Lomax et al. (2007), where *UCP1* expression was not detected in lambs by day four or five of age, respectively. On the other hand, some studies have found *UCP1* expression at older ages in sheep or even at younger ages in goats. A recent

study by Liu et al. (Liu et al., 2022b) in new-born goats, after 24 h of cold exposure, found (through RNAseq) an increase of expression of several regulatory genes for thermoregulation in BAT such as *ADRRB1* and *PPARGC1A* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), and in addition, observed an increase in the protein levels of *UCP1*. Furthermore, results from an RNAseq study on perirenal BAT of six-month-old lambs subjected to cold conditions for 25 days reported an expression of *UCP1* (Jiao et al., 2021). Another study using the same tissue from suckling lambs reported an expression of *UCP1* through RNAseq at 19−35 days of age (Suarez-Vega et al., 2018). Yuan et al. (2012) reported different levels of expression of this gene from 2 to 12 months of age, where lower levels were found in superficial fat, and higher levels were seen in deep fat deposits. This observation by Yuan et al. (2012) could explain in part why *UCP1* expression was not observed in this study on day three, due to the possibility that the samples utilised here were coming from the surface of perirenal BAT. Unfortunately, more information regarding the differences of gene expression within different parts of the adipose tissue are lacking. Other studies on this aspect focused more on differences between visceral and subcutaneous adipose tissue, which seem to have an independent metabolic function (Walker et al., 2007). Nevertheless, in the said studies there was no mention on the differences between sub-compartments of each tissue type. However, BAT is amongst the most vascularised tissues in the body (Brakenhielm & Cao, 2008), where there is a big interaction between adipocytes and vascular cells (Shamsi et al., 2021). For instance, vascular cells can express *VEGFA* (Marko & Damon, 2008), which boosts its proliferation and survival (Neufeld et al., 1999), where an overexpression of *VEGFA* is also considered a key factor responsible for the thermogenic response of BAT to cold exposure (Xue et al., 2009). Consequently, this interaction between vascular cells and adipocytes might have an impact on gene expression, where adipose tissue extracted from a more proximal location to the vascular vessels could provide different expression results, compared to the expression of a tissue extracted in a more distal location from the vessels.

The lack of differential expression of the β-adrenergic receptors in the present study could have impacted negatively by not inducing the expression of *UCP1* in BAT. In combination with these reports and the present study, it could be that the expression of *UCP1* comes and goes very rapidly during the first critical few days of life, but can also have a more prolonged and noticeable expression during cold conditions in older lambs. These discrepancies around the time where *UCP1* loses expression might also be caused by the different breeds used in thermogenesis studies, the techniques being used to measure the gene expression or other experimental environmental factors (Suarez-Vega et al., 2018). It could also be inferred that the conditions of the present study were not cold enough to induce a response. However, the enrichment analysis of the lambs of this study resulted in several Biological Process GO terms regarding response to stress, regulation of response to external stimulus, and cell communication and signalling, which were upregulated during cold conditions. These observations imply that the cold environment was in fact challenging the lambs, as has also been previously reported in mice indoors at 4 °C for three days (Marcher et al., 2015), and in goats kept indoors at 6 °C for 24 h (Liu et al., 2022b); therefore, one might conclude that the environment was cold enough to allow a physiological response. Consequently, it appears that there is a need for further studies to collect more information on the timing of *UCP1* expression.

The absence of differential expression of *UCP1* was accompanied by a decline or lack of expression of genes primarily associated with BAT and thermoregulation, such as the PPARs (peroxisome proliferator-activated receptors), where some of the roles of this group of genes are the control of fatty acid oxidation (van Raalte et al., 2004) and adipogenesis (Lowell, 1999), where their activation is subjected to transcriptional coactivation by *PPARGC1A* (Liang & Ward, 2006) and *PPARGC1B* (peroxisome proliferator-activated receptor gamma coactivator 1-beta) (Delezie et al., 2020). Thus, the lack of expression of *PPARA* or *PPARG* in this study was expected, as the expression of *PPARGC1A* was downregulated in thyroid tissue, and in BAT there was

no difference of expression, under neither cold nor ambient conditions, plus there was no differential expression of *PPARGC1B* recorded in any tissue/treatment. *PPARGC1A* has been proposed to be a master regulator of BAT differentiation and also an inductor of the *UCP1* gene (Puigserver et al., 1998; Liang & Ward, 2006), hence making this gene indispensable for proper thermogenesis (Wu et al., 1999). Consequently, the lack of expression of *PPARGC1A* in BAT and its downregulation in thyroid tissue could be impacting negatively on the thermogenic induction of *UCP1* and other thermogenic genes that it coactivates. Furthermore, there was a lack of expression in all tissues and treatments of several thermogenic genes that were previously reported to have an increased expression upon cold exposure. Genes regarded in those lines were: *ELOVL6* (Fatty Acid Elongase 6) as a regulator of fatty acid chain elongation (Tan et al., 2015; Liu et al., 2022b); *BMP7* (Bone Morphogenetic Protein 7), which promotes brown adipocyte differentiation (Tseng et al., 2008; Pope et al., 2014); *BMP8B* (Bone Morphogenetic Protein 8b) as an energy dissipator of BAT (Whittle et al., 2012; Rosell et al., 2014; Grefhorst et al., 2015); *CIDEA* (cell death-inducing DFFA-like effector A), which is considered as a marker of BAT in rodents (Li, 2004); and *CKB* (Creatine Kinase B), from the creatine cycle that drives the thermogenic respiration in fat cells (Kazak et al., 2015; Rahbani et al., 2021). In addition, some genes related to thermogenesis were differentially expressed and even downregulated after cold exposure in some cases, but were not seen upregulated after cold exposure in any tissues (Table 5.1), such as BAT adipogenesis genes *PDK4* (Isozyme 4) (Forner et al., 2009), *TGM2* (Transglutaminase 2) (Pope et al., 2014) and *FNDC5* (Fibronectin Type III Domain Containing 5) (Marcher et al., 2015); *ACSL5* (Acyl CoA synthetase 5), which encodes long-chain acyl CoA synthetase, a key enzyme for β-oxidation (Marcher et al., 2015); *CPT1A* (Carnitine Palmitoyltransferase 1A), which is involved in the transport of fatty acids into the inner mitochondrial membrane for β-oxidation (Daikoku et al., 2000; Yu et al., 2002; Wang et al., 2021); and *FABP3* (Fatty Acid Binding Protein 3), which is essential for accelerating fatty acid flux to its oxidation through *UCP1* (Yamashita et al., 2008).

According to previous reports, there are specific pathways which involve a group of genes with the principal objective to increase thermogenesis during cold adaptation. One of these pathways is the cGMP-PKG signalling pathway (Cyclic guanosine monophosphate—Protein Kinase G), which after activation through adrenergic receptors, can increase lipid uptake by BAT and further regulate thermogenesis (Haas et al., 2009; Bordicchia et al., 2012; Hoffmann et al., 2015). This pathway involves genes such as *PRKG1* (Protein Kinase CGMP-Dependent 1)*, NOS3* (Nitric Oxide Synthase 3)*, PDE3B* (Phosphodiesterase 3B), *VASP* (Vasodilator Stimulated Phosphoprotein) and *LPL* (Lipoprotein Lipase), which were previously recorded as upregulated in perirenal BAT of new-born goats after cold exposure (Liu et al., 2022b). Nevertheless, the said genes were not upregulated in BAT or thyroid tissue after cold exposure in this study, and in some cases, they were downregulated (*NOS3* in BAT and thyroid, and *VASP* in thyroid). Additionally, other pathways have been described around autonomous ways to produce heat from beige fat, such as the *PRDM16* (PR domain containing 16) pathway, which together with the expression of *EHMT1* (Euchromatic Histone-Lysine N-Methyltransferase 1) can activate beige adipocytes biogenesis, enhancing adipose tissue thermogenesis (Ohno et al., 2012; Ohno et al., 2013; Wang et al., 2022). Furthermore, another compensatory pathway of beige fat biogenesis has been described: the glycolytic beige fat, through the actions of *GABPA* (GA-Binding Protein alpha), which can be activated even in the absence of adrenergic receptor signalling (Chen et al., 2019). However, neither of the genes described in these alternative thermogenic pathways were recorded as upregulated in BAT or thyroid tissue after cold exposure in our study. In summary, each of the genes described here, which were characterised as thermogenic and previously observed as activated after cold exposure, were not being positively expressed in any tissue after the lambs of this study were in a cold environment for two days. Moreover, there was a total lack of differential expression, either upregulated or downregulated, of any of those genes when comparing each tissue between the cold and ambient groups. Therefore, these results can help visualise the

molecular state of BAT and thyroid tissue of new-born lambs exposed to cold, and how each and every character involved in thermogenesis is lacking its expression in order to produce heat in a non-shivering way.

One probable cause of this absence/loss of expression of these genes associated with thermoregulation might be the overall downregulation of *VEGFA* (vascular endothelium growth factor), as observed in both tissues and treatments in this study. Previous reports state that the overexpression of *VEGFA* promotes a "BAT-like" phenotype in the adipose tissue, and that it enhances the expression of BAT genes such as *PPARGC1A* and *UCP1* (Elias et al., 2013). Further, *VEGFA* can stimulate adrenergic receptors, which consequently induce these thermogenic genes (Xue et al., 2009), since the ablation of *VEGFA* leads to a downregulation of β-adrenergic signalling to BAT (Shimizu et al., 2014). Therefore, it might be expected that the downregulation of *VEGFA* could be a possible reason that the β-adrenergic receptors lacked expression, subsequently pulling down the expression of *PPARGC1A* and *UCP1*, thus reducing the thermogenic activity in these new-born lambs. Another possibility to explain the lack of expression of the key thermoregulator *UCP1* might be the overexpression of the *CYP1A1* gene (cytochrome P450 1A1), which was found in all tissues and treatments. The activity of the enzyme encoded by this gene can be stress-induced (Mufti & Shuler, 1996), which can be linked to the several BP GO terms regarding the response to stress that were seen in cold conditions, as previously described. Even though there were no records of stress-related terms in the lambs at ambient conditions, it could be presumed that there was some level of stress in these lambs due to the difference in temperature from birth. The ambient temperature utilised in this experiment is much lower than in the uterine environment, resulting in lambs needing to increase the rate of body heat production by up to fifteen times more than the fetal level to compensate for the heat loss (Dawes & Mott, 1959; Alexander, 1962a). Interestingly, this gene has been observed to be expressed in the inner mitochondrial membrane (Dong et al., 2009), exactly where *UPC1* acts producing heat, but in there *CYP1A1* releases arachidonic acid, which in turn suppresses the

mitochondrial activity (Fleckenstein-Elsen et al., 2016). For this reason, it might be the case that the over-expression of this gene might cut down the processes to produce heat by *UCP1*, therefore impairing thermogenesis. Unfortunately, little is known about the molecular actions that involve this gene and how it interacts with other thermogenic genes; further work is required to understand this process.

5.4.2 BAT and thyroid thermogenesis association

Thyroid hormones have a multifaceted contribution to thermogenesis, as there are about 8000 thyroid-hormone receptors per brown adipocyte cell (Bianco & Silva, 1987); hence, they would play an essential part in energy homeostasis during cold exposure as they activate heat production (Bianco & McAninch, 2013). However, evidence of thermogenic activity was not observed in the present study as previously discussed; thus, the lack of differential expression in main thyroidal genes was not unexpected. Thyroid hormone receptor A (*THRA*), which mediates the communication between thyroid hormone signalling and the sympathetic nervous system in BAT, was found not to be differentially expressed in all tissues and treatments, as well as the receptor B (*THRB*), which mediates the tri-iodothyronine hormone (T3) regulation of *UCP1* in BAT (Bianco & McAninch, 2013). Consequently, the lack of differential expression of these thyroid receptors suggests that there was neither induction of BAT through the hypothalamic pathway nor stimulation of the expression of *UCP1* by them. This missing thermogenic response has been previously observed in many mammals, including hypo-thyroidal new-born lambs (Schermer et al., 1996), new-born calves (Cabello, 1983; Vermorel et al., 1983; Barlow et al., 1987) and piglets (Berthon et al., 1993). In these studies it has been shown that an impairment in heat production occurs as BAT becomes unresponsive to noradrenaline stimulation in the absence of thyroidal hormones (Ribeiro et al., 2000). Furthermore, the type II iodothyronine deiodinase enzyme (*DIO2*) was not differentially expressed in all tissues and treatments in the present study. This enzyme

is responsible for the conversion of the inactive hormone, thyroxine (T4) to T3, which is the metabolically active form (Plush et al., 2016). If active, DIO2 would have increased the catecholamine response and *UCP1* expression (Rubio et al., 1995; Hellström et al., 1997), but in the present study these actions were not observed. In fact, a study on *DIO2* knockout mice embryos showed an impairment in thermogenesis, which was associated with decreased expression of *UCP1* and *PPARGC1A* (Hall et al., 2010). It could have been the case that the lack of expression of *ADRB3* found in the present study had a negative impact on *DIO2*, as it has been reported that the expression of *ADRB3* during cold exposure can stimulate the hormonal conversion of T4 to active T3 of *DIO2* (De Jesus et al., 2001; Cannon & Nedergaard, 2004). Moreover, in a study in humans, Kurylowicz et al. (2015) observed that a decrease in expression of *DIO2* resulted in a lower local conversion of T4 to T3, suggesting that those factors contributed to the reduced expression of *THRA* and *THRB* found in their study. These previous observations support the present study, where the importance of *DIO2* in heat production appears to be significant and it could have implications in the missing expression of *THRA* and *THRB* as found in this study. In summary, the possible absence of T3 production may have negatively affected lipolysis, as reported by both De Jesus et al. (2001) and Thrush et al. (2012). This observation could support this study, since a differential expression of the genes that encode lipases were missing in all tissues and treatments (*PNPLA2* (patatin-like phospholipase domain containing 2), *LIPE* (lipase E) and *MGLL* (monoglyceride lipase)). Given that these genes were not apparently active, a reduced availability of fatty acids might have occurred, therefore decreasing the possibility for heat production due to a lack of a suitable “fuel” for the *UCP1* machinery.

5.4.3 Transition from BAT to WAT

Within a matter of days after birth, the majority of BAT present in large mammals commences its transformation into WAT and shivering thermogenesis becomes the main response to cold exposure (Symonds et al., 1989; Symonds, 2013). This transition concludes with the loss of BAT and its thermogenic activity, where the β-adrenergic signalling has been found to be diminished (Finn et al., 1998; Shimizu et al., 2014). This latter observation may be in accordance with the present results and places the lambs at the beginning of this period, as there were no differences found in the expression of any of the ADRBs analysed (*ADRB1-3*) between treatments. Further, this lack of sensitivity to the adrenergic pathways seems to be interconnected with the lack of or missing expression of key thermogenic genes found here, such as *UCP1* and *DIO2*, and its transcription activators (PPARs) that were found to be not differentially expressed in the present study's lambs, or even downregulated as *PPARGC1A* in thyroid tissue in all lambs. Basse et al. (2015), utilising RNAseq in new-born lambs' perirenal adipose tissue, found not only the loss of *UCP1*, but also the loss of BAT-enriched factors such as *PPARGC1A*, *PPARG* and *DIO2*, which were reduced by day one and poorly expressed after day four. Moreover, another study (Lomax et al., 2007) observed that the sharp fall of *UCP1* on the first day of life was also accompanied by the decline of *PPARGC1A* and *PPARA*, even when the lambs were maintained below their thermoneutral zone. Lomax et al. (2007), showed that this cascade of lack or even loss of expression in these key BAT markers occurs soon after birth and marks the ontogenically programmed transformation of BAT to WAT.

Another probable leading factor in this transformation could be the overall underexpression of *VEGFA*. The downregulation of this gene could have imposed an opposite "BAT-like" phenotype in all lambs in this study, which impeded thermogenesis activation. Shimizu et al. (2014) observed that *VEGFA* knockout mice had lipid accumulation and mitochondrial dysfunction in adipose tissues, accompanied by an impairment of noradrenergic signalling, suggesting that the absence or decrease in this

gene is a primary factor that leads to BAT whitening. Additionally, Kotzbeck et al. (2018) stated that BAT whitening is not only induced by factors such as β-adrenergic signalling impairment, but to a lipase deficiency. Therefore, each of these factors, as the lack of differential expression of the ADRBs and lipases (*PNPLA2*, *LIPE*, *MGLL*) found in all lambs in the study between treatments, is capable of inducing macrophage infiltration and brown adipocyte death (Kotzbeck et al., 2018). In addition, *VEGFA* has anti-inflammatory properties in the adipose tissue (Elias et al., 2013); consequently, the reduction in its expression could be leaving BAT unprotected from the inflammation processes that takes place in its transformation to WAT. Consequently, this differentiation from brown to white adipocytes would be initiated, which could also be implied by the overexpression of *KI67* (monoclonal antibody KI67) found in all lamb tissues and treatments and the overexpression of *BMP4* (bone morphogenetic protein 4) found in both tissues under ambient conditions. It is known that these two genes are involved in WAT adipogenesis, where a rise in *BMP4* expression was previously observed with the loss of the BAT phenotype and the continuing differentiation of white adipocytes (Pope et al., 2014), implying that it might be marking the start of a change over to a WAT phenotype. Pope et al. (2014) also found an overexpression of *KI67* in the proliferative state of white adipocytes, where *UCP1* was not differentially expressed. The present results seem to be in agreement with the latter study, where the overexpression of key WAT genes mix in with the lack of differential expression of *UCP1* found here, resulting in the possible ending of BAT and the non-shivering thermogenesis period.

5.4.4 Downregulation of protein synthesis

Gene Ontology and pathway analysis showed that the majority of downregulated DEGs in the present tissues and treatments were enriched towards ribosome and protein synthesis and maturation terms. It is known that protein synthesis is energetically

expensive (Houlihan et al., 1995) and under stress conditions, a rapid attenuation or even a global shutdown of protein synthesis has been described (B. Liu et al., 2013). Since protein misfolding sets a major risk to health of cells and organisms, a protein quality control mechanism exists to maintain protein homeostasis (Frydman, 2001; Bukau et al., 2006; Hartl et al., 2011). Liu et al. (2013) observed that this quality control responds under adverse conditions with an inhibition of the translation initiation by halting ribosomes during early elongation. This pausing could represent a co-translational stress response in order to maintain intracellular protein homeostasis, while adapting to a change in environmental conditions (Hawkins, 1991; B. Liu et al., 2013). It might be expected to find this downregulation of protein synthesis in both tissues under cold conditions in the present study, as the lambs were subjected to cold and required energy expenditure (heat production) to thermoregulate. This halt in protein production was observed in both tissues under ambient conditions; however, it is possible that the lambs subjected to ambient conditions in the present study could also be experiencing some level of thermoregulatory stress and reconditioning after leaving the uterine environment. Therefore, it could be supposed that all lambs regardless of treatment might be experiencing decreasing protein production as a transient response to the differential environmental conditions post birth. It would be of interest in further studies to determine how long post-birth this activity occurs.

5.4.5 Highly expressed immune defence and cell cycle processes

Gene Ontology and pathway analysis also revealed that the majority of the upregulated DEGs in both tissue types and lamb treatments, were enriched towards immune defence response and cell cycle terms. These immune defence process findings are expected, as it is known that the days following birth are a very vulnerable period of life. The development of the immune system commences during the early stages of fetal life, but at birth, new-born lambs are immunologically naïve (Dwyer, 2008b), as immunoglobulins

are not passed through the ovine placenta (Hodgson et al., 1995; Gokce et al., 2014). Passive immunity is provided via colostrum intake, but as observed by Sanglid et al., (2000), the new-born's gut needs to be permeable to the passage of macromolecules, therefore leaving the lamb vulnerable to pathogen ingestion. In accordance with the multiple GO terms and pathways found, including those classified as being "defence response", "regulation of immune system process" and "response to virus", the lambs in the present study had their immune defence boosted above most other biological processes. This biological process likely helps prepare the lambs against possible viral infections and other harmful pathogens. In relation to the increase in cell cycle terms, this abundance in cell proliferation may come from the postnatal transformation of brown adipocytes to white adipocytes. Basse et al. (2015), who observed the transition from BAT to WAT in sheep, stated that it could be the case that the proliferation of white adipose precursor cells was in fact contributing to whitening. This statement potentially supports the idea that the rise in cell proliferation seen in the samples of this study might be linked to the whitening process, as this seems to be added as part of that cascade of events, which was previously discussed here.

5.5 Conclusions

This study provides an in-depth gene expression analysis of the main characters involved in the thermogenesis and whitening mechanisms that take place in the new-born lamb. The data from the present study add value to the understanding of the molecular processes that underlie these changes in first few days of life where currently little is known, and the interaction between these complex factors. This study shows that the heat production peak under cold exposure occurs in a very fast and immediate way, such that it may seem undetectable by day 3 of life. Moreover, these changes in expression might give way to the whitening of the adipose tissue, summing up the non-shivering thermogenesis period.

Chapter 6. Mass spectrometry-based lipidomics of brown adipose tissue and plasma of new-born lambs subjected to short-term cold exposure

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References and supplementary data of this chapter, as a published paper, have been moved to the sections "References" and "Appendices" sections, respectively, at the end of the thesis. Moreover, formatting has been changed from the published version to have a consistent style throughout the thesis. In addition, methodological information was included for this thesis version after the recommended PhD examiners' emendations.

Abstract

During cold exposure, brown adipose tissue (BAT) holds the key mechanism in the generation of heat, thus inducing thermogenic adaptation in response to cooler environmental changes. This process can lead to a major lipidome remodelling in BAT, where the increase in abundance of many lipid classes plays a significant role in the thermogenic mechanisms for heat production. This study aimed to identify different types of lipids, through liquid chromatography–mass spectrometry (LC-MS), in BAT and plasma during a short-term cold challenge (2-days), or not, in new-born lambs. Fifteen new-born Romney lambs were selected randomly and divided into three groups: Group 1 (n = 3) with BAT and plasma obtained within 24 h after birth, as a control; Group 2 (n = 6) kept indoors for two days at an ambient temperature (20–22 °C) and Group 3 (n = 6) kept indoors for two days at a cold temperature (4 °C). Significant differences in lipid composition of many lipid categories (such as glycerolipids, glycerophospholipids, sphingolipids and sterol lipids) were observed in BAT and plasma under cold conditions, compared with ambient conditions. Data obtained from the present study suggest that short-term cold exposure induces profound changes in BAT and plasma lipidome composition of new-born lambs, which may enhance lipid metabolism via BAT thermogenic activation and adipocyte survival during cold adaptation. Further analysis on the roles of these lipid changes, validation of potential biomarkers for BAT activity, such as LPC 18:1 and PC 35:6, should contribute to the improvement of new-born lamb survival. Collectively, these observations help broaden the knowledge on the variations of lipid composition during cold exposure.

6.1 Introduction

Brown adipose tissue (BAT) is the main orchestrator in mammals of thermogenic adaptation in response to a cold challenge (Leiria & Tseng, 2020). In such environments, BAT metabolism is considerably altered in order to maintain body temperature, boosting its thermogenic capacity by inducing non-shivering thermogenesis (Marcher et al., 2015; Simcox et al., 2017). There is higher BAT activity in winter (Saito et al., 2009; Yoneshiro et al., 2013) or after exposure to a cold environment (i.e., 4–5 °C) (Fuse et al., 2018). The key mechanism that leads to the generation of heat is the action of uncoupled mitochondrial respiration, through uncoupling protein 1 (UCP1) (Leiria & Tseng, 2020). This protein is able to disrupt adenosine triphosphate synthesis and allows protons to flow across the inner mitochondrial membrane to release energy as heat (Cannon & Nedergaard, 2004; Beck et al., 2007). Further, cold exposure leads to the stimulation of β-adrenergic receptors, which are normally found on the surface of brown adipocytes in BAT (Strosberg, 1997; Leaver & Pappone, 2002; Henry et al., 2017). This stimulus results in the activation of the lysis of triacylglycerols (TAGs) and the subsequent release of long-chain fatty acids (Forrest et al., 2007; Kurylowicz et al., 2015; Plush et al., 2016), which are the main substrate for UCP1 thermogenic function (Cannon & Nedergaard, 2004). The consequent distribution of heat produced through these processes appears to be facilitated by an increased vascularisation (Xue et al., 2009), to the already highly innervated and vascularised BAT (Marcher et al., 2015; Fuse et al., 2020). This increase in blood flow also ensures the continuous supply of metabolic substrates needed for thermogenesis (Cannon & Nedergaard, 2004). Hence, lipid metabolites that are produced or consumed by BAT could have an impact on the abundance or type of metabolites in plasma, and therefore could be used as predictors for BAT activity. A study by Boon et al. (2017) in humans linked an increase in blood serum of the glycerophospholipid lysophosphatidylcholine (LPC 16:0) to a high level of BAT activity when subjects were exposed to a short cold challenge. These authors suggested that

the lipid increase observed could be correlated with the thermogenic mechanisms of BAT, and could stimulate the actions of UCP1, making it a possible biological predictor. Furthermore, all of these changes during adaptive thermogenesis can result in lipidome remodelling in BAT, which can actively regulate the metabolic processes that take place under a cold challenge (Marcher et al., 2015; Leiria & Tseng, 2020). Evidence of lipidomic changes in thermogenic BAT have been previously recorded in humans, mice, goats and pigs (Marcher et al., 2015; Boon et al., 2017; Lu et al., 2017; Simcox et al., 2017; Lynes et al., 2018; Pan et al., 2019; Liu et al., 2022b). Hence, differences in lipidome composition of adipocytes may play a significant role in the rise of mitochondrial activity and thermogenic pathways, while enhancing energy communication with other tissues (S. Liu et al., 2013; Yore et al., 2014; Simcox et al., 2017; Leiria et al., 2019; Leiria & Tseng, 2020).

Previous studies (Fuse et al., 2020; Leiria & Tseng, 2020) have utilised liquid chromatography–mass spectrometry (LC-MS)-based lipidomic analysis as tool to quantify a broad range of lipid species, in order to improve the understanding of the physiological role of lipids that were previously unknown or had no correlated function. However, information regarding the lipidomic profile of thermogenic BAT and the function behind many of its lipid players remains scarce. No studies were identified that quantified the differences of lipid species of BAT in new-born lambs when exposed to cold vs. ambient temperature. Therefore, the aims of this study were to identify the impact of short-term cold exposure (2 days) on new-born lamb BAT and plasma lipid composition, and to search for potential biomarkers to predict BAT activity through mass spectrometry analysis.

6.2 Materials and methods

This study was undertaken in spring 2018 at the Massey University Animal Physiology Unit (APU) with new-born Romney type lambs born on Keeble Farm (40°24′ S, 175°36′ E), Palmerston North, New Zealand.

6.2.1 Animals and sampling

During the lambing period, which was outdoors under pastoral conditions, fifteen new-born Romney lambs (9 males and 6 females) were randomly procured. Lambs were born as twins, but only one lamb per dam was used (the heaviest of the set). Within 12–24 h after parturition, the selected lamb was tagged and the ewe together with both of its lambs were brought indoors. The selected lamb within each twin set was then weighed and allocated randomly to one of the three treatment groups: Group 1 (n = 3; two males weighing 4.8 kg and 5.2 kg, respectively, and a female weighing 4.4 kg) as control, Group 2 (n = 6; two females weighing 4.9 and 4.5 kg, respectively, and four males weighing 4.6, 6.0, 4.6 and 5.3 kg, respectively) that were kept indoors for two days at an ambient temperature (20–22 °C) and Group 3 (n = 6; two females weighing 5.5 and 6.1 kg, respectively, and four males weighing 5.4, 4.5, 5.4 and 5.2 kg, respectively) that were kept indoors for two days at a cold temperature (4 °C). This followed the previously described method of Marcher et al. (2015) to induce a cold challenge. Soon after being brought to the APU, all lambs in Group 1 were euthanised via captive bolt to provide a baseline lipidomic profile. Samples of brown adipose tissue (BAT) from around the kidneys were collected and stored at −80 °C. Lambs in Groups 2 and 3, together with their dams and siblings, were moved into indoor pens (2 m by 1 m) for two days. Blood samples (5 mL) from the jugular vein using a 22 G vacutainer needle from the lambs in Group 2 and 3 were collected into lithium heparinised tubes on days 0, 1 and 2. All blood tubes were centrifuged at 2000 × g at 4 °C for 15 min, whereafter the plasma was aliquoted into 2 mL tubes and stored at −80 °C. Siblings of the lambs in Group 3, that

were not subjects of the current study, were wrapped with wool covers (Woolover Limited, Christchurch, New Zealand) to enhance their comfort and to minimise the impact of the cold. During the two days of indoor retention, the ewes were fed commercial roughage (FiberEzy, Fiber Fresh Feeds Ltd., Reporoa, New Zealand) and commercial grain-based pellets (10%) (NRM Sheep Nuts, Northern Roller Mills, Christchurch, New Zealand) and had unrestricted access to water. The ewes and lambs were monitored at least 3 times per day during this period, to ensure successful ewe/lamb bonding and that the lambs were successfully suckling the ewe. On day 2, after 48 h exposure at respective temperatures, the 12 lambs of these two groups (i.e., 6 per group, in Groups 2 and 3) were weighed and euthanised by captive bolt. Samples of brown adipose tissue from around the kidneys were collected and stored at -80 °C. In all groups after the study lambs had been euthanised, their dams and remaining siblings were returned to Keeble Farm and to commercial farming conditions.

6.2.2 Sample preparation and randomisation

All BAT and plasma samples were submitted to the Metabolomics Platform at AgResearch (Lincoln Research Centre, Lincoln, New Zealand) for mass spectrometry analysis of lipids. Plasma samples were stored at −80 °C prior to analysis. A two-step randomisation batch was created using the random number function in Microsoft Excel, to account for the repeated measurements from each lamb, while minimising the potential effect of analytical instrument drift on the results (e.g., a sensitivity may be lower at the end of an analytical batch compared to the start). Therefore, in the first randomisation step the order of the lambs in the analytical batch was randomised, and within the second randomisation step the order of each repeated measurement (i.e., 0, 1, 2 days) within each individual lamb was randomised.

6.2.3 Extraction of lipids from brown adipose tissue

Brown adipose tissue was weighed and cut into 24 pieces while still frozen in a 4 x 6 matrix. Two grams of brown adipose tissue was placed into a centrifuge tube and placed at −80 °C for 1 h to refreeze the tissue. Forty mL of cold isopropanol (−20 °C) was added to the tube and the tissue was homogenised on ice using an Ultra Turrax homogeniser (T 25, IKA GmbH, Staufen, Germany). Ten microliters of the resulting brown adipose tissue slurry was added to a 2 mL microcentrifuge tube, along with 10 µL of an internal standard (Lipidomix Splash mix, Avanti Polar Lipids, Inc., Alabaster, AL, USA) and 1500 µL of butanol:methanol (1:1, v/v). The microcentrifuge tube was shaken in a bead shaker (Qiagen, Hilden, Germany) at 30 Hz for 5 min, and then sonicated for 1 h. The tube was then centrifuged at 16,000× g at room temperature for 10 min. The supernatant was then added to an amber chromatography vial for analysis.

6.2.4 Extraction of lipids from plasma

Plasma lipids were extracted using the method described by Huynh et al. (2019). Briefly, plasma samples were thawed at room temperature in the dark, mixed and 10 µL aliquoted into a 2 mL Eppendorf tube. Butanol/methanol with 5 mM ammonium formate (1:1, 95 µL) was added, along with 5 µL of an internal standard. Tubes were closed and vortexed in a multitube vortex mixer for 3 min at 2500 revolutions per minute at room temperature. The samples were then sonicated for 60 min and centrifuged at 16000 x g for 10 min at room temperature. The supernatant was transferred to amber chromatography vials prior analysis.

6.2.5 Lipidomic analysis by LC-MS

Detection of lipids in BAT and plasma was carried out using a Shimadzu LCMS 9030 LC-qTOF mass spectrometer. Separation was carried out through a Waters Acquity CSH C18 column (1.7 µm particle size, 2.1 × 100 mm ID) at 65 °C and eluted over a 17 min

gradient with a flow rate of 400 µL min−1. Mobile phase A was water:acetonitrile:isopropanol (50:30:20) with 20 mM ammonium formate, and mobile phase B was water:acetonitrile:isopropanol (1:9:90) with 20 mM ammonium formate. The gradient elution programme was as follows: 10–45% B (0–2.7 min), 45–53% B (2.7–2.8 min), 53–65% B (2.8–9 min), 65–89% B (9–9.1 min), 89–92% B (9.1–11 min), 92–100% B (11–11.1 min), held at 100% B (11.1–13.9 min), 100–10% (13.9–14 min), held at 10% B (14–17 min) (Su et al., 2019). The autosampler was held at 20 °C. Two microliters of plasma extract and 0.5 µL of BAT extract were injected onto the column. The mass spectrometer acquired data in “full scan” mode between m/z 50 and 1200, and in data independent acquisition MS/MS mode across the same range in 20 m/z windows to acquire fragmentation data on all lipids, with a collision energy ramp from 6 to 23 eV. Loop time for the MS method was 0.5 s.

6.2.6 Post-processing and analysis of lipidomic data

Data were converted into the mzML format and processed using MS DIAL (Tsugawa et al., 2015). BAT lipidomic data were normalised firstly by the weight of the tissue extracted. Afterwards, all data, BAT and plasma, were normalised based on the LOWESS algorithm in MS DIAL and exported for manual curation (checking for duplicate identifications, merging adducts with the same identification and data quality checking). Identification (lipid ontology, category, main and sub-class) was based on matching against the Lipidmaps database (Sud et al., 2007) in MS DIAL.

6.2.7 Statistical analysis

Statistical analyses were only undertaken for lipids with MS2 level identification and known ontology and lipid names. Boxplots were created from the raw logged features of lipid data for both BAT and plasma, which showed that a normal distribution was held between all samples and treatments (Figure S6.1). These boxplots were constructed in

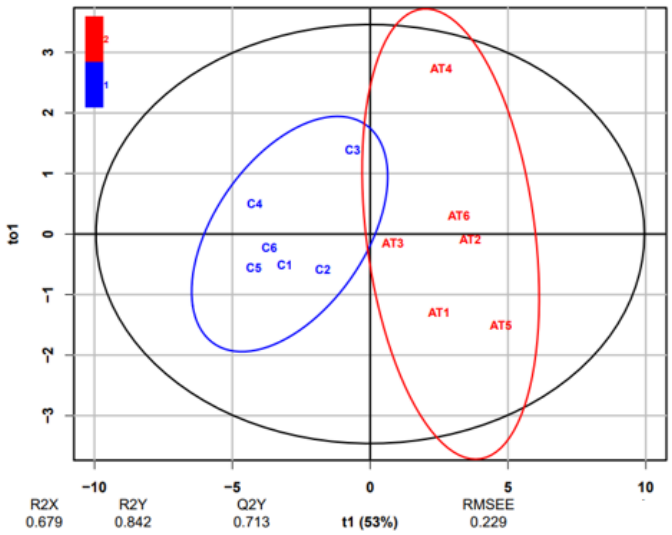
R (version 4.1.0) (R Core Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the “ggplot2” package (version 3.3.5) (Wickham, 2016). An average of the three BAT baseline samples from the control lambs (Group 1) was calculated. This average was used to correct all BAT lipidomics results from the lambs in Groups 2 and 3, by subtracting its value from each and all treatment values. Individual lamb plasma data recorded at day 1 and 2 (24 h and 48 h exposure, respectively) were baseline corrected with respective recordings of day 0. This baseline correction was carried out by subtracting the value recorded at day 0 to the correspondent values recorded at day 1 and day 2, for each sample accordingly. The basis behind this approach, instead of using the value of day 0 as a covariate, was because the statistical analysis was undertaken through the LIPID MAPS® online tool (www.lipidmaps.org/resources/tools/stats, accessed on 17 February 2022) (Fahy et al., 2007), which does not have a covariate option to use control BAT samples or plasma samples of day 0 to analyse the treatment samples. For that reason, the baseline corrected BAT and plasma lipidomic data were used for LIPID MAPS® analysis.

An analysis of variance (ANOVA) was utilised for the comparison between samples from ambient temperature lambs vs. those cold exposed (i.e., Group 2 vs. Group 3, respectively), with a P-value cutoff of 0.05. In addition, an orthogonal projection to latent structure discriminant analysis (OPLS-DA) was constructed via LIPID MAPS® to visualise the differences between cold vs. ambient temperature exposed lambs for: BAT, plasma day 1 and plasma day 2 samples. Plots of lipid class figures for BAT and plasma (days 1 and 2 of exposure) were constructed in R (version 4.1.0) (Team, 2013), inside RStudio (version 1.10.0) (RStudio-Team, 2021) with the “tidyverse” package (version 1.3.1) (Wickham et al., 2019), the “ggplot” package (version 3.3.5) (Wickham, 2016), the “ggnewscale” package (version 0.4.7) (Campitelli, 2022) and the “RColorBrewer” package (version 1.1.2) (Neuwirth & Neuwirth, 2014).

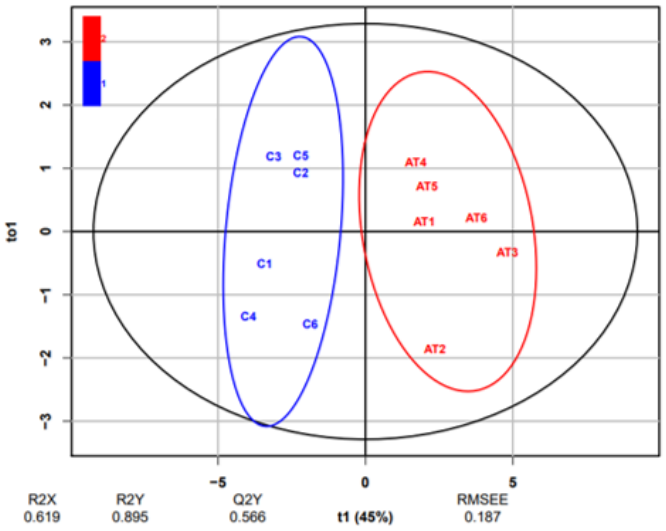
6.3 Results

A total of 1438 unique known lipids were found from LC-MS analysis within all BAT and plasma samples taken together from days 1 and 2 after exposure, with 101 being significantly different ($P < 0.05$) between samples collected from ambient temperature conditions (Group 2) vs. samples from cold conditions (Group 3) (Table S6.1 to S6.3). The 101 significantly different lipids were categorised as: glycerolipids (GLs), glycerophospholipids (GPs), sphingolipids (SPs) and sterol lipids (STs). These 101 significantly different lipids contributed the most to the separation observed in the OPLS-DA plot for BAT and plasma (days 1 and 2, after exposure), and between ambient temperature (Group 2) vs. cold conditions (Group 3) clusters (Figure 6.1).

(**A**)



(**B**)



(**C**)

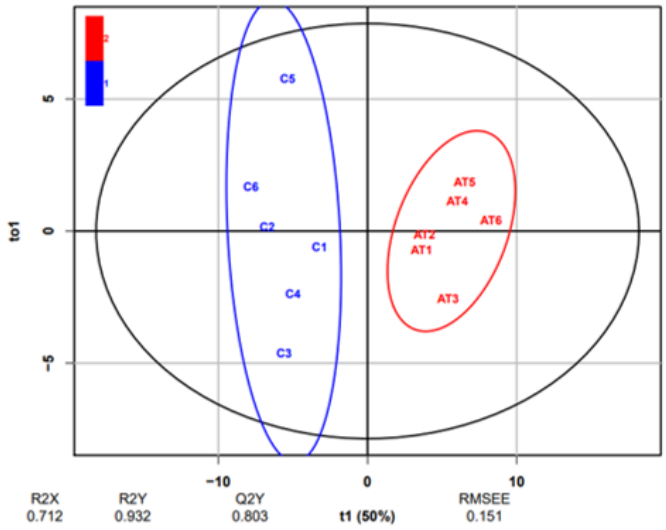


Figure 6.1. OPLS-DA score plot of (**A**) 20 different lipids (P < 0.05), between brown adipose tissue (BAT) samples from Group 2 (20–22 °C, AT in red) vs. Group 3 (4 °C, cold in blue), after 2 days of exposure; (**B**) 20 different lipids (P < 0.05), between plasma samples from Group 2 (20–22 °C, AT in red) vs. Group 3 (4 °C, cold in blue), after 1 day of exposure; (**C**) 74 different lipids (P < 0.05), between plasma samples from Group 2 (20–22 °C, AT in red) vs. Group 3 (4 °C, cold in blue), after 2 days of exposure. In each plot, the horizontal component (X-axis) of the OPLS-

DA score depicts the variation between the groups and the vertical component (Y-axis) depicts the variation within the groups. Class ellipses represent the 95% confidence regions for each group. R2X, variation of X-axis that is explained by the model; R2Y, variation of Y-axis that is explained by the model; Q2Y, goodness of model prediction; t01, first orthogonal component; t1, first principal component; RMSEE, root mean square error of estimation.

6.3.1 Brown adipose tissue (BAT)

A total of 291 unique known lipids were found from LC-MS analysis of BAT samples. The majority of lipids were glycerolipids (191 lipids, 67.52% of total), with the remainder being sphingolipids (56 lipids, 18.01% of total), glycerophospholipids (39 lipids, 12.86% of total), sterol lipids (2 lipids, 0.64% of total), prenol lipids (2 lipids, 0.64% of total) and fatty acyls (1 lipid, 0.32% of total) (Figure 6.2).

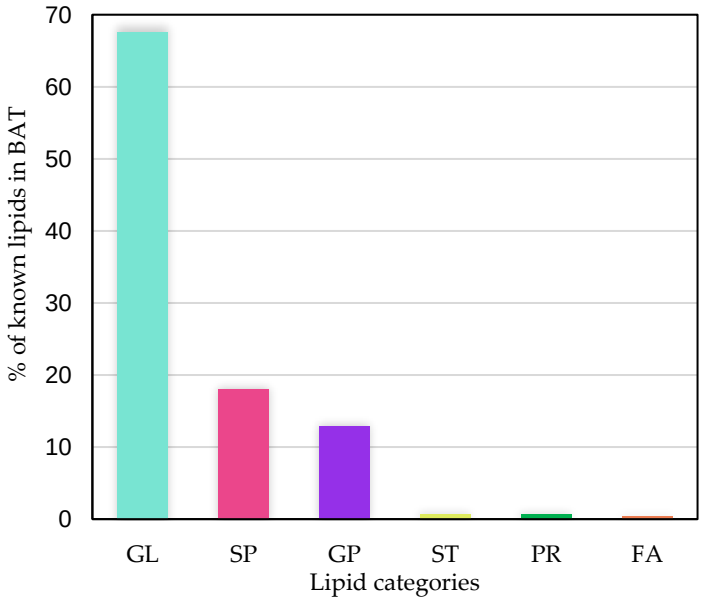


Figure 6.2. Percentages of the different categories of found known lipids in all lamb brown adipose tissue (BAT) samples. GL, glycerolipids; SP, sphingolipids; GP, glycerophospholipids; ST, sterol lipids; PR, prenol lipids; FA, fatty acyls.

Of the 291 unique known lipids detected in the BAT samples, 20 were significantly different ($P < 0.05$) between ambient temperature conditions (Group 2) vs. cold conditions (Group 3) (Figure 6.3). The majority of these 20 lipids comprised glycerophospholipids (13), followed by glycerolipids (6) and sphingolipids (1). Under cold conditions (Group 3), the sphingolipid Cer 50:9;4O|Cer 14:1;2O/36:8;2O and most of the glycerophospholipids in BAT were more abundant (in terms of ionic counts) compared with that in the ambient temperature conditions (Group 2). However, most glycerolipids and two glycerophospholipids (PC 36:4 and PC 38:5|PC 16:0_22:5) were scarcer under cold conditions, compared with the ambient temperature conditions.

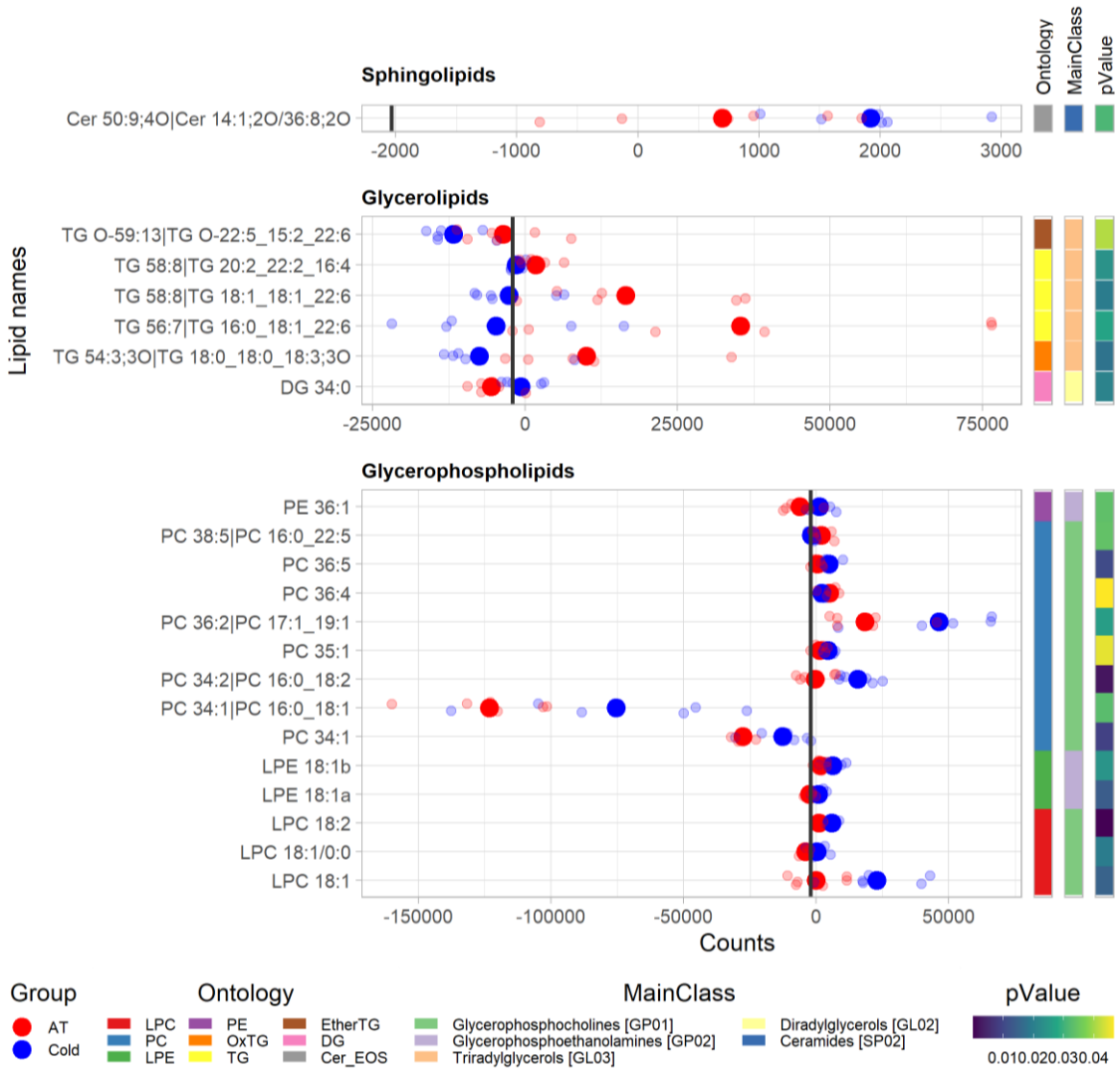


Figure 6.3. Plots of significantly different ($P < 0.05$) lipid classes within lipid categories in brown adipose tissue (BAT) samples from new-born lambs kept at ambient temperature (20–22 °C, AT in red) vs. those kept at cold temperature (4 °C, cold in blue) after 2 days of exposure. Each row of dots represents a lipid metabolite with respect to its ion counts obtained from the LC-MS analysis, where big dots represent the mean and smaller dots represent the individual values (n = 6 in each group). The vertical bold black line represents the average count value across all BAT datasets.

6.3.2 Plasma

A total of 1147 unique known lipids were found from LC-MS analysis of plasma samples. The majority of the known lipids were glycerolipids (498 lipids, 41.88% of total), with the

remainder being sphingolipids (331 lipids, 27.89% of total), glycerophospholipids (286 lipids, 27.50% of total), fatty acyls (20 lipids, 1.80% of total), sterol lipids (8 lipids, 0.63% of total) and prenol lipids (4 lipids, 0.31% of total) (Figure 6.4).

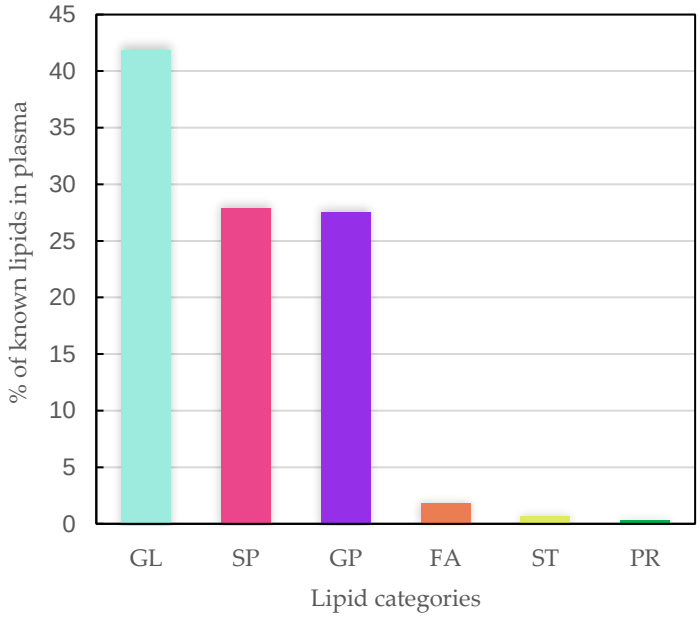


Figure 6.4. Percentages of the different categories of found known lipids in all lamb plasma samples. GL, glycerolipids; SP, sphingolipids; GP, glycerophospholipids; FA, fatty acyls; ST, sterol lipids; PR, prenol lipids.

Of the 1147 unique known lipids found in the plasma samples, 20 were significantly different ($P < 0.05$) between ambient temperature conditions (Group 2) vs. cold conditions (Group 3), after one day of exposure at the respective temperatures (Figure 6.5). Of these 20 lipids, most were glycerolipids (9), followed by sphingolipids (7) and glycerophospholipids (4). In the plasma of lambs under cold conditions (Group 3), after 1 day of exposure, all sphingolipids recorded and most glycerolipids and glycerophospholipids were increased when compared with the plasma samples from the lambs kept at ambient temperature (Group 2). However, two specific glycerolipids (TG 56:1|TG 20:0_20:0_16:1 and DGDG 30:2|DGDG 14:1_16:1) and one

glycerophospholipid (PC 34:1|PC 16:0_18:1) were decreased when compared with the plasma samples under ambient temperature conditions (Group 2).

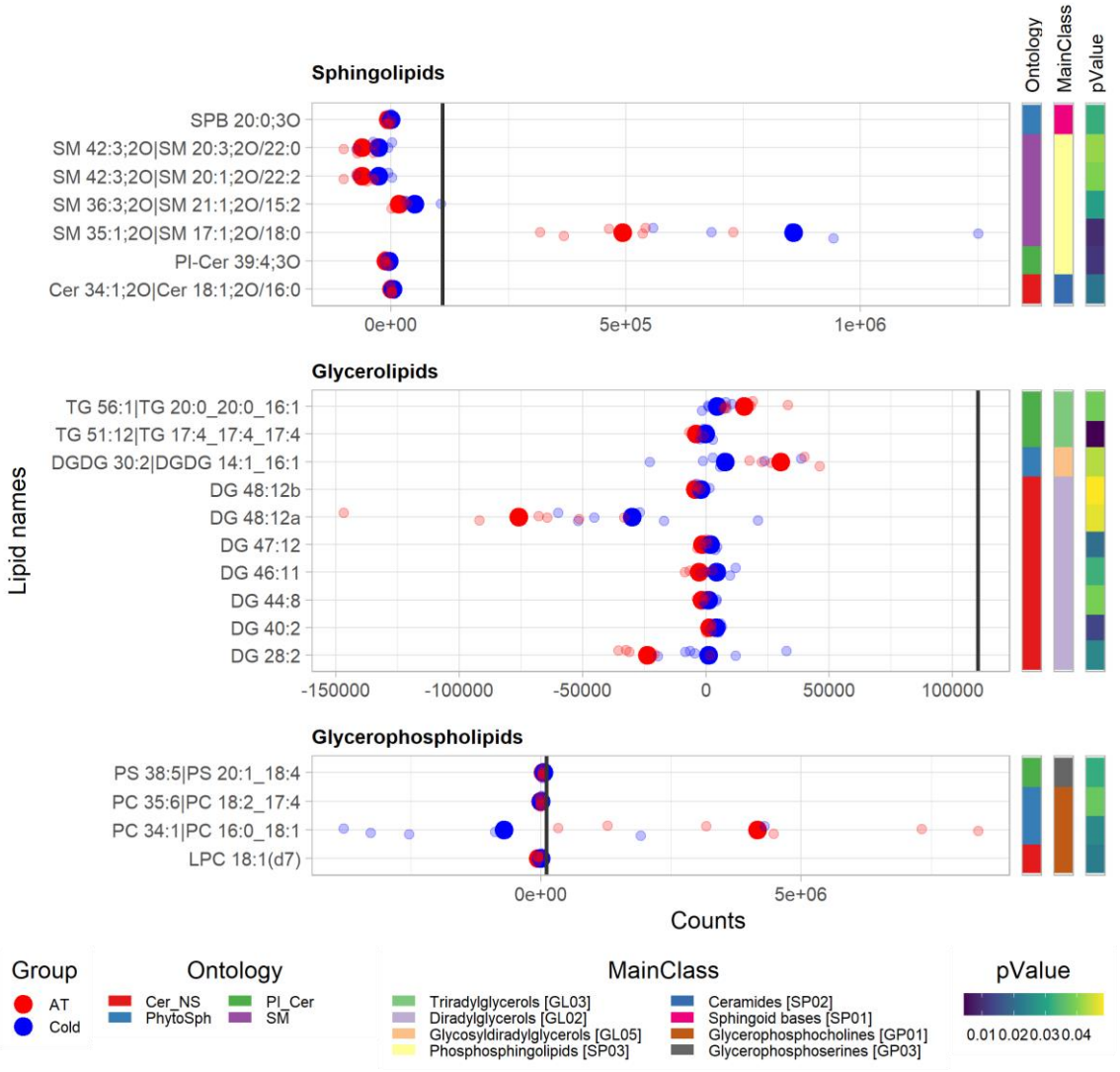


Figure 6.5. Plots of significantly different ($P < 0.05$) lipid classes within lipid categories in plasma samples from new-born lambs kept at ambient temperature (20–22 °C, AT in red) vs. those kept at cold temperature (4 °C, cold in blue), after 1 day of exposure. Each row of dots represents a lipid metabolite with respect to its ion counts obtained from LC-MS analysis, where the big dots represent the mean and smaller dots represent individual values (n = 6 in each group). The vertical bold black line represents the average count value across all plasma datasets, after 1 day of exposure.

Of the 1147 unique known lipids detected in plasma samples, 74 were significantly different ($P < 0.05$) between ambient temperature (Group 2) and cold (Group 3) conditions, after 2 days of exposure of lambs at the respective temperatures. The majority of the 74 lipids comprised sphingolipids (34 lipids), followed by glycerolipids (28 lipids), glycerophospholipids (10 lipids) and sterol lipids (2 lipids) (Figure 6.6). In plasma, under cold conditions, all sterol lipids and most sphingolipids, glycerolipids and glycerophospholipids were more abundant than in the plasma samples under ambient temperature conditions. However, four specific sphingolipids (HexCer 36:1;3O|HexCer 18:1;2O/18:0;O, HexCer 42:1;3O, AHexCer 44:2;3O and SM 32:2;2O|SM 14:1;2O/18:1), three glycerolipids (DG 45:12, DG 35:0 and DGDG 30:5|DGDG 15:2_15:3) and three glycerophospholipids (PC O-32:1, PE 36:2|PE 18:0_18:2 and PS 40:5|PS 18:0_22:5) were less prevalent, compared with those in plasma samples under ambient temperature conditions.

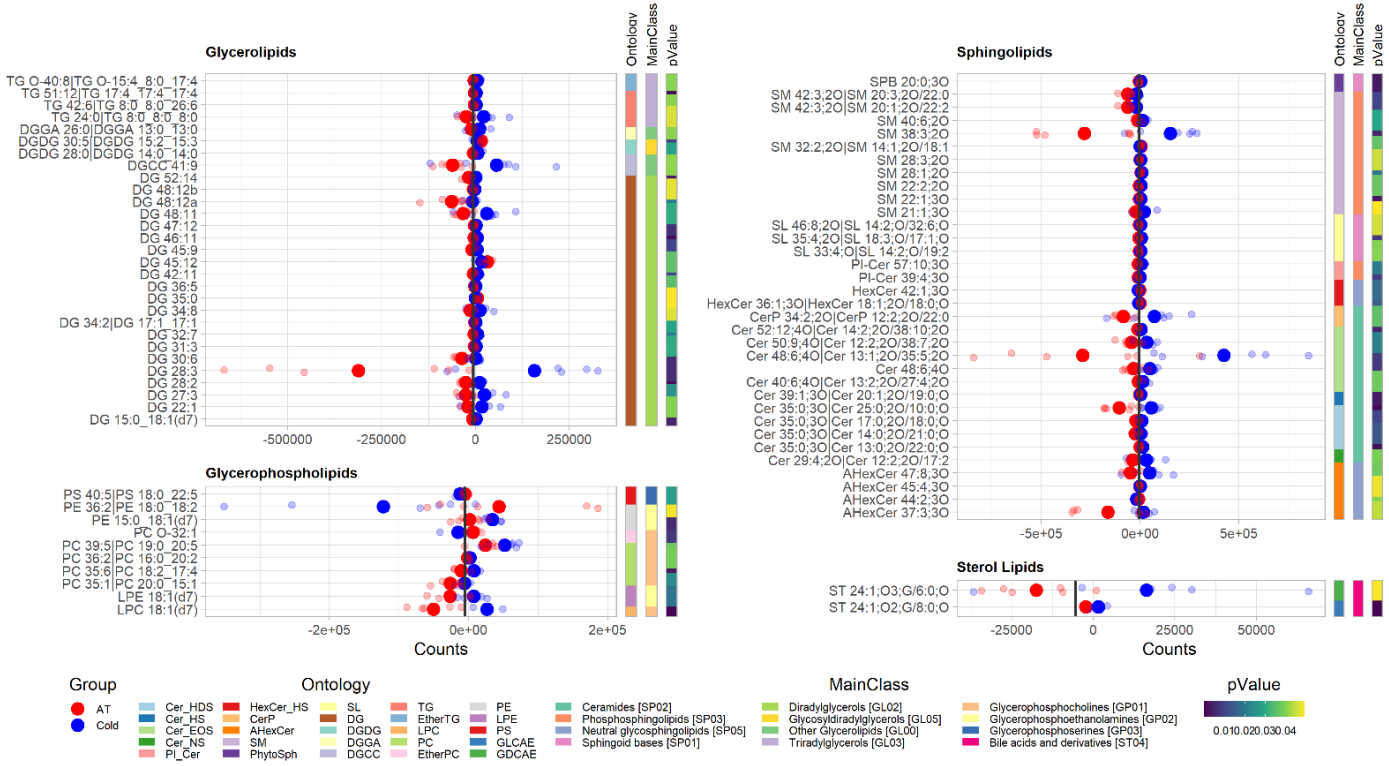


Figure 6.6. Plots of significantly different (P < 0.05) lipid classes within lipid categories in plasma samples from new-born lambs kept at ambient temperature (20–22 °C, AT in red) vs. those kept at cold temperature (4 °C, cold in blue), after 2 days of exposure. Each row of dots represents a lipid metabolite with respect to its ion counts obtained from LC-MS analysis, where big dots represent the mean and smaller dots represent the individual values (n = 6 in each group). The vertical bold black line represents the average count value across all plasma datasets, after 2 days of exposure.

6.4 Discussion

Exposure to cold leads to a significant cascade of events, where lipidome remodelling can influence and regulate adipocyte function towards thermogenesis (Lu et al., 2017). In the present study, lipid profiles of BAT and plasma were generated through LC-MS analysis, with the aim of determining the lipidomic changes that occur in new-born lambs exposed to cold conditions. The results, even with a relatively small number of lambs, demonstrated that this short cold challenge (2 days) induced significant changes in BAT and the plasma lipidome, by increasing the abundance of many of the lipid classes found.

Under the cold conditions of the present study, glycerolipids such as DGs were increased in plasma, and were more abundant over time, compared with the ambient temperature conditions. This increased abundance could be associated with lipid metabolism and TAG turnover (Grzybek et al., 2019), which seems to be connected with the decreased TAGs seen in BAT under cold conditions. These results are consistent with previous studies, where the most marked changes in the BAT lipidome under cold conditions were related to the hydrolysis of TGs into DGs (Bartelt et al., 2011; Lu et al., 2017; Xu et al., 2019). Therefore, this decline of TAGs in BAT could indicate that a hydrolytic action by lipases was occurring (Gibbons et al., 2000; Gilham & Lehner, 2004), resulting in the release of fatty acids. According to Bartelt et al. (2011), TAGs were decreased in activated BAT of mice under cold exposure, and TAG turnover was accelerated in plasma. This observation can be correlated to the abundance of diacylglycerols seen in plasma in the present study, which increased with time during the cold challenge. This lipolytic action of TAGs is triggered by the sympathetic stimulation of β-adrenergic receptors, induced by cold exposure (Haemmerle et al., 2006; Brasaemle & Wolins, 2012; Zechner et al., 2017), which is required in order to produce free fatty acids for the thermogenic activation of BAT (Schreiber et al., 2017; Shin et al., 2017). Moreover, mitochondrial activity becomes supported under thermogenic adaptation and the

released free fatty acids are used in BAT as the substrate for uncoupled oxidation by UCP1, thus generating heat (Cannon & Nedergaard, 2004; Leiria & Tseng, 2020).

The two types of sterol lipids detected, GLCAE and GDCAE, were observed to be greater in plasma after 2 days of cold exposure. These particular types of lipids correspond to a bile acid class. It is known that bile acids can regulate adipocyte physiology by taking part in lipolysis (Schmid et al., 2019), and in BAT they can induce thermogenesis (Broeders et al., 2015). Sterol lipids can also affect membrane fluidity and permeability (Ohvo-Rekilä et al., 2002), therefore the increase seen in these lipids could be boosting the lipolytic actions on TAGs as well as channeling the posterior free fatty acid uptake into BAT. In addition, all of the glycerolipids were significantly different in abundance between the cold vs. ambient environments contained very-long-chain fatty acyls. Previous studies with short-term cold challenges have associated an enrichment of very long acyl chains with TAGs (Marcher et al., 2015), glycerophospholipids and sphingolipids in the adipose tissue (Xu et al., 2019). The above-mentioned studies suggest that these lipid types could be functionally involved during thermogenic adaptation, as it is known that long-chain fatty acyls can activate UCP1 more efficiently (Fedorenko et al., 2012). Accordingly, not only did all glycerolipids, but all glycerophospholipids and sphingolipids, recorded and analysed here corresponded to very-long-chain fatty acyls.

Marked glycerophospholipid remodelling was observed during the 2-day exposure to cold, in BAT and plasma. The abundance of glycerophospholipids has been recorded in BAT (Lu et al., 2017; Grzybek et al., 2019), and reported to increase under cold exposure (Xu et al., 2019). Here, several lipid classes such as PC, LPC, PE and LPE were increased in the present study under cold conditions, compared with ambient temperature conditions. These observations are similar to the ones stated by Marcher et al. (2015), where short-term cold exposure in mice induced an increase in these lipid classes in BAT. This increase could be correlated with a response and adaptation to cold

exposure by having significant functional implications. Accordingly, Hoene et al. (2014) reported that there is a great abundance of phospholipids in heat-generating BAT that could have been correlated to a high density of mitochondria, since mitochondrial fission can increase its density within an hour when a big source of energy/heat is needed (Youle & Van Der Bliek, 2012). Additionally, phospholipid metabolism has been reported to be activated during both short and long cold exposure in mice (Marcher et al., 2015; Lynes et al., 2018), where it may have a supportive role for mitochondrial biogenesis. In addition, differences in the composition of acyl chains in the total mitochondrial phospholipids in BAT were previously observed, while adrenergic stimulation was occurring under the influence of long-term cold exposure (Ocloo et al., 2007). Therefore, considering these findings, the elevated content of glycerophospholipids seen in the current study could suggest that there is an increased mitochondrial content and function as a response to cold exposure, leading to an increase in the thermogenesis mechanisms.

As mentioned previously, all lipid categories that were significantly increased after cold exposure contained very-long-chain fatty acyls. Schweizer et al. (2019) reported that thermogenic adipocytes had higher contents of PC and PE constituted by unsaturated long chains of approximately 36 carbons. Similar observations of these lipid classes were seen in an adipose tissue comparison in mice, where a higher abundance of unsaturated long-chain PC and PE was found in BAT rather than in white adipose tissue (WAT) (Boon et al., 2017). It is known that glycerophospholipids are major components of cellular membranes, regulating fluidity, homeostasis and dynamics (Hishikawa et al., 2014), where PC and PE are key components that could determine the fate of adipocyte function and structure (Fuse et al., 2020). Consequently, this increase in unsaturated long-chain glycerophospholipids might have an influence on the membrane fluidity, particularly in mitochondria, thereby enhancing BAT uptake of the free fatty acids available through TAG lipolysis.

An indication of BAT activity that is correlated with cold-induced thermogenesis can be inferred by glycerophospholipid metabolites such as lysophophatidylcholines (Fuse et al., 2020). Previously, Boon et al. (2017) associated LPC 16:0 with BAT activity in humans that were exposed to cold, stating that the increase in this lipid could stimulate the actions of UCP1. In the current study, most LPCs and PCs increased in cold conditions both in BAT and plasma. Specifically, LPC 18:1 increased its mean value 11 times in plasma from 1 day compared with 2 days of exposure, similarly PC 35:6 increased twice in the same time span. These results suggest that this significant increase in both metabolites in plasma could be associated with a response to cold exposure seen through time, and therefore, generated as a consequence of BAT activity. Specifically, PC 35:6 and, more importantly, LPC 18:1, could serve as potential markers to predict BAT activity in a non-invasive way, since there are no methods known to the authors to either quantify the amount or the activity of BAT in lambs. Further studies would be needed to validate these biomarkers in plasma for BAT metabolic activity, which could be used to provide an indirect estimate of BAT activity and reserves in new-born lambs. If validated, they could contribute to further research and animal selection focused on improvement of new-born lamb survival under cold conditions.

In contrast to glycerophospholipids and glycerolipids, sphingolipids are not usually abundant in the body (Meikle & Summers, 2017). Nonetheless, a significant increase in many classes of sphingolipids was observed in BAT and plasma after cold exposure. These types of lipids are fundamental for cell proliferation (Tvrdik et al., 2000). They are mainly found in plasma membranes (Van Meer et al., 2008), where they are involved in cellular transport and signal transduction (Merrill 2011; Alexaki et al., 2017). Within this category, SMs are one of the biggest components of plasma membranes (Milhas et al., 2010), where they can be further metabolised into ceramides (Bartke & Hannun, 2009). Together with the actions of SM synthase, ceramides can utilise PC in order to increase the levels of SM and DGs (Milhas et al., 2010). It may be the case that these co-processes were occurring in the study animals, where not only SM, but several classes

of ceramides were seen jointly increased in the plasma of cold-exposed lambs. Moreover, as PC levels increased when exposed to cold, it may be that the SM synthase machinery was indeed active, inducing the increment of both SMs and DGs. As discussed above, DG levels were observed to be significantly increased in BAT and plasma after cold exposure in the present study. Therefore, it could be suggested that these observations had a correlation with this secondary result from sphingolipid biosynthesis, thus increasing DGs. Besides having a role as a major hub of sphingolipid metabolism (Leiria & Tseng, 2020), ceramides have an important physicochemical and structural function in cell membranes. According to Alexaki et al. (2017), sphingolipid metabolites and ceramides are bioactive and can change the cell activity by interaction between intracellular targets and cell-surface receptors. Sphingolipids and cholesterols compose up to 30% of the plasma membrane surface of adipocytes, which confers these cells with a detergent-resistant characteristic (Meshulam et al., 2011; Rutkowski et al., 2015). This ability to prevent detergent elements from compromising the cell structure and function seems to be essential. The main function of the adipocytes is to take up and release fatty acids, which are known to be mild detergents (Alexaki et al., 2017). In response to cold exposure, there will be an enhanced transport and uptake of fatty acids to BAT, as the basic substrate to thermogenesis. Therefore, it is imperative that the cell can manage this entire metabolic function. For that reason, Meshulam et al. (2011) implied that this detergent-resistant configuration of sphingolipids may protect the adipocyte membrane from deleterious effects when they are managing high concentrations of fatty acids, for transport or metabolism. Moreover, a previous study demonstrated that de novo sphingolipid biosynthesis is indispensable for adipocyte cell viability and normal metabolic function (Alexaki et al., 2017). A subsequent study on cold-induced inguinal WAT in mice (2019) linked cold exposure to the activation of de novo sphingolipid synthesis, as an increase in sphingomyelin and ceramides. These authors reasoned that this increase in sphingolipids was a consequence of the elevated availability of free fatty acids that were induced by cold exposure. In addition, free fatty

acids may trigger ceramide synthesis (Memon et al., 1998; Samad et al., 2006). Therefore, the present results can be paired up with these previous observations, where the increase in many sphingolipid classes such as ceramides and SMs observed in BAT could have had profound beneficial effects for the thermogenic adipocytes. This increase could provide a higher protection to the adipocyte's integrity, while still being able to utilise fatty acids as a means to produce heat when exposed to cold.

6.5 Conclusions

Collectively, these data demonstrate that in new-born lambs, short-term cold exposure (2 days) induces profound changes in BAT and the plasma lipidome. Significant increases in lipid composition of glycerolipids, glycerophospholipids, sphingolipids and sterol lipids seem to cooperate as one, in order to enhance lipid metabolism via BAT thermogenic activation and adipocyte survival during cold adaptation. Experiments which further analyse the roles of these lipid changes and validate potential biological markers for BAT activity, such as LPC 18:1 and PC 35:6, will provide additional insights into the mechanisms involved during cold adaptation and further contribute to the improvement of new-born lamb survival under cold conditions.

Chapter 7. General discussion

7.1 Introduction

New-born lamb mortality is a severe problem that causes both production and economic losses globally (Mellor & Stafford, 2004). In addition, these losses imply an animal welfare concern (Dwyer, 2008a), thus providing additional motivation to create efficient strategies to increase lamb survival. Direct selection for lamb survival is ineffective due to the low heritability of the trait (Lopez-Villalobos & Garrick, 1999; Safari et al., 2005; Everett-Hincks & Cullen, 2009), therefore, an alternative selection for a trait that is genetically correlated with lamb survival and has higher heritability is warranted. In this context, skin thickness, through its effect on cold tolerance, was genetically correlated with lamb survival (Soltani-Ghombavani et al., 2017). In addition, it is moderately heritable (Janssens & Vandepitte, 2004; Tait et al., 2015) and can be measured simultaneously with routine ultrasound-recorded production traits. Further, skin temperature, measured using infrared thermography, indicates heat loss into the environment and hence could be another potential indirect trait for lamb survival. There are just a few studies focussing on skin thickness variation in sheep (Tait et al., 2015; Soltani-Ghombavani et al., 2017), and to the author's knowledge, none of the past studies have looked at skin temperature variation in sheep. Hence, the first two chapters of this thesis were focussed on genetic variation (Chapter 3) regarding skin thickness, skin temperature and a few important production traits in FocusPrime, Texel, Romney and Highlander breeds, as well as genetic correlations (Chapter 4) among these traits.

Besides selecting for traits aimed at improving the lamb's insulation capability, the potential to produce heat is key for the new-born's survival. Through brown adipose tissue (BAT) activity, lambs can thermoregulate minutes after birth, so they can compensate for the increased heat loss at that moment, especially under cold conditions (Basse et al., 2015). This thermogenic capacity of BAT requires a multistep process, regulated by a complex network of genes and metabolic pathways. Moreover, this cascade of events triggered from cold exposure comes to an end quite rapidly over the

first few days of life (Bispham et al., 2002), as BAT transforms into white adipose tissue (WAT) as shivering becomes the main response to cold conditions (Symonds, 2013). Accordingly, investigating the factors that are involved in thermoregulation is essential, as well as the exploration of those factors involved in the fat-whitening transformation, as there appears to be minimal molecular information regarding this transition (Basse et al., 2015; Marcher et al., 2015). A ribonucleic acid sequencing (RNAseq) approach remains unexplored to further understand the underlying regulatory mechanisms controlling thermogenesis in new-born lambs. Hence, Chapter 5 of this thesis describes the transcriptome of BAT and thyroid gland in new-born Romney lambs exposed to either 20-22°C or 4°C for 2 days, using an RNAseq approach.

Beyond the complex network of factors that play a role in the differential expression of genes resulting in heat production, an investigation into the actual lipidome changes associated with an active BAT is fundamental. More so since information concerning the lipidomic profile of thermogenic BAT in lambs, and the function of its many lipid species is lacking. Differences in lipidome composition of adipocytes could imply a significant contribution to the increase of mitochondrial activity and further thermogenic pathways (Simcox et al., 2017; Leiria & Tseng, 2020). Moreover, lipid metabolites from BAT may influence the quantity or type of metabolites in plasma, therefore an increase of a certain lipid (or lipids) could be linked to a thermogenic mechanism of BAT, hence it could become a possible biological predictor for BAT activity. Lipidome remodelling in thermogenic BAT has been previously described in humans, mice, goats and pigs (Marcher et al., 2015; Boon et al., 2017; Pan et al., 2019; Liu et al., 2022a). However, to the author's knowledge, no research quantifying the differences in lipid species of BAT in new-born lambs exposed to cold vs. ambient temperature, nor suggesting biological markers for BAT activity has been undertaken. Therefore, a detailed mass spectrometry-based analysis of BAT and plasma lipids in new-born lambs exposed to either 20-22 °C

or 4 °C for 48 hours was undertaken as part of this doctoral project, the results of which were presented in Chapter 6.

The current chapter includes a short summary of the key findings of the four studies outlined above, as well as a brief discussion on their significance and potential application. Study limitations and scope for further research on these aspects are also elaborated on.

7.2 Summary of main findings

Chapter 3. Heritability estimates of skin thickness, skin temperature and production traits in FocusPrime, Texel, Romney and Highlander sheep

Skin thickness in lambs of five and 11 months of age showed a wide phenotypic variation and moderate heritability in all the four breeds investigated: FocusPrime, Texel, Romney and Highlander. Meanwhile, skin temperature at around 11 months of age, as an indicator of heat loss into the environment, was found to be lowly heritable overall besides being recorded as moderate to highly heritable only in the FocusPrime breed. Taken both skin traits into account, lambs that had thicker skin registered lower skin temperature. Thus, it would be suggested that thicker skinned lambs would have better insulation towards a cold challenge and hence, would lose less heat into the environment. However, further association studies are needed to support this notion. All things considered, skin thickness appears to be under genetic control in all the breeds studied, hence, it could be considered as an asset to the sheep industry as it could be utilized to improve lamb survival.

In brief, the heritability estimates for production traits in the four breeds studied here showed that fat depth and loin-eye muscle depth and width were mostly moderately heritable. Similarly, heritability estimates for body weight at weaning, six months and 12 months, were mostly moderate in all breeds. For these traits, higher heritability estimates

were found for the FocusPrime and Highlander breeds in particular. In addition, 12-month fleece weight was recorded as highly heritable in both breeds studied, Romney and Highlander.

Chapter 4: Genetic and phenotypic correlations of skin thickness and skin temperature with key production traits in FocusPrime, Texel, Romney and Highlander sheep

Based on the dataset for the traits studied in Chapter 3, further calculations were made with the aim to investigate the genetic and phenotypic correlations between them and how selection for skin thickness from measurements of five- and 11-month old lambs will potentially affect new-born lamb survival. Genetic and phenotypic correlations of skin thickness with skin temperature were moderately negative in all breeds, with particularly strong genetic correlations in the Texel and Romney breeds. This observation would suggest that selecting for a thicker skin should result in a decrease of heat escaping from the body surface and into the environment, which would potentially entail a positive effect on lamb survival. However, further studies are needed regarding this association in lambs at birth. Genetic and phenotypic correlations of skin thickness with fat depth and fleece weight were positive in all breeds, with a particularly strong genetic correlation with the fleece weight at 12 months in the Highlander. Additionally, genetic and phenotypic correlations of skin temperature with fat depth and fleece weight were negative in all breeds that had data available for calculations. A thicker skin associated with a greater fat deposition and a deeper coat would provide the new-born lambs with an increased tolerance to cooling and energy stored for additional thermogenic metabolism, when nutrition does not meet the metabolic demand. If the fat depth and fleece weight at birth were found to be genetically related to its measurements at older ages, then a positive survival response between the combination of greater skin thickness, fat depth and fleece weight would be expected in adverse weather conditions.

Further research is required to confirm this. Body weight traits had mainly a positive genetic and phenotypic association with skin thickness and a negative one with skin temperature across all breeds. A positive association between birth weight and skin thickness at birth has been established in previous studies (Jopson et al., 2000; Soltani-Ghombavani et al., 2021). Consequently, it would be possible to observe a greater body weight linked to a thicker skin in new-born lambs after selecting for skin thickness, providing additional body insulation to increase the likelihood of lamb survival in adverse weather conditions during lambing.

Overall genetic and phenotypic correlations of skin thickness at scanning age with fat depth, body weights and fleece weight traits were positive in all breeds. On the other hand, due to a disparity in between-breed results, a consensus was not reached regarding the genetic correlations of skin thickness with the muscle traits. Most genetic correlations between these two traits were positive, but negative values were found in the Highlander breed. However, genetic and phenotypic correlations of the muscle traits with skin temperature were generally negative across all breeds.

In brief, selecting for skin thickness would favourably affect other insulation traits, such as body weight, fat and wool, nevertheless, more research would be needed to corroborate how selection for skin thickness affects the muscle traits.

Chapter 5: RNAseq analysis of brown adipose tissue and thyroid of new-born lambs subjected to short-term cold exposure reveals signs of early whitening of adipose tissue

The transcriptome analysis of BAT and thyroid tissue after short-term cold exposure carried out in this chapter, showed no evidence of thermogenic activity at day three of life. There was no difference in the expression of the *UCP1* gene (uncoupling protein 1) or any of the ADRBs (β-adrenergic receptors) in all lambs under cold or ambient

temperature conditions. The absence of *UCP1* expression was followed by a decrease or absence of expression of several genes that are associated with BAT and thermoregulation, like *PPARGC1A* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), which has been considered to be a master regulator of BAT differentiation and inductor of *UCP1* (Puigserver et al., 1998; Liang & Ward, 2006).

Furthermore, other genes and pathways that are related to thermogenesis were observed to be downregulated after cold exposure in some cases. Besides, there was a lack of differential expression of the thyroid receptors, meaning that neither the hypothalamic route for BAT induction nor the expression of *UCP1* were stimulated by them, as opposed to its expected functions during a cold challenge (Bianco & McAninch, 2013). It could be the case that the overall downregulation of *VEGFA* (vascular endothelium growth factor) in every tissue/treatment may be promoting the contrary of a "BAT-like" phenotype, as opposed to its main functions as an enhancer of BAT thermogenic genes (Xue et al., 2009; Elias et al., 2013). Another probable cause of this scenario might be the overexpression of the *CYP1A1* gene (cytochrome P450 1A1), found in all tissues and treatments. This particular gene can suppress mitochondrial activity (Fleckenstein-Elsen et al., 2016), hence, it could be halting the heat production function of *UCP1*. Furthermore, the loss of BAT and its thermogenic activity, and the lack of β-adrenergic signalling could mean that the transition from BAT to WAT (white adipose tissue) has started, and that the non-shivering thermogenesis period has concluded. The results obtained in this chapter paint an overall picture of the molecular state of BAT and thyroid tissue of new-born lambs exposed to cold conditions, where each factor studied due to its involvement in thermogenesis is lacking its expression in favour to produce heat. It appears that by day three of age, the heat production peak under cold exposure has passed rapidly and seems undetectable, where consequently the expression of thermogenic genes gets replaced by other genes which would lead the transition of BAT to WAT.

Chapter 6: Mass spectrometry-based lipidomics of brown adipose tissue and plasma of new-born lambs subjected to short-term cold exposure

Lipidomic analysis revealed an increase of glycerolipids such as diacylglycerols (DGs) in plasma over the two-day cold exposure challenge. Sterol lipids from a bile acid class were also more abundant in plasma and a significant increase in many classes of sphingolipids was observed in BAT and plasma after exposure. Contrarily, triacylglycerols (TAGs) were decreased in BAT under cold conditions. This scenario may be a result from the known role of the type of sterols found in this chapter in lipolysis regulation (Schmid et al., 2019) by breaking down TGs into DGs, and their induction of BAT thermogenesis (Broeders et al., 2015), since the free fatty acids obtained from the breakdown would be utilized in BAT as the substrate for uncoupled oxidation by *UCP1*, thus generating heat (Leiria & Tseng, 2020). Moreover, the increase of sphingolipids after cold exposure could have provided the fat cells with higher integrity protection (Rutkowski et al., 2015), while still being able to utilise fatty acids to produce heat when exposed to cold conditions. Another major component of cellular membranes, the glycerophospholipids, were increased in BAT and plasma during day 2 of cold exposure. The elevated content of this type of lipid may have further influenced the membrane fluidity (Hishikawa et al., 2014), particularly in the mitochondria, thus improving BAT uptake of the free fatty acids available through TAG lipolysis. Interestingly, evidence of BAT activity associated with cold-induced thermogenesis can be deduced by glycerophospholipid metabolites such as lysophophatidylcholines (LPC) (Fuse et al., 2020). Results from this study showed a rapid escalation of LPC 18:1 (11 times) and PC (phosphatidylcholine) 35:6 (two times) in plasma, during the 24 to 48 h period of cold exposure. It is suggested that the rapid increase of these metabolites in plasma could be correlated with a response from the continuous cold exposure, and therefore, are produced due to BAT activity. Hence, the said metabolites could have the potential to be utilized as biomarkers to predict BAT activity in lambs. Collectively, the results described in this chapter suggest that short-term cold exposure causes profound changes in BAT

and plasma lipidome composition of new-born lambs, which could be reflective of lipid metabolism via BAT thermogenic activation and adipocyte survival during cold adaptation.

7.3 Significance and potential implications

As alternative strategies are required to improve lamb survival, selection for a trait that is cost-effective to measure and is correlated with survival might enable faster genetic progress and alleviate the high lamb mortality rates. Previous studies have indicated that a potential trait to indirectly improve lamb survival could be the skin thickness, as it was observed to be heritable in Romney lambs (Tait et al., 2015) and correlated with lamb survival up to weaning (Jopson et al., 2000; Soltani-Ghombavani et al., 2017). In Chapter 3, skin thickness has proven to be moderately heritable in other New Zealand breeds besides the Romney, such as FocusPrime, Texel and Highlander. Moreover, skin temperature was considered in this study as a novel trait for lamb survival improvement, as an indicator of heat loss into the environment. It was recorded as moderate to highly heritable in the FocusPrime breed, and lowly heritable in the Texel, Romney, and Highlander breeds. In the current study, as well as in the previous ones regarding skin thickness (Tait et al., 2015; Soltani-Ghombavani et al., 2021), the trait measurements were taken at an age where routine ultrasound measurements of production traits take place in New Zealand, between five to 12 months of age. A study by Soltani-Ghombavani et al. (2021) reported that scanning measurements of skin thickness at around eight months of age were a reliable indicator of the skin measurements at birth, and therefore, selecting for skin thickness from the measurements of older lambs should have a positive effect on the new-born's thermoregulation and survivability. This statement becomes crucial and indicates the benefit of adding skin thickness to routine ultrasound evaluated economic traits, such as fat and muscle traits. The slight additional cost and labour

required for skin thickness measurement could have a significant impact on heat insulation around birth and onwards for future generations.

Results in Chapter 4 revealed that the genetic correlations of skin thickness measured in lambs of around five and 11 months of age were negative with skin temperature and positive with fat depth, live weights at weaning, six months and at 12 months, and with fleece weight at 12 months in all breeds. Additionally, the genetic correlations between skin thickness and the muscle traits varied between breeds, but were mostly positive. In the case that a selection for skin thickness is made, it could have a positive impact in the new-born lamb survival, through improved insulation and thermoregulation, driven by these positive correlations with insulative traits and the decrease in heat loss to the environment.

Besides the potential positive effects on new-born lamb survival by skin thickness selection itself, impacts on the performance of older sheep could potentially be observed, as in the current study. It appears that breeding for a thicker-skinned lamb would also result in breeding for a fatter, heavier, and woollier lamb, thus, increasing overall body insulation. In the case of animals under pastoral outdoor conditions that live in cold environments, more layers of insulation would increase the chance of survival (Dwyer & Lawrence, 2005) and raise lamb numbers available for sale.

7.4 Limitations of the study

The dataset utilized for the estimation of genetic parameters described in Chapters 3 and 4 covered relatively a small number of animals, compared to other studies in the field. Even though a certain level of consistency was achieved when estimating correlations between traits, in some cases a disparity was found between breeds - for instance, the genetic correlations of skin thickness with loin-eye muscle depth and width. For most breeds, these correlations were positive, but negative values (and strong for

loin-eye muscle depth) were found in the Highlander breed. In other cases, some correlations could not be determined with the available dataset. This was mostly the case in the Romney breed, where it affected the calculation of the correlations of skin temperature with the muscle traits, 6-month live weight and 12-month fleece weight. Additionally, some associations were linked to large standard errors. Therefore, these inconsistent values of association, or lack thereof, did not allow for a clear interpretation of the results. It may be the case that a larger dataset would ease these errors and provide a better representation of the population. Nevertheless, sufficient evidence was gathered to advocate for the selection based on skin thickness and temperature to improve lamb survival, which was the primary goal of this research.

According to previous studies, lamb survival from birth to weaning is genetically correlated with skin thickness, presumably as a result of the effect of skin thickness on improved thermoregulation (Tait et al., 2015; Soltani-Ghombavani et al., 2017). Therefore, it was firstly planned to consider survival to weaning as a trait in this study, in order to estimate its correlation with the skin traits. Unfortunately, it was found that within the available dataset all the dead lambs were registered as “dummy dead lambs”. In those cases, for example, if a ewe pregnancy scan showed to be bearing twins, but no lambs were assigned to her at docking, two “dummy dead lambs” were registered (it was assumed the pregnancy scanner did assign the correct number of foetuses to the ewe), and a random sire would be assigned from the mate group she was mated in. As Focus Genetics relies on DNA parentage and does not allow for the collection of dead lambs during lambing, performing any heritability or correlation calculations with the available dead lambs’ data was deemed inaccurate, since there was no information on the death of the lamb, nor the exact sire linked to it. As survival or the cause of death is difficult to register in these types of flocks, if there is information on an excessive number of deaths, or the data might not have been entered correctly, or even if there was a weather event resulting in excessive lamb losses, information on said losses would be excluded,

preventing any calculations involving survival. This has been the case in many flocks throughout the years, mainly in the Highlander breed. At a closer look of the dataset, from 2017 onwards, all the lambs that have not survived until weaning in all the four breeds were tagged as “dummy dead lambs”. Prior to 2017, information from the breeders on actual records of dead lambs before weaning were only a handful, making any calculations regarding survival not feasible with this dataset.

To analyse the transcriptome of BAT and thyroid tissue in the new-born lambs in Chapter 5, only three control lambs could be procured for comparison between the two groups of lambs that experienced differential treatments, cold or ambient temperature exposure for two days. The controls were set up to help determine the effects of the treatment, therefore if there was a difference between the treatment and the control then a significance difference in the results of gene expression will be seen between the samples. During the preliminary analysis, heatmaps of raw counts for each treatment group; control, cold and ambient temperatures, for BAT and thyroid tissue were made to explore the data distribution and the clustering of the groups (Figure S5.4). These heatmaps were made in R, inside R studio (version 1.10.0) with the “pheatmap” package (version 1.0.12) and the “RColorBrewer” package (version 1.1-2). It’s evident from these figures that one control sample in both tissues to apparently have outlier counts overall, compared to all the other samples. It was concluded that it was best to discard this outlier control, to produce confident biological results. Therefore, the definitive analysis was performed with only two controls compared to either six cold exposed lambs or six ambient temperature exposed ones. This scenario is not ideal for analysis, and could have influenced the results of this chapter, since having fewer controls could increase the potential of unobserved biases that might exist or could lead into the false detection of a treatment effect. On the other hand, the expression results on the 38 selected genes were not significantly different if analysed as cold vs. ambient, cold vs. control, or ambient

vs. control. Hence, having only two controls did not seem to have interfered significantly with the results obtained in this chapter.

As previously detailed in the findings of Chapter 5, all lambs after cold exposure showed no difference in the expression of the key thermogenic characters, as *UCP1* or the ADRBs at day three of age. A study by Yuan et al. (2012) observed different levels of expression of *UCP1* according to the tissue planes, where lower levels of expression were found in superficial fat, as opposed to the higher levels observed in deep fat deposits. This said, it might be possible that the samples utilized for analysis in this chapter were coming from the surface of BAT, hence the expression of *UCP1* was not enough to be detected.

Another possible explanation could be that the cold challenge was inadequate to induce a response, however, the enrichment analysis from the lambs exposed to cold produced several upregulated biological systems regarding response to stress, regulation of response to external stimulus, and cell communication and signalling. In addition, BAT tissues of the animals utilized in this analysis were also processed for the lipids analysis in Chapter 6, where indications of thermogenic activity were observed. Therefore, these results suggested that the cold environment was challenging the animals, as it previously did in similar situations where it was cold enough to provoke a physiological response, such as in goats kept indoors at 6 °C for 24 h (Liu et al., 2022a) and in mice indoors at 4 °C for three days (Marcher et al., 2015). Nevertheless, previous reports by Basse et al. (2015) and Lomax et al. (2007), state that the expression of *UCP1* was not detected in lambs by day four or five of age, respectively. Therefore, it could be the case that the expression of *UCP1* observed in this chapter was already plummeting by day three of age, and this added to the fact that the tissue analysed may be superficial, then if a small expression was taking place at that moment it might have gone undetected.

A p-value analysis was utilized to explore the differences in lipid composition between treatment groups in Chapter 6, in BAT and plasma. This approach was utilized as a way to filter the data and focus on the lipids that were likely to best explain the differences. This analysis was chosen since lipid's features are a bit dependent between them. Some lipids could be represented by several ions, therefore, corrections such as Bonferroni or Benjamini–Hochberg can significantly overcorrect (Ni et al., 2023). For that reason, and since this chapter was more of a discovery-hypothesis generation experiment rather than looking for confirmation of certain lipids under cold exposure, using a false discovery rate was avoided as this test could be too stringent. Regardless, the univariate analysis done for each lipid between the treatment groups for BAT and plasma was supported by the results from multivariate analysis. These analysis through OPLS-DA plots showed in the chapter, seem to support the notion that there are in fact differences in the lipidome composition of BAT and plasma under cold conditions. Hence, these lipids do appear to be able to explain the differences, even though they were not adjusted to an FDR adjusted p-value, they would be important overall.

7.5 Future research

Through the course of both Chapters 3 and 4, selecting for skin thickness and temperature is considered as a tool to improve lamb survival under cold conditions. The ultimate goal would be to breed thicker skinned lambs with enhanced insulation and reduced heat loss into the environment. From the results in Chapter 4, skin thickness at around five and 11 months of age has a positive genetic correlation with fat depth, live weights and fleece weight. If the same correlations occurred at the new-born stage, then these lambs would be provided with increased insulation and thermoregulation, which would be crucial for survival. The possibility of this scenario is supported by Jopson et al. (2000) and Soltani-Ghombavani et al. (2021), where a positive correlation between birth weight and skin thickness at birth was observed. It could also be supported by Di

et al. (2011), where a positive correlation between pre-weaning staple length with its measurement with older lambs was found, and linked to fleece weight being correlated with staple length (Safari & Fogarty, 2003). Therefore, it could be possible that fleece weight measurement at an older age could be correlated to a pre-weaning one. These studies, provide the possibility of observing a larger body (birth size) and heavier fleece in the new-born when selecting for skin thickness, which should help with thermoregulation. However, direct studies which correlate post-weaning measurements of fat depth with its measurements at birth, or fleece weight in older lambs with fleece weight depth at birth is lacking. Further research is needed to connect these potential insulative traits from older lambs back to the new-born stage to accurately conclude that selecting for skin thickness will provide an increase in fat depth and fleece weight at birth.

Contrarily from the focus of this thesis, ruminants which live in hot climatic conditions can be affected by thermal stress, which can create a deterioration in their production indices (Silanikove, 2000). Under these conditions, the heat excess must be released through the skin into the environment (Bertipaglia et al., 2007). It is known that skin thickness variation could affect the sheep's heat adaptation (Castanheira et al., 2010), and that sheep with thinner skin appear to be better adapted to tropical climates (McManus et al., 2009). Future studies will be needed to investigate if selecting for a thicker skin to improve new-born survival under cold conditions will impact unfavourably on the animal's performance in hotter environments or during the summertime as they age. On the other hand, it would also be worthwhile to study if selection for a thinner skin will improve heat adaptation and further performance of sheep living under hot conditions. This latter research could be beneficial to improve ewe reproduction and longevity, since heat stress is known to impact on the reproduction cycle, impair the conception rate (Kipp et al., 2021) and have negative effects on offspring (Akbarinejad et al., 2017).

Besides selecting for skin thickness to improve lamb survival, it could be the case that an older sheep with thicker skin might be of interest to the leather industry. Therefore, studying the effect of hide quality when selecting for skin thickness might be worthwhile and provide further economic advantages for farmers, making the selection for this trait more attractive.

There was no indication of thermogenic activity in both BAT and thyroid tissues from the RNAseq analysis (Chapter 5). Previous research in lambs has detected the expression of the key thermogenic gene *UCP1* until day four or five of age (Lomax et al., 2007; Basse et al., 2015). Although it is not stated, it might be possible that the authors used bigger and/or deeper sections of the tissue for gene expression analysis, rather than superficial tissues as used in the present study. Therefore, future studies are needed to determine if having analysed only superficial tissues could have caused of the lack of expression of the thermogenic factors observed here. On the other hand, there is supporting evidence that the cold challenge might have been sufficient, from the lipidomic analysis results presented here, and from previous cold challenge studies (Marcher et al., 2015; Liu et al., 2022a). However, to achieve a stronger conclusion regarding the timing when the thermogenic factors reach their peak, it would be worthwhile to design a cold-challenge study where different lambs are slaughtered from day two, up until day four, by withstanding a degree or two lower than 4^0C. Most importantly, the tissues for analysis would need to be from deeper sections.

According to Young et al. (2010), long or highly expressed transcripts are more inclined to be identified as differentially expressed when compared to other lowly and/or short expressed transcripts. Therefore, it might be worthwhile to account for gene length bias when analysing differentially expressed genes. Unfortunately, no length bias was accounted for in this chapter (Chapter 5). It may be the case that if a method such as the GOseq (Young et al., 2010) was utilised to conduct Gene Ontology (GO) analysis, it

could have detected possible over-representation of some genes that were longer than others. In the case of Chapter 5, that could have meant the finding of differential expression of some thermogenic genes, that were possibly shadowed by longer non-thermogenic genes. Therefore, it would be recommended for future work that has the objective to further understand the molecular changes that occur during thermogenesis in the new-born lamb, to utilise the said or other methods that account for gene length bias.

Further, a more recent reference genome for sheep, ARS-UI_Ramb_v2.0 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_016772045.1/) has been created with improved continuity, fewer scaffolds, greater per-base accuracy and fewer insertions and deletions identified from mapped RNA sequence than previous assemblies (Davenport et al., 2022), when compared to the one - Oar_rambouillet_v1.0 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_002742125.1/) - utilised in Chapter 5. This new reference genome was created after the RNAseq analysis was performed in this chapter. Whilst this new version's genome metrics are better, the use of it as a reference genome for the reads from this chapter might not improve the results dramatically. By having around a hundred plus new genes, only slight changes could have occurred, and thermogenic activation would still be absent. Nevertheless, future RNAseq research in the sheep should utilise this new genome reference and check if the results change when utilising this one from a previous reference version.

Within Chapter 6, one of the goals was to identify lipids that could potentially be utilized as BAT activity markers. BAT is able to produce heat via non-shivering thermogenesis, where the resulting metabolites get circulated throughout the body (Fuse et al., 2020). The quantity and type of metabolites could depend on the degree of non-shivering thermogenesis within the BAT, and hence, could be used as indicators of BAT activity. Previous work in humans has correlated the abundance of LPC 16:0 after cold exposure

with BAT heat production mechanisms, since this lipid could be stimulating the actions of *UCP1* (Boon et al., 2017). In Chapter 6, LPC 18:1 was observed to increase its mean value 11 times in day 2 plasma, compared to the plasma on day one of cold exposure; the PC 35:6 level also rose two-fold during this time span. It is suggested that the increase observed in these metabolites could be a product of BAT metabolism as a response towards cold exposure over time. However, no methods were identified to either quantify the activity or the amount of BAT in lambs. Therefore, the validation of these metabolites would be needed in future studies, since they could serve as potential markers to predict BAT activity through a blood sample. If validated, such markers have multiple applications that would contribute to improved lamb survival by:

- Providing an indirect estimate of BAT reserves in new-born lambs, that is indicative of the ability of lambs to cope cold-stress.
- Identifying superior parents that are most likely to produce lambs with large BAT reserves.
- Assessing the effect of maternal nutrition during gestation on BAT reserves in lambs.

7.6 General conclusions

Skin thickness, and skin temperature as an indicator of heat loss to the environment, were heritable among the New Zealand sheep breeds assessed. This fact, taken together with previous studies that link skin thickness with lamb survival under cold conditions, makes this trait a potential tool to improve lamb survival through its insulative and thermoregulatory characteristics. The likely beneficial effect of skin thickness on lamb survival comes not only from just the insulation power that comes from having a thicker barrier between the body and the environment, but also from the positive correlations that this skin trait has with other insulative traits. Here, skin thickness has

been shown to have a positive genetic association with fat depth, body weight and fleece weight. Therefore, selection for skin thickness would enhance these traits and further improve body homeothermy, where a greater proportion of the heat produced would stay within the body with fewer losses into the environment. These outcomes would become key in outdoor pastoral systems under cold conditions, where these skin traits could become an asset to the sheep industry for lamb survival improvement.

Besides selecting for skin thickness to increase insulation, the ability to produce heat is crucial for survival. During the first few days of life, the new-born lamb produces heat through BAT via non-shivering thermogenesis, assisted by a complex network of factors and signals between tissues. From the molecular perspective, the heat production peak seems undetectable at day three of the lamb's life. No evidence of thermogenic activity from any of the key thermogenic factors was found, such as *UCP1* (uncoupling protein 1), β-adrenergic receptors, *PPARs* (peroxisome proliferator-activated receptors) or any of the thyroid receptors. These transcript-based results further imply that at this stage, the adipose tissue is starting to whiten, concluding the non-shivering thermogenesis period. On the other hand, from the lipidomic perspective, a significant increase in the prevalence of glycerolipids, glycerophospholipids, sphingolipids and sterol lipids were observed in BAT and plasma. In addition, a couple of specific lipids increased over time in plasma, such as LPC 18:1 and PC 35:6, identifying them as potential biomarkers for BAT activity. It seems that the potential expression of thermogenic genes that either occurred before day three of age or were occurring at that moment but were undetected in the study, was still having an effect on BAT lipid conformation. Overall, these lipid changes appeared to work as one, enhancing BAT thermogenic activation and adipocyte survival during cold adaptation.

The research conducted throughout this thesis presented significant findings that would provide a useful platform for future studies; from skin traits aiding selection for lamb

survival to further understanding the molecular and lipidomic changes under cold conditions.

References

Afolayan, R., Fogarty, N., Ingham, V., Gilmour, A. R., Gaunt, G., Cummins, L., & Pollard, T. (2007). Genetic Evaluation of Crossbred Lamb Production. 3. Growth and Carcass Performance of Second-Cross Lambs. *Australian Journal of Agricultural Research, 58*(5), 457-466.

Akbarinejad, V., Gharagozlou, F., & Vojgani, M. (2017). Temporal Effect of Maternal Heat Stress During Gestation on the Fertility and Anti-Müllerian Hormone Concentration of Offspring in Bovine. *Theriogenology, 99*, 69-78.

Al-Dalahmah, O., Sosunov, A. A., Shaik, A., Ofori, K., Liu, Y., Vonsattel, J. P., . . . Goldman, J. E. (2020). Single-Nucleus Rna-Seq Identifies Huntington Disease Astrocyte States. *Acta Neuropathol Commun, 8*(1), 19. doi:10.1186/s40478-020-0880-6

Alexaki, A., Clarke, B. A., Gavrilova, O., Ma, Y., Zhu, H., Ma, X., . . . Allende, M. L. (2017). De Novo Sphingolipid Biosynthesis Is Required for Adipocyte Survival and Metabolic Homeostasis. *Journal of Biological Chemistry, 292*(9), 3929-3939.

Alexander, G. (1961). Temperature Regulation in the New-Born Lamb. Iii. Effect of Environmental Temperature on Metabolic Rate Body Temperatures, and Respiratory Quotient. *Australian Journal of Agricultural Research, 12*(6), 1152-1174.

Alexander, G. (1962). Energy Metabolism in the Starved New-Born Lamb. *Australian Journal of Agricultural Research, 13*(1), 144-164.

Alexander, G. (1962a). Temperature Regulation in the New-Born Lamb. V. Summit Metabolism. *Australian Journal of Agricultural Research, 13*(1), 100-121.

Alexander, G. (1962b). Energy Metabolism in the Starved New-Born Lamb. *Australian Journal of Agricultural Research, 13*(1), 144-164.

Alexander, G. (1962c). Temperature Regulation in the New-Born Lamb. Iv. The Effect of Wind and Evaporation of Water from the Coat on Metabolic Rate and Body Temperature. *Australian Journal of Agricultural Research, 13*(1), 82-99.

Alexander, G. (1978). Quantitative Development of Adipose Tissue in Foetal Sheep. *Australian Journal of Biological Sciences, 31*(5), 489-504.

Alexander, G. (1979). Cold Thermogenesis. *International Review of Physiology. Environmental Physiology, 20*, 43-155.

Alexander, G. (1984). Constraints to Lamb Survival. In 'Reproduction in Sheep'. (Eds Dr Lindsay, Dt Pearce) Pp. 199–209. (Australian Academy of Science and the Australian Wool Corporation: Melbourne).

Alexander, G. (1988). *What Makes a Good Mother?: Components and Comparative Aspects of Maternal Behaviour in Ungulates.* Paper presented at the Proceedings of the Australian Society of Animal Production.

Alexander, G., & Bell, A. (1975). Maximum Thermogenic Response to Cold in Relation to the Proportion of Brown Adipose Tissue and Skeletal Muscle in the Body and to Other Parameters in Young Lambs. *Neonatology, 26*(3-4), 182-194.

Alexander, G., Bradley, L., & Stevens, D. (1993). Effect of Age and Parity on Maternal Behaviour in Single-Bearing Merino Ewes. *Australian Journal of Experimental Agriculture, 33*(6), 721-728.

Alexander, G., Lynch, J., Mottershead, B., & Donnelly, J. (1980). *Reduction in Lamb Mortality by Means of Grass Wind-Breaks: Results of a Five-Year Study.* Paper presented at the Proceedings of the Australian Society of Animal Production.

Alexander, G., & McCance, I. (1958). Temperature Regulation in the New-Born Lamb. I. Changes in Rectal Temperature within the First Six Hours of Life. *Australian Journal of Agricultural Research, 9*(3), 339-347.

Alexander, G., McCance, I., & Watson, R. (1955). Some Observations on Losses among Merino Lambs. Age at Death, Birth Weight and Duration of Gestation of the Lambs from One Flock. *Australian Veterinary Journal, 31*, 85-90.

Alexander, G., & Williams, D. (1966). Teat-Seeking Activity in New-Born Lambs: The Effects of Cold. *The Journal of Agricultural Science, 67*(2), 181-189.

Alexander, G., & Williams, D. (1968). Shivering and Non-Shivering Thermogenesis During Summit Metabolism in Young Lambs. *The Journal of Physiology, 198*(2), 251-276.

Alfageme, F. (2013). ¿ Cómo Utilizar Un Ecógrafo? Botonología. *Manual de ecografía cutánea, 1*.

Allain, D., Foulquié, D., Autran, P., François, D., & Bouix, J. (2014). Importance of Birthcoat for Lamb Survival and Growth in the Romane Sheep Breed Extensively Managed on Rangelands. *Journal of Animal Science, 92*(1), 54-63.

Amer, P., McEwan, J., Dodds, K., & Davis, G. (1999). Economic Values for Ewe Prolificacy and Lamb Survival in New Zealand Sheep. *Livestock Production Science, 58*(1), 75-90.

Arthur, G. (1967). Miscellaneous: Recent Advances in Bovine Obstetrics. *Obstetrical & Gynecological Survey, 22*(4), 624-628.

Arthur, G. H., Noakes, D. E., & Pearson, H. (1982). *Veterinary Reproduction and Obstetrics*: Ballière Tindall.

Aspinall, V., & Cappello, M. (2020). *Introduction to Animal and Veterinary Anatomy and Physiology*: CABI.

Aspres, N., Egerton, I. B., Lim, A. C., & Shumack, S. P. (2003). Imaging the Skin. *Australasian Journal of Dermatology, 44*(1), 19-27.

Atkins, K. (1980). The Comparative Productivity of Five Ewe Breeds. 1. Lamb Growth and Survival. *Australian Journal of Experimental Agriculture, 20*(104), 272-279.

Bailey, E. (2014). Heritability and the Equine Clinician. *Equine veterinary journal, 46*(1), 12-14.

Baker, R. C., Nikitina, Y., & Subauste, A. R. (2014). Analysis of Adipose Tissue Lipid Using Mass Spectrometry. In *Methods in Enzymology* (Vol. 538, pp. 89-105): Elsevier.

Bal, N. C., Maurya, S. K., Pani, S., Sethy, C., Banerjee, A., Das, S., . . . Kundu, C. N. (2017). Mild Cold Induced Thermogenesis: Are Bat and Skeletal Muscle Synergistic Partners? *Bioscience Reports, 37*(5).

Ballabriga, A. (1980). Immunity of the Infantile Gastrointestinal Tract and Implications in Modern Infant Feeding. *Pediatrics International, 24*(2), 235-239.

Barlow, R., Gardiner, A., Angus, K., Gilmour, J., Mellor, D., Cuthbertson, J., . . . Thompson, R. (1987). Clinical, Biochemical and Pathological Study of Perinatal Lambs in a Commercial Flock. *The Veterinary Record, 120*(15), 357-362.

Bartelt, A., Bruns, O. T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., . . . Waurisch, C. (2011). Brown Adipose Tissue Activity Controls Triglyceride Clearance. *Nature medicine, 17*(2), 200-205.

Bartke, N., & Hannun, Y. A. (2009). Bioactive Sphingolipids: Metabolism and Function. *Journal of lipid research, 50*, S91-S96.

Basse, A. L., Dixen, K., Yadav, R., Tygesen, M. P., Qvortrup, K., Kristiansen, K., . . . Hansen, J. B. (2015). Global Gene Expression Profiling of Brown to White Adipose Tissue Transformation in Sheep Reveals Novel Transcriptional Components Linked to Adipose Remodeling. *BMC Genomics, 16*, 215. doi:10.1186/s12864-015-1405-8

Beck, V., Jaburek, M., Demina, T., Rupprecht, A., Porter, R. K., Jezek, P., & Pohl, E. E. (2007). Polyunsaturated Fatty Acids Activate Human Uncoupling Proteins 1 and 2 in Planar Lipid Bilayers. *The FASEB Journal, 21*(4), 1137-1144.

Bedir, Ö., Gram, A., Dorsam, S. T., Grazul-Bilska, A. T., & Kowalewski, M. P. (2022). Plane of Nutrition and Fsh-Induced Superovulation Affect the Expression of Steroid Hormone Receptors and Growth Factors in Caruncular Tissue of Non-Pregnant Sheep. *Domestic animal endocrinology, 78*, 106683.

Beef + Lamb New Zealand. (2022). Mid-Season Update 2021–22 [Press release]. Retrieved from https://beeflambnz.com/sites/default/files/data/files/Mid-Season-Update-2021-22.pdf

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal statistical society: series B (Methodological), 57*(1), 289-300.

Berthon, D., Herpin, P., Duchamp, C., Dauncey, M., & Le, J. D. (1993). Modification of Thermogenic Capacity in Neonatal Pigs by Changes in Thyroid Status During Late Gestation. *Journal of developmental physiology, 19*(6), 253-261.

Bertipaglia, E. C. A., da Silva, R. G., Cardoso, V., & Fries, L. A. (2007). Hair Coat Characteristics and Sweating Rate of Braford Cows in Brazil. *Livestock Science, 112*(1-2), 99-108.

Bianco, & McAninch. (2013). The Role of Thyroid Hormone and Brown Adipose Tissue in Energy Homoeostasis. *The Lancet Diabetes & Endocrinology, 1*(3), 250-258. doi:10.1016/s2213-8587(13)70069-x

Bianco, & Silva. (1987). Nuclear 3, 5, 3′-Triiodothyronine (T3) in Brown Adipose Tissue: Receptor Occupancy and Sources of T3 as Determined by in Vivo Techniques. *Endocrinology, 120*(1), 55-62.

Bingham, C., & Hodge, A. (2022). Lamb Mortality and Clostridial Disease. *New Zealand Veterinary Journal, 70*(1), 49-54.

Bispham, J., Budge, H., Mostyn, A., Dandrea, J., Clarke, L., Keisler, D. H., . . . Stephenson, T. (2002). Ambient Temperature, Maternal Dexamethasone, and Postnatal Ontogeny of Leptin in the Neonatal Lamb. *Pediatric Research, 52*(1), 85-90.

Blighe, K., Rana, S., & Lewis, M. (2019). Enhancedvolcano: Publication-Ready Volcano Plots with Enhanced Colouring and Labeling. *R package version, 1*(0).

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics, 30*(15), 2114-2120.

Boon, M. R., Bakker, L. E., Prehn, C., Adamski, J., Vosselman, M. J., Jazet, I. M., . . . Rensen, P. C. (2017). Lysopc-Acyl C16: 0 Is Associated with Brown Adipose Tissue Activity in Men. *Metabolomics, 13*(5), 1-9.

Bordicchia, M., Liu, D., Amri, E.-Z., Ailhaud, G., Dessì-Fulgheri, P., Zhang, C., . . . Collins, S. (2012). Cardiac Natriuretic Peptides Act Via P38 Mapk to Induce the Brown Fat Thermogenic Program in Mouse and Human Adipocytes. *The Journal of clinical investigation, 122*(3), 1022-1036.

Boujenane, I., Chikhi, A., Lakcher, O., & Ibnelbachyr, M. (2013). Genetic and Environmental Factors Affecting Perinatal and Preweaning Survival of D'man Lambs. *Tropical Animal Health and Production, 45*(6), 1391-1397. doi:10.1007/s11250-013-0376-5

Bouzida, N., Bendada, A., & Maldague, X. P. (2009). Visualization of Body Thermoregulation by Infrared Imaging. *Journal of thermal Biology, 34*(3), 120-126.

Brakenhielm, E., & Cao, Y. (2008). Angiogenesis in Adipose Tissue. In *Adipose Tissue Protocols* (pp. 65-81): Springer.

Brasaemle, D. L., & Wolins, N. E. (2012). Packaging of Fat: An Evolving Model of Lipid Droplet Assembly and Expansion. *Journal of Biological Chemistry, 287*(4), 2273-2279.

Brash, L., Fogarty, N., & Gilmour, A. (1994). Genetic Parameters for Australian Maternal and Dual-Purpose Meatsheep Breeds. Iii. Liveweight, Fat Depth and Wool Production in Coopworth Sheep. *Australian Journal of Agricultural Research, 45*(2), 481-486.

Brash, L., Taylor, P., & Gilmour, A. (1997). *Estimates of Genetic Parameters and Environmental Effects for Production Traits in Young Merino Rams.* Paper presented at the Proceedings of the Association for the Advancement of Animal Breeding and Genetics.

Brien, F., Cloete, S., Fogarty, N., Greeff, J., Hebart, M., Hiendleder, S., . . . Kleemann, D. (2014). A Review of the Genetic and Epigenetic Factors Affecting Lamb Survival. *Animal Production Science, 54*(6), 667-693.

Brien, F. D., Hebart, M., Smith, D., Edwards, J. H., Greeff, J., Hart, K., . . . Behrendt, R. (2010). Opportunities for Genetic Improvement of Lamb Survival. *Animal Production Science, 50*(12), 1017-1025.

Brito, L. F., McEwan, J. C., Miller, S., Bain, W., Lee, M., Dodds, K., . . . Clarke, S. (2017). Genetic Parameters for Various Growth, Carcass and Meat Quality Traits in a New Zealand Sheep Population. *Small Ruminant Research, 154*, 81-91. doi:10.1016/j.smallrumres.2017.07.011

Broeders, E. P., Nascimento, E. B., Havekes, B., Brans, B., Roumans, K. H., Tailleux, A., . . . Deprez, B. (2015). The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity. *Cell metabolism, 22*(3), 418-426.

Brown, D. (2007). *Variance Components for Lambing Ease and Gestation Length in Sheep.* Paper presented at the Proceedings of the Association for the Advancement of Animal Breeding and Genetics.

Brown, D., & Swan, A. (2015). Genetic Parameters for Liveweight, Wool and Worm Resistance Traits in Multi-Breed Australian Meat Sheep. 2. Genetic Relationships between Traits. *Animal Production Science, 56*(9), 1449-1453.

Brown, D., Wolcott, M., & Crook, B. (2000). The Measurement of Skin Thickness in Merino Sheep Using Real Time Ultrasound. *Wool Technology and Sheep Breeding, 48*(4), 269-276.

Bukau, B., Weissman, J., & Horwich, A. (2006). Molecular Chaperones and Protein Quality Control. *Cell, 125*(3), 443-451.

Bunter, K., Brown, D., Gurman, P., Li, L., & Swan, A. (2023). The Genetic and Phenotypic Associations between Lamb Survival Outcomes and Other Traits Recorded at Lambing. *Animal Production Science*.

Burfening, P., & Carpio, M. (1993). Genetic and Environmental Factors Affecting Growth Rate and Survival of Junin Sheep in the Central Highlands of Peru. *Small Ruminant Research, 11*(3), 275-287.

Cabello, G. (1983). Endocrine Reactivity (T3, T4, Cortisol) During Cold Exposure in Preterm and Full-Term Lambs. *Neonatology, 44*(4), 224-233.

Cameron, N., & Bracken, J. (1992). Selection for Carcass Lean Content in a Terminal Sire Breed of Sheep. *Animal Science, 54*(3), 367-377. doi:10.1017/S0003356100020821

Campitelli, E. (2022). Ggnewscale: Multiple Fill and Colour Scales in 'Ggplot2'. *R package version 0.4.7*. doi:10.5281/zenodo.2543762

Cannon, B., & Nedergaard, J. (2004). Brown Adipose Tissue: Function and Physiological Significance. *Physiological reviews, 84*(1), 277-359.

Cassell, B. G. (2009). Using Heritability for Genetic Improvement. *Communications and Marketing, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University*.

Castanheira, M., Paiva, S. R., Louvandini, H., Landim, A., Fiorvanti, M. C. S., Dallago, B. S., . . . McManus, C. (2010). Use of Heat Tolerance Traits in Discriminating between Groups of Sheep in Central Brazil. *Tropical Animal Health and Production, 42*(8), 1821-1828.

Chen, Y., Ikeda, K., Yoneshiro, T., Scaramozza, A., Tajima, K., Wang, Q., . . . Brown, Z. (2019). Thermal Stress Induces Glycolytic Beige Fat Formation Via a Myogenic State. *Nature, 565*(7738), 180-185.

Chopra-Dewasthaly, R., Korb, M., Brunthaler, R., & Ertl, R. (2017). Comprehensive Rna-Seq Profiling to Evaluate the Sheep Mammary Gland Transcriptome in Response to

Experimental Mycoplasma Agalactiae Infection. *PLoS One, 12*(1), e0170015. doi:10.1371/journal.pone.0170015

Clarke, L., Buss, D., Juniper, D., Lomax, M., & Symonds, M. (1997). Adipose Tissue Development During Early Postnatal Life in Ewe-Reared Lambs. *Experimental Physiology, 82*(6), 1015-1027.

Cloete, J., Cloete, S., Hoffman, L., & Fourie, J. (2004). Slaughter Traits of Merino Sheep Divergently Selected for Multiple Rearing Ability.

Cloete, S., Misztal, I., & Olivier, J. (2009). Genetic Parameters and Trends for Lamb Survival and Birth Weight in a Merino Flock Divergently Selected for Multiple Rearing Ability. *Journal of Animal Science, 87*(7), 2196-2208.

Cloete, S., Schoeman, S., Coetzee, J., & Morris, J. d. V. (2001). Genetic Variances for Liveweight and Fleece Traits in Merino, Dohne Merino and South African Meat Merino Sheep. *Australian Journal of Experimental Agriculture, 41*(2), 145-153.

Collins, R., Eales, F., & Small, J. (1985). Observations on Watery Mouth in Newborn Lambs. *British Veterinary Journal, 141*(2), 135-140.

Conington, J., Bishop, S., Waterhouse, A., & Simm, G. (2004). A Bioeconomic Approach to Derive Economic Values for Pasture-Based Sheep Genetic Improvement Programs. *Journal of Animal Science, 82*(5), 1290-1304.

Contreras, C., Gonzalez, F., Ferno, J., Dieguez, C., Rahmouni, K., Nogueiras, R., & Lopez, M. (2015). The Brain and Brown Fat. *Ann Med, 47*(2), 150-168. doi:10.3109/07853890.2014.919727

Conway, J. R., Lex, A., & Gehlenborg, N. (2017). Upsetr: An R Package for the Visualization of Intersecting Sets and Their Properties. *Bioinformatics, 33*, 2938–2940.

Costa-Silva, J., Domingues, D., & Lopes, F. M. (2017). Rna-Seq Differential Expression Analysis: An Extended Review and a Software Tool. *PLoS One, 12*(12), e0190152.

Daikoku, T., Shinohara, Y., Shima, A., Yamazaki, N., & Terada, H. (2000). Specific Elevation of Transcript Levels of Particular Protein Subtypes Induced in Brown Adipose Tissue by Cold Exposure. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1457*(3), 263-272.

Dalton, D., Bigham, M., & Sumner, R. (1976). A Comparison between the Romney and Merino× Romney on Hill Country. *New Zealand journal of experimental agriculture, 4*(1), 27-33.

Dalton, D. C., Knight, T. W., & Johnson, D. L. (1980). Lamb Survival in Sheep Breeds on New Zealand Hill Country. *New Zealand Journal of Agricultural Research, 23*(2), 167-173. doi:10.1080/00288233.1980.10430783

Davenport, K. M., Bickhart, D. M., Worley, K., Murali, S. C., Salavati, M., Clark, E. L., . . . Murdoch, B. M. (2022). An Improved Ovine Reference Genome Assembly to Facilitate in-Depth Functional Annotation of the Sheep Genome. *Gigascience, 11*, giab096.

Dawes, G., & Mott, J. C. (1959). The Increase in Oxygen Consumption in the Lamb after Birth. *The Journal of Physiology, 146*(2), 295.

de Brun, V., Loor, J. J., Naya, H., Graña-Baumgartner, A., Vailati-Riboni, M., Bulgari, O., . . . Meikle, A. (2020). The Presence of an Embryo Affects Day 14 Uterine Transcriptome Depending on the Nutritional Status in Sheep. B. Immune System and Uterine Remodeling. *Theriogenology, 161*, 210-218.

De Jesus, L. A., Carvalho, S. D., Ribeiro, M. O., Schneider, M., Kim, S.-W., Harney, J. W., . . . Bianco, A. C. (2001). The Type 2 Iodothyronine Deiodinase Is Essential for Adaptive Thermogenesis in Brown Adipose Tissue. *The Journal of clinical investigation, 108*(9), 1379-1385.

Delezie, J., Gill, J. F., Santos, G., Karrer-Cardel, B., & Handschin, C. (2020). Pgc-1β-Expressing Pomc Neurons Mediate the Effect of Leptin on Thermoregulation in the Mouse. *Scientific reports, 10*(1), 1-12.

Dellman, H. (1993). Textbook of Veterinary Histology. Fouith Edition. *Lea and Febiger. Pennsylvania. USA*.

Deschamps-Francoeur, G., Simoneau, J., & Scott, M. S. (2020). Handling Multi-Mapped Reads in Rna-Seq. *Computational and Structural Biotechnology Journal, 18*, 1569-1576.

Di, J., Zhang, Y., Tian, K.-C., Liu, J.-F., Xu, X.-M., Zhang, Y.-J., & Zhang, T.-H. (2011). Estimation of (Co) Variance Components and Genetic Parameters for Growth and Wool Traits of Chinese Superfine Merino Sheep with the Use of a Multi-Trait Animal Model. *Livestock Science, 138*(1-3), 278-288. doi:10.1016/j.livsci.2011.01.005

Dikmen, S., Cole, J., Null, D., & Hansen, P. (2012). Heritability of Rectal Temperature and Genetic Correlations with Production and Reproduction Traits in Dairy Cattle. *Journal of dairy science, 95*(6), 3401-3405. doi:10.3168/jds.2011-4306

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., . . . Gingeras, T. R. (2013). Star: Ultrafast Universal Rna-Seq Aligner. *Bioinformatics, 29*(1), 15-21.

Dong, H., Dalton, T. P., Miller, M. L., Chen, Y., Uno, S., Shi, Z., . . . Nebert, D. W. (2009). Knock-in Mouse Lines Expressing Either Mitochondrial or Microsomal Cyp1a1: Differing Responses to Dietary Benzo[a]Pyrene as Proof of Principle. *Mol Pharmacol, 75*(3), 555-567. doi:10.1124/mol.108.051888

Duguma, G., Schoeman, S., Cloete, S., & Jordaan, G. (2002). Genetic Parameter Estimates of Early Growth Traits in the Tygerhoek Merino Flock. *South African Journal of Animal Science, 32*(2), 66-75.

Dutra, F., Quintans, G., & Banchero, G. (2007). Lesions in the Central Nervous System Associated with Perinatal Lamb Mortality. *Aust Vet J, 85*(10), 405-413. doi:10.1111/j.1751-0813.2007.00205.x

Dwyer, C. (2008a). Genetic and Physiological Determinants of Maternal Behavior and Lamb Survival: Implications for Low-Input Sheep Management. *Journal of Animal Science, 86*(suppl_14), E246-E258.

Dwyer, C. (2008b). The Welfare of the Neonatal Lamb. *Small Ruminant Research, 76*(1-2), 31-41.

Dwyer, C., & Morgan, C. (2006). Maintenance of Body Temperature in the Neonatal Lamb: Effects of Breed, Birth Weight, and Litter Size. *Journal of Animal Science, 84*(5), 1093-1101.

Dwyer, C. M., Conington, J., Corbiere, F., Holmoy, I. H., Muri, K., Nowak, R., . . . Gautier, J. M. (2016). Invited Review: Improving Neonatal Survival in Small Ruminants: Science into Practice. *Animal, 10*(3), 449-459. doi:10.1017/S1751731115001974

Dwyer, C. M., & Lawrence, A. B. (2005). A Review of the Behavioural and Physiological Adaptations of Hill and Lowland Breeds of Sheep That Favour Lamb Survival. *Applied Animal Behaviour Science, 92*(3), 235-260.

Dwyer, C. M., Lawrence, A. B., Bishop, S. C., & Lewis, M. (2003). Ewe–Lamb Bonding Behaviours at Birth Are Affected by Maternal Undernutrition in Pregnancy. *British Journal of nutrition, 89*(1), 123-136.

Eales, F., Gilmour, J., Barlow, R., & Small, J. (1982). Causes of Hypothermia in 89 Lambs. *The Veterinary Record, 110*(6), 118-120.

Eales, F., & Small, J. (1980). Summit Metabolism in Newborn Lambs. *Research in Veterinary Science, 29*(2), 211-218.

Eales, F., Small, J., & Gilmour, J. (1983). Neonatal Mortality of Lambs and Its Causes. *Proceedings-Easter School in Agricultural Science, University of Nottingham*.

Eales, F. A., & Small, J. (1986). *Practical Lambing. A Guide to Veterinary Care at Lambing*: Longman Group.

Earle, E., Boland, T., McHugh, N., & Creighton, P. (2017). Measures of Lamb Production Efficiency in a Temperate Grass-Based System Differing in Ewe Prolificacy Potential and Stocking Rate. *Journal of Animal Science, 95*(8), 3504-3512.

Elias, I., Franckhauser, S., & Bosch, F. (2013). New Insights into Adipose Tissue Vegf-a Actions in the Control of Obesity and Insulin Resistance. *Adipocyte, 2*(2), 109-112. doi:10.4161/adip.22880

Elliott, J., Sneddon, J., Lee, J. A., & Blache, D. (2011). Producers Have a Positive Attitude toward Improving Lamb Survival Rates but May Be Influenced by Enterprise Factors and Perceptions of Control. *Livestock Science, 140*(1-3), 103-110.

Ennen, S., Scholz, M., Voigt, K., Failing, K., & Wehrend, A. (2013). Puerperal Development of Ewes Following Dystocia: A Retrospective Analysis of Two Approaches to Caesarean Section. *Veterinary record, 172*(21), 554-554.

Everett-Hincks, J., Blair, H., Stafford, K., Lopez-Villalobos, N., Kenyon, P., & Morris, S. (2005a). The Effect of Pasture Allowance Fed to Twin-and Triplet-Bearing Ewes in Late Pregnancy on Ewe and Lamb Behaviour and Performance to Weaning. *Livestock Production Science, 97*(2-3), 253-266.

Everett-Hincks, J., & Cullen, N. (2009). Genetic Parameters for Ewe Rearing Performance. *Journal of Animal Science, 87*(9), 2753-2758.

Everett-Hincks, J., & Dodds, K. (2008). Management of Maternal-Offspring Behavior to Improve Lamb Survival in Easy Care Sheep Systems. *Journal of Animal Science, 86*(suppl_14), E259-E270.

Everett-Hincks, J., Dodds, K., & Wilson, T. (2007). *Lamb Survival: Agresearch Programme Update.* Paper presented at the Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association, Annual Seminar 2007.

Everett-Hincks, J., Dodds, K., & Wilson, T. (2007b). *Lamb Survival: Agresearch Programme Update.* Paper presented at the Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association, Annual Seminar 2007.

Everett-Hincks, J., Dodds, K. G., & Kerslake, J. I. (2007a). *Parturition Duration and Birthing Difficulty in Twin and Triplet Lambs.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Everett-Hincks, J., Lopez-Villalobos, N., Blair, H., & Stafford, K. (2005). The Effect of Ewe Maternal Behaviour Score on Lamb and Litter Survival. *Livestock Production Science, 93*(1), 51-61. doi:10.1016/j.livprodsci.2004.11.006

Everett-Hincks, J., Lopez-Villalobos, N., Blair, H., & Stafford, K. (2005b). The Effect of Ewe Maternal Behaviour Score on Lamb and Litter Survival. *Livestock Production Science, 93*(1), 51-61.

Everett-Hincks, J., Mathias-Davis, H., Greer, G., Auvray, B., & Dodds, K. (2014). Genetic Parameters for Lamb Birth Weight, Survival and Death Risk Traits. *Journal of Animal Science, 92*(7), 2885-2895.

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). Multiqc: Summarize Analysis Results for Multiple Tools and Samples in a Single Report. *Bioinformatics, 32*(19), 3047-3048.

Fahy, E., Sud, M., Cotter, D., & Subramaniam, S. (2007). Lipid Maps Online Tools for Lipid Research. *Nucleic acids research, 35*(suppl_2), W606-W612.

Falconer, D. S., & Mackay, T. F. C. (1996). *Introduction to Quantitative Genetics* (4th Edition ed.): Pearson Education Limited, United Kingdom.

Fedorenko, A., Lishko, P. V., & Kirichok, Y. (2012). Mechanism of Fatty-Acid-Dependent Ucp1 Uncoupling in Brown Fat Mitochondria. *Cell, 151*(2), 400-413. doi:10.1016/j.cell.2012.09.010

Ferguson, D., Schreurs, N., Kenyon, P. R., & Jacob, R. (2014). Balancing Consumer and Societal Requirements for Sheep Meat Production: An Australasian Perspective. *Meat Science, 98*(3), 477-483.

Ferreira, V., Francisco, N., Belloni, M., Aguirre, G., Caldara, F. R., Nääs, I., . . . Polycarpo, G. (2011). Infrared Thermography Applied to the Evaluation of Metabolic Heat Loss of Chicks Fed with Different Energy Densities. *Brazilian Journal of Poultry Science, 13*, 113-118.

Finn, D., Lomax, M., & Trayhurn, P. (1998). An Immunohistochemical and in Situ Hybridisation Study of the Postnatal Development of Uncoupling Protein-1 and Uncoupling Protein-1 Mrna in Lamb Perirenal Adipose Tissue. *Cell and tissue research, 294*(3), 461-466.

Fisher, M., & Mellor, D. (2002). The Welfare Implications of Shepherding During Lambing in Extensive New Zealand Farming Systems. *Animal welfare, 11*(2), 157-170.

Fisher, M. W. (2004). A Review of the Welfare Implications of out-of-Season Extensive Lamb Production Systems in New Zealand. *Livestock Production Science, 85*(2-3), 165-172. doi:10.1016/s0301-6226(03)00127-1

Fitzmaurice, S., Conington, J., Fetherstone, N., Pabiou, T., McDermott, K., Wall, E., . . . McHugh, N. (2020). Genetic Analyses of Live Weight and Carcass Composition Traits in Purebred Texel, Suffolk and Charollais Lambs. *Animal, 14*(5), 899-909. doi:10.1017/S1751731119002908

Fleckenstein-Elsen, M., Dinnies, D., Jelenik, T., Roden, M., Romacho, T., & Eckel, J. (2016). Eicosapentaenoic Acid and Arachidonic Acid Differentially Regulate Adipogenesis, Acquisition of a Brite Phenotype and Mitochondrial Function in Primary Human Adipocytes. *Molecular nutrition & food research, 60*(9), 2065-2075.

Fogarty, N., Hall, D., & Holst, P. (1992). The Effect of Nutrition in Mid Pregnancy and Ewe Liveweight Change on Birth Weight and Management for Lamb Survival in Highly Fecund Ewes. *Australian Journal of Experimental Agriculture, 32*(1), 1-10.

Fogarty, N., Ingham, V., Gilmour, A. R., Cummins, L., Gaunt, G., Stafford, J., . . . Banks, R. (2005). Genetic Evaluation of Crossbred Lamb Production. 1. Breed and Fixed Effects for Birth and Weaning Weight of First-Cross Lambs, Gestation Length, and Reproduction of Base Ewes. *Australian Journal of Agricultural Research, 56*(5), 443-453.

Fogarty, N., Safari, E., Taylor, P., & Murray, W. (2003). Genetic Parameters for Meat Quality and Carcass Traits and Their Correlation with Wool Traits in Australian Merino Sheep. *Australian Journal of Agricultural Research, 54*(7), 715-722. doi:10.1071/AR03047

Forner, F., Kumar, C., Luber, C. A., Fromme, T., Klingenspor, M., & Mann, M. (2009). Proteome Differences between Brown and White Fat Mitochondria Reveal Specialized Metabolic Functions. *Cell metabolism, 10*(4), 324-335.

Forrest, R., Hickford, J., & Frampton, C. (2007). Polymorphism at the Ovine B3-Adrenergic Receptor Locus (Adrb3) and Its Association with Lamb Mortality. *Journal of Animal Science, 85*(11), 2801-2806.

Forrest, R., Hickford, J., Wynyard, J., Merrick, N., Hogan, A., & Frampton, C. (2006). Polymorphism at the B3-Adrenergic Receptor (Adrb3) Locus of Merino Sheep and Its Association with Lamb Mortality. *Animal genetics, 37*(5), 465-468.

Fossceco, S., & Notter, D. (1995). Heritabilities and Genetic Correlations of Body Weight, Testis Growth and Ewe Lamb Reproductive Traits in Crossbred Sheep. *Animal Science, 60*(2), 185-195.

Fowler, D. (2007). Lamb Marking Performance for Ultrasound Scanned Ewes in Australian Sheep Flocks. *Final report AHW, 131*, 17.

Frydman, J. (2001). Folding of Newly Translated Proteins in Vivo: The Role of Molecular Chaperones. *Annual review of biochemistry, 70*(1), 603-647.

Fuse, S., Nirengi, S., Amagasa, S., Homma, T., Kime, R., Endo, T., . . . Yoneshiro, T. (2018). Brown Adipose Tissue Density Measured by near-Infrared Time-Resolved Spectroscopy in Japanese, across a Wide Age Range. *Journal of biomedical optics, 23*(6), 065002.

Fuse, S., Sugimoto, M., Kurosawa, Y., Kuroiwa, M., Aita, Y., Tomita, A., . . . Kime, R. (2020). Relationships between Plasma Lipidomic Profiles and Brown Adipose Tissue Density in Humans. *International Journal of Obesity, 44*(6), 1387-1396.

Garlid, K. D., Jabůrek, M., Ježek, P., & Vařecha, M. (2000). How Do Uncoupling Proteins Uncouple? *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1459*(2-3), 383-389.

Gartner, L. P., & Hiatt, J. L. (2007). Color Textbook of Histology . Saunders. In: Elsevier Philadelphia, PA, USA:.

Geenty, K. (1997). A Guide to Improved Lambing Percentage. *Wools of New Zealand and the New Zealand Meat Producers Board: Wellington*.

Geenty, K., Brien, F., Hinch, G., Dobos, R., Refshauge, G., McCaskill, M., . . . Savage, D. (2014). Reproductive Performance in the Sheep Crc Information Nucleus Using Artificial Insemination across Different Sheep-Production Environments in Southern Australia. *Animal Production Science, 54*(6), 715-726.

George, J. (1976). The Incidence of Dystocia in Dorset Horn Ewes. *Australian Veterinary Journal, 52*(11), 519-523.

Gibbons, G. F., Islam, K., & Pease, R. J. (2000). Mobilisation of Triacylglycerol Stores. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1483*(1), 37-57.

Gifford, D., Ponzoni, R., Hynd, P., Walkley, J., & Grimson, R. (1995). Genetic Studies on Wool Quality and Skin Characters of the Merino. *Skin, 1*, 16mo.

Gilham, D., & Lehner, R. (2004). The Physiological Role of Triacylglycerol Hydrolase in Lipid Metabolism. *Reviews in Endocrine and Metabolic Disorders, 5*(4), 303-309.

Gilmour, A., Gogel, B., Cullis, B., Welham, S., & Thompson, R. (2015). Asreml User Guide Release 4.1 Structural Specification. *Hemel hempstead: VSN international ltd*.

Gluckman, P. D., Grumbach, M. M., & Kaplan, S. L. (1981). The Neuroendocrine Regulation and Function of Growth Hormone and Prolactin in the Mammalian Fetus. *Endocrine reviews, 2*(4), 363-395.

Gniadecka, M., & Quistorff, B. (1996). Assessment of Dermal Water by High-Frequency Ultrasound: Comparative Studies with Nuclear Magnetic Resonance. *British Journal of Dermatology, 135*(2), 218-224.

Gokce, E., Atakisi, O., Kirmizigul, A. H., Unver, A., & Erdogan, H. M. (2014). Passive Immunity in Lambs: Serum Lactoferrin Concentrations as a Predictor of Igg Concentration and Its Relation to Health Status from Birth to 12 Weeks of Life. *Small Ruminant Research, 116*(2-3), 219-228.

Gowane, G., Chopra, A., Prakash, V., & Arora, A. (2010). Estimates of (Co) Variance Components and Genetic Parameters for Body Weights and First Greasy Fleece Weight in Malpura Sheep. *Livestock Science, 131*(1), 94-101. doi:10.1016/j.livsci.2010.03.006

Greeff, J., Safari, E., Fogarty, N., Hopkins, D., Brien, F., Atkins, K., . . . Van Der Werf, J. (2008). Genetic Parameters for Carcass and Meat Quality Traits and Their Relationships to Liveweight and Wool Production in Hogget Merino Rams. *Journal of Animal Breeding and Genetics, 125*(3), 205-215. doi:10.1111/j.1439-0388.2007.00711.x

Greenwood, P. L., Thompson, A. N., & Ford, S. P. (2010). Postnatal Consequences of the Maternal Environment and of Growth During Prenatal Life for Productivity of Ruminants. In *Managing the Prenatal Environment to Enhance Livestock Productivity* (pp. 3-36): Springer.

Greer, A., Corner-Thomas, R., Logan, C., Kenyon, P., Morris, S., Ridler, A., . . . Blair, H. (2015). Perceived Importance of Areas of Future Research: Results from a Survey of Sheep Farmers. *New Zealand Journal of Agricultural Research, 58*(4), 359-370.

Grefhorst, A., van den Beukel, J. C., van Houten, E. L. A., Steenbergen, J., Visser, J. A., & Themmen, A. P. (2015). Estrogens Increase Expression of Bone Morphogenetic Protein 8b in Brown Adipose Tissue of Mice. *Biology of sex differences, 6*(1), 1-13.

Gregory, I. (1982a). Genetic Studies of South Australian Merino Sheep. 3. Heritabilities of Various Wool and Body Traits. *Australian Journal of Agricultural Research, 33*(2), 355-362.

Gregory, I. (1982b). Genetic Studies of South Australian Merino Sheep. 4. Genetic, Phenotypic and Environmental Correlations between Various Wool and Body Traits. *Australian Journal of Agricultural Research, 33*(2), 363-373.

Gregory, N. (1998). Physiological Mechanisms Causing Sickness Behaviour and Suffering in Diseased Animals. *Animal welfare, 7*(3), 293-305.

Grzybek, M., Palladini, A., Alexaki, V. I., Surma, M. A., Simons, K., Chavakis, T., . . . Coskun, Ü. (2019). Comprehensive and Quantitative Analysis of White and Brown Adipose Tissue by Shotgun Lipidomics. *Molecular metabolism, 22*, 12-20.

Haas, B., Mayer, P., Jennissen, K., Scholz, D., Diaz, M. B., Bloch, W., . . . Pfeifer, A. (2009). Protein Kinase G Controls Brown Fat Cell Differentiation and Mitochondrial Biogenesis. *Science signaling, 2*(99), ra78-ra78.

Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., . . . Eder, S. (2006). Defective Lipolysis and Altered Energy Metabolism in Mice Lacking Adipose Triglyceride Lipase. *Science, 312*(5774), 734-737.

Hall, J. A., Ribich, S., Christoffolete, M. A., Simovic, G., Correa-Medina, M., Patti, M. E., & Bianco, A. C. (2010). Absence of Thyroid Hormone Activation During Development Underlies a Permanent Defect in Adaptive Thermogenesis. *Endocrinology, 151*(9), 4573-4582.

Han, X., & Gross, R. W. (2003). Global Analyses of Cellular Lipidomes Directly from Crude Extracts of Biological Samples by Esi Mass Spectrometry a Bridge to Lipidomics. *Journal of lipid research, 44*(6), 1071-1079.

Hanford, K. J., Van Vleck, L. D., & Snowder, G. (2006). Estimates of Genetic Parameters and Genetic Trend for Reproduction, Weight, and Wool Characteristics of Polypay Sheep. *Livestock Science, 102*(1-2), 72-82.

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular Chaperones in Protein Folding and Proteostasis. *Nature, 475*(7356), 324-332.

Hatcher, S., Atkins, K., & Safari, E. (2009). Phenotypic Aspects of Lamb Survival in Australian Merino Sheep. *Journal of Animal Science, 87*(9), 2781-2790.

Haughey, K. (1973). Vascular Abnormalities in the Central Nervous System Associated with Perinatal Lamb Mortality: Association of the Abnormalities with Recognised Lesions. *Australian Veterinary Journal, 49*(1), 9-15.

Haughey, K. (1993). Perinatal Lamb Mortality-Its Investigation, Causes and Control (Reprinted from Journal of the South-African Veterinary Association, Vol 62, Pg 78-91, 1991). *Irish Veterinary Journal, 46*(1), 9-28.

Haughey, K., George, J., & McGuirk, B. (1985). The Repeatability of Rearing Performance of Merino and Dorset Horn Ewes and Its Relationship with Mature Pelvic Size. *Australian Journal of Experimental Agriculture, 25*(3), 541-549.

Hawkins, A. (1991). Protein Turnover: A Functional Appraisal. *Functional Ecology*, 222-233.

Hellström, L., Wahrenberg, H., Reynisdottir, S., & Arner, P. (1997). Catecholamine-Induced Adipocyte Lipolysis in Human Hyperthyroidism. *The Journal of Clinical Endocrinology & Metabolism, 82*(1), 159-166.

Henry, B. A., Pope, M., Birtwistle, M., Loughnan, R., Alagal, R., Fuller-Jackson, J.-P., . . . Symonds, M. E. (2017). Ontogeny and Thermogenic Role for Sternal Fat in Female Sheep. *Endocrinology, 158*(7), 2212-2225.

Hickson, J., Kinghorn, B., Swan, A., & Piper, L. (1994). The Relationship between Hogget and Adult Production Traits in Merino Sheep.

Hight, G., & Jury, K. (1970). Hill Country Sheep Production: Ii. Lamb Mortality and Birth Weights in Romney and Border Leicester× Romney Flocks. *New Zealand Journal of Agricultural Research, 13*(4), 735-752.

Hinch, G. (1989). The Sucking Behaviour of Triplet, Twin and Single Lambs at Pasture. *Applied Animal Behaviour Science, 22*(1), 39-48.

Hinch, G., & Brien, F. (2014). Lamb Survival in Australian Flocks: A Review. *Animal Production Science, 54*(6), 656-666.

Hinch, G., Crosbie, S., Kelly, R., Owens, J., & Davis, G. (1985). Influence of Birth Weight and Litter Size on Lamb Survival in High Fecundity Booroola-Merino Crossbred Flocks. *New Zealand Journal of Agricultural Research, 28*(1), 31-38.

Hinch, G., Kelly, R., Owens, J., & Crosbie, S. (1983). *Paterns of Lamb Survival in High Fecundity Booroola Flocks.* Paper presented at the Proceedings of... annual conference-New Zealand Society of Animal Production.

Hishikawa, D., Hashidate, T., Shimizu, T., & Shindou, H. (2014). Diversity and Function of Membrane Glycerophospholipids Generated by the Remodeling Pathway in Mammalian Cells. *Journal of lipid research, 55*(5), 799-807.

Hodgson, J., Brebner, J., & Mckendrick, I. (1999). Efficacy of a Single Dose of Oral Antibiotic Given within Two Hours of Birth in Preventing Watery Mouth Disease and Illthrift in Colostrum-Deficient Lambs. *Veterinary record, 145*(3), 67-71.

Hodgson, J. C., Barclay, G. R., Hay, L. A., Moon, G. M., & Poxton, I. R. (1995). Prophylactic Use of Human Endotoxin-Core Hyperimmune Gammaglobulin to Prevent Endotoxaemia in Colostrum-Deprived, Gnotobiotic Lambs Challenged Orally with Escherichia Coli. *FEMS Immunology & Medical Microbiology, 11*(3), 171-180.

Hoene, M., Li, J., Häring, H.-U., Weigert, C., Xu, G., & Lehmann, R. (2014). The Lipid Profile of Brown Adipose Tissue Is Sex-Specific in Mice. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1841*(10), 1563-1570.

Hoffmann, L. S., Etzrodt, J., Willkomm, L., Sanyal, A., Scheja, L., Fischer, A. W., . . . Heeren, J. (2015). Stimulation of Soluble Guanylyl Cyclase Protects against Obesity by Recruiting Brown Adipose Tissue. *Nature communications, 6*(1), 1-9.

Holst, P., Fogarty, N., & Stanley, D. (2002). Birth Weights, Meningeal Lesions, and Survival of Diverse Genotypes of Lambs from Merino and Crossbred Ewes. *Australian Journal of Agricultural Research, 53*(2), 175-181.

Houlihan, D., Carter, C., & McCarthy, I. (1995). Protein Turnover in Animals.

Huisman, A., & Brown, D. (2008). Genetic Parameters for Bodyweight, Wool, and Disease Resistance and Reproduction Traits in Merino Sheep. 2. Genetic Relationships between Bodyweight Traits and Other Traits. *Australian Journal of Experimental Agriculture, 48*(9), 1186-1193. doi:10.1071/EA08120

Huisman, A., & Brown, D. (2009). Genetic Parameters for Bodyweight, Wool, and Disease Resistance and Reproduction Traits in Merino Sheep. 3. Genetic Relationships between Ultrasound Scan Traits and Other Traits. *Animal Production Science, 49*(4), 283-288.

Huisman, A., Brown, D., Ball, A., & Graser, H.-U. (2008). Genetic Parameters for Bodyweight, Wool, and Disease Resistance and Reproduction Traits in Merino Sheep. 1. Description of Traits, Model Comparison, Variance Components and Their Ratios. *Australian Journal of Experimental Agriculture, 48*(9), 1177-1185.

Huynh, K., Barlow, C. K., Jayawardana, K. S., Weir, J. M., Mellett, N. A., Cinel, M., . . . Meikle, P. J. (2019). High-Throughput Plasma Lipidomics: Detailed Mapping of the Associations with Cardiometabolic Risk Factors. *Cell Chem Biol, 26*(1), 71-84.e74. doi:10.1016/j.chembiol.2018.10.008

Jacobson, C., Bruce, M., Kenyon, P. R., Lockwood, A., Miller, D., Refshauge, G., & Masters, D. G. (2020). A Review of Dystocia in Sheep. *Small Ruminant Research, 192*. doi:10.1016/j.smallrumres.2020.106209

Janssens, S., Geysen, D., & Vandepitte, W. (2000). *Genetic Parameters for Live Weight in Belgian Texel Sheep.* Paper presented at the Proceedings of the 51st Annual Meeting of the European Association for Animal Production, Session.

Janssens, S., & Vandepitte, W. (2004). Genetic Parameters for Body Measurements and Linear Type Traits in Belgian Bleu Du Maine, Suffolk and Texel Sheep. *Small Ruminant Research, 54*(1-2), 13-24.

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., . . . Haw, R. (2020). The Reactome Pathway Knowledgebase. *Nucleic acids research, 48*(D1), D498-D503.

Jenkins, B., Ronis, M., & Koulman, A. (2020). Lc–Ms Lipidomics: Exploiting a Simple High-Throughput Method for the Comprehensive Extraction of Lipids in a Ruminant Fat Dose-Response Study. *Metabolites, 10*(7), 296.

Jiao, D., Ji, K., Liu, H., Wang, W., Wu, X., Zhou, J., . . . Yang, G. (2021). Transcriptome Analysis Reveals Genes Involved in Thermogenesis in Two Cold-Exposed Sheep Breeds. *Genes (Basel), 12*(3). doi:10.3390/genes12030375

Johnson, D., Clarke, J., Maclean, K., Cox, E., Amyes, N., & Rattray, P. (1982). *Level of Nutrition of the Ewe and Lamb Survival.* Paper presented at the Proceedings of the New Zealand society of animal production.

Johnson, D., Hight, G., Dobbie, J., Jones, K., & Wrigglesworth, A. (1995). Single Trait Selection for Yearling Fleece Weight or Liveweight in Romney Sheep—Direct Responses. *New Zealand Journal of Agricultural Research, 38*(1), 115-122.

Jones, B. F. (1998). A Reappraisal of the Use of Infrared Thermal Image Analysis in Medicine. *IEEE transactions on medical imaging, 17*(6), 1019-1027.

Jopson, N., Greer, G., Bain, W., Findlay, J., & McEwan, J. (2000). *Lamb Survival Traits in Coopworth Sheep Selected for High or Low Backfat Depth.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Kazak, L., Chouchani, E. T., Jedrychowski, M. P., Erickson, B. K., Shinoda, K., Cohen, P., . . . Hasenfuss, S. C. (2015). A Creatine-Driven Substrate Cycle Enhances Energy Expenditure and Thermogenesis in Beige Fat. *Cell, 163*(3), 643-655.

Keller, M., Meurisse, M., Poindron, P., Nowak, R., Ferreira, G., Shayit, M., & Lévy, F. (2003). Maternal Experience Influences the Establishment of Visual/Auditory, but Not Olfactory Recognition of the Newborn Lamb by Ewes at Parturition. *Developmental Psychobiology: The Journal of the International Society for Developmental Psychobiology, 43*(3), 167-176.

Kelly, R. (1982). Reproductive Performance of Commercial Sheep Flocks in South Island Districts: 1. Flock Performance and Sources of Wastage between Joining and Tailing. *New Zealand Journal of Agricultural Research, 25*(2), 175-183.

Kenyon, P., & Blair, H. (2014). Foetal Programming in Sheep–Effects on Production. *Small Ruminant Research, 118*(1-3), 16-30.

Kenyon, P., Stafford, K., Jenkinson, C., Morris, S., & West, D. (2007). The Body Composition and Metabolic Status of Twin-and Triplet-Bearing Ewes and Their Fetuses in Late Pregnancy. *Livestock Science, 107*(2-3), 103-112.

Kenyon, P. R., Revell, D. K., & Morris, S. T. (2006). Mid-Pregnancy Shearing Can Increase Birthweight and Survival to Weaning of Multiple-Born Lambs under Commercial Conditions. *Australian Journal of Experimental Agriculture, 46*(7). doi:10.1071/ea05329

Kenyon, P. R., Roca Fraga, F. J., Blumer, S., & Thompson, A. N. (2019). Triplet Lambs and Their Dams – a Review of Current Knowledge and Management Systems. *New Zealand Journal of Agricultural Research, 62*(4), 399-437. doi:10.1080/00288233.2019.1616568

Kerslake, J., Everett-Hincks, J., & Campbell, A. (2005). *Lamb Survival: A New Examination of an Old Problem.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Kerslake, J. I., Kenyon, P. R., Stafford, K. J., Morris, S. T., & Morel, P. C. H. (2009). The Effect of Offering Concentrate Supplement to Twin- and Triplet-Bearing Ewes Grazing a 60 Mm Herbage Sward Height on Lamb Birth Weight, Heat Production and Post-Natal Growth. *The Journal of Agricultural Science, 147*(5), 613-624. doi:10.1017/s0021859609990062

Kim, D., Paggi, J. M., Park, C., Bennett, C., & Salzberg, S. L. (2019). Graph-Based Genome Alignment and Genotyping with Hisat2 and Hisat-Genotype. *Nature biotechnology, 37*(8), 907-915.

Kipp, C., Brügemann, K., Zieger, P., Mütze, K., Möcklinghoff-Wicke, S., König, S., & Halli, K. (2021). Across-Generation Effects of Maternal Heat Stress During Late Gestation on Production, Female Fertility and Longevity Traits in Dairy Cows. *Journal of Dairy Research, 88*(2), 147-153.

Knight, T., Lynch, P. R., Hall, D., & Hockey, H. P. (1988). Identification of Factors Contributing to the Improved Lamb Survival in Marshall Romney Sheep. *New Zealand Journal of Agricultural Research, 31*(3), 259-271.

Kolde, R. (2018). R Package, Version 1.0.12; Package 'Pheatmap'. Retrieved from https://CRAN.R-project.org/package=pheatmap

Kotzbeck, P., Giordano, A., Mondini, E., Murano, I., Severi, I., Venema, W., . . . Cinti, S. (2018). Brown Adipose Tissue Whitening Leads to Brown Adipocyte Death and Adipose Tissue Inflammation. *J Lipid Res, 59*(5), 784-794. doi:10.1194/jlr.M079665

Krassowski, M. (2020). Complexupset: Create Complex Upset Plots Using Ggplot2 Components. *R package version 0.5, 18*.

Kurylowicz, A., Jonas, M., Lisik, W., Jonas, M., Wicik, Z. A., Wierzbicki, Z., . . . Puzianowska-Kuznicka, M. (2015). Obesity Is Associated with a Decrease in Expression but Not with the Hypermethylation of Thermogenesis-Related Genes in Adipose Tissues. *J Transl Med, 13*, 31. doi:10.1186/s12967-015-0395-2

Lahiri, B. B., Bagavathiappan, S., Jayakumar, T., & Philip, J. (2012). Medical Applications of Infrared Thermography: A Review. *Infrared Physics & Technology, 55*(4), 221-235.

Langmead, B., & Salzberg, S. L. (2012). Fast Gapped-Read Alignment with Bowtie 2. *Nature methods, 9*(4), 357-359.

Le Floch, E., Refshauge, G., van de Ven, R., Morgan, J., & Hopkins, D. (2010). *Border Leicester· Merino Dams Lose Fewer Lambs to Starvation Than Merino Dams.* Paper presented at the Australian Society of Animal Production 28th biennial conference, Armidale, NSW.

Leaver, E. V., & Pappone, P. A. (2002). B-Adrenergic Potentiation of Endoplasmic Reticulum Ca2+ Release in Brown Fat Cells. *American Journal of Physiology-Cell Physiology, 282*(5), C1016-C1024.

Leiria, L. O., & Tseng, Y.-H. (2020). Lipidomics of Brown and White Adipose Tissue: Implications for Energy Metabolism. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1865*(10), 158788.

Leiria, L. O., Wang, C.-H., Lynes, M. D., Yang, K., Shamsi, F., Sato, M., . . . Narain, N. R. (2019). 12-Lipoxygenase Regulates Cold Adaptation and Glucose Metabolism by Producing the Omega-3 Lipid 12-Hepe from Brown Fat. *Cell metabolism, 30*(4), 768-783. e767.

Li, B., & Dewey, C. N. (2011). Rsem: Accurate Transcript Quantification from Rna-Seq Data with or without a Reference Genome. *BMC Bioinformatics, 12*(1), 1-16.

Li, P. (2004). Cidea, Brown Fat and Obesity: Mechanisms of Ageing and Development. *Mechanisms of ageing and development, 125*(4), 337-338.

Li, P., Piao, Y., Shon, H. S., & Ryu, K. H. (2015). Comparing the Normalization Methods for the Differential Analysis of Illumina High-Throughput Rna-Seq Data. *BMC Bioinformatics, 16*(1), 1-9.

Liang, H., & Ward, W. F. (2006). Pgc-1α: A Key Regulator of Energy Metabolism. *Advances in physiology education*.

Liao, Y., Smyth, G. K., & Shi, W. (2014). Featurecounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features. *Bioinformatics, 30*(7), 923-930.

Lim, C.-K., & Lord, G. (2002). Current Developments in Lc-Ms for Pharmaceutical Analysis. *Biological and Pharmaceutical Bulletin, 25*(5), 547-557.

Lindsay, D., Nowak, R., Putu, I. G., & McNeill, D. (1990). Behavioural Interactions between the Ewe and Her Young at Parturition: A Vital Step for the Lamb. *Reproductive physiology of Merino sheep–concepts and consequences'.(Eds CM Oldham, GB Martin, IW Purvis) pp*, 191-205.

Liu, B., Han, Y., & Qian, S.-B. (2013). Cotranslational Response to Proteotoxic Stress by Elongation Pausing of Ribosomes. *Molecular cell, 49*(3), 453-463.

Liu, H., Hou, Y., Zhu, Q.-l., Xu, D., Wang, L., Li, J.-c., . . . Zhang, F.-c. (2017). A Preliminary Study of Skin Ultrasound in Diffuse Cutaneous Systemic Sclerosis: Does Skin Echogenicity Matter? *PLoS One, 12*(3), e0174481.

Liu, S., Brown, J. D., Stanya, K. J., Homan, E., Leidl, M., Inouye, K., . . . Hatano, B. (2013). A Diurnal Serum Lipid Integrates Hepatic Lipogenesis and Peripheral Fatty Acid Use. *Nature, 502*(7472), 550-554.

Liu, X., Tang, J., Zhang, R., Zhan, S., Zhong, T., Guo, J., . . . Zhang, H. (2022a). Cold Exposure Induces Lipid Dynamics and Thermogenesis in Brown Adipose Tissue of Goats. *BMC Genomics, 23*(1), 528.

Liu, X., Tang, J., Zhang, R., Zhan, S., Zhong, T., Guo, J., . . . Zhang, H. (2022b). Cold Exposure Induces Lipid Dynamics and Thermogenesis in Brown Adipose Tissue of Goats. *BMC Genomics, 23*(1), 1-12.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative Pcr and the 2− Δδct Method. *methods, 25*(4), 402-408.

Lomax, M. A., Sadiq, F., Karamanlidis, G., Karamitri, A., Trayhurn, P., & Hazlerigg, D. G. (2007). Ontogenic Loss of Brown Adipose Tissue Sensitivity to Beta-Adrenergic Stimulation in the Ovine. *Endocrinology, 148*(1), 461-468. doi:10.1210/en.2006-0918

Lopez-Villalobos, N., & Garrick, D. (1999). *Genetic Parameter Estimates for Lamb Survival in Romney Sheep.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

López, M., Varela, L., Vázquez, M. J., Rodríguez-Cuenca, S., González, C. R., Velagapudi, V. R., . . . Lage, R. (2010). Hypothalamic Ampk and Fatty Acid Metabolism Mediate Thyroid Regulation of Energy Balance. *Nature medicine, 16*(9), 1001-1008.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated Estimation of Fold Change and Dispersion for Rna-Seq Data with Deseq2. *Genome biology, 15*(12), 1-21.

Lowell, B. B. (1999). An Essential Regulator of Adipogenesis and Modulator of Fat Cell Function: Pparγ. *Cell, 99*(3), 239-242.

Loyau, T., Zerjal, T., Rodenburg, T., Fablet, J., Tixier-Boichard, M., Pinard-Van Der Laan, M., & Mignon-Grasteau, S. (2016). Heritability of Body Surface Temperature in Hens Estimated by Infrared Thermography at Normal or Hot Temperatures and Genetic Correlations with Egg and Feather Quality. *Animal, 10*(10), 1594-1601. doi:10.1017/S1751731116000616

Lu, X., Solmonson, A., Lodi, A., Nowinski, S. M., Sentandreu, E., Riley, C. L., . . . Tiziani, S. (2017). The Early Metabolomic Response of Adipose Tissue During Acute Cold Exposure in Mice. *Scientific reports, 7*(1), 1-11.

Lucas, A., Baker, B., & Cole, T. (1990). Plasma Prolactin and Clinical Outcome in Preterm Infants. *Archives of disease in childhood, 65*(9), 977-983.

Ludwig, N., Formenti, D., Gargano, M., & Alberti, G. (2014). Skin Temperature Evaluation by Infrared Thermography: Comparison of Image Analysis Methods. *Infrared Physics & Technology, 62*, 1-6.

Lynes, M. D., Shamsi, F., Sustarsic, E. G., Leiria, L. O., Wang, C.-H., Su, S.-C., . . . Chen, E. Y. (2018). Cold-Activated Lipid Dynamics in Adipose Tissue Highlights a Role for Cardiolipin in Thermogenic Metabolism. *Cell reports, 24*(3), 781-790.

Marcher, A. B., Loft, A., Nielsen, R., Vihervaara, T., Madsen, J. G., Sysi-Aho, M., . . . Mandrup, S. (2015). Rna-Seq and Mass-Spectrometry-Based Lipidomics Reveal Extensive Changes of Glycerolipid Pathways in Brown Adipose Tissue in Response to Cold. *Cell Rep, 13*(9), 2000-2013. doi:10.1016/j.celrep.2015.10.069

Marko, S. B., & Damon, D. H. (2008). Vegf Promotes Vascular Sympathetic Innervation. *American Journal of Physiology-Heart and Circulatory Physiology, 294*(6), H2646-H2652.

Marrif, H., Schifman, A., Stepanyan, Z., Gillis, M.-A., Calderone, A., Weiss, R. E., . . . Silva, J. E. (2005). Temperature Homeostasis in Transgenic Mice Lacking Thyroid Hormone Receptor-A Gene Products. *Endocrinology, 146*(7), 2872-2884.

Matheson, S., Bünger, L., & Dwyer, C. (2012). Genetic Parameters for Fitness and Neonatal Behavior Traits in Sheep. *Behavior Genetics, 42*(6), 899-911.

Maximini, L., Brown, D. J., Baumung, R., & Fuerst-Waltl, B. (2012). Genetic Parameters of Ultrasound and Computer Tomography Scan Traits in Austrian Meat Sheep. *Livestock Science, 146*(2-3), 168-174.

May, F. J., Baer, L. A., Lehnig, A. C., So, K., Chen, E. Y., Gao, F., . . . Stanford, K. I. (2017). Lipidomic Adaptations in White and Brown Adipose Tissue in Response to Exercise Demonstrate Molecular Species-Specific Remodeling. *Cell Rep, 18*(6), 1558-1572. doi:10.1016/j.celrep.2017.01.038

McCafferty, D. J. (2007). The Value of Infrared Thermography for Research on Mammals: Previous Applications and Future Directions. *Mammal Review, 37*(3), 207-223.

McCoard, S., Henderson, H., Knol, F., Dowling, S., & Webster, J. (2014). Infrared Thermal Imaging as a Method to Study Thermogenesis in the Neonatal Lamb. *Animal Production Science, 54*(9), 1497-1501.

McCorkindale, A. (1999). *Cf2000-a New Way of Looking at the Same Information.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

McCutcheon, S., Holmes, C., & McDonald, M. (1981). *The Starvation-Exposure Syndrome and Neonatal Lamb Mortality. A Review.* Paper presented at the Proceedings of the New Zealand Society of Animal Production.

McCutcheon, S., Holmes, C., McDonald, M., & Rae, A. (1983a). Resistance to Cold Stress in the Newborn Lamb 1. Responses of Romney, Drysdale× Romney, and Merino Lambs to Components of the Thermal Environment. *New Zealand Journal of Agricultural Research, 26*(2), 169-174.

McCutcheon, S., Holmes, C., McDonald, M., & Rae, A. (1983b). Resistance to Cold Stress in the Newborn Lamb 2. Role of Body Weight, Birth Rank, and Some Birth Coat Characters as Determinants of Resistance to Cold Stress. *New Zealand Journal of Agricultural Research, 26*(2), 175-181.

McDonald, I. W. (1962). Ewe Fertility and Neonatal Lamb Mortality. *New Zealand Veterinary Journal, 10*(3), 45-53. doi:10.1080/00480169.1962.33458

McEwan, J. C., Clarke, J., Hickey, S., & Knowler, K. (1993). *Heritability of Ultrasonic Fat and Muscle Depths in Romney Sheep.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

McFarlane, D. (1961). Perinatal Lamb Losses. *Australian Veterinary Journal, 37*(4), 105-109.

McHugh, N., Berry, D., & Pabiou, T. (2016). Risk Factors Associated with Lambing Traits. *Animal, 10*(1), 89-95.

McManus, C., Paludo, G. R., Louvandini, H., Gugel, R., Sasaki, L. C. B., & Paiva, S. R. (2009). Heat Tolerance in Brazilian Sheep: Physiological and Blood Parameters. *Tropical Animal Health and Production, 41*(1), 95-101.

McMillan, W. (1983). *Hogget Lamb Mortality.* Paper presented at the Proceedings of... annual conference-New Zealand Society of Animal Production.

McQueen, I. (1986). *Ewe Performance and Lamb Liveweight Gains Compared Following Autumn and Spring Lambing.* Paper presented at the Proceedings of the New Zealand Grassland Association.

McRae, K. M., Cooper, S. M., McEwan, J. C., Anderson, R., Bain, W. E., Baird, H. J., . . . Holmes, G. (2022). Genetic Variation in Skin Traits in New Zealand Lambs. *Journal of the Science of Food and Agriculture*. doi:10.1002/jsfa.11844

Medrado, B. D., Pedrosa, V. B., & Pinto, L. F. B. (2021). Meta-Analysis of Genetic Parameters for Economic Traits in Sheep. *Livestock Science, 247*, 104477. doi:10.1016/j.livsci.2021.104477

Meikle, B., Kimble, R. M., & Tyack, Z. (2022). Ultrasound Measurements of Pathological and Physiological Skin Thickness: A Scoping Review Protocol. *BMJ open, 12*(1), e056720.

Meikle, P. J., & Summers, S. A. (2017). Sphingolipids and Phospholipids in Insulin Resistance and Related Metabolic Disorders. *Nature Reviews Endocrinology, 13*(2), 79-91.

Mellor, D. (1983). Nutritional and Placental Determinants of Foetal Growth Rate in Sheep and Consequences for the Newborn Lamb. *British Veterinary Journal, 139*(4), 307-324.

Mellor, D., & Cockburn, F. (1986). A Comparison of Energy Metabolism in the New-Born Infant, Piglet and Lamb. *Quarterly Journal of Experimental Physiology: Translation and Integration, 71*(3), 361-379.

Mellor, D., & Murray, L. (1985a). Effects of Maternal Nutrition on Udder Development During Late Pregnancy and on Colostrum Production in Scottish Blackface Ewes with Twin Lambs. *Research in Veterinary Science, 39*(2), 230-234.

Mellor, D. J., & Stafford, K. J. (2004). Animal Welfare Implications of Neonatal Mortality and Morbidity in Farm Animals. *Vet J, 168*(2), 118-133. doi:10.1016/j.tvjl.2003.08.004

Melton, J., & Swanson, J. (1996). Dermatology Atlas. *URL: http://www. meddean. luc. edu/lumen/MedEd/medicine/dermatolo gy/melton/atlas. htm*.

Memon, R. A., Holleran, W. M., Moser, A. H., Seki, T., Uchida, Y., Fuller, J., . . . Feingold, K. R. (1998). Endotoxin and Cytokines Increase Hepatic Sphingolipid Biosynthesis and Produce Lipoproteins Enriched in Ceramides and Sphingomyelin. *Arteriosclerosis, thrombosis, and vascular biology, 18*(8), 1257-1265.

Merei, J. J., Rao, A., Clarke, I. J., & McMillen, I. C. (1993). Proopiomelanocortin, Prolactin and Growth Hormone Messenger Ribonucleic Acid Levels in the Fetal Sheep Pituitary During Late Gestation. *European journal of endocrinology, 129*(3), 263-267.

Merrill , A. H. (2011). Sphingolipid and Glycosphingolipid Metabolic Pathways in the Era of Sphingolipidomics. *Chemical reviews, 111*(10), 6387-6422.

Meshulam, T., Breen, M. R., Liu, L., Parton, R. G., & Pilch, P. F. (2011). Caveolins/Caveolae Protect Adipocytes from Fatty Acid-Mediated Lipotoxicity. *Journal of lipid research, 52*(8), 1526-1532.

Milhas, D., Clarke, C. J., & Hannun, Y. A. (2010). Sphingomyelin Metabolism at the Plasma Membrane: Implications for Bioactive Sphingolipids. *FEBS letters, 584*(9), 1887-1894.

Mishra, A., Arora, A., Kumar, S., Gupta, D., & Singh, V. (2007). Ewe Productivity Efficiency of Single and Multiple Lambs Bearing of Garole, Malpura and Their Crossbred Ewes. *The Indian Journal of Animal Sciences, 77*(8).

Mlosek, R. K., Migda, B., & Migda, M. (2021). High-Frequency Ultrasound in the 21 Century. *Journal of Ultrasonography, 20*(83), 233-241.

Morel, & Kenyon. (2006). *Sensitivity Analysis of Weaner Lamb Production in New Zealand.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Morel, P., Morris, S., & Kenyon, P. (2008). Effect of Birthweight on Survival in Triplet-Born Lambs. *Australian Journal of Experimental Agriculture, 48*(7), 984-987.

Morris, C., Hickey, S., & Clarke, J. (2000). Genetic and Environmental Factors Affecting Lamb Survival at Birth and through to Weaning. *New Zealand Journal of Agricultural Research, 43*(4), 515-524.

Morris, C., Jones, K., & Wilson, J. (1989). Heritability of Rectal Temperature and Relationships with Growth in Young Cattle in a Temperate Climate. *New Zealand Journal of Agricultural Research, 32*(3), 375-378. doi:10.1080/00288233.1989.10421755

Morris, S., & Kenyon, P. (2004). The Effect of Litter Size and Sward Height on Ewe and Lamb Performance. *New Zealand Journal of Agricultural Research, 47*(3), 275-286.

Morris, S. T. (2009). Economics of Sheep Production. *Small Ruminant Research, 86*(1-3), 59-62. doi:10.1016/j.smallrumres.2009.09.019

Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L., & Wold, B. (2008). Mapping and Quantifying Mammalian Transcriptomes by Rna-Seq. *Nature methods, 5*(7), 621-628.

Mortimer, S., & Atkins, K. (1997). *Improvement of Merino Reproductive Performance through Bloodline Substitution and Crossing.* Paper presented at the Proceedings of the Association for the Advancement of Animal Breeding and Genetics.

Mortimer, S., Hatcher, S., Fogarty, N., Van Der Werf, J., Brown, D., Swan, A., . . . Gaunt, G. (2017). Genetic Parameters for Wool Traits, Live Weight, and Ultrasound Carcass Traits in Merino Sheep. *Journal of Animal Science, 95*(5), 1879-1891.

Mostefai, E., Kouidri, M., & Selles, S. M. A. (2019). Causes of Sheep Dystocia in Djelfa Area (Algeria). *Revue Marocaine des Sciences Agronomiques et Veterinaires, 7*(2).

Moulk, G. (1954). Observations on Mortality Amongst Lambs in Queensland. *Australian Veterinary Journal, 30*(6), 153-171.

Mufti, N. A., & Shuler, M. L. (1996). Possible Role of Arachidonic Acid in Stress-Induced Cytochrome P450ia1 Activity1. *Biotechnology progress, 12*(6), 847-854.

Murphy, P., & Lindsay, D. (1996). *The Contribution of the Activity of the Newborn Merino Lamb to the Attachment to Its Mother and Subsequent Survival.* Paper presented at the The contribution of the activity of the newborn Merino lamb to the attachment to its mother and subsequent survival.

Neufeld, G., Cohen, T., Gengrinovitch, S., & Poltorak, Z. (1999). Vascular Endothelial Growth Factor (Vegf) and Its Receptors. *The FASEB Journal, 13*(1), 9-22.

Neuwirth, E., & Neuwirth, M. E. (2014). Package 'Rcolorbrewer'. *ColorBrewer Palettes*.

New Zealand Meat Classification Authority. (2004). New Zealand Meat Guide to Lamb and Mutton Carcass Classification. Retrieved from https://www.interest.co.nz/files/rural/lambgrade.pdf

Ni, Z., Wölk, M., Jukes, G., Mendivelso Espinosa, K., Ahrends, R., Aimo, L., . . . Bridge, A. (2023). Guiding the Choice of Informatics Software and Tools for Lipidomics Research Applications. *Nature methods, 20*(2), 193-204.

Nicoll, G., Dodds, K., & Aldeton, M. (1999). *Field Data Analysis of Lamb Survival and Mortality Rates Occurring between Pregnancy Scanning and Weaning.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Noakes, D. E., Parkinson, T. J., & England, G. C. (2018). *Arthur's Veterinary Reproduction and Obstetrics-E-Book*: Elsevier Health Sciences.

Nowak, R., Keller, M., Val-Laillet, D., & Lévy, F. (2007). Perinatal Visceral Events and Brain Mechanisms Involved in the Development of Mother–Young Bonding in Sheep. *Hormones and Behavior, 52*(1), 92-98.

Nowak, R., & Poindron, P. (2006). From Birth to Colostrum: Early Steps Leading to Lamb Survival. *Reprod Nutr Dev, 46*(4), 431-446. doi:10.1051/rnd:2006023

Nygren, H., Seppänen-Laakso, T., Castillo, S., Hyötyläinen, T., & Orešič, M. (2011). Liquid Chromatography-Mass Spectrometry (Lc-Ms)-Based Lipidomics for Studies of Body Fluids and Tissues. *Metabolic Profiling: Methods and Protocols*, 247-257.

Obst, J., & Evans, J. (1970). *Genotype-Environment Interactions in Lamb Mortality with Particular Reference to Birth Coat and Haemoglobin Type.* Paper presented at the Proceedings of the Australian Society of Animal Production.

Ocloo, A., Shabalina, I. G., Nedergaard, J., & Brand, M. D. (2007). Cold-Induced Alterations of Phospholipid Fatty Acyl Composition in Brown Adipose Tissue Mitochondria Are Independent of Uncoupling Protein-1. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 293*(3), R1086-R1093.

Ohno, H., Shinoda, K., Ohyama, K., Sharp, L. Z., & Kajimura, S. (2013). Ehmt1 Controls Brown Adipose Cell Fate and Thermogenesis through the Prdm16 Complex. *Nature, 504*(7478), 163-167.

Ohno, H., Shinoda, K., Spiegelman, B. M., & Kajimura, S. (2012). Pparγ Agonists Induce a White-to-Brown Fat Conversion through Stabilization of Prdm16 Protein. *Cell metabolism, 15*(3), 395-404.

Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P., & Slotte, J. P. (2002). Cholesterol Interactions with Phospholipids in Membranes. *Progress in lipid research, 41*(1), 66-97.

Ojha, S., Robinson, L., Yazdani, M., Symonds, M. E., & Budge, H. (2013). Brown Adipose Tissue Genes in Pericardial Adipose Tissue of Newborn Sheep Are Downregulated by Maternal Nutrient Restriction in Late Gestation. *Pediatr Res, 74*(3), 246-251. doi:10.1038/pr.2013.107

Orešič, M., Hänninen, V. A., & Vidal-Puig, A. (2008). Lipidomics: A New Window to Biomedical Frontiers. *Trends in biotechnology, 26*(12), 647-652.

Oshlack, A., Robinson, M. D., & Young, M. D. (2010). From Rna-Seq Reads to Differential Expression Results. *Genome biology, 11*, 1-10.

Pan, J., Tao, C., Cao, C., Zheng, Q., Lam, S. M., Shui, G., . . . Wang, Y. (2019). Adipose Lipidomics and Rna-Seq Analysis Revealed the Enhanced Mitochondrial Function in Ucp1 Knock-in Pigs. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1864*(10), 1375-1383.

Passman, A., & Sumner, R. (1987). Effects of Breed and Age at Slaughter on Leather Produced from Export Lambs Reared on Hill Country. *New Zealand journal of experimental agriculture, 15*(3), 309-316. doi:10.1080/03015521.1987.10425576

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon Provides Fast and Bias-Aware Quantification of Transcript Expression. *Nature methods, 14*(4), 417-419.

Petersson, C., & Danell, Ö. (1985). Factors Influencing Lamb Survival in Four Swedish Sheep Breeds. *Acta Agriculturæ Scandinavica, 35*(2), 217-232.

Pettigrew, E. J., Hickson, R. E., Blair, H. T., Griffiths, K. J., Ridler, A. L., Morris, S. T., & Kenyon, P. R. (2020). Differences in Lamb Production between Ewe Lambs and Mature Ewes. *New Zealand Journal of Agricultural Research*, 1-14. doi:10.1080/00288233.2020.1713177

Pickering, N., Elliott, D., & Bagley, D. (2018). *Focus Genetics – Summary of Breeding Programmes*. Paper presented at the Proceedings of the World Congress on Genetics Applied to Livestock Production.

Pinares-Patiño, C., Hickey, S., Young, E., Dodds, K., MacLean, S., Molano, G., . . . Hunt, C. (2013). Heritability Estimates of Methane Emissions from Sheep. *Animal, 7*, 316-321.

Plush, K. J., Brien, F. D., Hebart, M. L., & Hynd, P. I. (2016). Thermogenesis and Physiological Maturity in Neonatal Lambs: A Unifying Concept in Lamb Survival. *Animal Production Science, 56*(4). doi:10.1071/an15099

Pope, M., Budge, H., & Symonds, M. E. (2014). The Developmental Transition of Ovine Adipose Tissue through Early Life. *Acta Physiol (Oxf), 210*(1), 20-30. doi:10.1111/apha.12053

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., & Spiegelman, B. M. (1998). A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis. *Cell, 92*(6), 829-839.

Purser, A., & Karam, H. (1967). Lamb Survival, Growth and Fleece Production in Relation to Birthcoat Type among Welsh Mountain Sheep. *Animal Science, 9*(1), 75-85.

Quintela, F. D. (2007). A New Perspective in Perinatal Lamb Mortality. *Archivos Latinoamericanos de Produccion Animal, 15*(S1), 288-289.

R Core Team. (2013). R: A Language and Environment for Statistical Computing. Vienna, Austria.

Rahbani, J. F., Roesler, A., Hussain, M. F., Samborska, B., Dykstra, C. B., Tsai, L., . . . Spiegelman, B. M. (2021). Creatine Kinase B Controls Futile Creatine Cycling in Thermogenic Fat. *Nature, 590*(7846), 480-485.

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., & Vilo, J. (2019). G: Profiler: A Web Server for Functional Enrichment Analysis and Conversions of Gene Lists (2019 Update). *Nucleic acids research, 47*(W1), W191-W198.

Refshauge, G., Brien, F. D., Hinch, G. N., & van de Ven, R. (2016). Neonatal Lamb Mortality: Factors Associated with the Death of Australian Lambs. *Animal Production Science, 56*(4). doi:10.1071/an15121

Reimand, J., Isserlin, R., Voisin, V., Kucera, M., Tannus-Lopes, C., Rostamianfar, A., . . . Xu, C. (2019). Pathway Enrichment Analysis and Visualization of Omics Data Using G: Profiler, Gsea, Cytoscape and Enrichmentmap. *Nature protocols, 14*(2), 482-517.

Ribeiro, M. O., Carvalho, S. D., Schultz, J. J., Chiellini, G., Scanlan, T. S., Bianco, A. C., & Brent, G. A. (2001). Thyroid Hormone–Sympathetic Interaction and Adaptive Thermogenesis Are

Thyroid Hormone Receptor Isoform–Specific. *The Journal of clinical investigation, 108*(1), 97-105.

Ribeiro, M. O., Lebrun, F. L., Christoffolete, M. A., Branco, M., Crescenzi, A., Carvalho, S. D., . . . Bianco, A. C. (2000). Evidence of Ucp1-Independent Regulation of Norepinephrine-Induced Thermogenesis in Brown Fat. *American Journal of Physiology-Endocrinology And Metabolism, 279*(2), E314-E322.

Ridley, R. G., Patel, H. V., Gerber, G. E., Morton, R. C., & Freeman, K. B. (1986). Complete Nucleotide and Derived Amino Acid Sequence of Cdna Encoding the Mitochondrial Uncoupling Protein of Rat Brown Adipose Tissue: Lack of a Mitochondrial Targeting Presequence. *Nucleic acids research, 14*(10), 4025-4035.

Riggio, V., Finocchiaro, R., & Bishop, S. C. (2008). Genetic Parameters for Early Lamb Survival and Growth in Scottish Blackface Sheep. *J Anim Sci, 86*(8), 1758-1764. doi:10.2527/jas.2007-0132

Rooke, J., Arnott, G., Dwyer, C., & Rutherford, K. (2015). The Importance of the Gestation Period for Welfare of Lambs: Maternal Stressors and Lamb Vigour and Wellbeing. *Journal of Agricultural Science*.

Rosell, M., Kaforou, M., Frontini, A., Okolo, A., Chan, Y.-W., Nikolopoulou, E., . . . Turner, J. O. (2014). Brown and White Adipose Tissues: Intrinsic Differences in Gene Expression and Response to Cold Exposure in Mice. *American Journal of Physiology-Endocrinology And Metabolism, 306*(8), E945-E964.

Rowland, L. A., Bal, N. C., & Periasamy, M. (2015). The Role of Skeletal-Muscle-Based Thermogenic Mechanisms in Vertebrate Endothermy. *Biological Reviews, 90*(4), 1279-1297.

RStudio-Team. (2021). Rstudio: Integrated Development Environment for R. Rstudio, Pbc, Boston, Ma. *RStudio, PBC*. Retrieved from http://www.rstudio.com/

Rubio, A., Raasmaja, A., & Silva, J. E. (1995). Thyroid Hormone and Norepinephrine Signaling in Brown Adipose Tissue. Ii: Differential Effects of Thyroid Hormone on Beta 3-Adrenergic Receptors in Brown and White Adipose Tissue. *Endocrinology, 136*(8), 3277-3284.

Rutkowski, J. M., Stern, J. H., & Scherer, P. E. (2015). The Cell Biology of Fat Expansion. *Journal of Cell Biology, 208*(5), 501-512.

Sackett, D., Holmes, P., Abbott, K., Jephcott, S., & Barber, M. (2006). Assessing the Economic Cost of Endemic Disease on the Profitability of Australian Beef Cattle and Sheep Producers. *MLA Report AHW, 87*.

Safari, A., & Fogarty, N. (2003). Genetic Parameters for Sheep Production Traits: Estimates from the Literature. Tech. *Technical bulletin, 49*.

Safari, E., Fogarty, N., & Gilmour, A. R. (2005). A Review of Genetic Parameter Estimates for Wool, Growth, Meat and Reproduction Traits in Sheep. *Livestock Production Science, 92*(3), 271-289.

Safari, E., Fogarty, N., Gilmour, A. R., Atkins, K., Mortimer, S., Swan, A., . . . Van der Werf, J. (2007). Across Population Genetic Parameters for Wool, Growth, and Reproduction Traits in Australian Merino Sheep. 2. Estimates of Heritability and Variance Components. *Australian Journal of Agricultural Research, 58*(2), 177-184.

Sahu, A. R., Wani, S. A., Saxena, S., Rajak, K. K., Chaudhary, D., Sahoo, A. P., . . . Malla, W. A. (2018). Selection and Validation of Suitable Reference Genes for Qpcr Gene Expression Analysis in Goats and Sheep under Peste Des Petits Ruminants Virus (Pprv), Lineage Iv Infection. *Scientific reports, 8*(1), 1-11.

Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-Kobayashi, J., . . . Nakada, K. (2009). High Incidence of Metabolically Active Brown Adipose Tissue in Healthy Adult Humans: Effects of Cold Exposure and Adiposity. *Diabetes, 58*(7), 1526-1531.

Samad, F., Hester, K. D., Yang, G., Hannun, Y. A., & Bielawski, J. (2006). Altered Adipose and Plasma Sphingolipid Metabolism in Obesity: A Potential Mechanism for Cardiovascular and Metabolic Risk. *Diabetes, 55*(9), 2579-2587.

Samson, D. E., & Slee, J. (1981). Factors Affecting Resistance to Induced Body Cooling in Newborn Lambs of 10 Breeds. *Animal Science, 33*(1), 59-65.

Samuelson, D. (2007). Textbook of Veterinary Histology, Saunders Elsevier, St. *Louis, missouri*.

Sangild, P., Fowden, A., & Trahair, J. (2000). How Does the Foetal Gastrointestinal Tract Develop in Preparation for Enteral Nutrition after Birth? *Livestock Production Science, 66*(2), 141-150.

Sawalha, R., Conington, J., Brotherstone, S., & Villanueva, B. (2007). Analyses of Lamb Survival of Scottish Blackface Sheep. *Animal, 1*(1), 151-157.

Scales, G., Burton, R., & Moss, R. (1986). Lamb Mortality, Birthweight, and Nutrition in Late Pregnancy. *New Zealand Journal of Agricultural Research, 29*(1), 75-82.

Schermer, S. J., Bird, J. A., Lomax, M. A., Shepherd, D., & Symonds, M. E. (1996). Effect of Fetal Thyroidectomy on Brown Adipose Tissue and Thermoregulation in Newborn Lambs. *Reproduction, Fertility and Development, 8*(6), 995-1002.

Schmid-Wendtner, M.-H., & Dill-Müller, D. (2008). Ultrasound Technology in Dermatology. *, 27, 1, 27*(1), 44-51.

Schmid, A., Schlegel, J., Thomalla, M., Karrasch, T., & Schäffler, A. (2019). Evidence of Functional Bile Acid Signaling Pathways in Adipocytes. *Molecular and cellular endocrinology, 483*, 1-10.

Schreiber, R., Diwoky, C., Schoiswohl, G., Feiler, U., Wongsiriroj, N., Abdellatif, M., . . . Sedej, S. (2017). Cold-Induced Thermogenesis Depends on Atgl-Mediated Lipolysis in Cardiac Muscle, but Not Brown Adipose Tissue. *Cell metabolism, 26*(5), 753-763. e757.

Schweizer, S., Liebisch, G., Oeckl, J., Hoering, M., Seeliger, C., Schiebel, C., . . . Ecker, J. (2019). The Lipidome of Primary Murine White, Brite, and Brown Adipocytes—Impact of Beta-Adrenergic Stimulation. *PLoS biology, 17*(8), e3000412.

Shamsi, F., Wang, C.-H., & Tseng, Y.-H. (2021). The Evolving View of Thermogenic Adipocytes—Ontogeny, Niche and Function. *Nature Reviews Endocrinology, 17*(12), 726-744.

Sheath, G., Webby, R., Keeling, P., Thomson, R., Page, C., & Burton, G. (1999). *The Results and Success Factors of Nine Group Farm Monitoring Programmes.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Shimizu, I., Aprahamian, T., Kikuchi, R., Shimizu, A., Papanicolaou, K. N., MacLauchlan, S., . . . Walsh, K. (2014). Vascular Rarefaction Mediates Whitening of Brown Fat in Obesity. *J Clin Invest, 124*(5), 2099-2112. doi:10.1172/JCI71643

Shin, H., Ma, Y., Chanturiya, T., Cao, Q., Wang, Y., Kadegowda, A. K., . . . Shi, H. (2017). Lipolysis in Brown Adipocytes Is Not Essential for Cold-Induced Thermogenesis in Mice. *Cell metabolism, 26*(5), 764-777. e765.

Silanikove, N. (2000). Effects of Heat Stress on the Welfare of Extensively Managed Domestic Ruminants. *Livestock Production Science, 67*(1-2), 1-18.

Simcox, J., Geoghegan, G., Maschek, J. A., Bensard, C. L., Pasquali, M., Miao, R., . . . Kershaw, E. E. (2017). Global Analysis of Plasma Lipids Identifies Liver-Derived Acylcarnitines as a Fuel Source for Brown Fat Thermogenesis. *Cell metabolism, 26*(3), 509-522. e506.

Simoneau, J., Dumontier, S., Gosselin, R., & Scott, M. S. (2021). Current Rna-Seq Methodology Reporting Limits Reproducibility. *Briefings in Bioinformatics, 22*(1), 140-145.

Singh, A., Prasad, M., Mishra, B., Manjunath, S., Sahu, A. R., Bhuvana Priya, G., . . . Gandham, R. K. (2017). Transcriptome Analysis Reveals Common Differential and Global Gene Expression Profiles in Bluetongue Virus Serotype 16 (Btv-16) Infected Peripheral Blood Mononuclear Cells (Pbmcs) in Sheep and Goats. *Genom Data, 11*, 62-72. doi:10.1016/j.gdata.2016.12.001

Slee, J. (1978). The Effects of Breed, Birthcoat and Body Weight on the Cold Resistance of Newborn Lambs. *Animal Science, 27*(1), 43-49.

Slee, J. (1981). A Review of Genetic Aspects of Survival and Resistance to Cold in Newborn Lambs. *Livestock Production Science, 8*(5), 419-429.

Slee, J., Alexander, G., Bradley, L., Jackson, N., & Stevens, D. (1991). Genetic Aspects of Cold Resistance and Related Characters in Newborn Merino Lambs. *Australian Journal of Experimental Agriculture, 31*(2), 175-182.

Slee, J., Alexander, G., Bradley, L., Jackson, N., & Stevens, D. (1991a). Genetic Aspects of Cold Resistance and Related Characters in Newborn Merino Lambs. *Australian Journal of Experimental Agriculture, 31*(2), 175-182.

Smith, J., & Knight, T. (1998). Reproductive Management of Sheep. *Reproductive management of grazing ruminants in New Zealand, 12*, 113-144.

Soltani-Ghombavani, M., Dukkipati, V., & Blair, H. (2017). *Genetic Association of Skin Thickness with Lamb Survival from Birth to Weaning, and Growth and Wool Traits in New Zealand Romney Sheep.* Paper presented at the Proceedings of the 22nd Conference of the Association for the Advancement of Animal Breeding and Genetics, Townsville, Queensland, Australia.

Soltani-Ghombavani, M., Dukkipati, V. S. R., & Blair, H. T. (2021). *Skin Thickness as a Potential Indirect Trait for Genetic Improvement of Lamb Survival: A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Animal Science at Massey University, Palmerston North, New Zealand.* Massey University,

Southey, B., Rodriguez-Zas, S. L., & Leymaster, K. (2001). Survival Analysis of Lamb Mortality in a Terminal Sire Composite Population. *Journal of Animal Science, 79*(9), 2298-2306.

Speakman, J. R., & Ward, S. (1998). Infrared Thermography: Principles and Applications. *ZOOLOGY-JENA-, 101*, 224-232.

Stanford, K., Jones, S., & Price, M. (1998). Methods of Predicting Lamb Carcass Composition: A Review. *Small Ruminant Research, 29*(3), 241-254.

Stark, R., Grzelak, M., & Hadfield, J. (2019). Rna Sequencing: The Teenage Years. *Nature reviews genetics, 20*(11), 631-656.

Stewart, M., Webster, J., Schaefer, A., Cook, N., & Scott, S. (2005). Infrared Thermography as a Non-Invasive Tool to Study Animal Welfare. *Animal welfare, 14*(4), 319-325.

Stott, A., & Slee, J. (1987). The Effects of Litter Size, Sex, Age, Body Weight, Dam Age and Genetic Selection for Cold Resistance on the Physiological Responses to Cold Exposure of Scottish Blackface Lambs in a Progressively Cooled Water Bath. *Animal Science, 45*(3), 477-491.

Strosberg, A. D. (1997). Structure and Function of the B3-Adrenergic Receptor. *Annual review of pharmacology and toxicology, 37*(1), 421-450.

Su, M., Subbaraj, A. K., Fraser, K., Qi, X., Jia, H., Chen, W., . . . Roy, N. C. (2019). Lipidomics of Brain Tissues in Rats Fed Human Milk from Chinese Mothers or Commercial Infant Formula. *Metabolites, 9*(11), 253.

Suarez-Vega, A., Arranz, J. J., Perez, V., de la Fuente, L. F., Mateo, J., & Gutierrez-Gil, B. (2018). Early Adipose Deposits in Sheep: Comparative Analysis of the Perirenal Fat Transcriptome of Assaf and Churra Suckling Lambs. *Anim Genet, 49*(6), 605-617. doi:10.1111/age.12725

Sud, M., Fahy, E., Cotter, D., Brown, A., Dennis, E. A., Glass, C. K., . . . Subramaniam, S. (2007). Lmsd: Lipid Maps Structure Database. *Nucleic Acids Res, 35*(Database issue), D527-532. doi:10.1093/nar/gkl838

Suter, R. (2010). *A Sheep Mortality Survey – Experience with a Long-Term Victorian Program*.

Sykes, A., Griffiths, R., & Slee, J. (1976). Influence of Breed, Birth Weight and Weather on the Body Temperature of Newborn Lambs. *Animal Science, 22*(3), 395-402.

Symonds. (2013). Brown Adipose Tissue Growth and Development. *Scientifica (Cairo), 2013*, 305763. doi:10.1155/2013/305763

Symonds, Bird, J. A., Clarke, L., Gate, J. J., & Lomax, M. A. (1995). Nutrition, Temperature and Homeostasis During Perinatal Development. *Exp Physiol, 80*(6), 907-940. doi:10.1113/expphysiol.1995.sp003905

Symonds, M., Andrews, D., & Johnson, P. (1989). The Control of Thermoregulation in the Developing Lamb During Slow Wave Sleep. *Journal of developmental physiology, 11*(5), 289-298.

Symonds, M., Phillips, I., Anthony, R., Owens, J., & McMillen, I. (1998). Prolactin Receptor Gene Expression and Foetal Adipose Tissue. *Journal of Neuroendocrinology, 10*(11), 885-890.

Symonds, M. E., Stephenson, T., Gardner, D. S., & Budge, H. (2007). Long-Term Effects of Nutritional Programming of the Embryo and Fetus: Mechanisms and Critical Windows. *Reproduction, Fertility and Development, 19*(1), 53-63.

Tait, I., Hickson, R., Kenyon, P., & Blair, H. (2015). *Relationships among Skin Thickness, Fat Depth and Eye Muscle Depth in Sires and Their Progeny, and the Breeding Value for Survival of Their Progeny.* Paper presented at the Proceedings of the New Zealand Society of Animal Production.

Tait, R. G. (2016). Ultrasound Use for Body Composition and Carcass Quality Assessment in Cattle and Lambs. *Veterinary Clinics: Food Animal Practice, 32*(1), 207-218.

Tan, C. Y., Virtue, S., Bidault, G., Dale, M., Hagen, R., Griffin, J. L., & Vidal-Puig, A. (2015). Brown Adipose Tissue Thermogenic Capacity Is Regulated by Elovl6. *Cell reports, 13*(10), 2039-2047.

Tarbotton, I., & Webby, R. (1999). *Variation in Lamb Survival within Farm and between Farms: Results from Farmer Studies.* Paper presented at the PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION.

Taube, M., Andersson-Assarsson, J. C., Lindberg, K., Pereira, M. J., Gäbel, M., Svensson, M. K., . . . Svensson, P.-A. (2015). Evaluation of Reference Genes for Gene Expression Studies in Human Brown Adipose Tissue. *Adipocyte, 4*(4), 280-285.

Team, R. C. (2013). R: A Language and Environment for Statistical Computing.

Teixeira, A., Joy, M., & Delfa, R. (2008). In Vivo Estimation of Goat Carcass Composition and Body Fat Partition by Real-Time Ultrasonography. *Journal of Animal Science, 86*(9), 2369-2376.

Thrush, A., Gagnon, A., & Sorisky, A. (2012). Pkc Activation Is Required for Tsh-Mediated Lipolysis Via Perilipin Activation. *Hormone and Metabolic Research, 44*(11), 825-831.

Tseng, Y.-H., Kokkotou, E., Schulz, T. J., Huang, T. L., Winnay, J. N., Taniguchi, C. M., . . . Yamamoto, Y. (2008). New Role of Bone Morphogenetic Protein 7 in Brown Adipogenesis and Energy Expenditure. *Nature, 454*(7207), 1000-1004.

Tsugawa, H., Cajka, T., Kind, T., Ma, Y., Higgins, B., Ikeda, K., . . . Arita, M. (2015). Ms-Dial: Data-Independent Ms/Ms Deconvolution for Comprehensive Metabolome Analysis. *Nature Methods, 12*(6), 523-526. doi:10.1038/nmeth.3393

Tumas, J., Kvederaviciute, K., Petrulionis, M., Kurlinkus, B., Rimkus, A., Sakalauskaite, G., . . . Sileikis, A. (2016). Metabolomics in Pancreatic Cancer Biomarkers Research. *Medical Oncology, 33*(12), 133.

Turton, J., & Hooson, J. (1997). *Target Organ Pathology: A Basic Text*: CRC Press.

Tvrdik, P., Westerberg, R., Silve, S., Asadi, A., Jakobsson, A., Cannon, B., . . . Jacobsson, A. (2000). Role of a New Mammalian Gene Family in the Biosynthesis of Very Long Chain Fatty Acids and Sphingolipids. *The Journal of cell biology, 149*(3), 707-718.

Vadlejch, J., Knížková, I., Makovcová, K., Kunc, P., Jankovská, I., Janda, K., . . . Langrová, I. (2010). Thermal Profile of Rabbits Infected with Eimeria Intestinalis. *Veterinary parasitology, 171*(3-4), 343-345.

Van De Geijn, B., McVicker, G., Gilad, Y., & Pritchard, J. K. (2015). Wasp: Allele-Specific Software for Robust Molecular Quantitative Trait Locus Discovery. *Nature methods, 12*(11), 1061-1063.

Van der Merwe, D. A., Brand, T. S., Theron, P. G., Hoffman, L. C., & Jackson-Moss, C. A. (2021). Sheepskin Leather Quality Characteristics of South African Breeds. *Small Ruminant Research, 199*, 106365. doi:10.1016/j.smallrumres.2021.106365

Van Meer, G., Voelker, D. R., & Feigenson, G. W. (2008). Membrane Lipids: Where They Are and How They Behave. *Nature reviews Molecular cell biology, 9*(2), 112-124.

van Raalte, D. H., Li, M., Pritchard, P. H., & Wasan, K. M. (2004). Peroxisome Proliferator-Activated Receptor (Ppar)-A: A Pharmacological Target with a Promising Future. *Pharmaceutical research, 21*(9), 1531-1538.

Varela-Martinez, E., Corsi, G. I., Anthon, C., Gorodkin, J., & Jugo, B. M. (2021). Novel Circrna Discovery in Sheep Shows Evidence of High Backsplice Junction Conservation. *Sci Rep, 11*(1), 427. doi:10.1038/s41598-020-79781-2

Venus, M., Waterman, J., & McNab, I. (2010). Basic Physiology of the Skin. *Surgery (Oxford), 28*(10), 469-472.

Vermorel, M., Dardillat, C., Vernet, J., & Demigne, C. (1983). Energy Metabolism and Thermoregulation in the Newborn Calf [Neonatal Mortality, Thermogenesis, Calving Conditions]. *Annales de Recherches Veterinaires (France)*.

Vermunt, J., Stafford, K., & Thompson, K. (1995). Observations on Colostral Intake in Newborn Dairy Calves. *New Zealand Veterinary Journal, 43*(5), 205-206.

Villarroya, F., Iglesias, R., & Giralt, M. (2007). Ppars in the Control of Uncoupling Proteins Gene Expression. *PPAR Res, 2007*, 74364. doi:10.1155/2007/74364

Waldron, D., Clarke, J., Rae, A., Kirton, A., & Bennett, G. (1992). Genetic and Phenotypic Parameter Estimates for Selection to Improve Lamb Carcass Traits. *New Zealand Journal of Agricultural Research, 35*(3), 287-298. doi:10.1080/00288233.1992.10427506

Walker, G. E., Verti, B., Marzullo, P., Savia, G., Mencarelli, M., Zurleni, F., . . . Di Blasio, A. M. (2007). Deep Subcutaneous Adipose Tissue: A Distinct Abdominal Adipose Depot. *Obesity, 15*(8), 1933-1943.

Walker, S., Kleemann, D., & Bawden, C. (2002). Sheep Reproduction in Australia. *Current status and potential for improvement through flock management and gene discovery Meat and Livestock Australia. MS, 9*.

Wang, Q., Li, H., Tajima, K., Verkerke, A. R., Taxin, Z. H., Hou, Z., . . . Abe, I. (2022). Post-Translational Control of Beige Fat Biogenesis by Prdm16 Stabilization. *Nature*, 1-8.

Wang, Z., Gerstein, M., & Snyder, M. (2009). Rna-Seq: A Revolutionary Tool for Transcriptomics. *Nature reviews genetics, 10*(1), 57-63.

Wang, Z., Wang, Q. A., Liu, Y., & Jiang, L. (2021). Energy Metabolism in Brown Adipose Tissue. *The FEBS Journal, 288*(12), 3647-3662.

Wenk, M. R. (2005). The Emerging Field of Lipidomics. *Nature reviews Drug discovery, 4*(7), 594-610.

Whittle, A. J., Carobbio, S., Martins, L., Slawik, M., Hondares, E., Vázquez, M. J., . . . Rodriguez-Cuenca, S. (2012). Bmp8b Increases Brown Adipose Tissue Thermogenesis through Both Central and Peripheral Actions. *Cell, 149*(4), 871-885.

Whittle, A. J., López, M., & Vidal-Puig, A. (2011). Using Brown Adipose Tissue to Treat Obesity–the Central Issue. *Trends in molecular medicine, 17*(8), 405-411.

Wickham, H. (2016). *Ggplot2: Elegant Graphics for Data Analysis*: Springer-Verlag New York.

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D. A., François, R., . . . Hester, J. (2019). Welcome to the Tidyverse. *Journal of open source software, 4*(43), 1686.

Williams, A., & Thornberry, K. (1992). *The Skin Thickness of Medium Wool Merino Sheep and Its Relationship to Wool Production.* Paper presented at the Proceedings of the Australian Society of Animal Production.

Wilmer, J. L., Burleson, F. G., Kayama, F., Kanno, J., & Luster, M. I. (1994). Cytokine Induction in Human Epidermal Kerationocytes Exposed to Contact Irritants and Its Relation to

Chemical-Induced Inflammation in Mouse Skin. *Journal of Investigative Dermatology, 102*(6), 915-922.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., . . . Scarpulla, R. C. (1999). Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator Pgc-1. *Cell, 98*(1), 115-124.

Wuliji, T., Dodds, K., Land, J., Andrews, R., & Turner, P. (2001). Selection for Ultrafine Merino Sheep in New Zealand: Heritability, Phenotypic and Genetic Correlations of Live Weight, Fleece Weight and Wool Characteristics in Yearlings. *Animal Science, 72*(2), 241-250. doi:10.1017/S1357729800055739

Xu, Z., You, W., Zhou, Y., Chen, W., Wang, Y., & Shan, T. (2019). Cold-Induced Lipid Dynamics and Transcriptional Programs in White Adipose Tissue. *BMC biology, 17*(1), 1-21.

Xue, Y., Petrovic, N., Cao, R., Larsson, O., Lim, S., Chen, S., . . . Nedergaard, J. (2009). Hypoxia-Independent Angiogenesis in Adipose Tissues During Cold Acclimation. *Cell metabolism, 9*(1), 99-109.

Yamashita, H., Wang, Z., Wang, Y., Segawa, M., Kusudo, T., & Kontani, Y. (2008). Induction of Fatty Acid-Binding Protein 3 in Brown Adipose Tissue Correlates with Increased Demand for Adaptive Thermogenesis in Rodents. *Biochemical and biophysical research communications, 377*(2), 632-635.

Yapi, C., Boylan, W., & Robinson, R. (1990). Factors Associated with Causes of Preweaning Lamb Mortality. *Preventive veterinary medicine, 10*(1-2), 145-152.

Yoneshiro, T., Aita, S., Matsushita, M., Kayahara, T., Kameya, T., Kawai, Y., . . . Saito, M. (2013). Recruited Brown Adipose Tissue as an Antiobesity Agent in Humans. *The Journal of clinical investigation, 123*(8), 3404-3408.

Yore, M. M., Syed, I., Moraes-Vieira, P. M., Zhang, T., Herman, M. A., Homan, E. A., . . . Peroni, O. D. (2014). Discovery of a Class of Endogenous Mammalian Lipids with Anti-Diabetic and Anti-Inflammatory Effects. *Cell, 159*(2), 318-332.

Youle, R. J., & Van Der Bliek, A. M. (2012). Mitochondrial Fission, Fusion, and Stress. *Science, 337*(6098), 1062-1065.

Young, M. D., Wakefield, M. J., Smyth, G. K., & Oshlack, A. (2010). Gene Ontology Analysis for Rna-Seq: Accounting for Selection Bias. *Genome biology, 11*(2), 1-12.

Yu, X. X., Lewin, D. A., Forrest, W., & Adams, S. H. (2002). Cold Elicits the Simultaneous Induction of Fatty Acid Synthesis and B-Oxidation in Murine Brown Adipose Tissue: Prediction from Differential Gene Expression and Confirmation in Vivo. *The FASEB Journal, 16*(2), 155-168.

Yu, Y., Song, X., Du, L., & Wang, C. (2009). Molecular Characterization of the Sheep Cib1 Gene. *Molecular biology reports, 36*(7), 1799-1809.

Yuan, Y.-N., Liu, W.-Z., Liu, J.-H., Qiao, L.-Y., & Wu, J.-L. (2012). Cloning and Ontogenetic Expression of the Uncoupling Protein 1 Gene Ucp1 in Sheep. *Journal of applied genetics, 53*(2), 203-212.

Zaborski, D., Grzesiak, W., Szatkowska, I., Dybus, A., Muszynska, M., & Jedrzejczak, M. (2009). Factors Affecting Dystocia in Cattle. *Reproduction in domestic animals, 44*(3), 540-551.

Zanna, G., Fondevila, D., Ferrer, L., & Espada, Y. (2012). Evaluation of Ultrasonography for Measurement of Skin Thickness in Shar-Peis. *American journal of veterinary research, 73*(2), 220-226.

Zechner, R., Madeo, F., & Kratky, D. (2017). Cytosolic Lipolysis and Lipophagy: Two Sides of the Same Coin. *Nature reviews Molecular cell biology, 18*(11), 671-684.

Zhang, Z. H., Jhaveri, D. J., Marshall, V. M., Bauer, D. C., Edson, J., Narayanan, R. K., . . . Wray, N. R. (2014). A Comparative Study of Techniques for Differential Expression Analysis on Rna-Seq Data. *PLoS One, 9*(8), e103207.

Zhou, B., Xiao, J. F., Tuli, L., & Ressom, H. W. (2012). Lc-Ms-Based Metabolomics. *Molecular BioSystems, 8*(2), 470-481.

References

Zingaretti, M. C., Crosta, F., Vitali, A., Guerrieri, M., Frontini, A., Cannon, B., . . . Cinti, S. (2009). The Presence of Ucp1 Demonstrates That Metabolically Active Adipose Tissue in the Neck of Adult Humans Truly Represents Brown Adipose Tissue. *The FASEB Journal, 23*(9), 3113-3120.

References

Appendices

Appendix A. Supplementary data for Chapter 3

Supplementary tables

Table S3.1. Records available for each trait based on birth year, flock and sex for FocusPrime (**A**), Texel (**B**), Romney (**C**) and Highlander (**D**).

(**A**)

			Trait								
Breed	**Year**	**Flock**	**ST**	**Stemp**	**FD**	**EMD**	**EMW**	**WWT**	**LW6**	**LW12**	**FW12**
Focus Prime	2017	1	F 343	F 296	F 0	F 0	F 0	F 340	F 0	F 343	F 0
			M 459	M 400	M 458	M 454	M 458	M 455	M 457	M 459	M 0
		2	F 322	F 303	F 0	F 0	F 0	F 317	F 0	F 322	F 0
			M 103	M 100	M 103	M 103	M 103	M 102	M 103	M 103	M 0
	2019	1	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0
			M 861	M 0	M 861	M 857	M 858	M 860	M 861	M 445	M 0

(**B**)

			Trait								
Breed	**Year**	**Flock**	**ST**	**Stemp**	**FD**	**EMD**	**EMW**	**WWT**	**LW6**	**LW12**	**FW12**
Texel	2017	1	F 255	F 251	F 0	F 0	F 0	F 254	F 0	F 254	F 0
			M 231	M 203	M 230	M 229	M 229	M 229	M 230	M 231	M 0
	2019	1	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0
			M 246	M 0	M 264	M 246	M 246	M 244	M 264	M 162	M 0

(**C**)

			Trait								
Breed	**Year**	**Flock**	**ST**	**Stemp**	**FD**	**EMD**	**EMW**	**WWT**	**LW6**	**LW12**	**FW12**
Romney	2017	1	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0
			M 825	M 813	M 822	M 820	M 822	M 824	M 824	M 823	M 809

(**D**)

			Trait								
Breed	**Year**	**Flock**	**ST**	**Stemp**	**FD**	**EMD**	**EMW**	**WWT**	**LW6**	**LW12**	**FW12**
Highlander	2017	1	F 435	F 376	F 0	F 0	F 0	F 430	F 434	F 434	F 433
			M 236	M 236	M 236	M 236	M 236	M 233	M 236	M 236	M 236
		2	F 313	F 257	F 0	F 0	F 0	F 313	F 0	F 313	F 308
			M 180	M 180	M 177	M 176	M 176	M 175	M 177	M 180	M 176
	2019	1	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0	F 0
			M 637	M 0	M 637	M 637	M 637	M 634	M 637	M 0	M 0

ST: skin thickness, Stemp: skin temperature, FD: fat depth, EMD: eye-muscle depth, EMW: eye-muscle width, WWT: weaning weight, LW6: 6-month live weight, LW12: 12-month live weight, FW12: 12-month fleece weight. F: data available for females, M: data available for males.

Table S3.2. Estimates of heritabilities (± SE) for each trait in FocusPrime, Texel, Romney, Highlander, and all breeds considered as one, and their corresponding t-statistic and P-values.

Trait	Breed	Heritability	t-statistic= $h^2/SE(h^2)$	P-value
ST	**Focus Prime**	0.36 ± 0.06	6.00	1.16E-09
	Texel	0.23 ± 0.10	2.30	1.09E-02
	Romney	0.28 ± 0.10	2.80	2.61E-03
	Highlander	0.17 ± 0.05	3.40	3.44E-04
	All breeds	0.28 ± 0.04	7.00	1.43E-12
Stemp	**Focus Prime**	0.40 ± 0.11	3.64	1.40E-04
	Texel	0.16 ± 0.11	1.45	7.37E-02
	Romney	0.04 ± 0.03	1.33	9.19E-02
	Highlander	0.12 ± 0.06	2.00	2.28E-02
	All breeds	0.15 ± 0.04	3.75	8.93E-05
FD	**Focus Prime**	0.32 ± 0.08	4.00	3.28E-05
	Texel	0.33 ± 0.13	2.54	5.65E-03
	Romney	0.24 ± 0.10	2.40	8.31E-03
	Highlander	0.21 ± 0.08	2.63	4.31E-03
	All breeds	0.30 ± 0.05	6.00	1.05E-09
EMD	**Focus Prime**	0.39 ± 0.09	4.33	7.81E-06
	Texel	0.19 ± 0.11	1.73	4.20E-02
	Romney	0.40 ± 0.12	3.33	4.54E-04
	Highlander	0.33 ± 0.11	3.00	1.37E-03
	All breeds	0.39 ± 0.05	7.80	3.68E-15
EMW	**Focus Prime**	0.23 ± 0.07	3.29	5.09E-04
	Texel	0.17 ± 0.11	1.55	6.08E-02
	Romney	0.22 ± 0.09	2.44	7.45E-03
	Highlander	0.12 ± 0.07	1.71	4.37E-02
	All breeds	0.25 ± 0.04	6.25	2.21E-10
WWT	**Focus Prime**	0.49 ± 0.06	8.17	2.64E-16
	Texel	0.26 ± 0.10	2.60	4.76E-03
	Romney	0.28 ± 0.11	2.55	5.48E-03
	Highlander	0.42 ± 0.08	5.25	8.51E-08
	All breeds	0.43 ± 0.04	10.75	5.48E-27
LW6	**Focus Prime**	0.63 ± 0.09	7.00	1.72E-12
	Texel	0.20 ± 0.13	1.54	6.20E-02
	Romney	0.40 ± 0.12	3.33	4.54E-04
	Highlander	0.47 ± 0.09	5.22	9.98E-08
	All breeds	0.58 ± 0.05	11.60	4.73E-31

Trait	Breed	Heritability	t-statistic= $h^2/SE(h^2)$	P-value
LW12	**Focus Prime**	0.43 ± 0.08	5.38	4.14E-08
	Texel	0.26 ± 0.11	2.36	9.27E-03
	Romney	0.29 ± 0.10	2.90	1.92E-03
	Highlander	0.47 ± 0.10	4.70	1.40E-06
	All breeds	0.38 ± 0.05	7.60	1.73E-14
FW12	**Romney**	0.61 ± 0.14	4.36	7.33E-06
	Highlander	0.67 ± 0.10	6.70	1.39E-11
	All breeds	0.64 ± 0.08	8.00	9.24E-16

Appendix B. Supplementary data for Chapter 5

Supplementary tables

Table S5.1. Read numbers throughout the analysis and RIN values of RNA samples.

Sample ID	Tissue	Treatment	RIN	Reads from BGI	Reads mapped to the reference genome	Reads mapped to the reference genome (% of these to BGI reads)	Reads assigned to features	Reads assigned to features (% of these to BGI reads)
1	BAT	Cold Temp.	7.2	73066296	62678409	85.78	37025998	59.07
2	Thyroid		7.3	73703556	37847022	51.35	28612442	75.60
3	BAT	Cold Temp.	6.9	74057242	53767968	72.60	34382409	63.95
4	Thyroid		8.7	72994756	37261684	51.05	27779134	74.55
5	BAT	Cold Temp.	7.3	74135588	57856219	78.04	34575971	59.76
6	Thyroid		7.2	73555460	36284427	49.33	27222526	75.03
7	BAT	Cold Temp.	6.4	74207392	55011548	74.13	35464882	64.47
8	Thyroid		7.5	73503452	37107585	50.48	27728540	74.72
9	BAT	Cold Temp.	6.4	74281816	52448900	70.61	33246910	63.39
10	Thyroid		8.4	74298362	37245401	50.13	27846200	74.76
11	BAT	Cold Temp.	6.9	74622948	52364442	70.17	32844683	62.72
12	Thyroid		8.3	72844336	36158033	49.64	27240012	75.34
13	BAT	Ambient Temp.	6.9	73561744	56435097	76.72	33595377	59.53
14	Thyroid		7.3	73931808	37873413	51.23	28665528	75.69
15	BAT	Ambient Temp.	6.2	74028318	57368721	77.50	34191988	59.60
16	Thyroid		7.8	73546918	39006708	53.04	27689811	70.99
17	BAT	Ambient Temp.	6.0	73890946	55726034	75.42	34348089	61.64
18	Thyroid		7.8	73863628	38552528	52.19	30124871	78.14
19	BAT	Ambient Temp.	7.8	73655688	50330594	68.33	31556500	62.70
20	Thyroid		7.6	73344404	37385482	50.97	28086816	75.13

Sample ID	Tissue	Treatment	RIN	Reads from BGI	Reads mapped to the reference genome	Reads mapped to the reference genome (% of these to BGI reads)	Reads assigned to features	Reads assigned to features (% of these to BGI reads)
21	BAT	Ambient Temp.	5.8	74241580	59383107	79.99	34404783	57.94
22	Thyroid		7.6	72870850	38264692	52.51	27609747	72.15
23	BAT	Ambient Temp.	7.6	74590652	49348165	66.16	32529101	65.92
24	Thyroid		8.2	73279050	37064905	50.58	27701134	74.74
25	BAT	**Control**	7.5	74157486	58503089	78.89	34294146	58.62
26	Thyroid		7.9	73891274	37295112	50.47	28527034	76.49
27	BAT	**Control**	6.6	67153636	58065068	86.47	34523931	59.46
28	Thyroid		7.1	73762774	42849269	58.09	25062784	58.49
29	BAT	**Control**	6.0	74307724	50782515	68.34	32324089	63.65
30	Thyroid		8.3	73891614	36470965	49.36	28400537	77.87

Cold temp. is at 4 °C, and Ambient temp. is at 20-22 °C.

Table S5.2. DEGs list per tissue-treatment.

Table S5.3.; DESeq results for the 38 analyzed genes.

Table S5.4. GO terms and pathways per tissue-treatment.

These three tables are too large to be included in this thesis, therefore, for reasons of space, they are available online at: ***https://www.mdpi.com/article/10.3390/metabo12100996/s1****.*

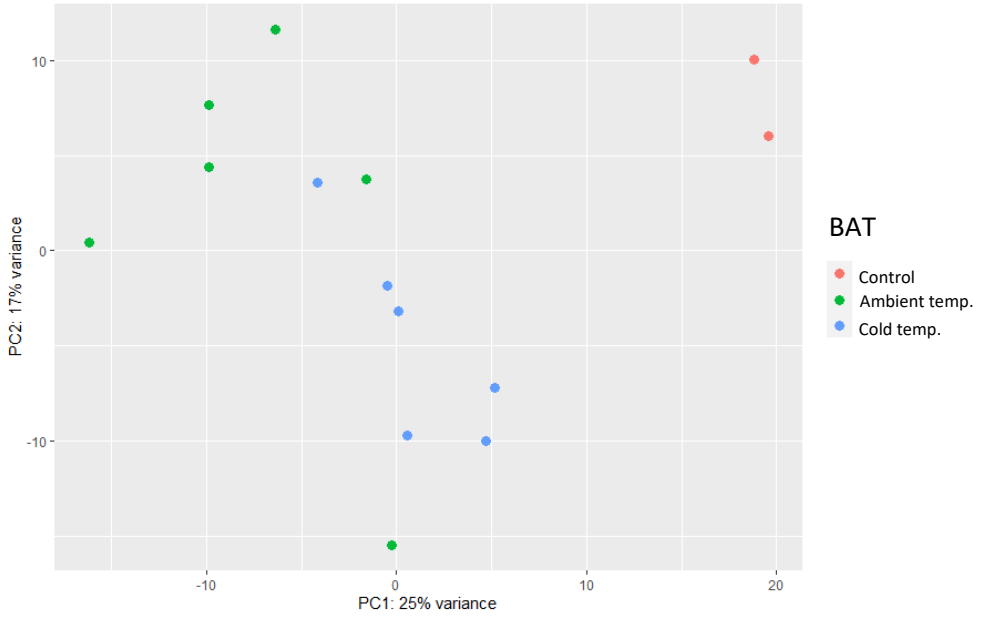
Table S5.5. Forward and reverse primer sequences and amplicon length of target and endogenous genes used for RT-qPCR validation.

Gene	Primer sequence Forward	Primer sequence Reverse	Amplicon length (bp)	Source
VEGFA	GGGCTGCTGTAATGACGAAAGT	CTGGCTTTGGTGAGGTTTGATC	94	Bedir et al. (2022)
PGC1A	TCGTGTTGGGCGAGAGAAAG	GGHGCCCTGGAATGGAAGTC	150	This paper
KI67	TGGCTTTGTTCTCTGCGTCT	CCTCGGAGCTGAAGTGTGAA	150	This paper
BMP4	TGACCAGGGTCTGCACAATG	CGCTCTACGTGGACTTCAGT	150	This paper
ADRB3	CGCCTCCAACATGCCCTAC	GCGTAGACGAAGAGCATCAC	81	Pope et al. (2014)
DIO2	AAGGCATTAGGAAGTGGCCC	GGGAAGTTGCAGACTGGGAA	150	This paper
ACTB	AGTCGGTTGGATCGAGCATC	AGAAGGAGGGTGGCTTTTGG	150	This paper
GAPDH	CTGCTGACGCTCCCATGTTTGT	TAAGTCCCTCCACGATGCCAAA	150	Yu et al. (2009)

Supplementary figures

Figure S5.1. Principal component analysis (PCA) plots of normalised sequence read counts in control vs. cold vs. ambient temperature groups for brown adipose tissue (BAT) (**A**) and Thyroid tissue (**B**).

(**A**)



(**B**)

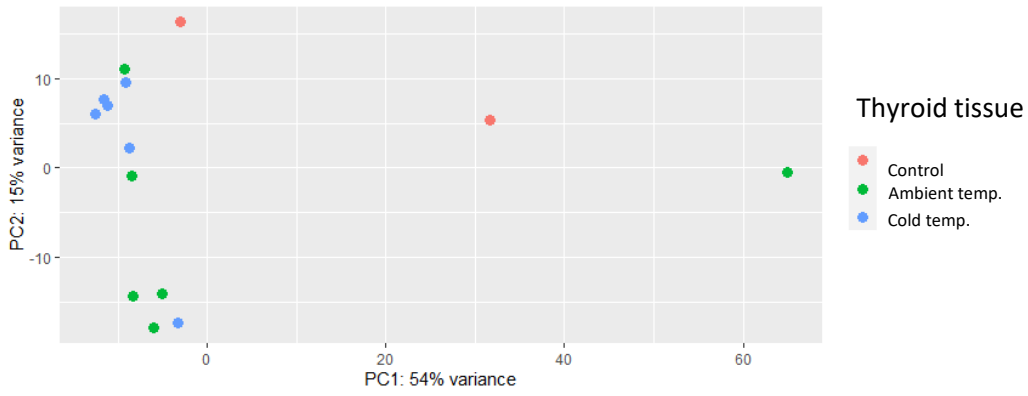
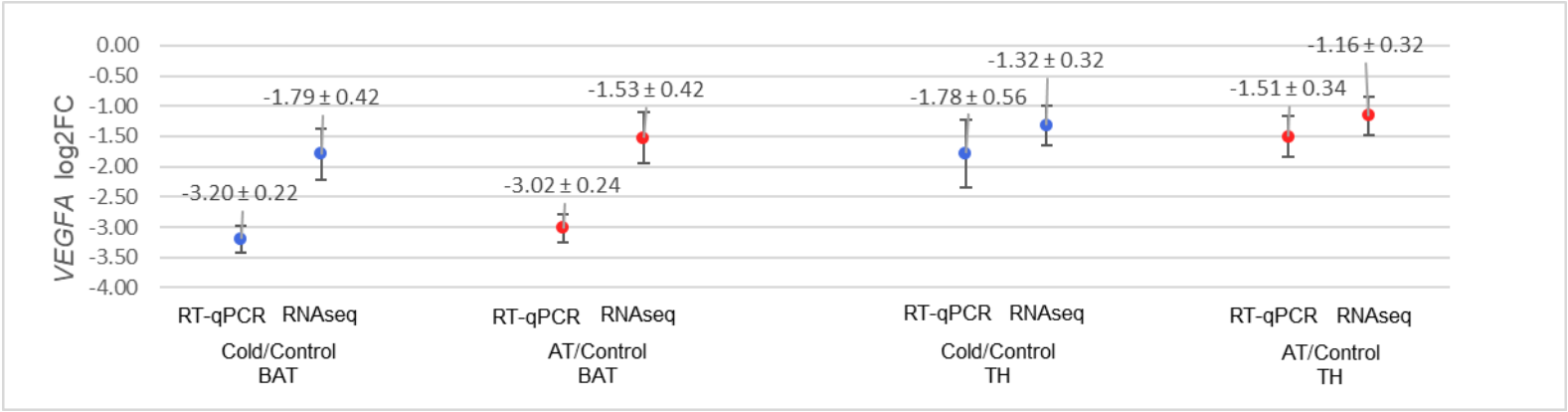
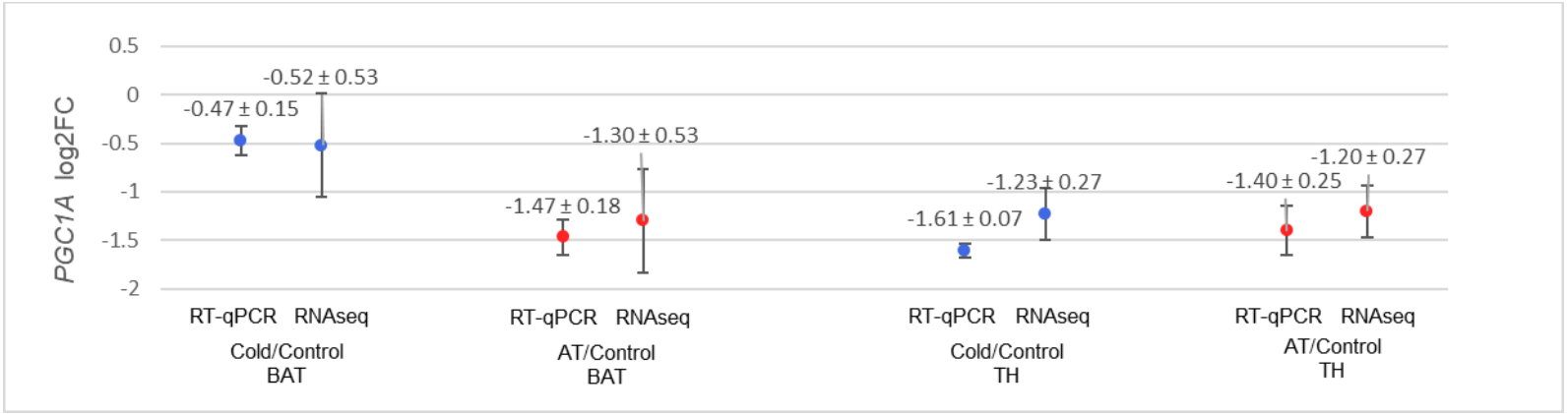
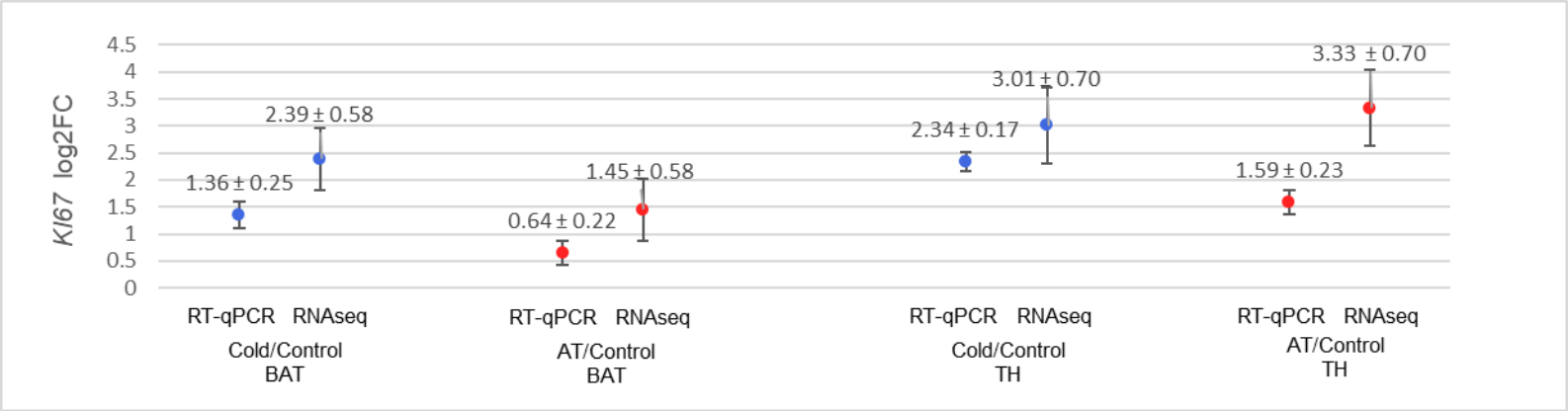
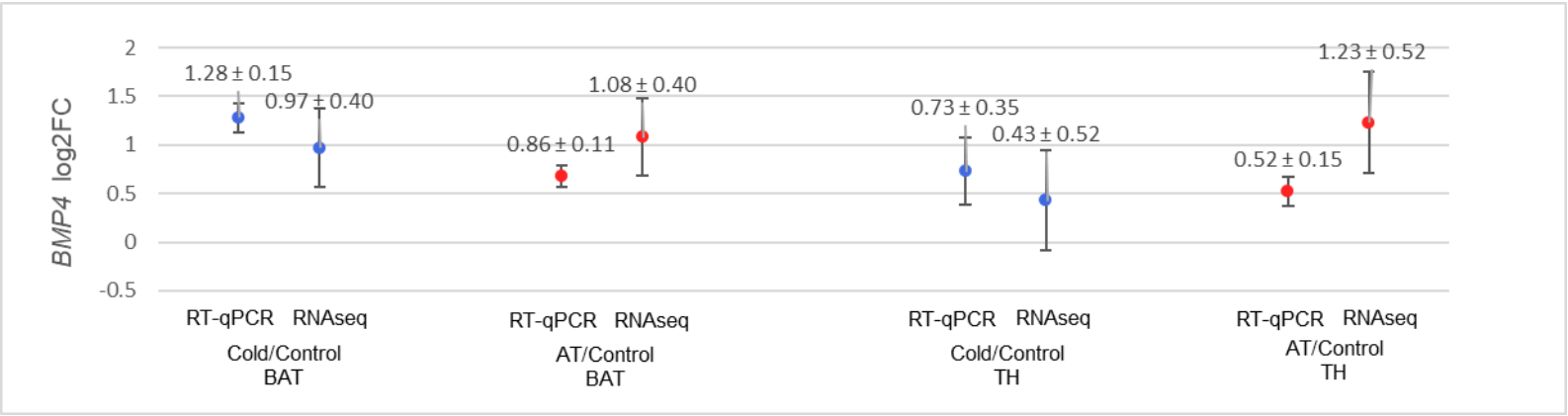


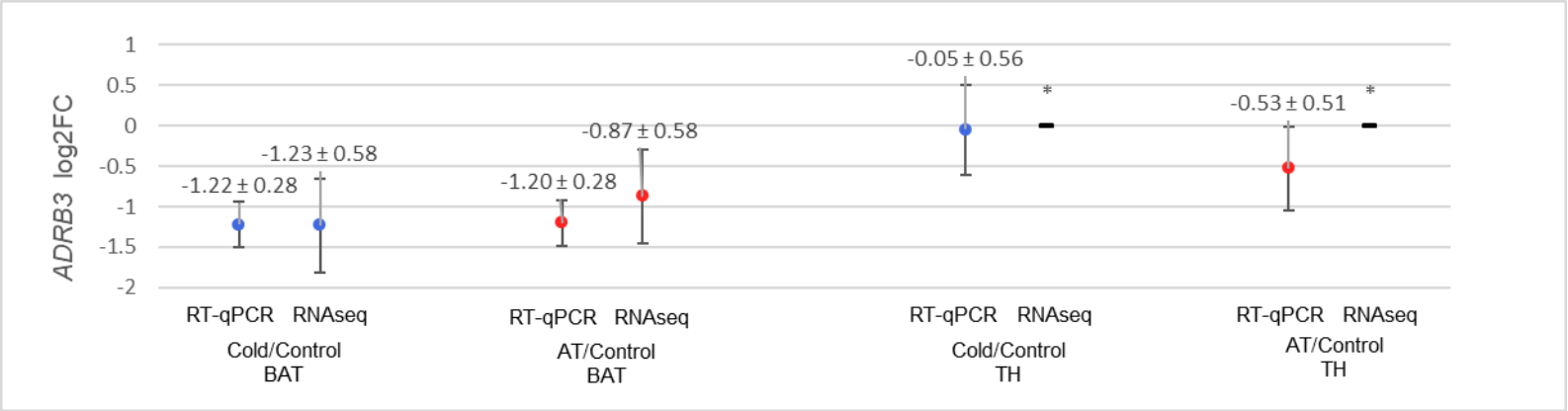
Figure S5.2. Fold change results of RT-qPCR target and endogenous genes compared to its corresponding RNAseq result (log2FC ± SE).

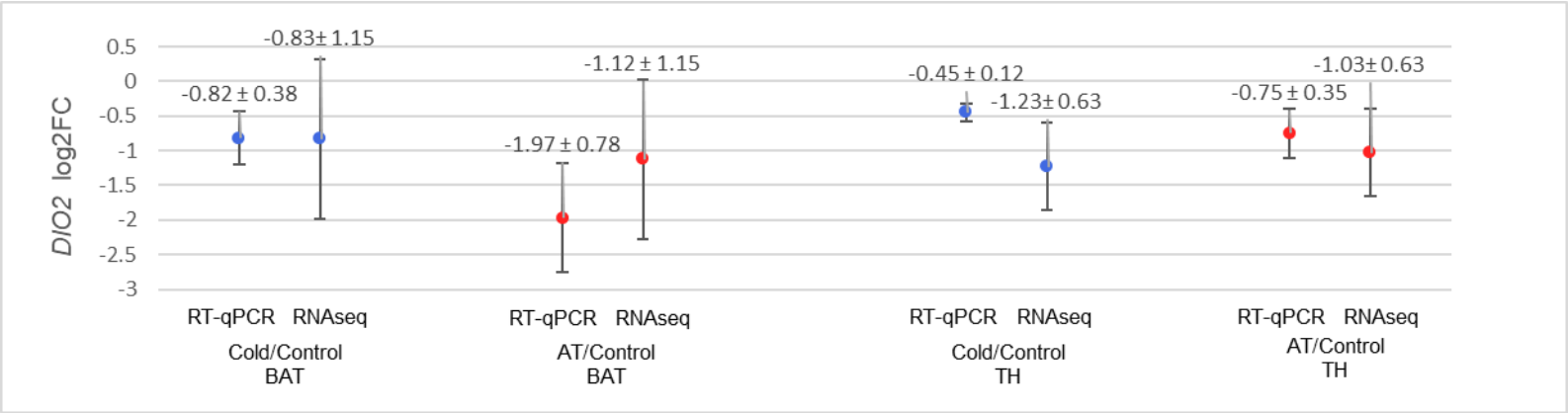





Ki67 log2FC
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1.36 ± 0.25
2.39 ± 0.58
0.64 ± 0.22
1.45 ± 0.58
2.34 ± 0.17
3.01 ± 0.70
1.59 ± 0.23
3.33 ± 0.70
RT-qPCR RNAseq
Cold/Control BAT
RT-qPCR RNAseq
AT/Control BAT
RT-qPCR RNAseq
Cold/Control TH
RT-qPCR RNAseq
AT/Control TH


BMP4 log2FC
2
1.5
1
0.5
0
-0.5
1.28 ± 0.15
0.97 ± 0.40
0.86 ± 0.11
1.08 ± 0.40
0.73 ± 0.35
0.43 ± 0.52
0.52 ± 0.15
1.23 ± 0.52
RT-qPCR RNAseq
Cold/Control BAT
RT-qPCR RNAseq
AT/Control BAT
RT-qPCR RNAseq
Cold/Control TH
RT-qPCR RNAseq
AT/Control TH





* Not enough counts for calculation of log2FC of ADRB3 in thyroid tissue via RNAseq

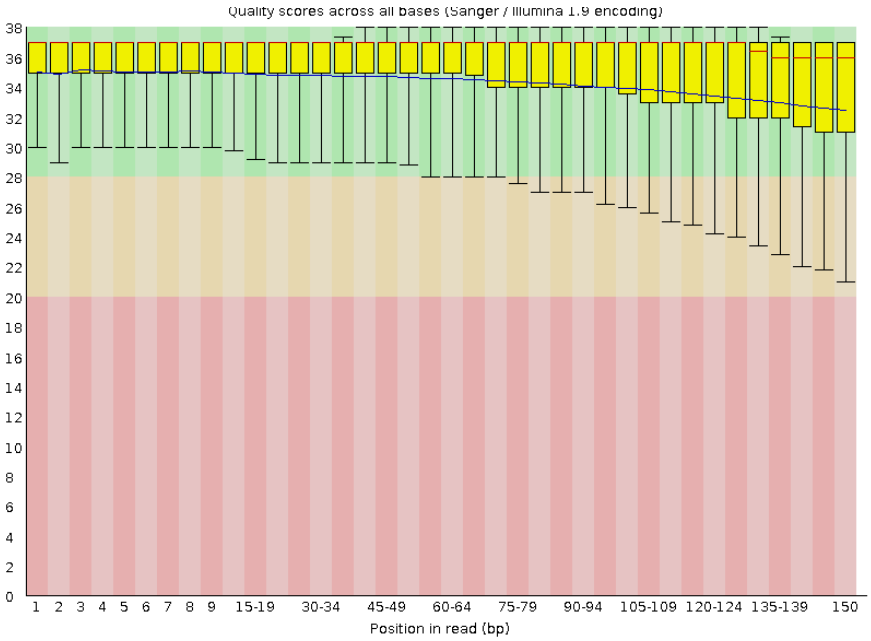
Summary of pipelines tested for quality control, mapping and reads quantification

Several workflows were tested in order to utilize the best one available for the present study's samples, according to the ones available and/or previously utilized in RNA-seq data analysis.

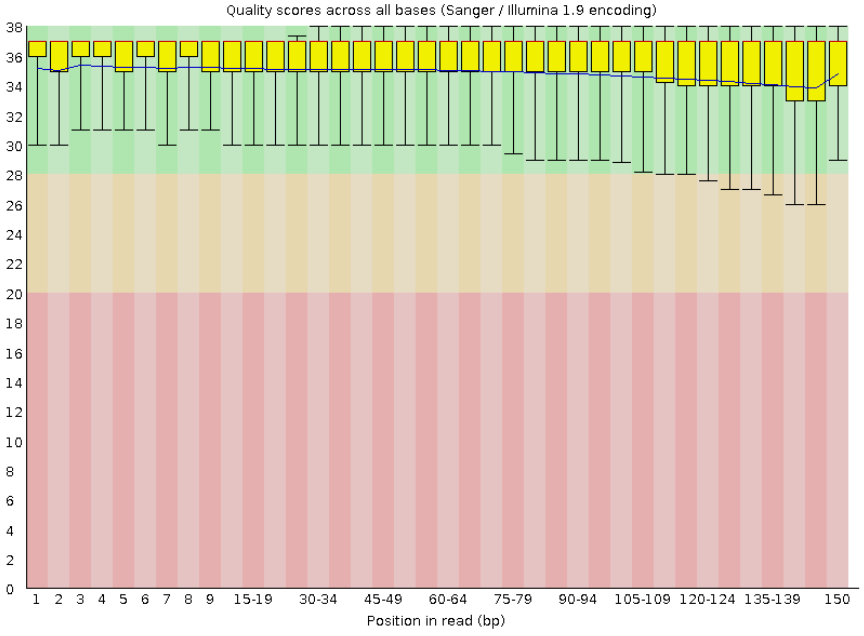
After checking the quality of the raw samples through FastQC and MultiQC, Trimmomatic was used to remove low quality bases and adapter sequences, already described in the Methods for Chapter 5, and was tested with different minimum lengths (MINLEN). The quality of reads was compared between raw (i.e., untrimmed), 25, 50 and 75 MINLEN (Figure S5.3), leaving all other Trimmomatic options the same, as described in the Methods section. The quality was visually improved with the further trims, but between MINLEN 50 and 75, the minimum and maximum quality didn't differ much, and it meant a loss of around 3% of the total reads (total reads for a forward pair from a random BAT sample as an example was 36.5 million), in addition to the 7.5% (2.7 million reads) of lost reads from raw to 50 MINLEN. Further investigation was done for this decision, where all reads from a random BAT and thyroid sample (from both forward and reverse sequence files), were processed with the two options, either 50 or 75 MINLEN and then mapped to the Rambouillet sheep reference genome using the mapping software STAR, as described in the Methods. This concluded in virtually the same mapping results for both options, therefore utilizing MINLEN 50 was the best quality option without losing too many reads.

Appendices

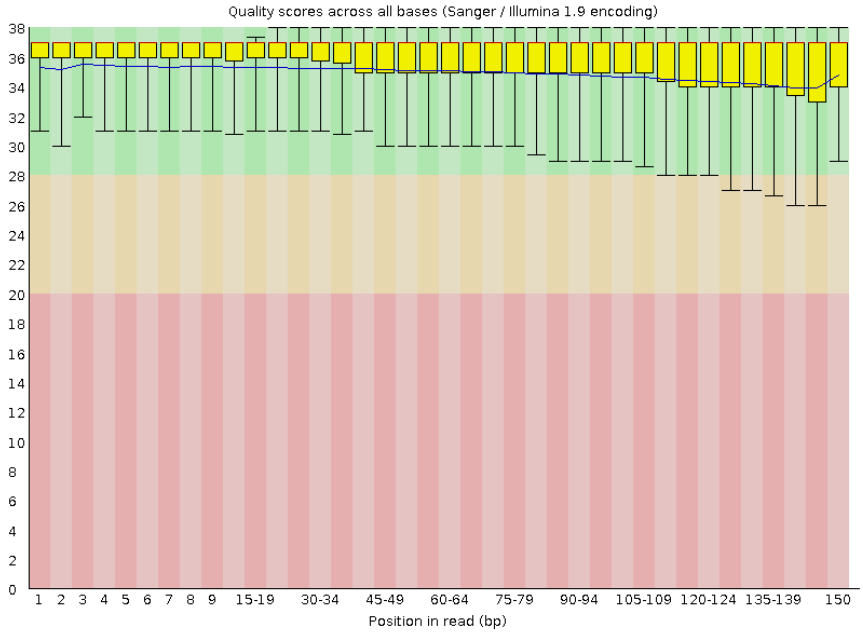
(**A**)



(**B**)



(**C**)



(**D**)

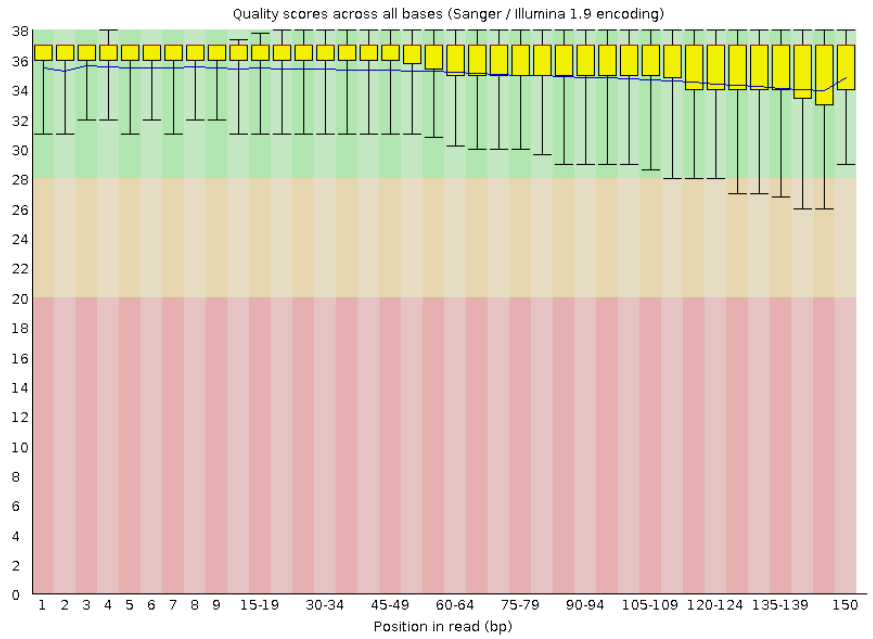


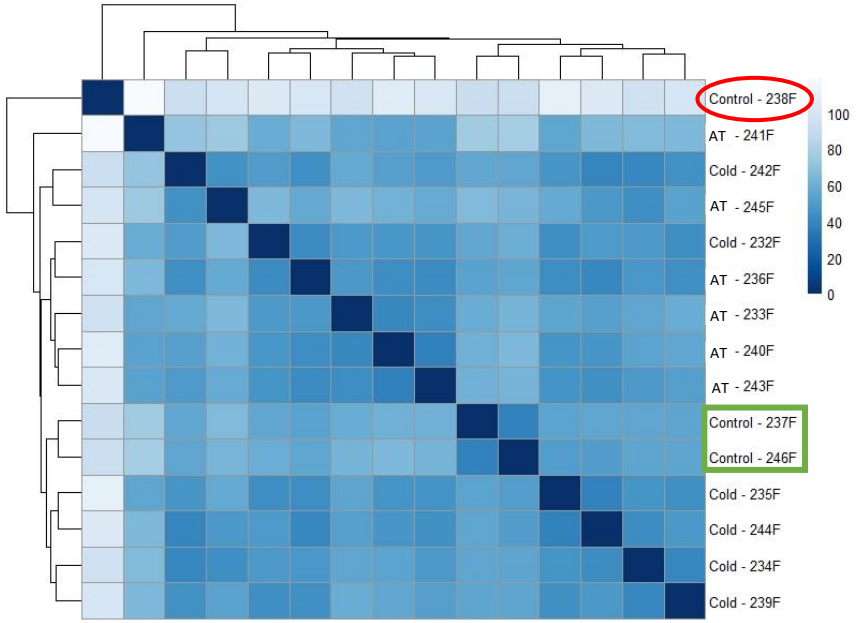
Figure S5.3. FastQC graphs for comparison of per base sequence quality on raw reads (**A**), raw reads processed with 25 (**B**), 50 (**C**) and 75 (**D**) MINLEN of the forward pair from the same random BAT sample. The Y-axis marks the quality scores across all bases (Sanger/Illumina 1.9 encoding), and the X-axis marks the position in the read (base pair).

A total of five mapping softwares and two quantification tools were tested, as they were described as mostly used in RNA-seq data analysis by Simoneau et al. (2021), among others previously used in RNA-seq data analysis. Other softwares were also investigated, but their performance or results were not satisfactory enough to be considered further and were thus excluded. The main mappers were STAR (Dobin et al., 2013), HISAT2 (Kim et al., 2019), BOWTIE2 (Langmead & Salzberg, 2012), Salmon (Patro et al., 2017) and Kallisto (Van De Geijn et al., 2015). All were used to map the reads from the present study's samples against the Rambouillet sheep reference genome (Oar_rambouillet_v1.0) from the RefSeq database (https://www.ncbi.nlm.nih.gov/assembly/GCF_002742125.1/).

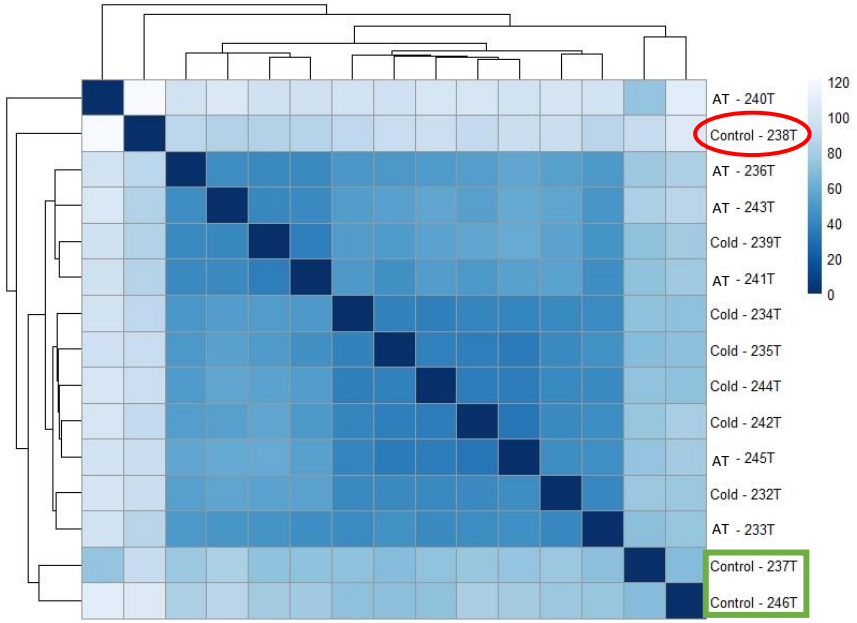
Results from mappers were quantified with either Subreads from the featureCounts package (Liao et al., 2014) or RSEM (Li & Dewey, 2011). The main reason behind this thorough testing was due to a multimapping issue (reads mapping to more than one location) that mostly occurred in the BAT samples of this study. Around half of the reads of any BAT sample were mapped to multiple loci, meanwhile only 10% were behaving similarly in the thyroid samples. There is no exact path to manage this, but according to Deschamps-Francoeur et al. (2020), a way to handle multi-mapped reads is to use the -M option in featureCounts, which will count all valid alignments for a read. As a default, featureCounts ignores multimapped reads, but as in the present study those reads were an important proportion, it was an issue if they were deleted in the final result. Finally, it was agreed that using the combination of STAR as a mapper and featureCounts with the -M option resulted in a much higher percentage of successfully assigned alignments and with no unassigned multimapped reads. Successful alignments in three random BAT samples increased from 18% on average to 60%, and from 63% to 77% for three random thyroid samples, making this workflow better suited for the samples used in this study.

Figure S5.4. Heatmaps of raw gene counts for each group; control, cold exposed (4 °C) and ambient exposed (AT) (20–22 °C), for brown adipose tissue (BAT) (**A**) and Thyroid tissue (**B**). Low read counts were prefiltered before these heatmaps were created, by removing rows that had fewer than ten reads in at least ten samples.

(**A**)



(**B**)



Appendix C. Supplementary data for Chapter 6

Supplementary tables

Table S6.1. Lipidomics data of BAT after 2 days of cold exposure.

				Cold		AT		
Category	**Main class**	**Lipid subclass**	**Lipid name**	**Mean**	**SD**	**Mean**	**SD**	**P-value**
Glycerophospholipids	Glycerophosphocholines	Lysophophatidylcholine	LPC 18:2	5974.00	1687.08	1079.67	961.96	1.05E-04
			LPC 18:1	22791.83	16200.12	4.50	9950.18	1.49E-02
			LPC 18:1/0:0	231.67	3440.08	-4003.17	1439.72	1.94E-02
	Glycerophosphocholines	Phosphatidylcholine	PC 34:2|PC 16:0_18:2	15626.33	6953.58	-370.50	6570.29	2.16E-03
			PC 34:1	-12647.67	10953.12	-27730.17	3141.55	8.84E-03
			PC 36:5	4907.17	2947.03	467.33	1828.05	1.06E-02
			PC 36:2|PC 17:1_19:1	46304.17	21425.57	18303.83	15196.17	2.60E-02
			PC 34:1|PC 16:0_18:1	-75432.33	42117.20	-123236.67	21488.71	3.27E-02
			PC 35:1	4430.67	2014.66	1469.00	2436.17	4.47E-02
			PC 36:4	2076.33	1311.96	5051.00	2929.80	4.66E-02
			PC 38:5|PC 16:0_22:5	-1893.50	1443.86	1915.67	3510.68	3.38E-02
	Glycerophosphoethanolamines	Lysophosphatidylethanolamine	LPE 18:1	879.00	2281.27	-2507.50	1602.92	1.39E-02
			LPE 18:1	6178.50	3735.62	1502.50	2155.45	2.41E-02
	Glycerophosphoethanolamines	Phosphatidylethanolamine	PE 36:1	1163.17	4413.84	-6266.00	5917.88	3.34E-02

Category	Main class	Lipid subclass	Lipid name	Cold		AT		P-value
				Mean	SD	Mean	SD	
Glycerolipids	Triradylglycerols	Oxidized triglyceride	TG 54:3;3O|TG 18:0_18:0_18:3;3O	-7529.50	7935.26	10069.00	12980.59	1.78E-02
		Triacylglycerol	TG 58:8|TG 18:1_18:1_22:6	-2603.83	6611.31	16520.83	15484.97	1.94E-02
			TG 58:8|TG 20:2_22:2_16:4	-1451.33	1041.17	1721.17	2727.60	2.38E-02
			TG 56:7|TG 16:0_18:1_22:6	-4761.33	14172.90	35353.67	35235.65	2.71E-02
		Ether-linked triacylglycerol	TG O-59:13|TG O-22:5_15:2_22:6	-11715.17	4700.65	-3596.17	7061.26	4.10E-02
	Diradylglycerols	Diacylglycerol	DG 34:0	-669.67	2909.83	-5577.83	3283.59	2.08E-02
Sphingolipids	Ceramides	Ceramide Esterified omega-hydroxy fatty acid-sphingosine	Cer 50:9;4O|Cer 14:1;2O/36:8;2O	1919.17	634.21	697.17	1012.42	3.12E-02

Data values are presented as Mean ± SD (n = 6 in each group). Means were considered different when $P < 0.05$.

Table S6.2. Lipidomics data of plasma after 1 day of cold exposure.

				Cold		AT		
Category	**Main class**	**Lipid subclass**	**Lipid name**	**Mean**	**SD**	**Mean**	**SD**	**P-value**
Glycerolipids	Triradylglycerols	Triacylglycerol	TG 51:12|TG 17:4_17:4_17:4	-196.83	1722.59	-3981.67	1848.51	4.32E-03
			TG 56:1|TG 20:0_20:0_16:1	4531.83	5021.07	15548.17	10144.95	3.84E-02
	Diradylglycerols	Diacylglycerol	DG 40:2	4139.50	2026.13	1439.17	871.74	1.34E-02
			DG 47:12	1747.17	2417.61	-1507.83	1589.54	2.03E-02
			DG 28:2	956.50	18471.78	-23707.50	13555.33	2.49E-02
			DG 46:11	4336.83	5809.48	-2864.50	4104.54	3.26E-02
			DG 44:8	1094.17	2480.48	-1648.83	1370.73	3.92E-02
			DG 48:12	-29984.67	29620.09	-75940.50	39790.93	4.66E-02
			DG 48:12	-2115.67	2006.42	-4317.83	1318.35	4.84E-02
	Glycosyldiradylglycerols	Digalactosyldiacylglycerol	DGDG 30:2|DGDG 14:1_16:1	7779.67	21211.57	30250.50	10792.76	4.33E-02
Sphingolipids	Phosphosphingolipids	Sphingomyelin	SM 35:1;2O|SM 17:1;2O/18:0	857449.83	237572.72	492847.33	146491.53	9.50E-03
			SM 36:3;2O|SM 21:1;2O/15:2	50040.17	28818.55	17578.83	11987.74	2.90E-02
			SM 42:3;2O|SM 20:1;2O/22:2	-26661.83	27230.99	-62278.17	24962.40	3.98E-02
			SM 42:3;2O|SM 20:3;2O/22:0	-26393.67	27217.45	-61829.00	25198.13	4.13E-02
	Phosphosphingolipids	Oxidized ceramide phosphoinositol	PI-Cer 39:4;3O	-4402.50	3757.53	-10901.17	3452.36	1.09E-02
	Ceramides	Ceramide non-hydroxyfatty acid-sphingosine	Cer 34:1;2O|Cer 18:1;2O/16:0	3541.33	2066.41	715.67	1476.32	2.14E-02

Category	Main class	Lipid subclass	Lipid name	Cold		AT		P-value
				Mean	SD	Mean	SD	
	Sphingoid bases	Phytosphingosine	SPB 20:0;3O	-84.50	3570.38	-6569.67	5290.15	3.21E-02
Glycerophospholipids	Glycerophosphocholines	Lysophophatidylcholine	LPC 18:1(d7)	2310.00	36880.07	-49907.33	30124.79	2.29E-02
		Phosphatidylcholine	PC 34:1|PC 16:0_18:1	-710101.67	3206269.44	4158387.50	3222603.07	2.55E-02
			PC 35:6|PC 18:2_17:4	3537.33	7118.45	-8341.67	9822.60	3.74E-02
	Glycerophosphoserines	Phosphatidylserine	PS 38:5|PS 20:1_18:4	54129.17	17676.46	33585.33	9767.15	3.19E-02

Data values are presented as Mean ± SD (n = 6 in each group). Means were considered different when $P < 0.05$.

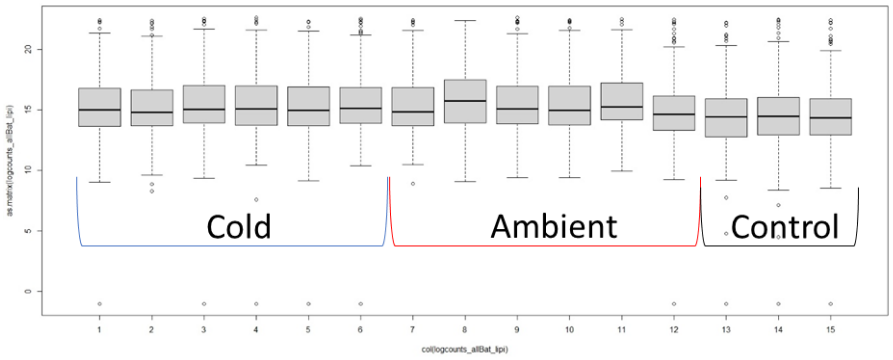
Table S6.3. Lipidomics data of plasma after 2 days of cold exposure.

This table is too large to be included in this thesis, therefore, for reasons of space, it is available online at: **https://www.mdpi.com/article/10.3390/ani12202762/s1**

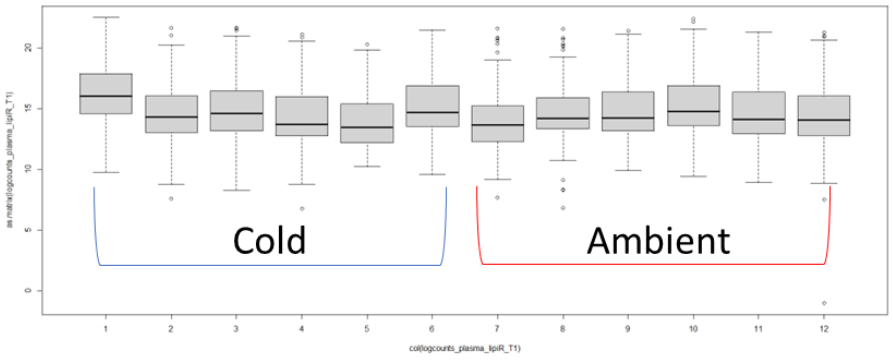
Supplementary figures

Figure S6.1. Boxplots from raw logged features of the lipid data for brown adipose tissue (BAT) after two days of exposure to either a cold temperature (4 °C) or ambient temperature (20–22 °C) (**A**); plasma after one day of exposure to either a cold temperature (4 °C) or ambient temperature (20–22 °C) (**B**); and (**C**) plasma after two days of exposure to either a cold temperature (4 °C) or ambient temperature (20–22 °C). Control samples in BAT boxplots belong to lambs euthanised soon after birth that provided a baseline lipidome. Plasma boxplots are already baseline corrected to its values at Time 0 (i.e., at the beginning of the study) as detailed in the methodology of this chapter.

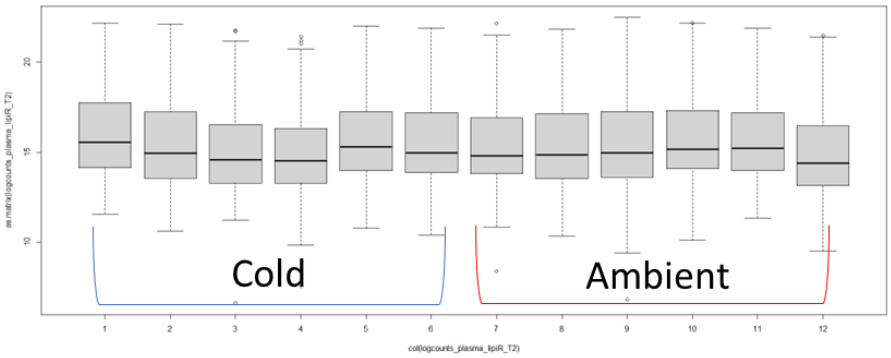
(**A**)



(**B**)



(**C**)


Cold
Ambient
as.matrix(logcounts_plasma_lpiR_T2)
col(logcounts_plasma_lpiR_T2)

Appendix D. Statements of contribution

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

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:	Andrea Grana
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- Describe the contribution that the candidate has made to the manuscript/published work:

Data curation, formal analysis, software, interpretation of results, visualization, writing - original draft, writing - review & editing

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