UNIVERSITY OF NEW ENGLAND

The effects of mild prenatal stress during mid- and late-pregnancy, on lamb vigour traits.

A Dissertation submitted by

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Certification of Dissertation

I certify that the ideas, experimental work, results, analyses, software and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

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20/09/2017

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21/09/2017

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21/09/2017

Signature of Supervisor/s Date

This thesis is dedicated to Marie-Louise, Odette and Yvan

Whom I know would have been very proud to see me complete this work and would have loved to see me thrive doing what I love.

« Le simple travail ne suffit pas pour finir ce qu'on entreprend, c'est de l'assiduité du travail que dépend la réussite » *Ménandre ; Fragments - IVe s. av. J.-C.*

« Work alone is not is not enough to complete what we began, it is from the assiduity and commitment of work that success depends »*Menandre ; Fragments - 4hc. b. J.-C.*

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Abstract

Shearing during pregnancy has been shown to increase lamb birthweight but the underlying mechanisms remain unknown.

This thesis aims to examine the effects of prenatal husbandry-like and cold stressors during mid- (pregnancy day 85-95) and late-pregnancy (pregnancy day 120-130) on lamb vigour traits. The long-term effects of prenatal mild stress on lamb vigour traits were assessed using lamb growth and body dimensions, neonatal behaviour and reactivity after a cold challenge and the lambs thermoregulatory abilities when exposed to cold. Maternal glucose response to the stressors and lamb metabolic responses were also examined.

It was hypothesized that increased birthweights were a result of a maternal stress response to the husbandry-like stressors involved with shearing, particularly handling, and/or the subsequent cold exposure of shearing. It was also hypothesized that this maternal stress response would impact on lamb vigour traits such as behaviour and thermoregulation. This thesis examined the effects of a range of mild stressors commonly experienced by ewes during husbandry procedures during mid-pregnancy (pregnancy day 85-95) or during late-pregnancy (pregnancy day 120-130) such as yarding, handling, shearing, transport as well as cold exposure. The effects of these stressors on lamb vigour traits were assessed using a separation test for reactivity, before and after a 1 hr separation and cold challenge and thermal imaging during the cold challenge to determine the ability of the lamb to maintain body surface temperature.

Shearing during mid-pregnancy increased lamb body dimensions and weight at birth while other stressors did not, which suggests that the causal mechanisms are something other than psychological and cold stress. Cold exposure during both mid- and late-pregnancy (cold exposure and/or cold exposure resulting from shearing) negatively impacted the reactivity levels of newborn lambs by increasing their latency to bleat, stand and return to ewe during a separation test. Cold exposure combined with acute stressors in keeping with husbandry procedures resulted in improved body surface temperature maintenance in newborn lambs when exposed to cold. The presence of effects of mild stressors for both stages of pregnancy on all three lamb vigour traits tested, suggests a number of alternate mechanisms are triggered by maternal-pregnancy stress impacting foetal development. Understanding how to modulate these effects could allow flock management to be modified to improve lamb survival.

Chapter 4
Labeur, L., Small, A H., Hinch, G. N., McFarlane, J. R., & Schmoelzl, S. (2020). Mid- and late-pregnancy ewe shearing affects lamb neonatal reactivity and vigour. *Applied Animal Behaviour Science*, In Press 10 June 2020.
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Chapters 6
Labeur, L., Villiers, G., Small, A H., Hinch, G. N., & Schmoelzl, S. (2017). Infrared thermal imaging as a method to evaluate heat loss in newborn lambs. *Research in Veterinary Science*, 115, 517-522. https://doi.org/10.1016/j.rvsc.2017.09.023

Chapter 3 and 5

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Chapter 1: **General Introduction**

Lamb survival is a multifactorial variable and the contributions of the ewe and the lamb have been widely studied and this background provides the framework for this thesis (Haughey 1981; Alexander 1984; Nowak 1996; Dwyer and Lawrence 2005; Nowak and Poindron 2006; Hinch and Brien 2014). For commercial sheep flocks, poor weaning rates represent a major commercial issue and economic loss for the sheep industry which has been estimated to add up to at least $A56 million/year (Sackett *et al.* 2006). However, it also represents additional challenges as it results in reduced options for selection, decreased possibilities to change the flock structure and also ultimately reduced option for sale animals (Alexander 1984). Finally, poor lamb survival also embodies an animal welfare concern for both the ewe and the lamb which is of major importance in the current context where customers are increasingly concerned about welfare of farm animals.

Although poor lamb survival has been recognised since the early years of Australian settlement and has consistently been studied in the last century (Moulk 1954; Haughey 1983), average lamb marking rates in Australia have improved only slightly. A study, conducted by Fowler (2007), of lamb losses in Australian NSW commercial sheep flocks, reported 16% of single lambs and 31% of twin lambs died before marking levels not unlike those reported decades earlier by Atkins (1980); Jordan and Feuvre (1989); and Kleemann *et al.* (1991). Hinch and Brien (2014) have provided an extensive review of lamb survival in Australian flocks and report highly variable lamb mortality levels, between 5-70% but most commonly averaging between 20 and 25%. They also reported that in Australian commercial flock mortality rates below 10% for single and 30% for twins were rare.

Similar survival rates are found in Europe where rearing systems contrast with Australian sheep production systems which are characterised by large flock sizes and extensive outdoor grazing. An extensive study conducted in France between 2004 and 2015 on 44200 lambs showed an average lamb loss between 10 and 20% for a relatively intense rearing system (Corbière *et al.* 2012). More generally, Gautier and Corbière (2011) reported that international average lamb loss before weaning were of 15-20% across all systems.

Many studies reported that twins losses are generally 2-2.5 times that of singles, which was confirmed by Fowler (2007), with 16.5% for singles and 31.5% for twins for both merinos and crossbred. Similar figures were reported by Refshauge *et al.* (2016), as most of the autopsied lambs in their study were twins (47%) and triplets (25%).

While welfare of the ewe is a primary concern, the exposure of pregnant ewes to environmental (cold and heat stress), physiological (undernutrition) and psychological stressors (yarding, shearing, transport) is aa normal part of production systems and may be linked to long term behavioural and physiological changes in neonatal lamb. Understanding these effects and the underlying mechanisms that link pregnancy stress with neonatal outcomes is crucial as an understanding of these may provide management strategies to improve lamb survival. Most of the research to date has focused on management strategies targeting ewe nutrition (Alexander 1962a; Fogarty *et al.* 1992; Banchero *et al.* 2004a; Banchero *et al.* 2004b), providing a favourable environment (Alexander 1962b; Lynch *et al.* 1980) and more recently the use of prenatal shearing to increase lamb birthweight (Morris *et al.* 1999; Kenyon *et al.* 2002b; deNicolo *et al.* 2008; De Barbieri *et al.* 2014).

The following review will focus particularly on lamb factors involved in adaptation to the environment by the neonatal lamb and will investigate current knowledge on the effects of prenatal stress on this adaptability.

Chapter 2: **Literature Review**

2.1 Lamb survival

2.1.1 Timing of lamb losses

Alexander (1984) in his review suggested that ‘core loss’ in most flocks is closely linked to the birth process and to the birthweight. The trauma at birth resulting particularly from the transition from placental to pulmonary respiration makes the first few hours after birth crucial for lamb survival (Hinch and Brien 2014). Hinch and associates profiled in two papers the chronology of lamb losses in Merinos and crossbred flocks and found that the majority of lamb death (>80%) occurred within the first 48 hours during and after birth (Hinch *et al.* 1985; Hinch *et al.* 1986) and similar results were reported in a French flock by Corbière *et al.* (2012). They also found that the proportion of lambs dying after a week of life was less than 15%.

In a recent study the majority of the lambs (53%) died on the day of their birth, 19% 1 day after, 16% 2 days after, 8% 3 days, 3% 4 days and 1% 5 days (Refshauge *et al.* 2016). Similar figures were obtained by Hall *et al.* (1995) with 67% of lambs dying within the first day of birth while Hughes *et al.* (1964), found that death at partum or immediately postpartum accounted for 55% of the dead lambs and that 23% more died after having walked.

2.1.2 Causes of death

Haughey (1973), suggested that identifying and defining the major causes of lamb deaths was the primary step towards improving lamb survival and there have been many studies since that time examining causes of lamb loss. Most recently, Refshauge *et al.* (2016) reported an extensive autopsy study conducted on 3832 Merinos and 796 crossbred lambs across different sites in Australia. Their work provides the framework for the key causes of loss including: birth injury, dystocia and stillbirth, starvation-mismothering and exposure.

2.1.2.1 Dystocia, Birth Injury and Still birth

Birth injuries, mostly due to birth trauma and prolonged birth, were defined by Haughey (1973) as significant lesions to the central nervous system and Refshauge *et al.* (2016), reported that birth injury directly accounted for 18% of the lamb postnatal deaths. Other studies have shown that longer birth times increase central nervous system lesions (Haughey 1982; Haughey 1983). In past studies, birth injuries have been reported in conjunction with dystocia and have been included in deaths classified as resulting from the birth process. They can represent up to half of the losses (Haughey 1980; Refshauge *et al.* 2016) and as such are considered as a major contributor to lamb loss and a focus for improving lamb survival (Haughey 1982). Dystocia has been defined by the presence of subcutaneous oedema generally around the head and neck (McFarlane 1965) usually resulting from mal-presentation or foeto-pelvic disproportions (Haughey 1983). Refshauge *et al.* (2010) confirmed that dystocia and birth injuries were closely linked with birthweight as heavier lambs were affected. As birth weight increased, cause of death was likely to change from other causes to dystocia.

In the study of Refshauge *et al.* (2016), stillbirth, as defined by high level of central nervous system lesions and no apparent metabolisation of fat reserves, accounted for 21% of the lamb deaths. They reported triplet and single Merino lambs had the same probabilities of being stillborn, however, Purser and Young (1964) reported that, in Blackface and a Welsh Mountain lambs triplets were more likely to die from stillbirth than twins and this might be due to the lower average birth weight,

The proportion of lamb deaths due to dystocia range widely from 9% to 54% (Hughes *et al.* 1964; Everett-Hincks *et al.* 2014; Refshauge *et al.* 2016). Refshauge *et al.* (2016)

reported that single lambs were twice as likely to die from dystocia as twins and triplets confirming these findings of Haughey (1973) and Purser and Young (1964).

The likely impact of the birth process and associated trauma on lamb behaviour, viability and thermoregulatory capacities has been argued by Haughey (1983) but Alexander (1984) questioned the importance of this impact highlighting the part played by the ewes in the establishment of the lamb-ewe bond and the prevention of the SME complex. Recently, Morton *et al.* (2014) have concluded that lambs were a major contributor to the bonding process.

2.1.2.2 Starvation and mismothering

Starvation is usually identified as a cause of death when no or insufficient amounts of milk are present in digestive tract. Poor ewe-lamb bonding, poor maternal behaviour, poor lamb mobility and sucking abilities, trauma resulting from birth process, increased litter size and separation between lamb and ewe can all be responsible for lamb starvation. Furthermore, poor ewe nutrition, which can result in reduced colostrum production and slow milk secretion rate, increased starvation risks (Murphy et al. 1996). Nowak and Poindron (2006) also mentioned that a lack of colostrum could induce a lack of transferred immunity. However, Hinch and Brien (2014) noted that even when pregnant ewes had good body condition scores, starvation still represented a major cause of lamb death. Because of the link between ewe behaviour and lamb starvation, starvation is often combined with mismothering and exposure in the SME complex (Starvation-Mismothering-Exposure) (McCutcheon et al. 1981; Hinch and Brien 2014). Haughey (1983) concluded that birth injury was associated with most lambs in the SME complex who fail to suck and thermoregulate normally which could be explained by damage to the central nervous system impacting both neonatal behaviour and physiology. In contrast, Alexander (1984) argued that separation of ewe and lamb was a the major reason for starvation. Recent evidence seems to suggest that birth injury contributes to an increased likelihood of separation.

2.1.2.3 Exposure

In Australia, exposure deaths have been reported to range from 5 to 10% (Hughes *et al.* 1964; Refshauge *et al.* 2016). While exposure and hypothermia might not account for most of deaths, they are a primary concern in regions where lambing occurs during Winter and early Spring. Deaths due to exposure have been identified by the presence of subcutaneous oedema around the head and the neck in animals that have evidence of being mobile (Refshauge *et al.* 2016). Eales *et al.* (1982) found that death by hypothermia within 12 hours of birth was often associated with hypoxia and immaturity. In Refshauge *et al.* (2016), body mass index and birth type increased birth difficulty which might impact the ability to thermoregulate, however it did not affect the probabilities of lambs dying from hypothermia..

2.1.3 Factors contributing to lamb survival

With the diversity of causes of death, the factors contributing to lamb losses can be classified as environmental, behavioural and physiological. Furthermore, it is widely understood and accepted that both the ewe and the lamb are involved in determining survival (O’Connor *et al.* 1992; Dwyer and Lawrence 1999). Nevertheless, the relative contribution from mother and offspring may vary with breed (Nowak 1989) and Nowak (1996) for instance attributed the high loss rates of Merino lambs to poor maternal behaviour.

Figure 2.1 summarises the factors that have been identified as contributing to lamb survival.

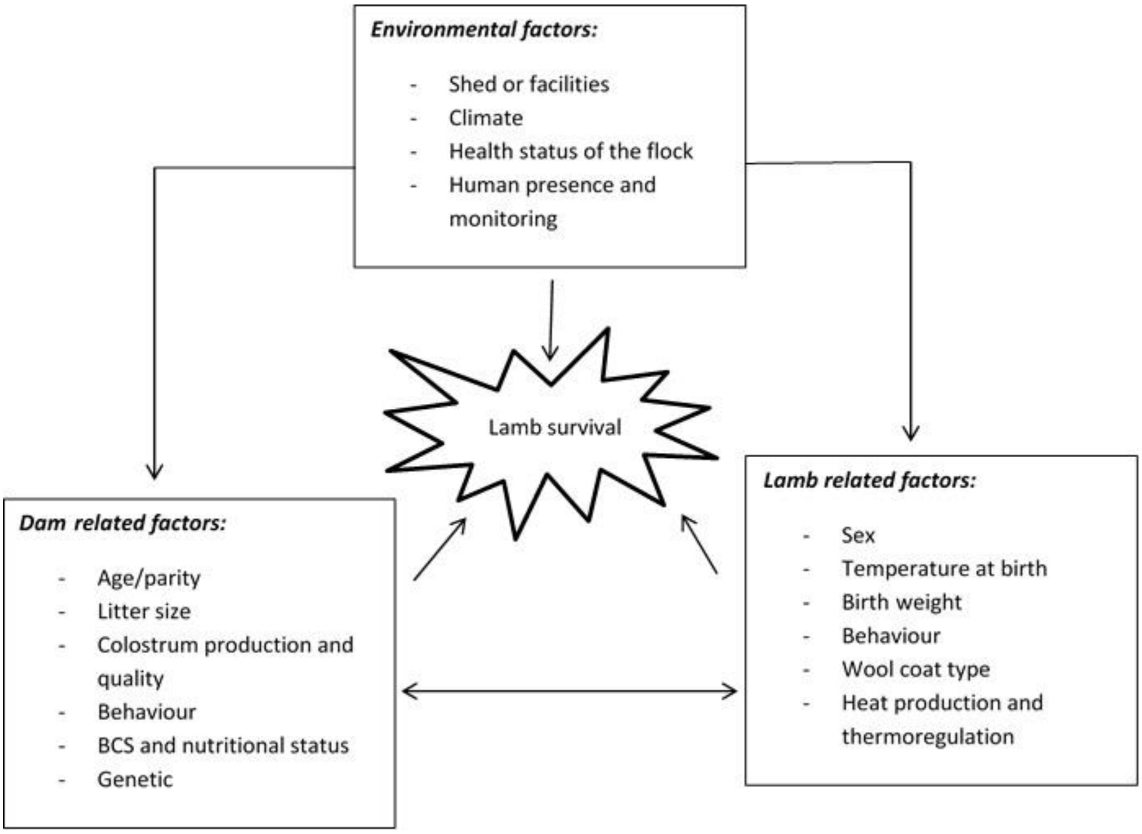

Figure 2.1 Factors contributing to lamb survival (adapted from Gautier and Corbière (2011))

2.1.3.1 Ewe factors

Some causes of lamb mortality are directly linked to the mother and Southey *et al.* (2001) suggested that the maternal factors linked to the lamb survival and maternal care were mostly crucial for the lambs early life when it is completely dependent on the dam. In this section, we describe factors relevant to our study: ewe body condition score and nutrition, parity and behaviour

2.1.3.1.1 Ewe body condition score and nutrition

The effect of undernutrition on lamb birthweight has been extensively studied and several reviews have been written on this topic (Mellor 1983; Robinson *et al.* 1999; Corner 2007b).

In utero, the foetal lamb solely relies on the ewe for energy and trace elements supplies, which they receive through placental transfer. Ewe nutrition and condition prior to and during pregnancy has significant effects on the birthweight of lambs. Therefore, poor ewe nutrition, as evidenced by a poor body condition score during pregnancy, can jeopardise subsequent survival (Alexander 1984; Fogarty *et al.* 1992; Everett-Hincks *et al.* 2005; Gardner *et al.* 2007; Corner *et al.* 2008). Mellor and Murray (1981) also, established that undernutrition during pregnancy may result in reduced placental size and efficiency. Similar placental insufficiency is observed with increasing litter size as there is a decrease in the size of the placenta and therefore nutrient availability per foetus (Alexander 1978; Mellor and Murray 1981; Mellor 1983; Dwyer *et al.* 2005).

Foetal growth is major and rapid during the last trimester of pregnancy, accounting for more than 85% of weight and dimension changes. Therefore it is vital that the ewe obtains good levels of nutrition at this time as inadequate nutrition during late pregnancy has detrimental effects on foetal development especially in twins (Mellor 1983; Egan 1984).

However, maternal undernutrition during pregnancy has also been shown to affect the lamb after birth as the neonatal lamb relies on the dam for milk and any insufficiency may result in reduced nutrient supplies and physiological and metabolic impairment in neonates. Mellor and Murray (1985); Banchero *et al.* (2006) have demonstrated that low body condition ewes have decreased mammary development and reduced colostrum and milk production.

Dwyer (2003) has also shown that ewes maintaining their body condition score by mobilising less backfat, gave birth to lambs that reached early behavioural milestones quicker than those born to ewes that were mobilising more backfat.

2.1.3.1.2 Parity

Several studies have reported that lambs born to primiparous ewes have a higher risk of mortality (Owens *et al.* 1985; Gama *et al.* 1991; Morris *et al.* 2000a). This could be explained by longer labour and higher risk of dystocia, less colostrum and/or colostrum of poorer quality (Cloete *et al.* 2002; Nowak and Poindron 2006; Dwyer 2008). Furthermore, primiparous ewes usually give birth to lighter lambs (Dalton *et al.* 1980) and lamb birth weight increases notably between the 1st and the 2nd gestation (Cloete *et al.* 2002). Finally, even under the best conditions, primiparous ewes are usually less maternally competent than experienced ewes and may display delayed grooming of the lamb (Arnold and Morgan 1975; Dwyer and Lawrence 1998), fearful behaviour towards the lamb, or just delayed maternal behaviour which can lead to abandonment (Nowak and Poindron 2006). Consequently, poor maternal behaviour and abilities often found in primiparous ewes could also account for the losses observed.

2.1.3.1.3 Maternal behaviour and establishment of the ewe-lamb bond

The ewe-lamb bond plays a key role in lamb survival as the neonate depends on the dam for nutrient supply, particularly colostrum in the first hours of life to provide energy and immunoglobulins. Dwyer (2014) commented that maternal care provides lambs with survival strategies amongst which, social learning, is as important as the provision of nutrition and protection. The bond called a “life insurance” by Nowak and Poindron (2006) normally ensures maternal care and prevents mismothering. The ewe bonds with her lamb/s at the lambing site where she usually remains for several hours after birth. The time spent by the ewe at the birth site varies between breeds and can last up to 24 hrs, but in Merinos is usually less than 2 hrs (Murphy *et al.* 1994a; Murphy *et al.* 1994b; Bickell *et al.* 2010). Maternal care can be defined as the ability of ewes to display caring behaviours such as, grooming and active cooperation while the lamb is sucking. Deficient maternal care can be a result of poor lamb vigour, particularly in twins where bonding can be 'uneven' resulting in the death of one or both lambs. Differences in maternal abilities have been observed in different breeds (Dwyer and Lawrence 2005). In the Merino for instance, their highly gregarious nature increases chances of permanent separation of ewes from their lambs by up to 50% in singles. In twin-bearing ewes, because some ewes are content with only one of the lambs following, the risks of a temporary separation of one or both lambs increases by another 30% (Alexander *et al.* 1983).

Furthermore, in presence of other parturient ewes, interference and disturbance in the establishment of the ewe-lamb bond can happen, particularly in Merinos ewes where it has been reported to occur in up to 20% of a flock. This can be a result of grooming of a lamb to allowing the lamb to follow and "stealing" (Arnold and Morgan 1975). Subsequently the ewe might "abandon" the stolen lamb when she gives birth to their own. The risks of mismothering, lamb stealing and disrupted lambing events increase with greater stock density (Alexander 1984; Poindron *et al.* 1997) but in intensive systems the offer of cubicles during lambing period decreases these risks (Nowak and Poindron 2006).

The conditions of and mechanisms underpinning the establishment of the bond between the dam and her lamb have been extensively reviewed in the last two decades (Kendrick *et al.* 1997; Goursaud and Nowak 1999; Nowak and Poindron 2006; Poindron *et al.* 2007; Dwyer *et al.* 2015). Essentially, the bond between the dam and her lamb is based on the lamb's distinctive "olfactory signature" (Poindron *et al.* 1992) and the attachment to the lamb scent and segregation from others is established within 30-60 min after birth (Poindron *et al.* 2007). Grooming can occur within 2-3 min of birth (Alexander and Peterson 1961) and is an essential step for the establishment of the ewe-lamb bond as the ewe dries, cleans and stimulates the newborn (Poindron and Le Neindre 1980). This process also provides the ewe with the smell and taste of the lamb's amniotic fluids

(Kendrick *et al.* 1997; Poindron *et al.* 2007) without which the bond may not be established or at least delayed (Poindron *et al.* 2010). However, Levy *et al.* (1983); Arnould *et al.* (1991) point out that while these cues are essential for the establishment of the bond, attraction to these cues develops during the perinatal period and might not exist a few days before lambing. Cooperation of the ewe during the sucking process by adopting a slightly hunched posture to facilitate the lamb's access to the udder also helps the establishment of the bond (Lynch *et al.* 1992; Goursaud and Nowak 1999).

Throughout the establishment of the bond, low pitched bleats or rumbling noises with a closed mouth are emitted exclusively in the presence of the lamb and decline over time with the grooming (Dwyer *et al.* 1998). While quietening the lambs these noises also provide cues for later recognition of the dam who will communicate using high pitched open mouth bleats (Dwyer 2014). Recently, Morton *et al.* (2014) have concluded that lambs were a major contributor to the bonding process, as they reported a maternal preference for stronger and more responsive vocalising lambs.

Alexander (1988) suggested selecting ewes on that maternal abilities care traits, however those temperament traits have a poor heritability and selecting for this trait represent a slow process. Nevertheless, the selection process ran by Cloete and Scholtz (1998) over 12 years has been shown to be quite efficient as a way to improve maternal ability in ewes.

2.1.3.2 Lamb risk factors

The lamb's contribution to its own survival depends on numerous factors (Figure 2.1). In this section, we will examine the importance of: birth weight, litter size behaviour and metabolic maturity on neonatal lamb survival. Lamb ability to produce heat and thermoregulate is essential during the transition from *in* to *ex utero* and is described in paragraph 2.2.2.

2.1.3.2.1 Birth Weight

Many authors have identified birth weight as the single most important determinant of lamb mortality (Dalton *et al.* 1980; Hinch *et al.* 1983). With a U-shaped relationship between birthweight and survival being traditionally used to describe probability of survival, both heavy, and low weight, lambs have lower chances of survival than “optimal” weight lambs. In heavy lambs, feto-pelvic disproportion causes higher risks of dystocia and birth trauma whereas, in light-weight lambs, the high surface area to volume ratio increases the risks of exposure and hypothermia and lower fat reserves increase the risk of starvation (Dalton *et al.* 1980; Nowak and Poindron 2006; Refshauge *et al.* 2010).

2.1.3.2.2 Litter size

Associated with their lighter birthweights twin lambs are at a higher risk of both abandonment, exposure and starvation. Nowak and Poindron (2006) estimated that mortality rate amongst twins is 1.5 to 3 times higher than in singles, but within similar weight ranges mortalities are similar, which confirms the importance of birthweight for survival (Purser and Young 1964). Twin neonates are also disadvantaged through competition for colostrum. Colostrum is essential for the newborn survival and it has been reported that up to 30% of twin bearing ewes do not produce enough colostrum for the requirements of twin lambs with the subsequent decrease in chances of survival (Nowak and Poindron 2006).

2.1.3.2.3 Lamb behaviour

Early lamb behaviours such as standing, finding the udder (through smell and touch) and sucking are crucial milestones for survival and usually occur within the first hour or two of life. Latencies to display such behaviours vary with breed, litter size, gender and birthweight (Alexander and Williams 1966a; Dwyer *et al.* 1996; Dwyer *et al.* 1998;

Dwyer and Lawrence 1999). Owens *et al.* (1985) established that, the chances of survival drop by 1% for every minute that these behaviours are delayed. Therefore, latencies to display early milestones have commonly been used to assess lamb viability and neonatal vigour (Owens *et al.* 1985; Slee and Springbett 1986; Cloete 1993; Dwyer 2003; Dwyer *et al.* 2005; Dwyer and Morgan 2006; Pfister *et al.* 2006a; Pfister *et al.* 2006b; Matheson *et al.* 2011). However, as vocalisations in lambs attest a strong communication-based dam-lamb bond (Nowak 1990), latency to emit the first bleat (Darwish and Ashmawy 2011), the bleating rate (Nowak 1990; Hernandez *et al.* 2009) and bleat response following tagging (Brien *et al.* 2009; Brien *et al.* 2014) have also been used as measures of lamb vigour.

Zeskind and Lester (2001); Zeskind (2013) reported that latency to vocalize when separated from the dam represents a communication responsiveness which is a reflex behaviour suggestive of neurobiological status, and although it has a low heritability (0.13 +/- 0.02), latency to bleat has been shown to be genetically correlated to lamb survival (Brien *et al.* 2010). Other studies have focused on assessing lamb cognition and behaviour in older lambs (1-3 months old) using tests (eg. the aptitude to reunite with their dams or discriminate an alien one with barrier and maze tests) or exploratory behaviour and emotional reactivity (startle test, isolation test and human presence test)(Roussel et al. 2005; Roussel et al. 2006; Roussel-Huchette et al. 2008).

Successful first sucking is very rewarding for the lamb and triggers the sucking drive in newborn lambs (Nowak and Poindron 2006). If colostrum ingestion is delayed for more than six hours after birth, the chances of successful sucking and maintenance of sucking drive are reduced with the likely consequence of starvation (Alexander 1958, 1964; Alexander and Williams 1966a). Sucking has also non-nutritive functions as it facilitates the establishment of the bond while triggering a neurophysiological cascade responsible for the lamb behavioural development (Nowak and Giraud 2006).

2.1.3.2.4 Lamb metabolic maturity

At birth the lamb goes through a cascade of physiological changes to adapt from the intra-uterine to the extra-uterine life. ‘Metabolic or physiological maturity’ describes the end result of this process and the neonate’s ability to adapt to these changes (Thompson *et al.* 2006). One of the most important adjustments required is the shift from a passive placental supply of amino acids and carbohydrate to an independent feeding of milk which contains less carbohydrate and is high in fat (Greenwood *et al.* 2002).

Independent regulation of energy supply through milk requires a functioning oxidative metabolism and glycogenolysis and gluconeogenesis that ensure energy availability for mobility, thermoregulation and neurological functions are met. The main storage locations of glycogen are the liver and skeletal muscles and Bassett (1989) reported that rapid development of gluconeogenesis is crucial as the glycogenolysis of the initial glycogen stored is not sufficient for the neonate energy requirements for other than a short period of hours. Because lipids do not cross the placenta as well as glucose and amino acids, lambs are born with limited fat deposits (Alexander and Bell 1975). However, lipid metabolism is essential for neonate survival as it provides the lamb with energy and substrate for non-shivering thermogenesis (Alexander 1962a).

During parturition, neonatal lambs undergo a rapid transition from the protective intrauterine to the external environment which is challenging (Mellor 1988). This transition requires adaptation of several physiological processes such as feeding, adaptive behaviour and heat production. Therefore, the lamb survival chances rely on its ability to respond and adapt adequately to the new environment. We have described some of factors involved in the adaption process and while ewe and lamb contributions are linked and overlapping, in the remaining of this literature review we will only focus on the ability of the lamb to cope with its environment and the effects of stress during pregnancy on foetal development and lamb adaptive abilities.

2.2 Adapting to a new environment during lambing

2.2.1 Cold resistance in newborn lambs

2.2.1.1 The transition to the outside environment at birth.

In New Zealand, McCutcheon *et al.* (1981) reported that approximately 30% of lamb losses could be attributed to cold weather, but on the tablelands of eastern Australia, Alexander *et al.* (1980a) found losses due to cold were less than 10% although losses due to hypothermia have been reported to be as high as 52% under extreme weather conditions (Arnold and Morgan 1975)

At birth, lambs experience a strong contrast in temperatures, the outside temperature being significantly cooler than the warm uterine environment, and while sheep are thought to be born with a well-developed thermoregulatory mechanism (Alexander and McCance 1958), lambs must increase their heat production by up to fifteen times to compensate for the heat loss to the environment they experience (Alexander 1962b; McCutcheon *et al.* 1981). After birth, a lamb's temperature will normally drop to between 32 and35°C, and then within several hours of birth increased heat production increases body temperature to a range between 38-41°C (Alexander and McCance 1958). McCutcheon *et al.* (1981) showed that if a lamb's rectal temperature fell below 30°C, then hypothermia would become too difficult to overcome and death was highly probable. Therefore heat production in lambs has been identified as a potential major contributor to lamb survival as the ability of a lamb to generate heat is crucial for survival in inclement cold weather conditions (Dwyer and Morgan 2006).

In order to increase heat production and maintain core temperature the newborn has to metabolise brown fat and increase muscular activity by shivering (Alexander and Williams 1968). Non-shivering thermogenesis is a rapid method to generate heat associated with brown fat metabolism, which has been reported to account for approximately 40% of heat produced (Alexander and Williams 1968). Under benign conditions the contribution of non-shivering thermogenesis to heat production decreases over time and will be exhausted by around 32 days (Gemmell *et al.* 1972). However, lambs born with limited energy reserves h can be sustained for up 3 to 5 days; but, in cold weather this period can be dramatically reduced (McCutcheon *et al.* 1981). Therefore apart from the period immediately after birth, susceptibility to hypothermia peaks between 13 and 36 hours post-partum when all the energy reserves of the lambs can be depleted (Eales *et al.* 1982). In some of the earliest experiments, Alexander and Williams (1966b) showed that cold exposure have a significant impact on neonatal behaviour and the establishment of the ewe-lamb bond. Their study also reported that teat seeking activity was inhibited by cold, wet and windy conditions and particularly if a lamb's rectal temperature dropped lower than 38°C. Similarly, Dwyer and Morgan (2006), reported that lambs with lower rectal temperatures 1 hour after birth showed delayed early behaviours and reduced post-natal vigour. Consequently, lower milk ingestion has also been observed in lambs in cold conditions (-1°C) (Thompson 1983). Generally, Wassmuth *et al.* (2001) linked thermoregulatory capacities with lamb vigour, and trauma at birth or exposure to extreme weather has been shown to impair neonatal adaptation.

2.2.1.2 Environmental conditions impact the cold resistance in neonates

The cooler the environment gets the more heat needs to be produced to maintain homeothermy of the lamb. Environmental factors such as wind velocity, humidity and the amount of fluids (amniotic fluids or rain) on the lamb's coat can heighten the heat loss (Nowak and Poindron 2006). These factors increase loss of radiated heat and as such increase risks of hypothermia thus decreasing lamb survival chances (Obst and Day 1968; Obst and Ellis 1977; Alexander *et al.* 1980b; Lynch *et al.* 1980; Martin 1999). Alexander (1962b) illustrated that humidity and coat wetness were the greatest threat to a lamb's homeothermy (Figure 2.2.) and Egan *et al.* (1972) identified that high wind speed combined with rain represented the most detrimental conditions positive impact on lamb survival of shelter in cold conditions, particularly for large litter sizes was clearly demonstrated (Lynch and Alexander 1977). However, they also reported that unshorn adult ewes did not seek shelter unless the wind speed was over 40 km.m^{-1}, but that pre-lambing shearing could increase shelter seeking in Merino ewes and could improve lamb survival by up to 50% in high chill situations (Alexander and Lynch 1976; Alexander *et al.* 1980b; Lynch *et al.* 1980).

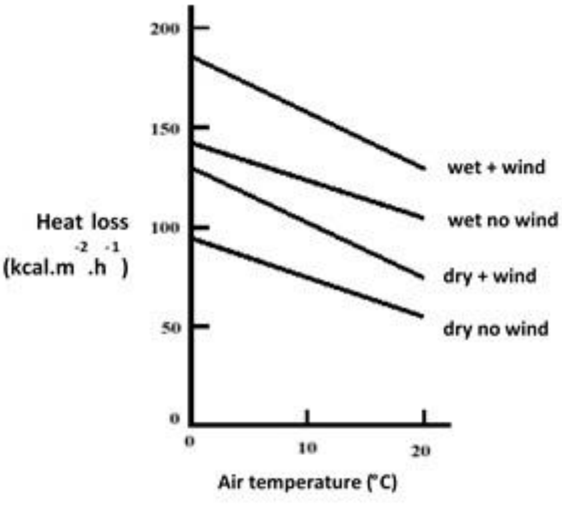

Figure 2.2 Heat production of lambs over ambient temperature depending on environmental conditions (adapted from Martin (1999))

2.2.2 Thermoregulation in neonates: BAT localisation and hormonal regulation

Alexander (1962c) defined cold-induced summit metabolic rate as the highest level of heat production while maintaining normal temperature and without voluntary muscle activity. The ability of a lamb to reach summit metabolic rate and thus prevent hypothermia is essential for lamb survival in cold conditions; however, differences across breeds can be observed for the latency and the level of the summit metabolic rate (Slee 1981; Slee *et al.* 1991).

Brown adipose Tissue (BAT) is the major energy source for newborn lambs but represents only 2-4.5% of the body weight. Brown adipose tissue is the primary source of heat production from lipid oxidation in newborn lambs. Major BAT depots are located in the peri-renal abdominal and inguinal regions of lambs (Gemmell *et al.* 1972; Alexander and Bell 1975; Everett-Hincks and Duncan 2008); however, subcutaneous fat in the prescapular and hind limb regions are also functional BAT and non-shivering thermogenesis is also apparent in cardiac and skeletal muscles (Foster and Frydman 1978). Fat reserves and BAT have been shown to decrease in lambs of ewes undernourished during pregnancy (Nowak and Poindron 2006).

The high vascularisation of BAT ensures the high demand in oxygen for rapid oxidation of fat is met as well as helping with the heat dissipation to the organs. Furthermore, BAT contains many mitochondria associated with uncoupling proteins (Trayhurn 1993), and is unique in that they allow energy produced by the proton gradient to be dissipated as heat instead of being used to produce Adenosine Tri Phosphate (ATP) (Himms-Hagen 1985).

When exposed to cold, the parasympathetic nervous system is stimulated, which in turn triggers the release of noradrenaline. Noradrenaline binding with plasma membrane receptors launches a cascade of reactions which result in mobilisation of the triglycerol within adipose cells. These fatty acids are then oxidised in the BAT mitochondria and this oxidation, which is uncoupled from Adenosine Tri Phosphate synthesis releases heat (Himms-Hagen 1985). This mechanism is controlled by uncoupled proteins which allow the oxidative phosphorylation in the mitochondria activated by the release of noradrenaline (Cannon *et al.* 1977).

According to Trayhurn (1993), the capacity of BAT for thermogenesis depends on the amount of uncoupled proteins which increases with cold exposure as a response to improve the ability to maintain thermoneutrality (Clarke and Symonds 1998).

Noradrenaline also stimulates the activity of iodothyronine-5'-deiodinase which is a selenium based enzyme (Berry *et al.* 1991) catalysing the conversion of thyroxine (T4) to Triiodothyronine (T3) (Arthur *et al.* 1990; Trayhurn 1993). Triiodothyronine (T3) is essential for thermoregulation as it controls the transcription of uncoupled proteins and as such the fatty acid oxidation (Cannon *et al.* 1977). The ability of a lamb to maintain its core temperature when exposed to cold has been directly linked to the level of circulating thyroid hormones (Caple *et al.* 1985); higher levels of Triiodothyronine (T3) occurring during cold-exposure (Himms-Hagen 1985).

Thyroxine (T4) has been shown to play an important role during re-warming after a cold challenge (Caple *et al.* 1985) and although selenium deficiency, and low circulating thyroxine (T4) reduced the BAT mobilisation in lambs, selenium deficiency was not found by Donald *et al.* (1994) to affect the cooling or warming rates during a cold challenge.

Cold stimulus and cutaneous cooling are essential for the initiation of non-shivering thermogenesis (Gunn *et al.* 1991) and lambs at birth have a very rapid onset of non-shivering thermogenesis. If the lamb does not suck within the first hours after birth, BAT can become atrophied which will reduce thermoregulatory capacities (Alexander 1962a).

Sucking and birthweight seemed to be factors linked with BAT metabolism and thermogenic capacities. Dwyer and Morgan (2006) reported higher levels of thyroid hormones and greater amount of BAT have also been found in heavier lambs. Clarke and Symonds (1998) have also shown that feeding colostrum increased thermogenic capacities in lambs and this could be due to a leptin concentration which enhances sympathetic activity.

2.2.3 Assessing thermogenesis

2.2.3.1 Methods to induce and assess cold stress

The ability to display an appropriate physiological response to cold conditions is a strong measure of lamb vigour and a cold component has been shown to be essential to trigger non-shivering thermogenesis (Gunn *et al.* 1991). Previous studies on thermoregulation and cold resistance have used methods such as cooling baths, climate controlled chambers and pharmacological treatments to produce controlled cooling and non-shivering thermogenesis. In this section, the strengths and weaknesses of these methods as well as infrared thermography to measure the impact of a cold challenge are examined

2.2.3.1.1 Cooling baths

One method to induce a cold challenge to lambs is the use of a cooling baths with the lamb immersed in water at a thermo-neutral temperature. The water temperature is then gradually decreased until the rectal temperature, monitored regularly to prevent hypothermia (set between 35°C to 37°C), reaches a defined threshold (Eales and Small 1980; Slee *et al.* 1980; Samson and Slee 1981). Latency to acclimate, to drop to threshold temperature, the critical temperature and latency to rewarm can be measured and compared to assess resistance to cold. Slee *et al.* (1990) concluded that fast cooling and self-rewarming was a satisfactory method to induce non-shivering thermogenesis in neonatal lambs, but this process requires specialised equipment. An earlier study by Slee *et al.*

(1980) established that although not mimicking environmental conditions such as wind, differences in moisture, as well as being very variable between animals and across breeds, the water bath method was 95% repeatable. However, recently Plush (2013) has suggested that the complete immersion of lambs might interfere with the wool coat insulation properties. The method does appear to be useful in differentiating the capacity to maintain body temperature of different breeds and also potential genetic differences within a breed.

2.2.3.1.2 Pharmacological treatments

In order to standardise the cold resistance assessment injections of pharmacological components such as noradrenaline, adrenaline (Alexander 1969), isoprenaline (Slee et al. 1987), and thyroid hormones (Alexander 1970), have been used to simulate cold exposure by stimulating the activity of the BAT. Consistent rectal temperature response was observed following noradrenaline injections (Slee et al. 1987) and Alexander and Williams (1968) also observed an increased heat production in the tissue identified as BAT when noradrenalin was injected. Although producing a strong thermoregulatory response without the need of an actual cold exposure and allowing a better understanding of the underlying mechanism/s of heat production, pharmacological injections might not be a very appropriate model to study cold stress induced non-shivering thermogenesis as lambs are not exposed to the array of environmental conditions, such as wind which has been identified as a major cause to hypothermia, that might trigger other heat production processes such as shivering as well as potentially inducing physiological stress. Additionally, inappropriate dosage might result in responses outside the normal physiological range (Hergenhan 2012).

2.2.3.1.3 Climate chambers

Climate chambers, allowing precise control over moisture, air movement and temperature have been used to mimic all the environmental conditions (rain, cold and wind)

(Alexander and McCance 1958; Alexander 1961b, 1961a; Alexander and Peterson 1961; Alexander 1962b; Alexander and Williams 1962). Climate chambers allowed measurement of oxygen usage via indirect calorimetry providing an indication of changes in metabolic rate (Alexander and Williams 1968). Although, climate chambers allow complete environmental control and therefore are able to reproduce field conditions the equipment is costly and does not allow studies that include large numbers of animals. However, similarly to pharmacological treatments it can allow an intense study of mechanism/s activated during non-shivering thermogenesis.

2.2.3.1.4 Cold challenges

It has been suggested that to properly assess thermogenesis, the test used should be controllable, repeatable, economic, simple and allow testing of a large number of animals (Slee 1981). Most of the methods previously described require specialised equipment and often separation of the lamb from its dam. An alternative to pharmacological injections to stimulate BAT and trigger non-shivering thermogenesis while maintaining the lamb in the presence of the ewe is the use of an ice vest. Fitting lambs with ice vest seemed to fulfil both objectives and the response observed was different than the one using noradrenaline injections (Hergenhan 2012). However, it was also highlighted that the efficiency of ice vest depended on ambient temperature and while it allows the lamb to stay with the dam it might still present some threats to the establishment of the lamb-ewe bond (Hergenhan 2012).

2.2.3.2 Assessing heat production in newborn lambs

2.2.3.2.1 Metabolic and physiological changes resulting from cold stress

Traditionally, thermogenesis has been assessed by monitoring core body temperature but this measurement alone does not account for the heat radiated from the skin surface.

Another method to assess cold resistance and heat production is oxygen consumption which is an indicator of the metabolic rate. Metabolic rate is the inverse of rectal temperature as it gradually increases until summit metabolic rate is reached.

Higher concentrations of glucose, lactate and free fatty acids were observed in lambs exposed to cold (Alexander and Mills 1968; Alexander *et al.* 1968), evidence of heightened metabolism activity and hence an increase in metabolic rate . An increase in the concentration of Triiodothyronine (T3) and thyroxine (T4) was also observed during cold exposure, and the peak was reached within an hour of exposure (Cabello 1983). However, Plush *et al.* (2016) did not observe an increase in Triiodothyronine (T3) production during their cold exposure using water bath cooling but reported higher Triiodothyronine (T3) concentrations once the lamb had recovered from the cold exposure. This could be explained by the difference in nature, temperature and length of the cold stress to which the lambs were exposed. as Wrutniak and Cabello (1989) used a moderate cold exposure (4°C for 4hrs) compared to Plush *et al.* (2016) who used a chilled water bath resulting in a more acute cold stress (from 36°C to 15°C for 1hr).

2.2.3.2.2 Use of Infrared Thermography to measure thermoregulation in lambs

Infrared thermography (IRT) is a technology that allows the detection of temperature by capturing an image of the infrared radiation emitted from an animal or object in the infrared region of the light spectrum. IRT has been shown to be a safe, accurate and non-invasive method for mapping radiated temperature of an object or building surface (Clark *et al.* 2003).

IRT has been used in humans to identify and visualise breast cancer (Ng 2009), neonatal monitoring (Abbas and Leonhardt 2014) and to study energy expenditure in preterm infants (Adams *et al.* 2000). Veterinary and animal sciences have also made use of this technology to detect diseases (Poikalainen *et al.* 2012), lameness (Nikkhah *et al.* 2005), stress and more generally to assess animal welfare (Schaefer *et al.* 1988; Stewart *et al.* 2005; Stewart *et al.* 2009). In bats and voles, IRT has been used to study torpor and thermogenesis (Lancaster *et al.* 1997; Jackson *et al.* 2001) and when compared to other methods such as calorimetry (Shuran and Nelson 1991; Adams *et al.* 2000) or use of standard equations, IRT has proven to be a very useful method to quantify heat loss. In comparison to measurement of the core body temperature which does not account for the infrared heat loss radiating from the skin of the animal, infrared thermography can record thermal loss and thermogenesis linked to subcutaneous brown fat depots by measuring the body surface temperature in the infrared frequency band of the light spectrum. Although Hergenhan (2012) postulated that the use of IRT might not provide measures of absolute temperature due to the need of adjustments associated with atmospheric and object parameters, McCoard *et al.* (2014) has recently shown that continuous recordings using IRT was a suitable and non-invasive method to study thermogenesis in the neonatal lamb. Hergenhan (2012) reported that IRT snapshots were a good method to identify the areas of heat production in lambs and in field voles, Jackson *et al.* (2001), showed that IRT could highlight regions of the skin with underlying BAT, but suggested that IRT might not be sensitive enough to quantify BAT activity.

While a few studies have used IRT to assess heat production no standardised method have been described allowing assessment of heat production. McCoard *et al.* (2014) used continuous recordings and assessed changes in body surface temperature across the back surface of one individual. Similarly, while Hergenhan (2012) used dorsal temperature profiles collected using thermograms capturing the entire lamb back midline, they also focused on the body surface temperature changes of one individual undergoing a noradrenaline challenge.

Finally, IRT has to be used cautiously in lambs as the coat type and length, which represent a thermo-insulant might also interfere with IRT readings and the emissivity of lamb body surface has not been accurately determined.

2.3 Prenatal stress and foetal development.

Sometimes foetal lambs have to cope with their environment prior to their birth while in-utero. Environmental, physiological and psychological stressors can occur during pregnancy, thus potentially affecting the foetal lamb. Understanding the effects of prenatal stress and the consequences on lamb survival is crucial. In this section, we will define the stress and the stress response of interest and review current knowledge on the effects of prenatal stress on lamb ability to cope with its environment and survival traits.

2.3.1 Stress, stress response

For the purpose of this review we will use “a subjective experience induced by a novel, potentially threatening or distressing situation, and the behavioural and neurological reaction to it” (Weinstock 1997) as the definition of stress. Moberg and Mench (2000) have reviewed and defined the response to a stress in animals. Basically, the first and least biologically expensive stress response is a change in behaviour and such a response can minimise the stress, assist with the recovery, prevent additional stress and overall participate in the protection of the animal. The response to stress in animals is usually in two stages. The initial response to stress which allows the animal to react quickly to preserve homeostasis and which is produced by the autonomic system (Moberg and Mench 2000). The sympathetic nervous system, which controls the body’s responses to a perceived stress and is responsible for the “fight” or “flight” response, produces the cathecolamines, adrenaline and to a lesser extent noradrenaline. These hormones result in an increased heart-rate, respiration-rate, blood pressure and increased plasma concentrations of glucose, fatty-acids and lactate. If the challenge persists, the body needs to adapt to make the stress tolerable via the neuroendocrine system. The activation of the hypothalamic-pituitary-adrenal axis stimulates the glucocorticoids production under the control of adrenocorticotropic hormone. Elevated levels of corticosteroids increase the production of glucose from protein resources, increase glycogen deposition in the liver as well as inhibiting glycogenolysis and conversion of amino acids to proteins and fatty acids to triglycerides (Holst 1998). As a result, plasma cortisol has been measured extensively to assess the effect of stressors but neuroendocrine assays were also used to asses chronic stress in sheep (Dwyer and Bornett 2004).

The two response stages lead to the definition of two duration types of stress events: acute and chronic. While the first represents a transient physiological situation with a return to baseline and homeostasis, the later representing a persistent stressor to which the animal cannot adapt, and which may result in an elevated “new” baseline. In humans, Elliott and Eisdorfer (1982) have defined stressors according to duration and course (discreet vs continuous) including five categories: the *Acute time-limited stressors which* involve laboratory challenges ; *Brief naturalistic stressors*, which are normal real-life short-term challenges which are nevertheless stressful ; S*tressful event sequence,* a focal event, which gives rise to a series of related challenges ; *Chronic stressors*, leading to an altered homeostasis allowing an adaptation and *Distant stressors* which are long-lasting trauma. While not all categories can be applied to animals and particularly to livestock, husbandry-related stressors could be considered as brief naturalistic, “artificial” stressors, not previously experienced by animals and when performed under “laboratory” conditions, acute time-limited stressors, commonly called acute stressors and finally chronic stressors.

2.3.2 Effects of prenatal stress

2.3.2.1 Hormonal and physiological changes impact on foetal development

Braastad (1998) defines prenatal stress as “a stress experienced by the pregnant mother which affects the offspring” and mentions that the effect of stress depends upon the perception of the stress by the dam. The hypothalamic-pituitary-adrenal axis plays a crucial role in mediating an animal’s ability to cope with stress (Bohus 1987) and the physiological changes promoting adaptation to acute stressors might also affect prenatal lamb development.

During pregnancy, it has been suggested that hormones such as glucocorticoids and catecholamines were likely mediators of stress transported from the maternal blood to the placenta as there are no direct neural connection between the mother and the foetus (Weinstock *et al.* 1988).

Therefore changes in maternal cortisol concentrations can result in altered foetal plasma cortisol concentrations (Otten *et al.* 2004; Seckl and Meaney 2006) which in turn could alter the development of the foetal hypothalamic pituitary adrenal axis (Challis *et al.* 2000; Symonds *et al.* 2001). Jensen *et al.* (2002) showed that elevated maternal cortisol in late-pregnancy resulted in increased foetal cortisol concentrations, which suggest that the lamb is under a physiological stress status.

In the last third of pregnancy, glucocorticoids cross the placenta easily (Wood and Rudolph 1984; Seckl 2004), cortisol can thus be transferred from the maternal to the placental circulation and be carried to the foetal brain and other major organ systems (Weinstock 1997). Therefore, foetal exposure to excess maternal glucocorticoids at different stages of pregnancy might influence embryonic development of the hypothalamic pituitary adrenal axis functions differently. High levels of cortisol were observed when dams had high glucocorticoid levels (Matthews 2000; Sloboda and Newnham 2000;

Sloboda *et al.* 2002) and the levels in lambs born to ewes subjected to an acute stress were different from the control lambs (Matthews 2000). Piquer *et al.* (2017) also showed that under sympathetic stress conditions (when sympathetic nervous system is stimulated), increased noradrenaline plasma levels during pregnancy were correlated with decreased noradrenaline transporter functionality which was responsible for changes in the development of progeny.

In goats and sheep, some studies have found that prenatal stress has a positive impact on the cortisol levels in offspring (Duvaux-Ponter *et al.* 2003; Roussel *et al.* 2004), but this has not been consistent (Roussel-Huchette *et al.* 2008).

The effects of prenatal stress on foetal growth and development seems to depend on the level and the duration of stress and consequently the amount of cortisol and adrenaline reaching the foetus and/or the subsequent metabolic cascade. The effects observed include both reduced and increased birthweight, decreased post-weaning weight gain, altered reproductive function, abnormal postnatal endocrinology, increased emotional reactivity and behavioural responses to stress (Morris *et al.* 1999; Roussel *et al.* 2004; Rutherford *et al.* 2012). Entringer *et al.* (2010) showed that in humans, stressful events during pregnancy, had a negative influence on the developing brain and thus could compromise cognitive and emotional process. In lambs, stress during the last third of pregnancy has been reported to decrease learning performance of the lambs when tested in a maze test, to reduce locomotor activity, in a test of human interaction, impair spatial learning and working memory, increase emotional reactivity and induce pessimistic-like biased judgement (Coulon *et al.* 2011; Coulon *et al.* 2015). When the prenatal stress treatment used was aversive human interaction, lambs displayed an increased fear of humans (Coulon *et al.* 2014). In lambs and kids increased exploratory behaviours were observed in lambs born to repeatedly isolated or transported dams (Roussel *et al.* 2005; Roussel-Huchette *et al.* 2008).

2.3.2.1.1 On the maternal traits

In rodents, stress during pregnancy was reported to result in a reduction in maternal care towards offspring (Patin *et al.* 2002; Smith *et al.* 2004). In contrast in sheep, improved maternal behaviour was observed subsequent to stress during pregnancy, Roussel *et al.* (2006) observed that regularly isolated ewes stayed close to lambs, when in presence of humans, for longer than control ewes and those exposed to transport. In primiparous ewes, negative and unpredictable handling during late-pregnancy resulted in an increase in grooming behaviour when compared to gentled control ewes (Hild *et al.* 2011). The extent of the effects of stress during pregnancy on maternal traits and behaviour as well as the mechanisms mediating maternal behaviour have not been well documented.

2.3.2.1.2 On the offspring traits

Similarly to maternal traits, the consequences of prenatal stress on lambs behavioural and cognitive traits are not well documented. It seems that the effect of prenatal stress on lamb traits depends on the nature of the stress, the pregnancy stage and the severity of the stress but also possibly genetics (Weinstock *et al.* 1988). In rats, daily restrain in a small cage during 240 minutes for three consecutive days and excess circulating maternal stress hormones, on foetal brain development have produced a neurotoxic effect of prenatal stress on offspring foetal development (Fujioka *et al.* 1999; Weinstock 2008). However, similar stress treatment applied for only 30 minutes for three consecutive days resulted in an increased learning ability and a reduced emotional reactivity in pups born to dams submitted to mild prenatal stress. Fujioka *et al.* (2001) concluded that mild prenatal stress might not be of a magnitude great enough to have a neurotoxic effect.

2.3.2.2 *Effects of chronic stress*

As discussed previously, the difference between acute and chronic stress lies in the intensity and duration of the stress, chronic stress usually occurs when animals are unable to deal with persistent stressors or when several stressors are present concurrently and when the chronic elevation of the stress mediators can incur a significant biological cost to the individual (Moberg and Mench 2000). Prolonged gestational stress has been shown to cause permanent modifications (foetal programming), that predispose animals to disease in adulthood (Godfrey and Barker 2000, 2001). In sheep, exposure to chronic stress, has been shown to alter the growth of lambs and to negatively affect the function of the immune and reproductive system (Dwyer and Bornett 2004), and similar results have been observed in rats (Freide and Weinstock 1984; Weinstock *et al.* 1988; Weinstock 1997). Coulon *et al.* (2015) reported that prenatally chronically stressed lambs did not perform as well as control lambs in a maze test and showed pessimistic-like judgment in a cognitive bias test.

It is widely understood and agreed that the effects of chronic stress during pregnancy are detrimental and that such situations should be avoided. However, the effects of mild and/ or acute stressors such as those experienced during husbandry procedures are not fully understood.

2.3.3 The effects of stressors during management practices on lamb vigour traits

On farms, even under best management and welfare practices, animals are subjected to stress throughout their life. An understanding of the effect of stressors such as transport (Lay *et al.* 1997a; Lay *et al.* 1997b; Roussel *et al.* 2006), shearing (Kenyon *et al.* 2003), undernutrition (Bell 2006), hyperthermia (Mellado *et al.* 2000) and isolation (Roussel *et* *al.* 2004) on foetal growth and development are essential to ensure the best chance of survival of offspring and adapt management strategies to optimise survival chances.

While more than 85% of foetal growth occurs during the last third of pregnancy (Mellor 1983), foetal brain development takes place in three main stages which all contribute to an increase in brain weight: neuroblast multiplication (early-pregnancy), neuroglial multiplication (mid-pregnancy) and myelination process and growth of the cerebellum (late-pregnancy) (McIntosh *et al.* 1979).

Therefore, in most of the following sections, three pregnancy stages will be considered, early-pregnancy (pregnancy days 30-60), mid-pregnancy (pregnancy days 70-100) and late-pregnancy (pregnancy days 115-145).

2.3.3.1 Shearing during pregnancy

Shearing exposes the ewes to several stressors inducting both acute and chronic stress responses in sheep. The acute stress elicited by the mustering, time off pasture and fastening, handling, tactile stimulus and exposure to dogs, vehicle and noise produced elevated cortisol levels and heart rate responses which were greater than those to crutching, isolation or handling (Hargreaves and Hutson 1990c, 1990b, 1990e, 1990d, 1990a). Chronic stress potentially results from the effects of cold exposure resulting from by the fleece removal.

2.3.3.1.1 Impact of shearing on lamb physical measurements

In many studies, mid and late-pregnancy shearing has been reported to impact on the foetal development with larger and heavier lambs (Symonds *et al.* 1986; Black and Chestnutt 1990; Morris and McCutcheon 1997; Morris *et al.* 2000a; Revell *et al.* 2002). However, a few studies while observing live weight differences in the pregnant ewes, have reported no differences in lamb size (girth and crown-rump circumference). Kenyon *et al.* (2003)

concluded that responses to pregnancy shearing were very inconsistent and with a wide range in magnitude. However, Kenyon *et al.* (2002c) reported that increased birthweights could only be seen if ewes were destined to give birth to lightweight lambs (growth restricted conditions) and had the "potential" to produce a heavier lamb,

Higher birthweight implies higher lamb survival rate especially, if the increase is in lightweight multiple litter lambs (Morris *et al.* 1999; Kenyon *et al.* 2002a; Kenyon *et al.* 2006a). However; it has been suggested that to have a positive impact of the increased birthweight on survival chances, lambs must be otherwise destined to be born within a birth weight range below the optimum (3.5 to 5.5 kg for Merino lambs) (Atkins 1980; Hatcher *et al.* 2009; Geenty *et al.* 2014). An increase of birthweight in already big lambs increased their risk of dystocia and trauma at birth (Dalton *et al.* 1980) and a few studies have failed to find a birth weight effect (Stott and Slee 1985; Dabiri *et al.* 1994; Morris *et al.* 2000a; Kenyon *et al.* 2002c; Kenyon *et al.* 2006b).

2.3.3.1.2 *Other impacts of shearing on lambs: thermoregulation and behaviour*

Even if the results were inconsistent under New Zealand pastoral conditions, studies have shown an increased amount of BAT in lambs born to ewes shorn during late-pregnancy (Symonds *et al.* 1992a) and an enhanced cold resistance in housed shorn sheep (Symonds *et al.* 1992b). Similarly shearing followed by a 2 weeks cold exposure during late-pregnancy (14 days before expected lambing) resulted in lambs with a greater capacity for non-shivering thermogenesis (Stott and Slee 1985) and it seems that summit metabolic rate may be increased in such situations (Revell *et al.* 2002). However, some studies reported no improvement in thermoregulatory capacities in lambs born to ewes shorn during mid-pregnancy when exposed to cold water and air flow (Kenyon *et al.* 2002c, 2002b), which could be linked to the timing of shearing and different stages of pregnancy. Late-pregnancy shearing and cold exposure could potentially impact BAT deposition.

No direct effect of mid- or late-shearing was observed on lamb survival to weaning (Cloete *et al.* 1994; Morris *et al.* 1999; Kenyon *et al.* 2002a; Kenyon *et al.* 2006b) and it seems that the consequences of pregnancy shearing on neonatal behaviour have not been studied in any detail (Corner *et al.* 2007a, 2007b). Dwyer (2003); and Roussel *et al.* (2004) have hypothesised that altered hypothalamic-pituitary-adrenal axis function in the lamb could impair neonatal behaviour with resulting poor lamb survival but this has not been tested.

2.3.3.1.3 Conditions and timing of shearing

Shearing ewes during mid-pregnancy increased lamb birth weight of both singles and twins in grazing situations (Morris and McCutcheon 1997; Morris *et al.* 2000a; Kenyon *et al.* 2002a; Revell *et al.* 2002; Sherlock *et al.* 2003; Kenyon *et al.* 2004; Corner *et al.* 2006; Corner *et al.* 2007b; De Barbieri *et al.* 2012) and also when ewes were housed (Symonds *et al.* 1986; Black and Chestnutt 1990). Under housed conditions increased birthweights have been observed in both winter (Symonds *et al.* 1986; Dýrmundsson 1991) and Summer conditions, within the thermos-neutral zone for shorn sheep (temperatures for the 10 days following shearing, average temperature 20°C, min 15°C and max 27°C) (Council 1981; deNicolo *et al.* 2008) suggesting that stress effects from cold may not be the only stressor effecting foetal growth.

Several studies have noted a positive impact of shearing on lamb birthweight when the shearing occurred during mid-pregnancy (Morris and McCutcheon 1997; Banchero *et al.* 2010; De Barbieri *et al.* 2014) and in the experiment of Kenyon *et al.* (2006b) shearing effects were only seen for a mid-pregnancy shearing but not for late-pregnancy. If this effect is consistent it might suggest that the shearing stress is impacting on placental development and nutrient partitioning.

2.3.3.1.4 Possible mechanisms explaining physiological and behavioural impacts of shearing during pregnancy

Higher glucose plasma concentrations in shorn ewes have been observed to impact on foetal growth (Symonds *et al.* 1988; Clarke *et al.*, 1997; Morris *et al.*, 2000). To compensate for the loss of insulation due to wool removal, ewes have to increase their heat production by up to 25-30% (Davey and Holmes 1977; Symonds *et al.* 1986; Symonds *et al.* 1988b), which occurs due to the oxidation of maternal reserves and therefore an increased maternal and foetal plasma glucose concentrations (Thompson *et al.* 1982). As placental glucose diffusion depends on the glucose gradient between maternal and foetal concentrations (Takata and Hirano 1997), it is unclear if there is an increase in glucose in the lamb or if it is due to placental glucose transfer. Symonds *et al.* (1988b) have shown that shearing had no effect on the uterine blood flow and the utilisation of glucose which would suggest an increase in foetal glucose per se. Furthermore lower blood insulin concentrations have been found in housed shorn ewes (Symonds *et al.* 1986) and a lower insulin response to a glucose challenge could indicate a reduced insulin release capacity or an increase of the maternal sensitivity (Revell *et al.* 2002).

Shearing of sheep during pregnancy can also result in an elevation in ewe plasma concentrations of cortisol (Pierzchala *et al.* 1983; Hargreaves and Hutson 1990a; Mears *et al.* 1999) and this can be transferred through the placenta to the foetal brain and other organs (Zarrow *et al.* 1970; Weinstock 1997).

Cold stress resulting from shearing produces a cascade of hormonal and metabolic adaptations in the ewe (Clarke *et al.* 1997), amongst which are an elevation of thyroid hormones T3 and T4 (Symonds *et al.* 1988a, 1989; Morris *et al.* 2000a; Sherlock *et al.* 2003) which can last between 20-50 days (Symonds *et al.* 1988a; Morris *et al.* 2000a). As mentioned previously the exposure of the foetus to excess glucocorticoids may influence postnatal function of the hypothalamic pituitary adrenal axis which in turn can affect thyroid gland activity and Revell *et al.* (2002) reported increased foetal thyroid gland and decreased adrenal gland activities. Bell *et al.* (1989) have suggested that the increase in thyroid hormones and the altered hypothalamic pituitary adrenal axis function could possible account for increased birth weights, but hormone treatments did not increased birthweight in the studies of (Kenyon *et al.* 2005; Corner *et al.* 2007a). Regardless, the altered hypothalamic-pituitary-adrenal axis activity could explain the increased thermoregulatory capacity of lambs born to shorn ewes. Alternatively, the increased birth weights and increased foetal glucose, evidence of greater reserves, may help to explain the effect. Finally, another potential explanation to the increased thermoregulatory capacity observed in lambs born to shorn ewes could be an increase in maternal plasma non esterified fatty acid concentration (Elvidge and Coop 1974), but this increase has not been observed in all studies (Symonds *et al.* 1989).

It has been postulated that the acute stress component of shearing is responsible for birthweight increases as repeated isolation during pregnancy has also resulted in heavier birthweights (Roussel *et al.* 2004). However, in other studies, repeated isolation, cortisol injection and sham shearing have failed to reproduce similar results and cortisol injections were reported to decrease birthweight (Corner *et al.* 2010).

Shearing during pregnancy can reduce heat stress in ewes and prolong gestation (Revell *et al.* 2002; Kenyon *et al.* 2003; Corner *et al.* 2006; Keady *et al.* 2007), the later potentially accounting for at least some of the increased birthweight. However, in other studies where an increase birthweight was reported, no difference in gestation length have been observed (Kenyon *et al.* 2002b; Corner *et al.* 2010) so this potential mechanism is open to question. Kennedy (1985) observed an increased feed intake in housed shorn animals and the increased feed intake was suggested as a possible cause for birthweight increase

(Symonds *et al.* 1986; Black and Chestnutt 1990; Keady *et al.* 2007) but this observation was not observed under grazing conditions (Husain *et al.* 1997; Kenyon *et al.* 2002b; Revell *et al.* 2002). All studies agreed on the need for maintenance level nutritional conditions and Kenyon *et al.* (2002c) have suggested that under poor nutritional conditions, increased birthweight resulting from mid-pregnancy shearing might not occur.

2.3.3.2 The effects of prenatal cold stress on lambs

Stott and Slee (1985) reported that the cold exposure of pregnant ewes during late pregnancy increased BAT deposition in the foetus. When comparing shorn and unshorn pregnant ewes exposed to cold, cold exposure during late-pregnancy positively impacted thermogenic activity in BAT in lambs, increasing metabolic non-shivering thermogenesis at 1 day of life (Symonds *et al.* 1992a). The exposure to cold exposed the ewe to a sympathetic stress but did not impair hypothalamic pituitary adrenal axis function which in turn produced an adrenergic overload in lambs potentially affecting foetal development (Benedict *et al.* 1979; Pacak *et al.* 1998). Similarly it was suggested that in adult sheep severe cold exposure, defined by the attainment of summit metabolic rate, resulted in a rapid body fat reserve mobilisation and ultimately an increase in fatty acids subsequently used for heat production in liver and skeletal muscles (Slee and Halliday 1968), subsequently reducing the nutrient reserves available to foetus by placental diffusion and thus potentially impacting foetal development.

Finally, according to Pääkkönen and Leppäluoto (2002) cold exposure has to be more acute than 3 hrs at -5°C to trigger a response and affect the plasma adrenaline levels. However, when the cold exposure was combined with a cognitive task 1.5 hrs at 4°C was sufficient to raise noradrenaline levels.

2.3.3.3 The impact of other stressors during pregnancy on lambs: isolation, yarding and transport

Several studies have explored whether the effects of exposure to common stressors such as yarding, isolation and transport during pregnancy can mimic the effects of pregnancy shearing.

In cattle repeated transportation in early up to mid-pregnancy resulted in increased foetal calf weights (Lay *et al.* 1997b). Roussel *et al.* (2004) reported that repeated 1 hr social isolation twice a week during the last 5 weeks of pregnancy (day 112 to 145) resulted in increased lamb birthweight. They recorded a decrease in plasma cortisol concentrations following repeated isolation indicating habituation of the ewe to the stressor which was confirmed in a later study of Corner *et al.* (2006). Roussel-Huchette *et al.* (2008) compared isolation and transport stressor during late-pregnancy and lambs born to repeatedly isolated pregnant ewes were heavier at 3 months than those born to transported and control pregnant ewes. In the same study, lambs born to transported ewes were less fearful when tested at 1 and 3 months using a human interaction and novel object test and had less escape attempts during isolation and novelty object tests compared to the control lambs. This result seems contrary to observations in rodents, where pups born to stressed dams had decreased exploratory behaviour (Weinstock 2008). Differences observed between lambs born to transported and isolated ewes might be due to different habituation rate to these two stressors.

Twenty four-hour fasting followed by intense yarding in mid-pregnancy of ewes in good body condition did not produce an effect on the lamb birthweight (Corner *et al.* 2007a). The mid-pregnancy yarding also had no significant impact on lamb behaviour at 12 hours of life although Corner *et al.* (2006) reported that, yarded ewes were more likely to emit low pitch bleats which have been described as the ‘care giver’ communication by Dwyer *et al.* (1998).

The aims of this thesis were to:

- ✓ Understand the mechanisms underpinning increased birthweights in lambs born to mid-pregnancy shorn ewes using alternate husbandry stressors and cold stress.
- ✓ Assess the effects of mid-pregnancy (day 85-95 of pregnancy) shearing on lamb behavioural reactivity and heat production.
- ✓ Assess the effects of mid-pregnancy stressors combination (yarding, transport, hosing and cold exposure) on lamb’s body dimension, behavioural reactivity and heat production.
- ✓ Assess the effects of late-pregnancy (day 120 -130 of pregnancy) shearing and stressors combination on lamb’s body dimension, behavioural reactivity and heat production. Assess the role played by cold stress in the effects of late-pregnancy shearing on lamb vigour traits.
- ✓ Assess the impact of prenatal stressors on lamb response to a challenge.

This thesis investigates the impact of husbandry-like prenatal stress during mid- and late-pregnancy on lamb traits associated with vigour. We focused particularly on three traits: morphometric, behavioural and thermoregulatory. A series of three experiments was conducted to address the following hypothesis “Exposure to mild prenatal stress during mid- and late-pregnancy alters lamb birthweight and dimensions, behaviour and thermogenic capacities”. A different flock of ewes were used for each experiment, however all three experiments the same experimental design with only different treatments. Same protocols were used across the experiments to assess the morphometric, behaviour and thermogenic effects. The results of these experiments are presented in this thesis grouped by lamb vigour traits: lamb body dimension (Chapter 3), lamb behaviour and reactivity (Chapter 4) and finally lamb thermoregulatory abilities (Chapter 5). We dedicated a chapter to the description of the method used to assess and discriminate heat production in newborn lambs (Chapter 6), which for practical and analysis purpose focused on only one treatment group in one experiment. While the same experiments and animal pools are described in the experimental chapters, numbers of lambs assessed and analysed may vary between chapters as due to time and experimental constraint only lambs born during the observation lambing period and which could be assessed as per protocol were enrolled. The experimental and method chapters in this thesis have been written as journal articles for publication and therefore have some commonalities. However, references for the whole thesis were kept separate and are all displayed at the end of the thesis.

Chapter 3: **Impact of prenatal stress on ewe metabolism and lamb birthweight and body dimensions.**

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ABSTRACT

Previous research has shown that shearing during pregnancy can increase birthweight of lamb; however, the contributing roles played by stress or by the cold exposure of ewes resulting from shearing have yet to be fully understood. This trial, divided into three experiments, was designed to examine the effect of cold stress resulting from shearing, cold exposure, and combined stressors including cold exposure of ewes during different stages of pregnancy on: ewe blood glucose and lactate levels, and live weight and body dimensions (crown-rump length and girth circumference) of lambs in three separate experiments. For the first experiment, pregnant ewes were subjected to either shearing or handled, as a sham treatment, during mid-and late-pregnancy (D90 and D130) to highlight the effects of cold stress, and shorn ewes were wetted using sprinklers on three occasions the week following shearing to exacerbate the effect of cold. In a second experiment, during late-pregnancy ewes (D130) were transported and cold exposed for 3 hrs on three occasions alongside a control group. Finally, in a third experiment, pregnant ewes were exposed to a combination of stressors designed to mimic the effects of shearing (yarding, transport, wetting and cold exposure), on five occasions during mid- and late-pregnancy (D90 and D125). Cold stress during pregnancy (cold stress and/or cold stress resulting from shearing) during both mid- and late-pregnancy increased the size of lambs at birth (*Experiment 1* $P<0.01$ for girth circumference and $P<0.05$ for crown-rump length; *Experiment 2* $P<0.02$ for crown-rump length and $0.05 \leq P<0.1$ for girth circumference), and shearing at mid-pregnancy significantly increased birthweights ($P<0.05$). No positive effects on lamb dimensions and birthweights were observed with the combined stressors. Stress resulting from shearing and sham-shearing during mid-pregnancy increased blood glucose in ewes ($P<0.01$) but decreased it when the treatment was applied during late-pregnancy ($P<0.01$). Similarly, late-pregnancy cold exposure and control treatments decreased blood glucose (*P*<0.01 for both). Finally, in the third experiment, repeated combination of stressors resulted in a reduction of blood glucose levels during both mid and late-pregnancy (P<0.01). We concluded that under our experimental conditions mid- and late-pregnancy stress combinations did not produce a similar effect to shearing and late-pregnancy cold exposure despite having similar effects on maternal glucose.

Keywords: pre-lamb shearing, prenatal stress, cold stress, lamb live weight, maternal glucose

3.1 Introduction

Managing pregnant ewes to optimise lamb birthweight is an important target for current management practices. It is recognised that across breeds and litter sizes birthweights of 3.5 to 5.5 kg lead to best survival for newborn Merino lambs (Atkins 1980; Hatcher *et al.* 2009; Geenty *et al.* 2014). Despite using optimum welfare management practices pregnant ewes will experience stressful husbandry practices amongst which are yarding, shearing, fasting, isolation and transport and understanding their effects on lamb morphometric traits relating to survival is crucial. Shearing during pregnancy under both pastoral and housed conditions has been shown to result in an increase in lamb birthweight (Dýrmundsson 1991; Kenyon *et al.* 2003; Sphor *et al.* 2011; De Barbieri *et al.* 2012, 2014). Increased birthweight is consistently observed in both single (Morris *et al.* 2000a; Sherlock *et al.* 2003; Corner *et al.* 2006; Corner *et al.* 2007a) and multiple born lambs (Morris and McCutcheon 1997; Smeaton *et al.* 2000; Kenyon *et al.* 2004; Corner *et al.* 2006; Corner *et al.* 2007b) when shearing is applied during mid-pregnancy.

In some studies, the increased birthweights resulting from shearing have led to increased survival rates, regardless of the timing of shearing (Morris *et al.* 1999; Kenyon *et al.* 2003; Kenyon *et al.* 2006a; Keady and Hanrahan 2009), and Montossi *et al.* (2005) observed differences in survival rates following shearing without any birthweight differences.

When investigating the mechanisms underlying increased birthweight, cold stress of the ewe resulting from fleece removal was first considered as a causal factor as most shearing occurred during winter. However, similar birthweight effects were also observed in summer shorn ewes (deNicolo *et al.* 2008), and manipulation of thyroid hormones to replicate changes associated with cold stress failed to produce similar effects (Kenyon *et al.* 2005), suggesting that cold stress might not be solely responsible. The effects of acute, repeated and chronic stress were also investigated, with inconsistent results. While Roussel *et al.* (2004) observed increased birthweight in lambs born to ewes exposed to repeated isolation during the last 5 weeks of pregnancy, differences could be accounted for by different breeds or the selection criterion for the study which was reactivity level. Although ewes were selected for either a high or a low reactivity level, the authors reported that the differences in reactivity were no longer apparent after the stress was applied, and no such effect was observed by Corner *et al.* (2010).

Similarly, inconclusive results were found for the effects of transport stress on birthweight. Repeated transport of pregnant cows during early and mid-pregnancy has been reported to result in increased birthweight in calves (Lay *et al.* 1997a) while Roussel-Huchette *et al.* (2008) did not find birthweight differences in lambs born to ewes transported in isolation during the last 6 weeks of pregnancy. Others investigated stressors such as yarding, fasting and handling, as repeated or single events, and these did not produce effects similar to those of shearing (Corner *et al.* 2006; Corner *et al.* 2007b, 2010).

In most studies of the effects of stress, unexpected psychological stressors such as road transport for 1 or 2 hours and yarding or movement to an unfamiliar environment resulted in an increased blood glucose in adult sheep (Reid and Mills 1962). Similar results were evident following adrenaline infusions at 3 different rates (40, 15 or 3 μ/kg/h) in normal and hypercortisolaemic sheep (Bolton and Weekes 1986). In pregnant ewes, increased glucose concentration has been observed in shorn ewes compared to unshorn ewes (Symonds *et al.* 1988a; Clarke *et al.* 1997; Morris *et al.* 2000b) although differences were not evident in all studies (Sherlock *et al.* 2003) and lamb birthweights were not always reported. Past studies have shown that, in lambs, high glucose and lactate levels are usually associated with higher birthweights, often associated with stressful parturition and fetal asphyxia (Dutra and Banchero 2011). However, the effects of prenatal stress on offspring metabolites and their link with birth weight have yet to be fully examined.

The present study aimed to investigate the effects of mild husbandry-like stressors such as, yarding, handling and cold exposure, individually or combined during different stages of pregnancy (mid and late-pregnancy) on lamb body dimensions at birth. The effects of those stress treatments on glucose levels in pregnant ewes and the relationships between ewe blood glucose and lamb body dimensions were also examined.

It was hypothesized that, a combination of stressors in mid-pregnancy would result in increased birthweights, mimicking the effects of shearing. and that late-pregnancy stressors (shearing, cold exposure or combined stressors) would also have a positive effect on lamb body dimensions. It was also hypothesized that, increased birthweights would be associated with high metabolites levels in lambs.

3.2 Material and methods

The study was carried out in 3 experiments to assess the effects of stressors of pregnant ewes on lamb morphometric traits. *Experiment 1* examined the effects of shearing stress and the associated cold exposure in mid-pregnancy and in late-pregnancy. *Experiment 2* was designed to examine the effect of cold exposure during late-pregnancy, and *Experiment 3* investigated the effects of a combination of stressors during mid or late-pregnancy. The experimental protocols were reviewed and approved by CSIRO FD McMaster Laboratory Animal Ethics Committee (Approval numbers 14/17, 14/30 and 15/08).

3.2.1 Experimental animals and design

All the experiments were conducted at the CSIRO Chiswick site located in Armidale, New South Wales, Australia and all animals included in the experiments were of apparent good health throughout the experiments and were individually identified with side-branded numbers applied with livestock-marking spray.

Table 3.1 details number of animals per treatment groups for each experiment.

Experiment 1, conducted from August to September of 2014, used 60 pregnant Merino ewes and 64 lambs. Ewes were multiparous and selected on pregnancy status at 80 and 110 days after ultrasound scanning. Gestation lengths ranged between 139 and-156 days, as calculated from estimated pregnancy day on scanning day. In total, 62 lambs were assessed and 4 cases of dystocia were observed where lambing assistance was required.

Experiment 2, conducted in December of 2014, used 61 pregnant Merinos ewes, in their last 2 weeks of pregnancy, 125-135 days of pregnancy, determined using ultrasound scanning. Gestation lengths ranged between 142-164 days, as calculated from estimated pregnancy day on scanning day. Due to staffing issues and shift in lambing dates the experimental data set only included 27 ewes from all those originally enrolled and 30 lambs were assessed. Six cases of dystocia were observed where lambing assistance was required.

Experiment 3, conducted from July to early September of 2015, used 108 multiparous pregnant Merino mature ewes selected on their pregnancy status at 80 and 110 days based on ultrasound scanning and 84 lambs assessed. Gestation lengths were 130-146 days. One case of dystocia was observed.

For all lambing events, in cases of observed dystocia, lambing was assisted and lambs were visually monitored after birth to confirm successful suckling of colostrum.

Successful suckling of colostrum was visually monitored for each lamb. Three lambs from *Experiment 1,* two from *Experiment 2* and two from *Experiment 3* with insufficient suckling activity were deemed to be too weak to be enrolled in the respective experiments. All five of those lambs received manual latching support, but died within the following 24 hrs.

3.2.2 Housing

For all three experiments, the ewes were grazed ad libitum on improved native pasture and supplemented with 150g/day of sheep pellets (based on wheat, millrun and lucerne; 17.5% protein, 2.5% fat, 17% fibre, 20% ADF, 34% NDF) for *Experiment 1* and *2 and* for *Experiment 3* the ewes were fed ad libitum hay and 100g/animal/day of corn grain.

During indoor periods animals were fed a 50:50 lucerne and oaten chaff mix supplemented with a 3:2 sheep pellet and corn ration (200 g/day; sheep pellets based on wheat, millrun and lucerne; 17.5% protein, 2.5% fat, 17% fibre, 20% ADF, 34% NDF). Throughout all three experiments, nutritional management aimed to maintain a BCS of ewes between 2.5 and 3.5.

For all 3 experiments, around 10 days before the expected lambing date, ewes were housed on straw bedding in indoor lambing pens, in an open-sided animal house, at a density of 3-4 ewes per pen (1.5 m^2 per ewe) on straw bedding and under continuous artificial lighting 24 hrs/day. Once ewes showed signs of parturition, they were moved to separate pens as much as possible to allow intimate ewe-lamb bonding. Ewes were visually checked at least every 30 min and video cameras were used to continuously monitor for parturition and early behaviours. At any moment, each camera captured a view of the all lambing pens. The cameras were connected to digital video recorders and footage captured using IVMS4200 software (Hangzhou Hikvision Digital Technology Co., Ltd).

Experiment 1 lambing took place between 29th September and 17th October 2014 and average temperature were on average 12˚C (2˚C/24˚C min/max temperature; 56% humidity on average). *Experiment* 2 lambing took place between 6th December 2014 and 13th January 2015 and average temperatures were on average 18.5˚C (10˚C/29˚C min/max temperature; 75% humidity on average). *Experiment 3* took place between 14th and 24th September 2015 and average temperature were on average 9˚C (0˚C/19˚C min/max temperature; 74% humidity on average).

3.2.3 Ewe Stress Treatments

Ewes were assigned to treatment groups according to Table 3.1.

Experiment 1 - Shearing and Handling

At D90 or D125 of pregnancy, over the treatment of 7 days, pregnant ewes from both the shorn and control groups, were housed indoor in group pens (3 m^2/ewe) on a slatted floor, separated according to treatment group. Animals were acclimated in the shed for 5 days before the beginning of the experiment. On the first day (D0), the treated groups were shorn (MID-S & LATE-S) while the control groups were sham-handled for 2 min (MID-C & LATE-C) alongside the shorn group by handling similar to shearing (seated on haunches, manual stroking of both sides of the body and rump). Following shearing, shorn ewes, but not control ewes, were wetted using ceiling sprinklers for 30 min, on 3 occasions over 7-day period (D0, D3 & D7).

Experiment 2 - Cold exposure

During the last 2 weeks of pregnancy (D130-D140 of pregnancy) one group of ewes were cold exposed (CE) on 3 occasions over 5 days (D0, D2, D5) and a control group was transported but not cold exposed (TR). On those 3 occasions, both CE and TR groups were transported from the paddock to the cold room facility using a stock trailer. For practical reasons, each group was divided in 4 subgroups of 7-8 animals and each subgroup was exposed to 5 min of loading and transport. At the cold room facility, the CE ewes were unloaded and were individually wetted for 2 min using a jet hose in order to enhance the cold treatment. For the wetting, ewes were sat on their rump and bare skin areas were hosed to ensure wool free areas around legs and groin were wet. The CE ewes were subsequently put in a 3˚C cold room for 3 hrs. At the same time the TR ewes were unloaded and held on a slatted floor pen and left without any interaction for the 3 hrs. At the completion of the treatment all animals were returned to their paddock.

Experiment 3 – Combination of stressors

At D90 (MID) or D125 (LATE) of pregnancy both the treated groups (MID and LATE) were exposed to a stress treatment on 5 occasions over a period of 11 days (D0, D2, D5, D8 & D10 or D11). In this experiment, late-pregnancy stage was considered at D125 instead of D130 as in *Experiment 2* to allow completion of the stress treatment before beginning of lambing. The stress treatment consisted of ten minutes of yarding (humans only), ten minutes of transport in a stock trailer, ten minutes of high water pressure hosing and then three hours in a 3˚C cold room. During these treatments, the control group (CTRL) remained in a paddock with as little human interaction as possible.

Table 3.1 Summary of treatment groups, stress treatments and number of animals (ewes and lambs) per treatment group for experiment 1, 2 and 3.

Group code	**Treatment applied**	**Number of ewes**	**Total number of lambs**	**Resultant number of lambs**	
				Singles	**Twins**
Experiment 1					
MID-S	Shorn at 90 days of pregnancy, the wetted three times using ceiling sprinklers during the following week	15	16	8	8
MID-C	Sham-shorn at 90 days of pregnancy, no shearing comb in the handpiece, not wetted during the following week	15	19	8	11
LATE-S	Shorn at 120 days of pregnancy, the wetted three times using ceiling sprinklers during the following week	15	14	10	4
LATE-C	Sham-shorn at 120 days of pregnancy, no shearing comb in the handpiece, not wetted during the following week	15	15	11	4
Experiment 2					
CE	Unshorn, wetted on the belly and groin then placed in a cold room at 3 °C for 3 hrs, on three occasions over a 5-day period	17	18	16	2
CTR	Transported alongside the CE ewes, but held at environmental temperature for the 3- hr period	16	19	13	6
Experiment 3					
MID	At 90 days of pregnancy, transported, yarded, hosed under high pressure cold water for 10 min, then placed in a cold room at 4 °C for 3 hrs	36	24	16	8
LATE	At 120 days of pregnancy, transported, yarded, hosed under high pressure cold water for 10 min, then placed in a cold room at 4 °C for 3 hrs	36	28	11	17
CTRL	Remained in the paddock with minimal human interference throughout pregnancy	36	33	17	16

3.2.4 Lambing

All live-born lambs born during the experiments were individually identified shortly after birth and physical morphometric measurements were taken within the first 4 hrs post-partum.

For all 3 experiments body dimensions measured included: body weight (placing the lamb in a bucket on top of laboratory scales), girth (chest) circumference- (measured with a soft measuring tape behind the front legs) and crown – rump length (distance between the cross point of a line between the left horn bud and the right eye and a line between the right horn bud and the left eye to the base of the tail).

3.2.5 Blood glucose levels

For each experiment, blood samples were collected from ewes before the beginning of the stress treatment – baseline (D0) and after the last stress of the treatment (D5; D7 or D11) to compare blood glucose level evolution resulting from the stress treatment. For *Experiment 1* the baseline samples were taken on D0 and the after-stress samples on D7. For *Experiment 2* the baseline samples were taken on D0 and after-stress treatment samples on D5. Finally, for *Experiment 3* the baseline samples were taken on D0 and after stress treatment samples on D11. Ewe blood samples were taken by jugular venepuncture using a 16-gauge needle and a 10 mL heparin vacutainer.

For each experiment, blood samples were also collected from lambs close to the time of birth, leaving some time for the dam to lick the lamb and establish the ewe-lamb bond but before latching (T0). Lamb blood samples were taken by jugular venepuncture using a 20-gauge needle and a 5 mL heparin vacutainer prior to the lamb feeding.

For E*xperiments 1* and *2*, blood samples were centrifuged and plasma glucose and lactate were determined using a Siemens Dimension Xpand plus clinical analyser (Bayswater Vic Australia 3153) after samples had been frozen at -20°C.

For *Experiment 3,* blood glucose analysis was determined using the Accu-chek Performa system (Accu-chek, Roche Diabetes Care, Roche Products Pty, Basel, Switzerland). This glucose-meter allowed a measurement in the field immediately after the bleeding. Similarly to *Experiment 1* and *2*, plasma lactate levels were determined using a Siemens Dimension Xpand plus clinical analyser (Bayswater Vic Australia 3153) after samples had been frozen at -20°C.

Blood glucose levels were given in mmol/L by the glucometer and plasma lactate and glucose levels were obtained in mg/dL using the clinical analyser. For glucose the two methods were calibrated to obtain a conversion ratio of 18 between the two units which is the conversion ration previously used by Chase (2006). We used this conversion (1 mmol = 18 mg/dL of glucose) to homogenise the data to allow a direct comparison.

3.2.6 Statistical analysis

All statistical analyses were performed using R software (version 3.1.0, The R foundation for Statistical Computing). For normally distributed morphometric traits an analysis of variance using a linear mixed model was used to test the effects of treatment, timing of treatment, litter size and their interactions and with lamb random effect. For blood and plasma glucose data that were normally distributed, a repeated measures analysis of variance using the linear mixed model was used to analyse the effects of treatments and litter size. For each model, fixed effects initially included sex of the lambs but this was subsequently removed due to the absence of any significant effects ($P > 0.09$).

When data were not normally distributed, non-parametric Kolmogorov-Smirnov tests were used to assess differences between treatment and litter-size groups. Results are shown as LS means ± standard deviation (S.D.). Results with an associated probability less or equal to 0.05 were considered significant and those less to 0.10, a tendency.

3.3 Results

3.3.1 Blood and plasma glucose in ewes

Experiment 1 At baseline (D0), blood glucose levels were significantly lower in mid pregnancy compared to late pregnancy (D0 MID than D0 LATE ewes (P < 0.0001; 51.0±1.65 vs 65.9 ±1.65 mg/dL).

No significant treatment effects were apparent at either time point (MID or LATE); however, stress effects differed when applied at mid or late-pregnancy (Figure 3.1) and this was observed for both shearing and sham shearing. Overall, LATE-C treatment was significantly lower than MID-C (49.86±4.31 vs 63.02±4.31 mg/dL; P=0.0189) and LATE-S tended to be lower than MID-S (51.54±4.31 vs 61.20±4.31 mg/dL; P=0.0815).

At mid pregnancy, both shearing and sham shearing treatments increased glucose levels, whereas at late pregnancy, either treatment lowered glucose levels. For both MID-C and MID-S ewes, blood glucose level was significantly higher for D7 compared to D0 measurements (overall means respectively, 76.27±18.2 vs 51.80±7.78 mg/dL *P* < 0.001; 74.28±33.3 vs 50.14±8.92 mg/dL P=0.001)

whereas for both LATE-C and LATE-S ewes, plasma glucose was significantly lower for the D7 compared to D0 measurement (overall means respectively, 35.67±9.69 vs 66.07±8.61 mg/dL, *P* < 0.001; 39.40±17.2 vs 65.70±11.0 mg/dL, *P* < 0.001).

At mid pregnancy, both shearing and sham shearing treatments increased glucose levels, whereas at late pregnancy, either treatment lowered glucose levels. For both MID-C and MID-S ewes, blood glucose level was significantly higher for D7 compared to D0 measurements (overall means respectively, 76.27±18.2 vs 51.80±7.78 mg/dL *P* < 0.001; 74.28±33.3 vs 50.14±8.92 mg/dL P=0.001)

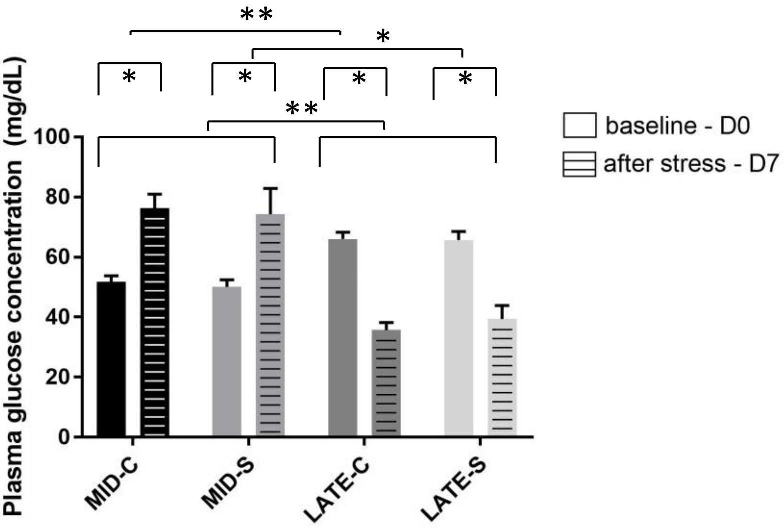

Figure 3.1 Maternal plasma glucose levels at (baseline D0 (full bar) and after stress D7 (striped bar) of ewes either shorn at mid or late pregnancy (MID-S& LATE-S) or sham shorn at mid or late-pregnancy (MID-C & LATE-C). Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

Experiment 2

No significant treatment differences were observed in ewe glucose levels when comparing D5 and D0 measurements (Figure 3.2). For both CE and TR maternal blood glucose levels were lower for D5 than D0 (overall means respectively, 67.50±16.2 vs 81.38±13.5 mg/dL P<0.001; 61.82±10.0 vs 82.87±10.8 mg/dL P<0.001).

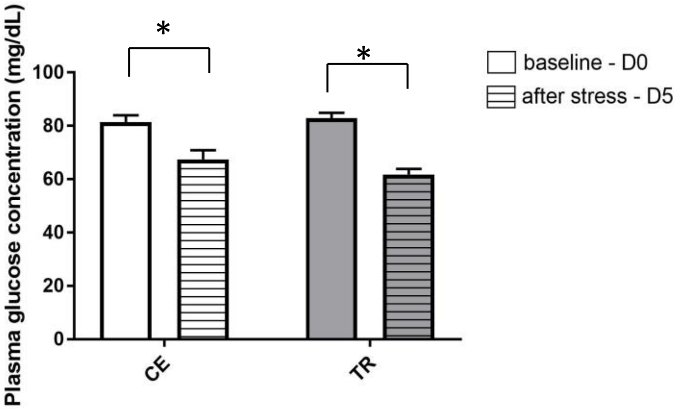

Figure 3.2 Maternal plasma glucose levels at baseline D0 (full bar) and after stress D5 (striped bar) of ewes either cold-exposed at 3 times during late-pregnancy (CE) or control (TR). Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

Experiment 3

At D0, LATE and MID glucose levels were both significantly different (P<0.0032) from that of the CTRL ewes (59.51±1.21 & 58.59±1.26 vs 64.60±11.88 mg/dL; P=0.0032 & P=0.0007) but no significant difference was seen between stages of pregnancy. The D11 blood glucose level in the LATE ewes were significantly lower than MID (58.07±1.58 vs 71.89±1.66 mg/d; P<0.001) and significantly lower than CTRL (58.07±1.58 vs 75.85±1.55 mg/dL; P<0.001) (Figure 3.6).

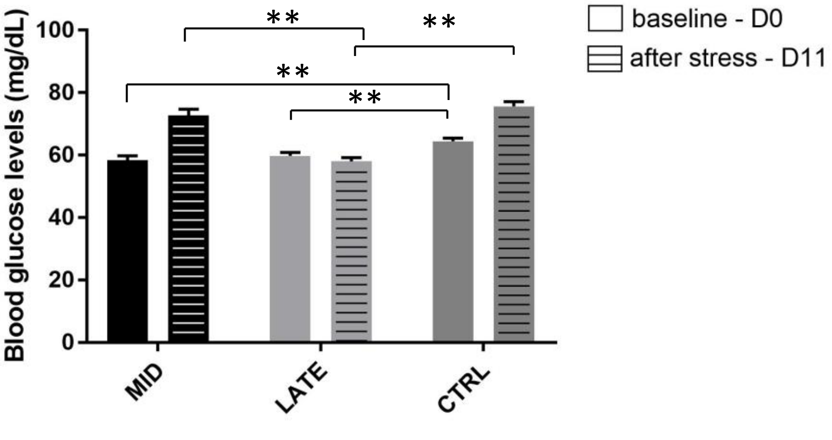

Figure 3.3 Maternal blood glucose levels at baseline D0 (full bar) and after stress D11 (striped bar) of ewes subjected to a combination of stressors (yarding, transport, hosing and cold exposure) during mid-pregnancy (MID), late-pregnancy (LATE) and control ewes (CTRL). Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

3.3.2 Lamb birthweight

Experiment 1 twins were significantly lighter than singles for all treatment groups (MID-S, P=0.0007; LATE-S P=0.0001; MID-C P=0.0010; LATE-C P=0.0001). *Experiment 1* MID-S singles lambs were significantly heavier (+510g) at birth than MID-C single lambs (P=0.0431) and while not significant a similar trend (+430g) was seen in twins (P=0.0661; Figure 3.4 (a)). In *Experiment 1,* no significant difference was observed between LATE-S and LATE-C ewes, however LATE-S singles tended to be lighter (+410g) than MID-S singles (P=0.0863) (Figure 3.4 (a)).

In *Experiment 2,* no significant treatment or litter size effect were observed (Figure 3.4 (b)).

Experiment 3 CTRL lambs were significantly heavier (+370g) at birth than MID lambs (P=0.0109) and LATE lambs tended to be heavier (+270g) than MID lambs (P=0.0583; Figure 3.4 (c)).

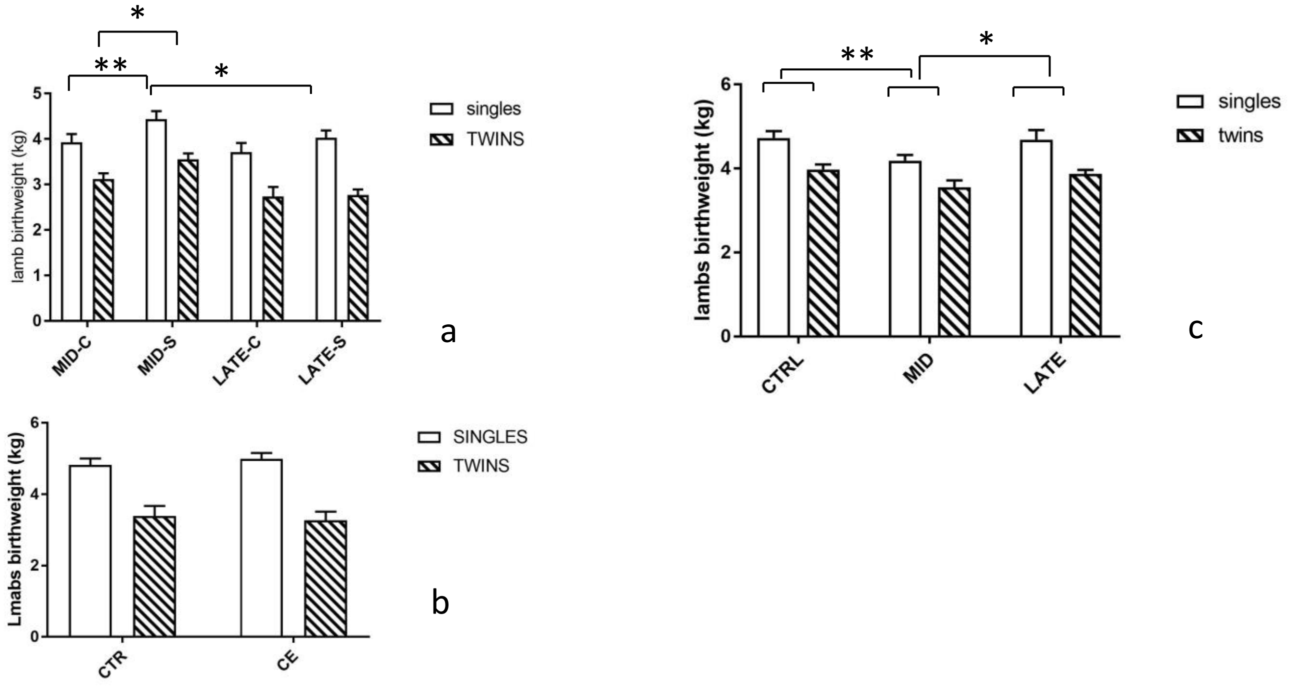

Figure 3.4 Weight at birth of single and twin lambs born to ewes prenataly stressed. In Experiment 1 (a), ewes were shorn either in mid- or late-pregnancy (MID-S & LATE-S) or sham shorn at mid- or late-pregnancy (MID-C & LATE-C). In Experiment 2 (b), ewes were ewes cold exposed during late-pregnancy (CE) or control (CTR). In Experiment 3 (c), ewes were exposed to a combination of stressors (yarding, transport, hosing and cold exposure) at mid- (MID) or late-pregnancy (LATE) or control ewes (CTRL). Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

3.3.3 Girth circumference

In *Experiment 1* singles lambs from shorn ewes (MID-S and LATE-S) had a greater girth (+ 3 cm) than control animals (MID-C and LATE-C) (P=0.0036), but the difference was not significant for twins (Figure 3.5 (a)).

In *Experiment 1,* there was also no significant difference between stages of pregnancy treatment.

Experiment 2 singles CE lambs tended to have greater girth circumferences (+ 2 cm) than CTR single lambs (P=0.0774; Figure 3.5 (b)).

Experiment 3 MID lambs had smaller girth circumferences (- 1 cm) than CTRL lambs (P=0.0391; Figure 3.5(c)).

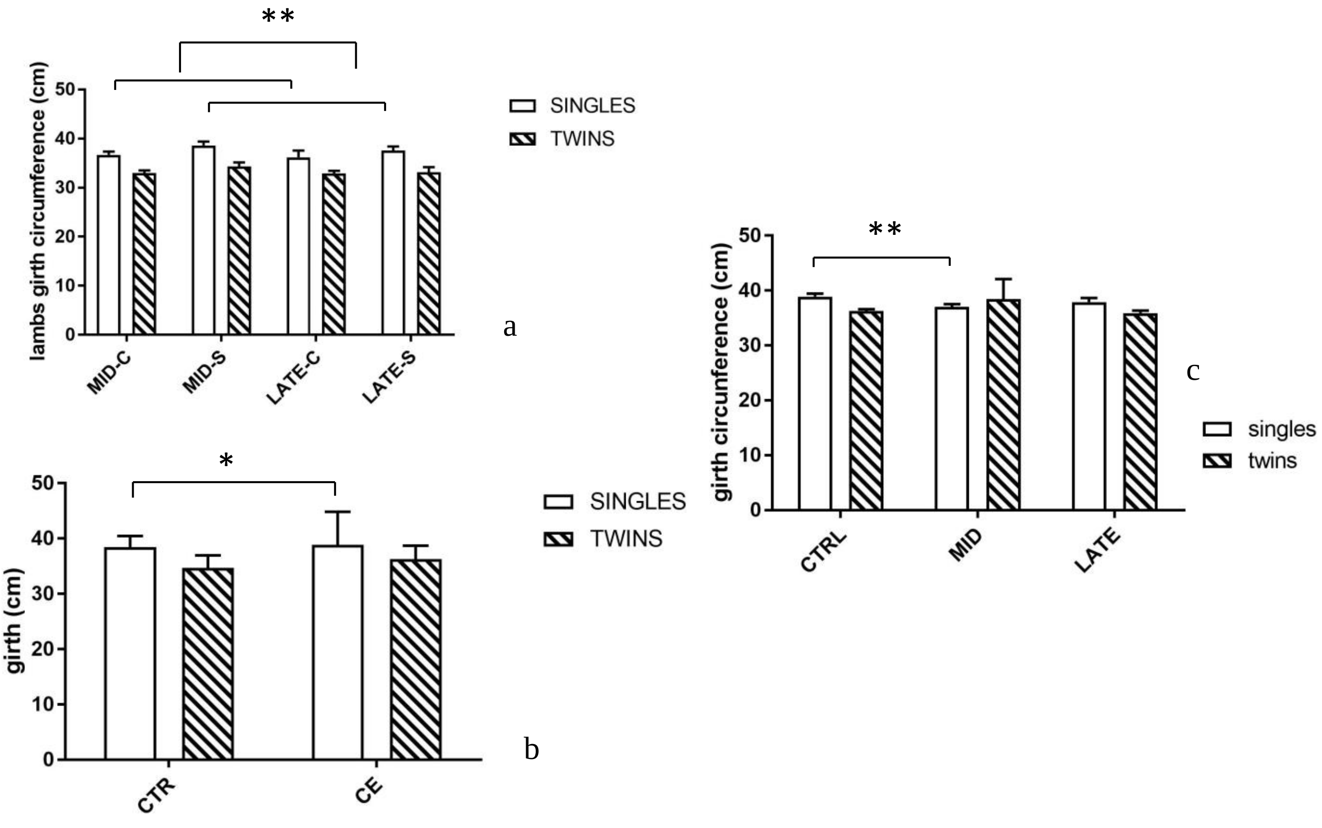

Figure 3.5 Girth circumference, measured behind the forelimbs, at birth of single and twin lambs born to ewes prenatally stressed. In Experiment 1 (a), ewes were shorn either in mid- or late-pregnancy (MID-S & LATE-S) or sham shorn at mid- or late-pregnancy (MID-C & LATE-C). In Experiment 2 (b), ewes were ewes cold exposed during late-pregnancy (CE) or control (CTR). In Experiment 3 (c), ewes were exposed to a combination of stressors (yarding, transport, hosing and cold exposure) at mid- (MID) or late-pregnancy (LATE) or control ewes (CTRL).
Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

3.3.4 Crown-Rump length

In *Experiment 1,* similarly to girth circumference, differences between stages of pregnancy were not significant for lamb crown-rump length. The *Experiment 1* twin control lambs (MID-C & LATE-C) were shorter (- 3 cm) than twin shorn lambs (MID-S & LATE-S) (P=0.0362) but differences were not significant for singles (Figure 3.6 (a)).

Experiment 2 CE lambs had significantly longer (+ 3 cm) crown-rump lengths than CTR lambs (P=0.0127; Figure 3.6 (b)) while for *Experiment3* there was no significant differences between treatment groups (Figure 3.6 (c)).

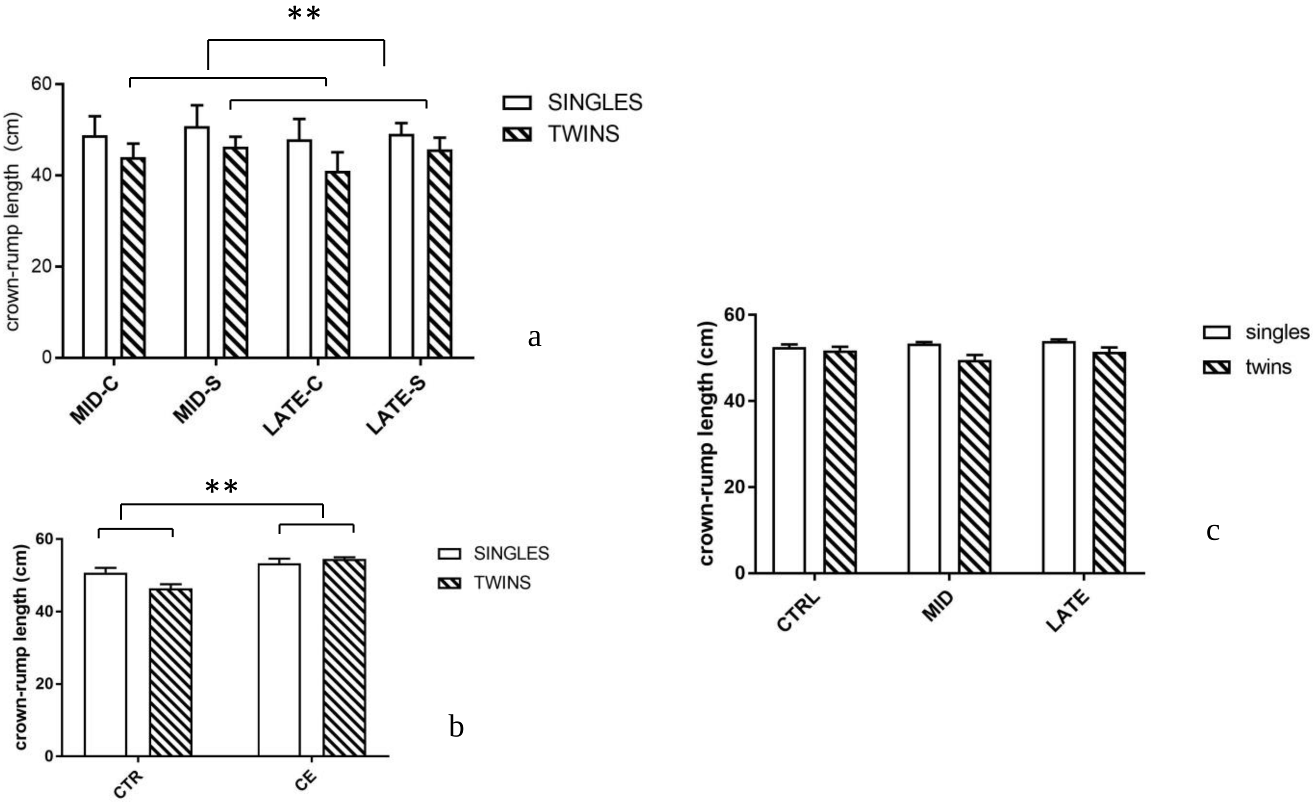

Figure 3.6 Crown-rump length, measured between the crown and the tail bending point, at birth of single and twin lambs born to ewes prenatally stressed. In Experiment 1 (a), ewes were shorn either in mid- or late-pregnancy (MID-S & LATE-S) or sham shorn at mid- or late-pregnancy (MID-C & LATE-C). In Experiment 2 (b), ewes were ewes cold exposed during late-pregnancy (CE) or control (CTR). In Experiment 3 (c), ewes were exposed to a combination of stressors (yarding, transport, hosing and cold exposure) at mid- (MID) or late-pregnancy (LATE) or control ewes (CTRL).
Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

3.3.5 Lambs blood and plasma glucose and lactate

In *Experiment 1* no significant treatment effect was observed on lamb plasma glucose and lactate levels at birth. In *Experiment 2*, No significant litter size effect was observed on lamb plasma glucose and lactate levels at birth, however, TR lambs displayed a significantly lower glucose level at birth than CE lambs (P=0.0468; 47.30 ±4.6 vs 33.45 ±4.8 mg/dL). Finally, in *Experiment 3*, MID lambs had significantly higher plasma lactate levels at birth than LATE lambs and tended to have lower lactate levels at birth compared to the CTRL lambs (back-transformed means; 47.9 vs 34.9 and 47.9 vs 36.5 mg/dL; P=0.0304 & P=0.0539). Blood glucose of CTRL twin lambs was lower than singles (54.36±8.28 vs 79.92±8.28 mg/dL; P=0.0329) and similar tendency was observed for the levels of the MID lambs (55.08±12.24 vs 80.82±8.28 mg/dL; P=0.0865). No significant difference was noted in the LATE lambs.

3.4 Discussion

The results of this study indicate that cold exposure during pregnancy (cold exposure per se and/or cold exposure resulting from shearing), during both mid- and late-pregnancy, impacts the size of lambs, as seen by increased girth and crown-rump (in single lambs). When mid-pregnancy shearing was compared to mid-pregnancy handling alone, to highlight the effects of subsequent cold exposure, lambs born to shorn ewes were heavier than control lambs, which supported the hypothesis that cold exposure played a role in the effects of shearing on lamb birthweights. Shearing has been shown to expose the ewes to several stressors inducting both acute and chronic stress responses in sheep. The acute stress elicited by the mustering, time off pasture and fastening, handling, tactile stimulus and exposure to dogs, vehicles and noise has been reported to produce elevated cortisol levels and heart rate responses which were greater than those to individual stressors of crutching, isolation or handling (Hargreaves and Hutson 1990c, 1990b, 1990e, 1990d, 1990a). Chronic stress potentially results from the effects of cold exposure resulting from fleece removal and raises the. No effect on lamb size was observed when cold exposure was combined with husbandry stressors including yarding and transport which aimed to reproduce the stress response observed to shearing. However, late-pregnancy cold exposure following transport and hosing, while not producing any effects on birthweights, did alter lamb size supporting the hypothesis that "husbandry like" acute stressors might not be the only reason for increased birthweights observed in lambs born to shorn ewes (Corner *et al.* 2006; Corner *et al.* 2010). In the past, studies have examined different potential underlying mechanisms contributing to increases in lamb birthweight after shearing, but without success. Previously, Corner *et al.* (2006) compared lambs born to shorn ewes with lambs born to yarded ewes and to control (unstressed) ewes, allowing the stress from yarding to be eliminated as a potential mechanism responsible for the increased birthweight. While they observed an effect from mid-pregnancy shearing, no difference in lamb birthweight resulted from mid-pregnancy yarding when compared to control treatment (Corner *et al.* 2006). Chronic cold exposure and thyroid hormones changes subsequent to shearing have also been rejected as sole mechanisms underpinning increased birthweights (Kenyon *et al.* 2005). Corner (2007a) assessed the effects of both chronic stress and acute stress on lambs body dimensions and did not observe effects but in the present series of studies we had hypothesized that cold stress/exposure had to be combined with acute stressors to increase lamb size. However, the absence of such effects in the third experiment, in which we observed smaller and lighter lambs born to ewes stressed during mid-pregnancy when compared to those born to control ewes or ewes exposed to this stress combination during late-pregnancy. Although the magnitude of the effects of shearing on lamb birth weight have been inconsistent, and some studies have failed to find a birth weight effect (Stott and Slee 1985; Dabiri *et al.* 1994; Morris *et al.* 2000a; Kenyon *et al.* 2002c; Kenyon *et al.* 2006b) no negative effects have been reported. This variation in response may be due to the nature of the stressors being either too mild to produce any physiological response or “severe” enough to trigger an adaptive response. The expected increase in lamb dimensions associated with increased live weight has not always occurred in mid pregnancy shearing studies (Sherlock *et al.* 2003; Kenyon *et al.* 2005), confirming the possibility that there are different underlying mechanisms responsible of size (skeletal size) and weight (muscle).

As noted in previous studies, the greatest effects on lamb size were observed when ewes were exposed to shearing during mid-pregnancy (Corner *et al.* 2006; Corner *et al.* 2007a; deNicolo *et al.* 2008; De Barbieri *et al.* 2014).

Glucose is the primary energy source for foetal tissues and their growth and development (Bell and Bauman 1997) and as such may be a significant contributor to birthweight changes in response to shearing. In general, we found that shearing and stress during pregnancy resulted in changes in maternal blood glucose concentrations, the direction of change being dependant on the stage of pregnancy. Stress resulting from shearing and sham-shearing during mid-pregnancy increased blood glucose level which was an expected result as hyperglycemia resulting from acute stress had been highlighted previously by Reid and Mills (1962). However, shearing in mid-pregnancy, have been shown to have inconsistent effects on maternal glucose production as some studies have found an increased blood glucose concentrations in shorn ewes compared to unshorn (Symonds et al. 1988a; Clarke et al. 1997; Morris et al. 2000b), while others have reported similar concentrations (Symonds et al. 1988b; Sherlock et al. 2003).

This increase might be expected as maternal glucose production increases as gestation advances to accommodate uterine and foetal requirements (Wilson et al. 1983) and glucose production rate has been shown to increase particularly in late-pregnancy and to reach its maximum at parturition (Steel and Leng 1973a; Wilson *et al.* 1983; Bell and Bauman 1997; El-Sherif and Assad 2001). Clarke *et al.* (1997) reported higher plasma glucose concentrations in shorn ewes between 126 and 140 days of gestation although no lamb body dimensions were reported. Furthermore, exogenous glucose intravenous infusions into foetuses during late-pregnancy produced heavier lambs (Stevens et al. 1990) This contrasts with our findings that shearing and cold-exposure during late-pregnancy decreased maternal plasma and blood level, witnessing a physiological impact of the prenatal treatments. The lack of such observations in *Experiment 3* for both mid and late-pregnancy treatments, seem to suggest that a threshold trigger to initiate a maternal glucose decrease might be the reason for the unexpected reduced birthweight observed.

In the early studies of Steel and Leng (1968), Steel and Leng (1973a), Steel and Leng (1973b), Lindsay (1970), Bergman (1973) and Chaiyabutr *et al.* (1982) increased blood glucose levels in late-pregnancy were explained by an increase in feed intake; however, more recent studies have confirmed the increase in blood glucose levels but without any increase in energy intake (MacRae and Egan 1983; Wilson *et al.* 1983) or even with restricted diet during the last 6 weeks of pregnancy (Husted *et al.* 2008). In shorn ewes that display increased glucose concentration there have been no differences observed in blood flow across uterine tissues, or on the extraction and utilisation of glucose (Symonds *et al.* 1988b) or on placental weight (Revell *et al.* 2002). It has been suggested that gestational diabetes resulting from increased glucose production, especially during late-pregnancy, might impact nutrient partitioning towards the utero-placental-foetal unit (Bell *et al.* 2005). These observations have led to the suggestion that placental/foetal glucose transport might be the key mechanism responsible for any birthweight increases (Symonds *et al.* 1988b).

Without comparing blood glucose values across all three experiments, as they were obtained in different environments and animals, the values obtained were in similar range (50-85 mg/dL) and the effects of the different stress treatments on blood glucose were consistent which confirms that the stress treatments were coherent and of relatively close magnitude. Shearing during mid-pregnancy produced heavier lambs than shearing during late-pregnancy which could be partially explained by differences observed in maternal blood glucose levels and as such the quantity of glucose permeating to the foetus, as glucose diffusion to the placenta is dependent on the maternal-placental gradient in glucose concentration (Takata and Hirano 1997).

Although shearing, sham-shearing and late-pregnancy cold exposure did not impact glucose and lactate blood levels in newborn lambs, the combination of stressors negatively impacted newborn lactate levels. This impact was even more negative when the combination of stressors was applied in late-pregnancy compared to mid-pregnancy. Past studies have associated high lactate levels with high birthweights and stressful parturition resulting in fetal asphyxia (Dutra and Banchero 2011).

For a long time, birthweight has been used as sole indicator of lamb survival chances and has been linked to offspring metabolic profiles at birth. While mid-pregnancy shearing has been shown to increase birthweight, numerous studies examined the effects of other husbandry related stressors. However, the results observed were very inconsistent and, at present, the underlying mechanism/s remain unknown. In our study, shearing during mid-pregnancy impacted lambs body dimensions in a way that no other stressors (husbandry-like or cold) which leads to the suggestion that the mechanism lies within something other than husbandry-like stressors and cold stress as we had previously hypothesized. Both shearing and handling stress increased blood glucose levels in lambs mid pregnancy from baseline levels, while at late pregnancy, both shearing and handling stress and cold exposure lowered blood glucose in comparison to baseline.

The different impact of prenatal stress during pregnancy on ewe metabolic profile, while potentially explaining the different effects of pregnancy stress on birthweight and body size when stress was applied during different stages of pregnancy, does not help to explain the increased weight resulting from shearing and increased size resulting from shearing and late-pregnancy cold exposure. Further research needs to investigate different nature, levels, duration and timing of stressors and their effects on the ewe/foetal unit metabolic profile and foetal development. The effects of stress hormones injections such as cortisol and noradrenaline could also be used to eliminate the stressors nature component and could help understanding the underlying mechanism/s.

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STATEMENT OF ORIGINALITY

Chapter 3 - Impact of prenatal stress on ewe metabolism and lamb birthweight and body dimensions.

We, the Research Master/PhD candidate and the candidate’s Principal Supervisor, certify that the following text, figures and diagrams are the candidate’s original work.

Type of work	Page number/s
All figures, diagrams and text	

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Candidate

[redacted] 21/09/2017

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University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

Chapter 3 - Impact of prenatal stress on ewe metabolism and lamb birthweight and body dimensions.

We, the Research Master/PhD candidate and the candidate's Principal 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 in the *Statement of Originality*.

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Chapter 4: **Impact of mid- and late-pregnancy prenatal stress on lamb neonatal reactivity and vigour.**

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ABSTRACT

Poor lamb survival is an important animal welfare issue, and the lamb must express complex behaviours to successfully transition to life ex-utero. Even under best management, sheep husbandry practices, such as shearing, yarding and transport can be stressful for animals, and when they take place during pregnancy the effect of these stressors on lamb early behaviour is yet to be fully understood. This study, divided in three experiments, examined and compared the effects of diverse prenatal stressors, in keeping with husbandry procedures, on neonatal lamb behaviour. For the first experiment, pregnant ewes were subjected to either shearing or were handled only as a sham treatment, during mid-and late-pregnancy and to highlight the effects of cold exposure, the shorn ewes were wetted using sprinklers on three occasions the week following shearing. In a second experiment, during late-pregnancy ewes were transported and exposed to cold for 3 hrs on three occasions and compared to a non "stressed" control group. Finally, in a third experiment, pregnant ewes were exposed to a combination of stressors designed to mimic the effects of shearing (yarding, transport, wetting and cold exposure), on five occasions during mid- and late-pregnancy. Lamb behaviour was assessed using a separation test and lamb vigour related behaviours: latency to bleat; latency to stand; and latency to return to ewe, before and after a 1 hr cold challenge at 4°C. Overall, in all three experiments, lambs born to prenatally stressed ewes displayed longer latencies to bleat than control born lambs (*Exp1*, $P=0.05$; *Exp2*, $P=0.01$; *Exp3* $P=0.05$). Stressors combinations during pregnancy impacted lambs' latency to bleat and stand, when the treatment was applied during late-pregnancy lambs' latency to bleat and stand tended to be shorter than when stress occurred during mid-pregnancy (bleat, $P=0.05$ and stand, $P=0.09$) which was also shorter than latency to stand for control lambs ($P<0.01$). However, prenatal shearing did not impact lambs' latency to stand nor did stressor combinations on lamb latency to return to the ewe. Regardless of treatment groups single lambs exhibited slower latencies than twin lambs (*Exp1,* P=0.02 for latency to stand; *Exp2,* P<0.001 for latency to bleat). Lambs also tended to react faster after a cold challenge than before (*Exp1,* P<0.001; *Exp3,* P=0.004 both for latency to stand. Faster lambs displayed lower blood glucose levels both before and after cold challenge. Prenatal stress during mid or late pregnancy affected neonatal behaviours, and lambs' response to a cold challenge. Differences between treatment groups and particularly between the timing of the stress treatment suggest different mechanisms underlying altered neonatal behaviour resulting from 'mild' prenatal stress

Keywords: prenatal stress; lamb vigour; lamb behaviour; cold challenge; lamb survival; foetal programming

4.1 Introduction

The dam-lamb bond is a key element to lamb survival as the neonate depends on maternal care for nutrient supply and social learning (Dwyer 2014). In particular in Merinos, where dams leave the lambing site within hours of lambing event, rapid bonding and the a lambs ability to follow the dam are essentials to ensure regular suckling by the lamb and hence lamb survival (Nowak *et al.* 1997; Val-Laillet *et al.* 2004).

Early lamb milestone behaviours such as standing, seeking and finding the udder (through smell and touch) and suckling, usually occurring within the first hour or two of life, have commonly be used to assess lamb viability and neonatal behavioural status (Owens *et al.* 1985; Slee and Springbett 1986; Cloete 1993; Dwyer 2003; Dwyer *et al.* 2005; Dwyer and Morgan 2006; Pfister *et al.* 2006a; Pfister *et al.* 2006b; Matheson *et al.* 2011). According to Owens *et al.* (1985), the chances of survival drop by 1% for every minute that these behaviours are delayed. However, as vocalisations in lambs attest a strong communication-based dam-lamb bond (Nowak 1990), latency to emit the first bleat (Darwish and Ashmawy 2011), the bleating rate (Nowak 1990; Hernandez *et al.* 2009) and bleat response following tagging (Brien *et al.* 2009; Brien *et al.* 2014) have also been also measured to assess lamb vigour.

Although it has a low heritability (0.13 +/-0.02), latency to bleat has been shown to be genetically correlated to lamb survival (Brien *et al.* 2010). Even under best management practices, general livestock husbandry such as transport, shearing, yarding, fasting and isolation are stressors which impact on prenatal development negatively. Braastad (1998) defined prenatal stress as “a stress experienced by the pregnant mother which affects the offspring”. While the effects of prenatal stress on neonatal behavioural and cognition have been widely studied in lambs, the effects on livestock of husbandry stressors and their interaction with environmental stressors (heat or cold) are not well documented.

Although the offspring response depends on the level, the duration and the timing of the stressful event during pregnancy, most studies examining prenatal stress in lambs reported negative effects. Daily aversive events, such as isolation, dog handling, transport and sham-shearing, inducing “mild” stress (stress which requires an adaptative physiological and behavioural response to maintain homeostasis) during the last third of pregnancy have been reported to decrease learning performance of lambs in a maze test; to reduce locomotor activity in a test of human interaction while in social isolation, to produce a deficit in spatial learning and working memory; to increase emotional reactivity; and to induce pessimistic-like biased judgement (Coulon *et al.* 2011; Coulon *et al.* 2015). Furthermore, when pregnant dams were stressed using aversive human interaction, lambs displayed an increased fear of humans (Coulon *et al.* 2014). These studies all suggest that chronic prenatal stress has negative effect on lamb behavioural performances. Nevertheless, in a different study, husbandry related stress treatments during pregnancy did not appear to have a negative impact on lambs and were speculated to be beneficial (Rooke *et al.* 2015). In lambs and kids increased exploratory behaviours were observed in offspring born to repeatedly isolated or transported dams (Roussel *et al.* 2005; Roussel-Huchette *et al.* 2008). Additionally, lambs born to ewes shorn during gestation displayed increased vigour and earlier reaching of behaviour milestones compared to control lambs as well as increased survival at 20 days independently of birthweight (Banchero *et al.* 2010). These latter studies, provide evidence that husbandry- like short-term (brief naturalistic stress - Elliott and Eisdorfer (1982)) mild prenatal stress, which, are not detrimental to lamb behaviour and might even be beneficial. Literature seems to suggest that there is not one response to prenatal stressors but that the response may vary according to magnitude of the stress, timing of stress treatment and assessment type and timing. Most of the studies presented earlier assessed lamb behaviour, either directly at birth for neonatal vigour or at

1 month old for tests involving learning, spatial awareness and emotional reactivity and none of the tests are very practical nor appropriate to be performed under field conditions. This study aimed to assess the impact of mild husbandry-like stressors such as, shearing, handling and cold stress, individually or combined during different stages of pregnancy on lamb neonatal vigour and their reactivity after a cold challenge with separation from the dam. It is accepted now that chronic stress should be avoided during pregnancy hence we focused our investigation on stress resulting from standard husbandry procedures and circumstances which ewes have already experienced. We hypothesized that lambs born to prenatally mildly stressed ewes would display improved thermoregulation when they were submitted to a 1 hr cold challenge.

It was firstly hypothesized that lambs born to pregnant ewes exposed to mild husbandry-like stressors would display altered vigour-related behaviours and that the resulting changes in behaviours would be suggestive of an improved vigour. Finally, a subordinate hypothesis was that lambs born to prenatally stressed ewes would have an altered behaviour after the separation and cold challenge suggestive of a better coping mechanism.

4.2 Material and methods

Based on the observation that prenatal shearing improved lambs reaching of early milestones we questioned the role played by the acute and cold stress component of the shearing experience. The study was carried out in three experiments all designed to assess the effects of a range of prenatal stressors on neonatal lamb behaviour and ability to response to a challenge. In a first experiment (*Experiment 1*), we compared the effects of shearing and mock-shearing, during mid- and late-pregnancy to assess the effect of cold stress resulting from fleece removal. With a similar objective, a second experiment (*Experiment 2*) focused on the effects of pure cold stress during late-pregnancy. Finally, a third experiment (*Experiment 3*) investigated the impact of combined acute stressors and cold stress during mid-and late-pregnancy and aimed to replicate the effects of shearing. Neonatal vigour and behaviour was assessed measuring behavioural reactivity and cognition in a lamb vigour test in newly born lambs at 4 hrs after birth before and after a 1 hr cold challenge at 4˚C when 4 hrs old.

The experimental protocols were reviewed and approved by CSIRO ethics committee under numbers 14/17, 14/30 and 15/08.

4.2.1 Experimental animals and design

All the experiments were conducted at the CSIRO property Chiswick, Armidale, New South Wales, Australia and all ewes included in the experiments were in good health throughout the experiments and were individually identified with side branded numbers applied with livestock-marking spray. Only lambs where data sets were complete were used in the behavioural analysis. All ewes were enrolled to only one experiment and were naïve to long-term housing.

Experiment 1, conducted from August to September of 2014, used 60 pregnant Merino ewes and 64 lambs. Ewes were multiparous and selected on pregnancy status at 80 and 110 days after ultrasound scanning. The 60 ewes were divided into 4 treatment groups: mid-pregnancy (pregnancy day 90) shorn and subsequently wetted (MID-S), mid-pregnancy handled, as sham treatment (MID-C), late-pregnancy (pregnancy day 130) shorn and subsequently wetted (LATE-S), late-pregnancy handled, as sham treatment (LATE-C). There were 15 ewes per group and 16 MID-S lambs (8 single and 8 twins), 18 MID-C lambs (7 singles, 8 twins and 3 triplets), 13 LATE-S lambs (9 singles and 4 twins) and 15 LATE-C lambs (11 singles and 4 twins) were assessed. Gestation lengths ranged between 139 and-156 days, as calculated from estimated pregnancy day on scanning day and 4 cases of dystocia were observed where lambing assistance was required.

Experiment 2, conducted in December of 2014, used 61 pregnant Merinos ewes, in their last 2 weeks of pregnancy (D130) and these were divided into two treatment groups: wetted and cold exposed (CE) and a positive control (TR). Due to staffing issues and shift in lambing dates the experimental data set only included 14 CE and 13 TR ewes from all those originally enrolled; 15 CE lambs (13 singles and 2 twins) and 15 TR lambs (11 singles and 4 twins). Gestation lengths ranged between 142 and 64 days. Six cases of dystocia were observed requiring lambing assistance.

Experiment 3, conducted from July to early September of 2015, used 108 pregnant Merino mature ewes selected on their pregnancy status at 80 and 110 days based on ultrasound scanning. The ewes were divided into three treatments groups: mid-pregnancy (pregnancy day 88-92) stressed (MID), late-pregnancy (pregnancy day 117-123) stressed (LATE) and control (CTRL). In this experiment, 84 lambs were assessed, 32 CTRL lambs (16 singles and 16 twins), 28 LATE lambs (11 singles and 17 twins) and 24 MID lambs (16 singles and 8 twins). Gestation lengths ranged between 130 and 146 days. One case of dystocia was observed and lambing assisted.

For all lambing events, in cases of observed dystocia, lambing was assisted and lambs were visually monitored after birth to confirm successful suckling of colostrum.

Successful suckling of colostrum was visually monitored for each lamb. Three lambs from *Experiment 1*, two from *Experiment 2* and two from *Experiment 3* with insufficient suckling activity were deemed to be too weak to be enrolled in the respective experiments. All five of those lambs received assistance to bond with their dam, but died within the following 24 hrs.

4.2.2 Housing

For all three experiments, the ewes were grazed ad libitum on improved native pasture and supplemented with 150g/day of sheep pellets (based on wheat, millrun and lucerne;

17.5% protein, 2.5% fat, 17% fibre, 20% ADF, 34% NDF) for *Experiment 1* and *2 and* for *Experiment 3* the ewes were fed ad libitum hay and 100g/animal/day of corn grain. During indoor periods animals were fed a 50:50 lucerne and oaten chaff mix supplemented with a 3:2 sheep pellet and corn ration (200 g/day; sheep pellets based on wheat, mill-run and lucerne; 17.5% protein, 2.5% fat, 17% fibre, 20% ADF, 34% NDF). Throughout all three experiments, nutritional management aimed to maintain a BCS of ewes between 2.5 and 3.5.

For all 3 experiments, around 10 days before the expected lambing date, ewes were housed on straw bedding in indoor lambing pens, in an open-sided animal house, at a density of 3-4 ewes per pen (1.5 m^2 per ewe) on straw bedding and under continuous artificial lighting 24 hrs/day. Once ewes showed signs of parturition, they were moved to separate pens as much as possible to allow intimate ewe-lamb bonding. Ewes were visually checked at least every 30 min and video cameras were used to continuously monitor for parturition and early behaviours. At any moment, each camera captured a view of the all lambing pens. The cameras were connected to digital video recorders and footage captured using IVMS4200 software (Hangzhou Hikvision Digital Technology Co., Ltd).

Experiment1 lambing took place between 29th September and 17th October 2014 and average temperature were on average 12˚C (2˚C/24˚C min/max temperature; 56% humidity on average). *Experiment* 2 lambing took place between 6th December 2014 and 13th January 2015 and average temperatures were on average 18.5˚C (10˚C/29˚C min/max temperature; 75% humidity on average). *Experiment 3* took place between 14th and 24th September 2015 and average temperature were on average 9˚C (0˚C/19˚C min/max temperature; 74% humidity on average).

4.2.3 Treatments

Experiment 1 - Shearing and Handling stressors

At D90 or D125 of pregnancy, over the treatment of 7 days, pregnant ewes from both the shorn and control groups, were housed indoor in group pens (3 m^2/ewe) on a slatted floor and separated according to treatment group. Ewes were acclimated in the shed 5 days before the beginning of the experiment. On the first day (D0), the treated groups were shorn (MID-S & LATE-S) while the control groups were sham-handled for 2 min (MID-C & LATE-C) alongside the shorn group by handling similar to shearing (seated on haunches, manual stroking of both sides of the body and rump). Following shearing, shorn ewes, but not control ewes, were wetted using ceiling sprinklers for 30 min, on 3 occasions during a 7-day period (D0, D3 & D7).

Experiment 2 - Cold exposure stressor

During the last two weeks of pregnancy (D130-D140 of pregnancy), one group of ewes was transported and cold exposed (CE) on three occasions over 5 days (D0, D2, D5) and a control group was transported but not cold exposed (TR). During the three wetting occasions, both CE and TR groups were transported from the paddock to the cold room facility using a stock trailer. For practical reasons, each group was divided in 4 subgroups of 7-8 animals and each subgroup was exposed to 5 min of loading and transport. At the cold room facility, the CE ewes were unloaded and were individually wetted for 2 min using a jet hose in order to enhance the cold treatment. For the wetting, ewes were sat on their rump and bare skin areas were hosed to ensure wool free areas around legs and groin were wet. The CE ewes were subsequently put in a 3˚C cold room for 3 hrs. At the same time the TR ewes were unloaded and held on a slatted floor pen for the duration of 3 hrs. At the completion of the treatment all animals were returned to their paddock

Experiment 3 – Combination of stressors

At D90 (MID) or D125 (LATE) of pregnancy both the treated groups (MID and LATE) were exposed to a stress treatment on 5 occasions over a period of 11 days (D0, D2, D5, D8 & D10 or D11). In this experiment, late-pregnancy stage was considered at D125 instead of D130 as in *Experiment 2* to allow completion of the stress treatment before beginning of lambing. The stress treatment was designed to mimic shearing and consisted of ten minutes of yarding, ten minutes of transport in a stock trailer, ten minutes of high water pressure hosing and then three hours in a 3˚C cold room. During these treatments, the control group (CTRL) remained in a paddock with as little human interaction as possible.

4.2.4 Lambing

All live-born lambs born during the experiments were individually identified using electronic and visual ear tags shortly after birth and included in the behavioural assessments. Behaviour assessment was conducted 4 hrs after birth when lambs were separated from their mothers, tested for neonatal behaviour, subjected to a 1 hr cold challenge, tested again for behaviour and finally reunited with their dam. The overall test + cold challenge period lasted between 90 and 120 min.

4.2.4.1 Cold challenge

Following the initial behaviour testing lambs were placed into a temperature controlled room at 4 °C for 1 hr. They were transported (less than 5 min) to the cold room in a plastic box to minimise human contact and immediately prior to being placed in the cold room, all lambs had their abdominal, inguinal and axillar areas manually wetted (tap water in half-filled 20 l bucket, temperature 8-10°C) to ensure all lambs were equally wet, mimicking amniotic fluid cover immediately after birth. Lambs were then placed inside a temperature-controlled room (without fan-forced air movement) set at 4°C, held in a cradle to limit movement (Figure 4.2). The cradle was designed to support the lamb ventrally to maintain an upright position and consisted of a fabric mat suspended between two longitudinal metal bars, with four holes cut into the fabric to accommodate the legs. If required, the lamb was secured with a bandage around the shoulder girdle, although we found that once placed into the cradle, lambs settled into position and remained quiet. To monitor for potential hypothermia, lamb's rectal temperatures were measured every 10 min with a digital thermometer (accuracy +/- 0.1 °C from 35-42 °C, Flexible Rapid 10 Second Thermometer DT K101A; Medshop Australia, Preston, VIC). Lambs were removed if temperature dropped below 36.5 °C at which time they were given warm milk. Overall 24 lambs were pulled out of the cold room before the cold challenge was complete (no lambs removed prior 40 min of cold challenge). On the return from the cold room lambs were subjected again to the behaviour test prior to returning to their mothers when they were monitored to ensure the ewe-lamb bond was intact. Lambs pulled out early from the cold room were enrolled in the behaviour test afterwards while recorded as hypothermic.

To reduce the psychological stress induced by transport and handling as much as possible and reduce bias, handling was limited during testing and a plastic box was used to transport the lamb as it has been established that tactile stimulation in lambs could alter normal vocal and motor behaviour (Vince *et al.* 1985; Nowak 1991) as well as impacting lamb body temperature. Furthermore, to ensure best response from lambs during the separation and the tests, dam-lamb bonding was facilitated with an intimate and undisturbed environment during the 4 hrs after birth.

4.2.4.2 Blood samples

For each experiment, blood samples were collected from lambs before (BCC) and after the cold challenge (ACC), approximately 90 min apart to measure blood glucose and lactate levels. Blood samples were taken by jugular venepuncture using 20 gauge needles and 5 mL heparin vacutainers. Plasma was stored in 1 ml aliquots at -20°C.

For E*xperiments 1 and 2,* blood samples were centrifuged and plasma glucose and lactate were analysed using a Siemens Dimension Xpand plus clinical analyser (Bayswater Vic Australia 3153) after samples had been frozen at -20°C. For *Experiment 3,* blood glucose analysis was determined using the Accu-chek Performa system (Accu-chek, Roche Diabetes Care, Roche Products Pty, Basel, Switzerland). This glucose-meter allowed a measurement in the field immediately after the bleeding. Lactate plasma levels were determined using a Siemens Dimension Xpand plus clinical analyser (Bayswater Vic Australia 3153), after samples had been frozen at -20°C.

Blood glucose levels were given in mmol/L by the glucometer and plasma lactate and glucose levels were obtained in mg/dL using the clinical analyser. For glucose the two methods were calibrated to obtain a conversion ratio of 18 between the two units which is the conversion ration previously used by (Chase 2006). Having confirmed a similar relationship using samples from experiment 3 we used this conversion (1 mmol = 18 mg/dL of glucose) to homogenise the data to allow a direct comparison.

4.2.4.3 Behaviour test

For all 3 experiments, behaviour tests were carried out in a covered 3 m long and 1.2 m wide, concrete-floored race, adjacent to the lambing shed test arena (Figure 4.1). Prior to the test for *Experiments 1 and 3,* ewes were brought to the test arena and put in the 1 m^2 retaining pen at the end of the test race. At the beginning of the test lambs were gently restrained for 5 s in a lateral position on the floor, at a marked position 2 m away from the retaining pen. When lambs were released the test started, and the latencies to bleat, to stand and to return to the ewe were recorded. For *Experiment 2*, to reproduce field situations and remove the maternal behaviour component, no ewes were used and only the latencies to bleat and to stand were recorded.

Latency to bleat was the time taken by the lamb to initiate the first open mouth vocalisation and was manually recorded using a stopwatch to the nearest tenth of a second. If lambs vocalised while restrained by the user (rarely encountered), the restrain continued until the vocalisation was finished. If lambs did not vocalise with 90 s, the measurement was recorded as a fail (see statistical analysis).

Latency to stand was the time taken by the lamb to stand on four legs for at least 5 s without falling.

Latency to return to the ewe was the time taken by the lamb to reach the contact zone within 0.5 m away from the pen retaining the ewes. If lambs failed to return to the dam within 180 s, the measurement was recorded as a fail.

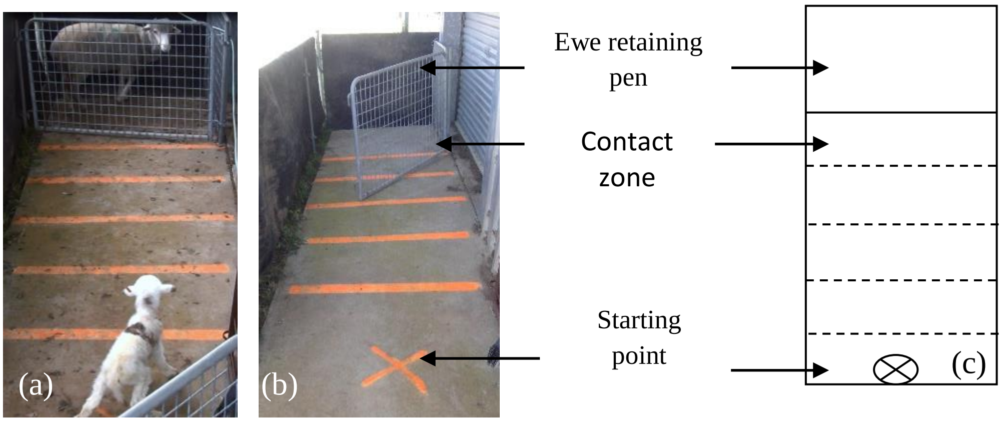


Figure 4.1 Behaviour test arena. (a) behaviour test performed with ewe in retaining pen. (b) and (c) diagram and picture of the test arena empty. Ewe retaining pen was located 2m away from the starting point. Lambs were gently restrained for 5 seconds in a lateral position on the floor, at a marked position and released when the test started.

4.2.5 Statistical analysis

All statistical analysis was performed using the R software (version 3.1.0, The R foundation for Statistical Computing). For normally distributed latencies and data t-tests were used and non-parametric Mann-Whitney tests were used when data sets were not normally distributed. In past studies, husbandry-like prenatal stress has been shown to result in increased birthweights which have been linked to neonatal behaviour. We attempted to analyse those data using a repeated measures ANOVA mixed model with treatment, location, time-point, wool length, litter size, sex and their interactions as fixed effects, birthweight as covariate and lamb ID as a random effect when data were normally distributed. However, despite using box cox procedures to determine the optimal transformation and reach normality for non-normally distributed data, this model or the deriving models did not fulfill the assumptions and hence could not be used.

Lambs were excluded from the analysis if the ewes failed to lamb during the experiment period (accurate lambing time not known), if the lamb died within the first 4 hrs after birth or if a foetus died in utero. All dystocic and hypothermic lambs were included in the analysis.

For lambs which failed to perform in the given time, the maximum value was used, ie 90 s for latency to bleat and 180s for latencies to stand and return to the ewe. Hypothermic lambs were tested as per normal protocol while recorded as hypothermic and removed later if sending out as outliers.

In *Experiment 1* twin lambs born to ewes treated during late-pregnancy were too few (LATE-S 4; LATE-C 4) to allow powerful statistical analysis and were not included.

Results are expressed as LS means ± standard deviation of the mean (S.D.). Results with an associated probability less or equal to 0.05 were considered significant and those above and less or equal to 0.09, a tendency.

4.3 Results

4.3.1 Behaviour

Experiment 1

<u>BCC vs ACC (Before Cold Challenge vs After Cold Challenge)</u>

A comparison before and after cold challenge showed that latencies to stand ACC were significantly shorter than BCC across all 4 treatments (P=0.009; 11.41±31.82 s vs 12.99±18.56 s). A tendency was also observed for latency to bleat in single lambs (P=0.09). Similarly, MID-S singles lambs were faster ACC to bleat, stand and return to the ewe than BCC (respectively P=0.04; 0.04; 0.03; Figure 4.2B). A similar pattern was seen for latency to stand for LATE-S single lambs (P=0.02), latency to bleat for LATE-C lambs (P=0.03) and a trend for latency to return to dams for MID-C twin lambs (P=0.07; Figure 4.2B).

<u>BCC (Before Cold Challenge)</u>

In the BCC sampling, across treatments and treatment times, twins were faster to stand than singles (P=0.02; 6.20±4.372s vs 17.7±22.84s; Figure 4.2A) but no such difference was observed for latencies to bleat and return to the ewe. BCC, in the MID-C group, singles were faster to bleat than twins (P=0.02), while in the corresponding treatment group MID-S, single lambs tended to be slower to bleat than twins (P=0.07).

BCC across treatment times, lambs born to shorn ewes (MID-S and LATE-S) tended to be slower to bleat than lambs born to control ewes (MID-C and LATE-C) (P=0.05). This difference was significant when shearing was done during mid-pregnancy, MID-S lambs were significantly slower to bleat than MID-C (P=0.03; Figure 4.2B). However, when looking specifically at twins, an opposite trend was observed for twin MID-S and twin MID-C lambs for the latency to bleat and return to the ewe (respectively P=0.08 and P=0.05, Figure 4.2A).

No significant difference between time of stress treatment was observed in BCC tests. However, BCC, MID-S single lambs were significantly slower to bleat than LATE-S singles (P=0.01) and a similar trend was observed for the latency to return to ewes (P=0.09; Figure 4.2A).

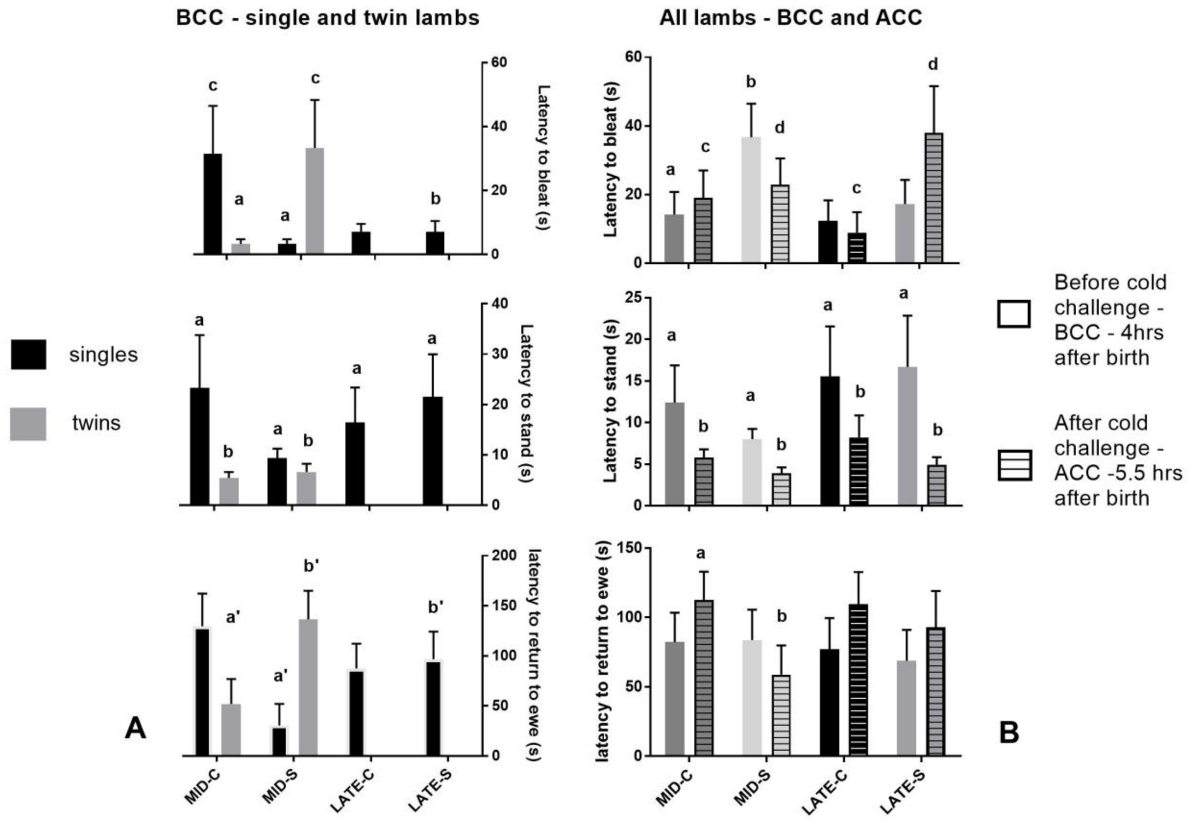


Figure 4.2 Lamb latencies to bleat, stand and return to the dam for LATE-C, LATE-S, MID-C and MID-S lambs. (A) latencies at 4 hrs after birth – Before the cold challenge (BCC) for single and twin lambs (resp black and grey). For LATE groups (LATE-C and LATE-S) only the single lambs are represented. (B) Latencies for all lambs before (clear) and after (striped) a 1 hr cold challenge. Letters represent significant differences (P<0.05) within each graph.

ACC (After Cold Challenge)

Similarly to BCC, ACC, lambs born to shorn ewes were significantly slower to bleat than lambs from the control group (P=0.04) but lambs born to control ewes tended to be slower to return to the dam than the shorn treatment lambs (Figure 4.2B pooled data singles & twins).

ACC, LATE-C lambs tended to be faster to bleat than LATE-S (P=0.06) and MID-S single lambs were significantly faster to return to the ewe than LATE-S (P=0.006). MID-S lambs were also significantly faster to return to the ewe than MID-C lambs (P=0.04). (Figure 4.2B)

Experiment 2

Twins lambs were significantly faster to bleat than single lambs regardless of the timing of the tests (P=<0.001; 2.75±4.72 s vs 20.03±30.46 s). Similiarly, across testing time, CE born lambs were slower to bleat than TR born lambs (P=0.01; 18.52±29.83s vs 14.63±27.69 s).

Across treatment groups lambs were faster to bleat BCC than ACC (P=0.02; 10.54±22.2s vs 22.61±32.3s). When looking at individual treatment groups, TR born lambs were significantly slower ACC than BCC (P=0.02) but no such difference was observed for CE born lambs.

BCC, CE born lambs were significantly slower to bleat than TR born lambs (P=0.02) but no difference was observed between treatments ACC.

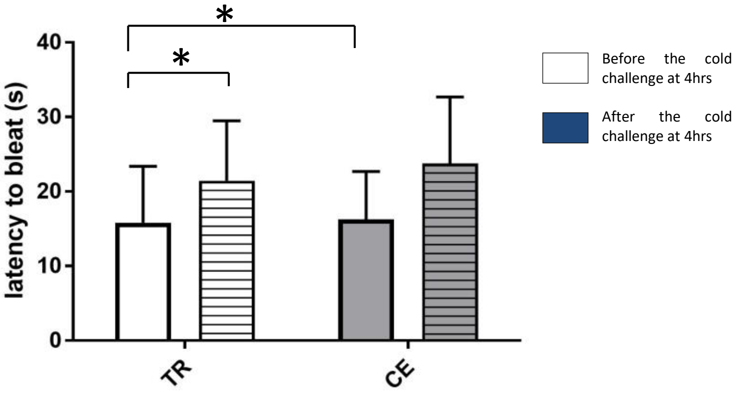


Figure 4.3 Lamb latency to bleat for all lambs for CE (transported, wetted and cold exposed) and TR (transported, control group) treatment groups before (clear) and after (striped) a 1 hr cold challenge. Asterisks represent significant differences (P<0.05).

Experiment 3

BCC vs ACC

Regardless of prenatal treatment, cold challenge resulted in lambs being significanlty faster to stand ACC than BCC (P=0.004; 3.53±4.25 s vs 6.11±11.25 s; Figure 4.4) and there was a similar tendency for latency to bleat (P=0.09; 4.92±15.05 s vs 6.25±15.5 s).

LATE lambs tended to be slower to bleat BCC than ACC (P=0.06) and a similar pattern was observed for the latency to stand BCC and ACC for CTRL and MID lambs (resp. P=0.04; P=0.002).

However, no significant difference between testing time was observed for the latency to return to the ewe.

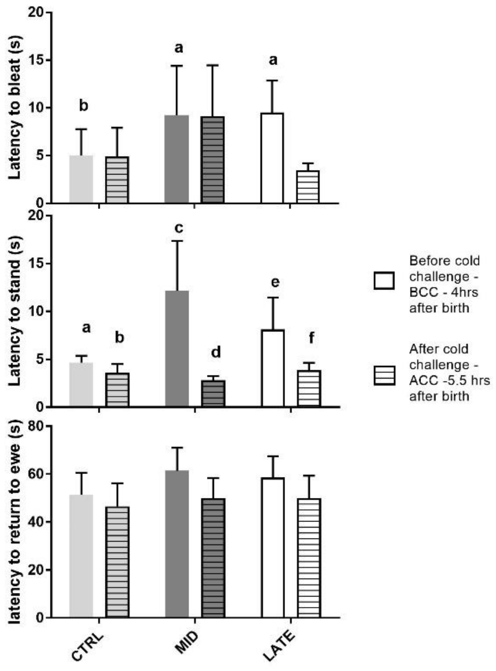
BCC

BCC, lambs born to stressed ewes (MID and LATE pooled) were significantly slower to bleat than lambs born to control ewes (CTRL) (P=0.049 while lambs born to CTRL ewes were significanlty faster to bleat than lambs born to LATE ewes (P=0.004; 5.13±16.26s vs 6.28±13.1s).

For both latency to bleat and to stand, MID lambs tended to be slower than LATE lambs (resp. P=0.05; P=0.09).

ACC

After cold exposure, no treatment differences were observed.



All lambs

Figure 4.4 Behavioural latencies to bleat, stand and return to the dam before (clear) and after a 1 hr cold challenge (striped) at 4 hrs after birth of lambs born to ewes stressed during mid-pregnancy (MID), during late-pregnancy (LATE) and control ewes (CTRL). Letters represent significant differences (P<0.05) within each graph.

4.3.2 Lambs blood glucose and lactate

4.3.2.1 Experiment 1- GLUCOSE

Overall, singles lambs tended to have lower blood glucose levels than twins (treatments groups pooled; P=0.05; 137.4±71.44 vs 156.6±65.19 mg/dL). Lambs born to shorn ewes displayed significantly higher blood glucose levels than control born lambs (P=0.01; 165.3±72.97 vs 133.4±60.67 mg/dL).

When comparing BCC and ACC, regardless of treatments and treatment times, blood glucose levels were significantly higher ACC than BCC (P<0.001; 123.4±61.88 vs 170.9±65.2 mg/dL). For both MID-C and LATE-C lambs, blood glucose levels BCC were significantly lower than ACC (P<0.001 for both; 81.91±30.49 vs 142.6±54.74 mg/dL for MID-C, 127.6±55.31 vs 177.6±65.29 mg/dL for LATE-C). A similar trend was also found for MID-S lambs (P=0.09).

BCC, MID-C lambs had significantly lower blood glucose levels than MID-S (81.2±30.49 vs 141.1±69.16 mg/dL; P=0.02) and than LATE-C lambs (P=0.02, 81.2±30.49 vs 127.6±55.31). ACC, MID-C lambs also tended to have lower blood glucose levels than MID-S (142.6±54.75 vs 180.2±65.97 mg/dL; P=0.06).

4.3.2.2 Experiment 1 – LACTATE

No treatment differences were observed between BCC and ACC; however, BCC lactate levels were significantly lower than ACC (P<0.001; 40.87±13.88 vs 53.41±19.44 mg/dL) and this difference was also observed for MID-C and LATE-C lambs (respectively 36.3±12.55 vs 50.11±24.75, P=0.03;40.73±8.82 vs 54.39±19.02 mg/dL, P=0.02) and a similar tendency was observed for MID-S (P=0.06).

4.3.2.3 Experiment 2 - GLUCOSE

No treatment differences in lambs blood glucose values were observed at BCC and ACC; however, across all data points, blood glucose levels were higher for ACC than BCC (P=0.006; 116.8±53.62 vs 77.69±37.6 mg/dL). CE lambs had significantly higher blood glucose levels ACC than BCC (P=0.01), but this was not seen for TR lambs.

4.3.2.4 Experiment 2 - LACTATE

No treatment differences in lamb plasma lactate was observed at BCC and ACC but, lactate plasma levels were higher ACC than BCC (P=0.006; 42.92±12.28 vs 32.78±9.43 mg/dL).

4.3.2.5 Experiment 3 - GLUCOSE

While no treatment difference was observed BCC; ACC LATE twin lamb blood glucose levels were significantly higher than levels of CTRL twin lambs (112.86±40.14 vs 77.69±37.6, P=0.03).

Overall, ACC blood glucose levels were significantly higher than BCC (P<0.001; 123.3±49.5 vs 87.84±37.26 mg/dL).

4.3.2.6 Experiment 3 - LACTATE

BCC, MID twin lambs had significantly higher blood lactate levels than LATE twins (29.16±2.25 vs 25.75±6.80P=0.02) and the pattern while not significant was similar for ACC (P=0.06).

Overall, BCC plasma lacate levels were significantly higher than ACC (P<0.001; 31.47±7.88 vs 26.04±5.65 mg/dL).

4.4 Discussion

The results of this study indicate that mild stress implemented during mid and late pregnancy affects the neonatal lamb behavioural development, as seen by the reduction in vocalisation and locomotor reactivity and abilities in lambs born to stressed ewes. In all three experiments, lambs born to stressed ewes were significantly slower to reach the measured behaviours than lambs born to control ewes and this effect was particularly clear for latency to bleat.

In this study, we used brief husbandry-like (naturalistic) stressors during different stages of pregnancy. These stressors aimed to represent a transient, short-term stressful experience which sheep would commonly experience during on-farm husbandry procedures. Lambs born to ewes stressed during pregnancy displayed overall slower behaviours than control lambs. In *Experiment 1,* shearing stress resulted in significantly decreased lamb reactivity when stress treatment took place during mid-pregnancy. Lambs born to mid-pregnancy shorn ewes were slower to bleat and to return to ewe than mid-pregnancy handled animals but, no such effects were evident when the stress treatment occurred during late-pregnancy. However, the results from *Experiment 3*, which aimed to mimic shearing stress, for late-pregnancy obtained similar outcomes to *Experiment 2*, with lambs born from stressed ewes being slower to bleat when compared to control lambs. Previous studies on rats have suggested a neurotoxic effect of prenatal stress, consisting of daily restrain in a small cage during 240 minutes for three consecutive days and excess circulating maternal stress hormones, on foetal brain development (Fujioka *et al.* 1999; Weinstock 2008) which would be consistent with such effects. These observations also align with previous research by Coulon et al. (2011); (2014; 2015) where it was found that prenatal stress negatively impacted lamb neonatal behaviour. In those sheep studies, prenatal stress, induced using daily aversive uncontrollable events such as restraint, mixing groups and transport during the last third of pregnancy, decreased lamb learning performance in a maze test, decreased locomotion in a response to 'human' test, increased pessimistic-like biased judgement and increased fear of humans (Coulon et al. 2011; Coulon et al. 2014; Coulon et al. 2015).

In *Experiment 3*, lambs from mid-pregnancy stressed ewes were faster to stand than control lambs which supports the findings of Rooke *et al.* (2015), who in their review, suggest positive effects of transport and isolation of the dams, with lambs exhibiting increased exploration and decreased fear (Roussel *et al.* 2005; Roussel-Huchette *et al.* 2008). Shearing during mid and late-pregnancy (pregnancy day 70; 80 and 120) produced lambs that were faster to succeed at first suckling (Corner *et al.* 2006; Banchero *et al.* 2010). Our findings contrast with previous findings on the consequences of husbandry -like mild pregnancy stressors on neonatal behaviour as no negative neonatal behavioural outcomes had been reported in lambs born to ewes exposed to husbandry stressors during pregnancy. After observing improved foetal brain development, increased learning abilities and reduced emotionality in rat pups born to mildly stressed pregnant dams, Fujioka *et al.* (2001) concluded that mild prenatal stress might not be of a magnitude great enough to have a neurotoxic effect. In Coulon et al. (2011); (2014; 2015), while the stressors used could be associated to husbandry-like stressors (mixing, isolation, transport and handling), the overall stress treatment was considered chronic due to the length and number of occurrences which could account for the negative consequences on lamb behaviour observed. However, in both goats and sheep, similar stressors (transport and isolation) and shearing when applied once or occasionally, had a positive effect on offspring behaviour (Roussel *et al.* 2005; Corner *et al.* 2006; Roussel-Huchette *et al.* 2008; Banchero *et al.* 2010). Aversive human interaction used as a prenatal treatment resulted in increased fear towards human (Coulon *et al.* 2014; Coulon *et al.* 2015) whereas prenatal repeated isolation resulted in increased exploratory behaviours (Roussel-Huchette *et al.* 2008). All those findings seem to suggest that the contrasting effects of prenatal stress treatments on offspring behavioural development could be accounted for by stressors of different nature, durations and magnitude, which would impact on the amount of cortisol reaching the foetal brain. However, in our experiments, mild transient husbandry-like stressors, overall, resulted in negative effects which might highlight divergences in methods or other mechanisms triggered.

In *Experiment 1*, the contrast between the effects of shearing stress and handling stress could be associated with the cold exposure component. This lead us to question the role played by the cold stress resulting from fleece removal during shearing. However, late-pregnancy cold e and a short transport in *Experiment 2* obtained similar outcomes to late-pregnancy treatment in *Experiment 3*, which aimed to mimic shearing stress, with lambs born from stressed ewes being slower to bleat when compared to control lambs. We thus concluded that the differences observed in Experiment 1 could not be accounted for by the cold exposure itself but has to rely on mechanisms that could be triggered by both cold and husbandry stressors. At this stage, more investigations are required before the effects of pregnancy cold exposure on neonatal lamb behaviour are clear. However, while our overall findings tend to disagree with previous observations of improved brain development in rats resulting from mild prenatal stress, we remain cautious as studies conducted in rats present a more standardised testing procedure and greater test subject number.

The timing (mid or late pregnancy) of the stressor appeared to change the impact of the stress on lamb behaviours. In *Experiment 1*, shearing stress reduced lamb reactivity only when it occurred during mid-pregnancy, whereas latencies to bleat and return to ewe were reduced compared to mid-pregnancy when shearing occurred during late-pregnancy. Additionally, late-pregnancy shorn treatment lambs were faster to bleat and return to ewe than mid-pregnancy shorn treatment lambs but this difference was not apparent for mid- and late-pregnancy handling treatment suggesting that shearing stress and handling stress elicited different adaptive mechanisms or levels of stress hormones however, there is no data to support this and further studies are required to investigate those mechanisms. In *Experiment 3*, increase in speed of standing for lambs born in the mid-pregnancy stress treatment group, and increased latency to bleat when stress treatment occurred during late-pregnancy was observed. Contrary to *Experiment 1*, in *Experiment 3*, mid-pregnancy stressed lambs were faster to bleat and stand than late-pregnancy stressed lambs. These findings point towards different mechanisms affected by stress during different foetal brain and cognitive development stages. Good candidates to account for these differences are placental insufficiency and foetal hypoxemia. Indeed, Mallard *et al.* (1998) reported that prolonged period of placental insufficiency during the last third of pregnancy could affect neurodevelopmental processes and result in damaged neural connections and altered behaviour.

Foetal brain development takes place in three main stages which all contribute to an increase in brain weight: neuroblast multiplication (early-pregnancy), neuroglial multiplication (mid-pregnancy) and myelination process and growth of the cerebellum (late-pregnancy) (McIntosh *et al.* 1979). Along with damaged neural connection, Mallard *et al.* (1998) observed increased cell death and reduced dendritic growth in the cerebellum as well as decreased cerebral white matter which are all evidence of retarded or slowed neural connections, all resulting from prenatal stress during the last trimester of pregnancy. In other studies, chronic mild placental insufficiency and hypoxemia have been shown to result in deficits in neural connectivity and myelination (Rees *et al.* 1999; Rees and Inder 2005). Although no study has investigated the effects of mild prenatal stress on placental efficiency, environmental heat stress can lead to the development of placental insufficiency and ultimately to intrauterine growth restriction (Marai *et al.* 2007). While remaining cautious, we could conceive that mild prenatal stress resulted in placental insufficiency and hypoxemia which in turn result in slowed neural connection and behaviour.

In both *Experiments 1* and *2*, prenatal stress affected both twin and single lambs with twins being significantly faster to display measured behaviours (bleat and stand) than singles which parallels the observations of Dwyer *et al.* (2005) who observed that twin lambs tended to be faster to reach their initial behaviour milestones than singles.

In their study, Dwyer *et al.* (2005) found that lamb latency to stand was correlated to placental and total cotyledon weights and negatively correlated to placental efficiency. As mentioned previously, placental insufficiency can result in deficits in foetal brain development such as decreased neuroblast multiplication and myelination or slowed neural connections (Mallard *et al.* 1998; Rees *et al.* 1999; Mallard *et al.* 2000). These deficits can result in altered neurological processing and subsequently altered behaviour (Mallard *et al.* 1998; Rees *et al.* 1999; Mallard *et al.* 2000). In our study twins displayed greater reactivity than singles, during a separation test, which could be explained by better in utero placental efficiency and hence potentially improved neurological and brain development.

Cold exposure and separation from the dam can be common occurrences for lambs in paddock situations. Situations such as isolation or separation from the dam or situations inducing fear or anxiety are highly stressful for sheep (Price and Thos 1980; Porter *et al.* 1995; Ligout *et al.* 2011; Rault *et al.* 2011) and the lamb's ability to respond accordingly when facing stressful situation is crucial at the risk of losing the ewe-lamb bond. In this experiment, the cold challenge was designed to assess the ability of newborn lambs to react after a stressful situation. In both *Experiments 1* and *3*, latencies to bleat, stand and return were shorter after the cold challenge than before suggesting that the stressor had a positive effect on behaviours. Confounding effects of age and experience need to be examined further to be sure this is the case and in *Experiment 2* the opposite pattern was seen and more precise experimental designs are needed to clarify these differences.

Differences in latencies after a cold challenge to the lamb were observed only in *Experiment 1* and, looking at the latency to return to ewe specifically, mid-pregnancy shorn treatment lambs were faster to return than control ones. Why such differences might exist is unclear as while poor behavioural response can be linked to increased behavioural inhibition and cognitive abilities (Takahashi 1992; Hofer 1996; Braastad 1998; Korte and De Boer 2003; Weinstock 2008; Cox *et al.* 2012) there is no reason to assume that the control animals should be negatively impacted so as to reduce cognitive ability. However, it is worth considering that the improved reactivity after cold challenge compared to before observed in *Experiment 1* and *3* could also be accounted for by the difference in experience, between the discovery of the test before cold challenge and the repetition after cold challenge, as well as the age of the lambs. Indeed, while the overall challenge and testing could only take up to 90 minutes, Morton *et al.* (2014) have shown that latency to bleat improved over the twelve hours after birth as a result of maturation and recovery from the lambing event so time maybe a confounding factor.

Glucose and lactate are the primary energy supply for foetal tissues (Bell and Bauman 1997), and might be a primary contributor to maintenance of normal neonatal behaviour. The effect of treatments seemed to be consistent on both latencies to bleat and return to the dam with high glucose extremes associated with longer latencies. This observation is consistent with previous findings by Morton (2015) who found that risks of delayed vocalisations increased by 4% for every unit increase in blood glucose values in newborn lambs.

Traditionally, the assessment of the impact of prenatal stress on postnatal behaviour in lambs and kids have been carried out either in newborn lambs of 12 hours or greater age using vigour test such as the timing of early behavioural milestones (i.e. latency to first bleat, stand, reach the udder and sucke) (Owens *et al.* 1985; Dwyer 2003; Pfister *et al.* 2006a; Pfister *et al.* 2006b; Darwish and Ashmawy 2011; Matheson *et al.* 2011) or measure of bleat rate (Nowak 1990; Hernandez *et al.* 2009), or in older lambs (1-3 months old) using tests (i.e. the aptitude to reunite with their dams or discriminate an alien one with barrier and maze tests) or exploratory behaviour and emotional reactivity (startle test, isolation test and human presence test)(Roussel *et al.* 2005; Roussel *et al.* 2006; Roussel-Huchette *et al.* 2008). However, this study used a protocol adapted from Morton *et al.* (2017), based on communication behaviour and locomotion abilities and was performed 4 hours after birth. The difference in timing is probably crucial in lambs as it has been shown that latency to bleat decreases rapidly over time and particularly within the first 12 hours of birth, suggesting developmental maturation (Morton *et al.* 2014). Such differences in timing of the tests could explain the variability of findings in different experiments.

Of the three latencies measured in the present experiments (to bleat, to stand and to return to the dam), latency to bleat presented the most consistent effects and significance. The differences observed between these measurements could be accounted for by the different mechanism involved. On one hand, latency to vocalize when separated from the dam represents a communication responsiveness which is a reflex behaviour suggestive of neurobiological status (Zeskind and Lester 2001; Zeskind 2013). On the other hand, latencies to stand and to return to the dam are associated with motor abilities and represent the ability to return to the dam in case of separation and were shown to be essential to survival (Calamandrei *et al.* 2004).

Although potentially involving different mechanisms, we observed an overlap between latencies to bleat, to stand and to return to ewe which is in agreement with previous findings that lambs presenting poor mother reunion due to poor exploratory behaviour were likely to display delayed vocalisations (Nowak 1994; Morton *et al.* 2017).

The dam-lamb bond is crucial to lamb survival as the neonate is dependent on maternal nutrient supply and protection. At present, the mechanism/s contributing to the altered behaviours resulting from prenatal stress remains unknown. This study aimed to address this gap by exploring the effects of prenatal stress on lambs' neonatal behaviour and reactivity, and their ability to cope with a challenge. Although the stressors used in our study were considered mild we observed an impact on neonatal behaviour during a separation test. The main finding of this study was that cold stress during pregnancy (cold stress and/or cold stress resulting from shearing) associated with acute husbandry-like stressors, negatively impacted the reactivity levels of lambs by increasing their latency to bleat stand and return to ewe during a separation test. However, effects of prenatal stress on lamb behaviour varied with the nature of the stressor and the timing during pregnancy suggesting several underlying mechanisms. Future research needs to investigate the effects of different levels of severity and duration of husbandry stressors on lamb early behaviour and reactivity to establish a response curve. The effects of stress hormones such as cortisol and noradrenaline on lamb early behaviour and reactivity could also provide some insights on the underpinning mechanisms. Finally, an assessment of the effects of prenatal mild stress on lamb cognition at an early age using cognitive tests such as a maze could reinforce present knowledge of the effects of prenatal stress on lamb survival. Greater understanding of how mild prenatal stress impacts lamb behaviour and cognition is vital in the future, as it could improve husbandry procedures and flock management and increase survival chances.

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STATEMENT OF ORIGINALITY

Chapter 4 - Impact of mid- and late-pregnancy prenatal stress on lamb neonatal reactivity and vigour.

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	Page number/s
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STATEMENT OF AUTHORS' CONTRIBUTION

Chapter 4 - Impact of mid- and late-pregnancy prenatal stress on lamb neonatal reactivity and vigour.

We, the Research Master/PhD candidate and the candidate's Principal 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 in the *Statement of Originality*.

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Chapter 5: **Impact of prenatal stress on lamb neonatal ability to maintain body surface temperature in cold conditions**

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ABSTRACT

Exposure to cold and subsequent hypothermia is one of the greatest threat to survival for newborn lambs in extensive systems. In this context, the ability to thermoregulate, including non-shivering thermogenesis is essential. Previous studies have observed improved thermogenic abilities and an increase in brown adipose tissue deposition in lambs born to ewes cold exposed during late-pregnancy. This study aimed to assess the effects of diverse prenatal stressors on newborn lamb thermogenesis. For the first experiment, we tested the effects of shearing of ewes on lamb thermogenesis by subjecting pregnant ewes during mid-pregnancy (PD90), either to shearing or to mock shearing (handling resembling the actions of shearing), and the shorn ewes were in addition wetted using sprinklers. In a second experiment, ewes were cold exposed for 3 hrs on three occasions during late-pregnancy, while in a third experiment, pregnant ewes were exposed to a combination of stressors on five occasions during mid or late-pregnancy, the stressor combination designed to mimic shearing (ie yarding, transport, wetting and cold exposure). Lambs were subjected to a cold challenge of 1 hr, 4 hrs after birth, during which time, thermography images were taken every 10 min at a fixed distance from the dorsal midline. In all lambs, on average body surface temperature decreased and prenatally stressed lambs tended to have higher body surface temperatures than control lambs. Body surface temperature tended to be higher for lambs born to ewes shorn and wetted during mid-pregnancy than for lambs handled during mid-pregnancy (P= 0.0726) and they also tended to maintain their body surface temperature during the 1 hr cold challenge (P= 0.0715). Similarly, lambs born to ewes exposed to the combination of stressors during late-pregnancy displayed higher body surface temperature than lambs born to both mid-pregnancy stressed and control ewes (P<0.05 for both). However mid-pregnancy stress group lambs maintained their body surface temperature better than both late-pregnancy shorn and control groups lambs (P<0.01 for both). Wool length was in two of the experiments, indicating that in contrast to prior results coat length can interfere with IRT measurements. Overall, we concluded that under our experimental conditions cold stress in combination with acute stress can lead to observable differences in lamb thermogenesis. Furthermore, we conclude from our results that mild transient prenatal stress during mid- and late-pregnancy might affect subsequent postnatal thermogenesis in lambs via two different underlying mechanisms.

Keywords: thermoregulation; cold challenge; prenatal stress; neonate; sheep; thermogenesis

5.1 Introduction

At birth, lambs experience a rapid transition from the warm and protective womb to the external environment; a change that represents a radical thermoregulatory challenge. Exposure to environmental factors such as wind, rain and cold temperatures increases heat loss and thus the chance of hypothermia (Obst and Day 1968; Obst and Ellis 1977; Alexander *et al.* 1980a; Lynch *et al.* 1980). Heat production has been identified as a potential major contributor to lamb survival (Dwyer and Morgan 2006) and deaths resulting from hypothermia/exposure have been reported to account for 5-10% of lamb losses in Australia (Hughes *et al.* 1964; Refshauge *et al.* 2016).

Heat production in lambs is achieved by either shivering and muscular activity or non-shivering thermogenesis associated with brown fat metabolism (Alexander and Williams 1968). In newborn lambs, brown fat, which is the primary source of heat production from lipid oxidation, is mostly deposited in the peri-renal abdominal and inguinal regions of lambs (Gemmell *et al.* 1972; Alexander and Bell 1975). It can be argued that increased amount of brown fat could be positively related to survival in cold conditions. Non-shivering thermogenesis has been reported to account for approximately 40% of heat produced by the neonatal lamb (Alexander and Williams 1968) but the mechanisms involved are difficult to study in vivo (Hergenhan 2012). Recently, infrared thermography has been identified as a technology suitable for the measurement and discrimination of levels of thermogenesis in lambs (McCoard *et al.* 2014; Labeur *et al.* 2017).

Previous studies have found positive effects of prenatal shearing and cold exposure on lamb thermoregulatory abilities, and late-pregnancy winter-shearing of ewes has been reported to increase brown fat reserves in newborn lambs, leading to enhanced cold resistance (Symonds *et al.* 1992a; Symonds and Lomax 1992). Stott and Slee (1985) reported greater non-shivering thermogenesis capacities in lambs born to ewes exposed to cold after being shorn two weeks before expected lambing date and Symonds *et al.* (1992a) also reported positive effects of cold stress in late-pregnancy on brown fat activity and non-shivering thermogenesis in new born lambs. However, other studies did not observe improved thermoregulatory abilities in lambs after prenatal shearing of the ewes (Kenyon *et al.* 2002c, 2002b).

The aim of the present study was to better understand the effects of both acute stressors and/or cold stress commonly associated with shearing, at different stages of pregnancy, on the subsequent thermoregulatory ability of the neonate. The study included three experiments: in the first experiment, the effects of shearing and mock-shearing/ handling during pregnancy were compared to assess whether the effects of fleece removal and subsequent cold exposure differed from the effect of handling stress. In the second experiment the effects of cold exposure during late-pregnancy were assessed while in a third experiment the impact of a combination of acute stressors and cold exposure during pregnancy (mimic of stressors associated with shearing) was assessed. It was hypothesized that lambs born to prenatally stressed ewes would display improved thermoregulatory ability when they were submitted to a 1 hr cold challenge.

5.2 Material and methods

Experiment 1 examined the effects of shearing stress and the associated cold exposure during mid-pregnancy. *Experiment 2* was designed to examine the effect of cold exposure during late-pregnancy. *Experiment 3* investigated the effects of a combination of stressors during mid- or late-pregnancy. Lamb thermoregulation was assessed using infrared thermography images taken at 10 min intervals (ensuring temperature in the cold room were minimised) during a 1 hr cold challenge of 4˚C in an environment controlled cold room when lambs were 4 hrs old. For some experiments the numbers of lambs assessed did not match the number of ewes treated during pregnancy as only the lambs for which accurate birth time was known and which could be assessed at 4 hrs ± 15 min were used.

The experimental protocols were reviewed and approved by CSIRO ethics committee under numbers 14/17, 14/30 and 15/08.

Weather observations were obtained from CustomWeather (Time and Date AS 1995–2017. All rights reserved).

5.2.1 Experimental animals

All the experiments were conducted at the CSIRO Chiswick site, Armidale, Australia and all ewes included in the experiments were of apparent good health throughout the experiments and were individually identified with side-branded numbers applied with livestock-marking spray.

Experiment 1 used 30 pregnant Merino multiparous ewes and 34 lambs. Ewes were selected on pregnancy status at 80 days after ultrasound scanning. The 30 ewes were divided into 2 treatment groups: mid-pregnancy (pregnancy day 90) shearing followed by wetting (SH) and mid-pregnancy handling as a sham treatment (HA). There were 15 ewes per group and 17 SH lambs (9 singles and 8 twins) and 18 HA lambs (7 singles, 8 twins and 3 triplets) were assessed. Gestation lengths ranged between 139 and 156 days, as calculated from estimated pregnancy day on scanning day. The experiment incorporated a late pregnancy treatment for which data were used to describe the thermal imaging analysis method and are presented in Chapter 6 (Labeur *et al.* 2017).

Experiment 2 used 61 pregnant Merinos mature ewes after natural mating, in their last 2 weeks of pregnancy (D130) and these were divided into two treatment groups: wetted and cold exposed (CE) and a positive control group (TR). In this experiment, due to staffing issues and shift in lambing dates only 17 CE ewes; 18 CE lambs (16 singles and 2 twins), 16 TR ewes and 19 TR lambs (13 singles and 6 twins) could be used. Gestation lengths ranged between 142 and 164 days.

Experiment 3 used 108 pregnant Merino mature ewes selected on their pregnancy status at 80 and 110 days based on ultrasound scanning. The ewes were divided into three treatments groups: mid-pregnancy (pregnancy day 88-92) stressed (MID), late-pregnancy (pregnancy day 117-123) stressed (LATE) and control (CTRL). Seventy-eight lambs were tested, 27 CTRL lambs (13 singles and 14 twins), 25 LATE lambs (9 singles and 16 twins) and 24 MID lambs (16 singles and 8 twins). Gestation ranged between 130 and 146 days.

5.2.2 Housing

For all three experiments, ewes were grazed ad-libitum on improved native pasture and for *Experiment 1 and 2* were supplemented with sheep pellets (based on wheat, millrun and lucerne; 17.5% protein, 2.5% fat, 17% fibre, 20% ADF, 34% NDF). For *Experiment 3* the ewes were fed ad- libitum hay and 100g/animal/day of maize grain. During indoor periods animals were fed a 50:50 lucerne and oaten chaff mix supplemented with a 3:2 sheep pellet and corn ration (200 g/day; sheep pellets based on wheat, millrun and lucerne; 17.5% protein, 2.5% fat, 17% fibre, 20% ADF, 34% NDF). Throughout all three experiments, nutritional management aimed to maintain a BCS of ewes between 2.5 and 3.5.

In all 3 experiments, at around 10 days before the expected lambing date, ewes were moved into indoor lambing pens at 3-4 ewes per pen (1.5 m^2 per ewe) on straw bedding, and kept under continuous artificial lighting 24 hrs/day. Ewes were visually checked at least every 30 min and video cameras were used to continuously monitor the parturition.

Cameras were connected to digital video recorders and footage captured using IVMS4200 software (Hangzhou Hikvision Digital Technology Co., Ltd).

5.2.3 Treatments

Experiment 1 - Shearing and Handling stressors

At D90 of pregnancy, over the treatment of 7 days, pregnant ewes from both the shorn (SH) and handling control groups (HA), were housed indoor in group pens (3m^2/ewe) on a slatted floor and separated into treatment group. Ewes were acclimated in the shed 5 days before the beginning of the experiment. On the first day (D0), the treated group was shorn (SH) while the control group was sham-shorn for 2 min (HA), by mimicking the handling associated with shearing (seated on haunches, manual stroking of both sides of the body and rump). In the 7-day post shearing the shorn ewes, but not control ewes, were wetted using ceiling sprinklers for 30 min (water temperature 8-10˚C), on 3 occasions (D0, D3 & D7).

Experiment 2 - Cold exposure stressor

During the last two weeks of pregnancy (D130-D140 of pregnancy), one group of ewes was transported and cold exposed (CE) on three occasions over 5 days (D0, D2, D5) and a control group was transported but not cold exposed (TR). During the three wetting occasions, both CE and TR groups were transported from the paddock to the cold room facility using a stock trailer. For practical reasons, each group was divided into four subgroups of 7-8 animals and each subgroup was exposed to 5 min of loading and transport. At the cold room facility, CE ewes were unloaded and individually wetted for 2 min using a jet hose. During the wetting, ewes were sat on their rump and bare skin areas were hosed to ensure wool free areas around legs and groin were wet. The CE ewes were subsequently held in a 4˚C cold room for 3 hrs, without use of air fans. During this period, the TR ewes were unloaded and held on a slatted floor pen for the duration of 3 hrs (ambient temperature of 15-21°C; Table 5.1). At the completion of the cold treatment all animals were transported back to their paddock.

Experiment 3 – Combination of stressors

At D90 (MID) or D125 (LATE) of pregnancy both treatment groups (MID and LATE) were exposed to a stress treatment on 5 occasions over a period of 11 days (D0, D2, D5, D8 & D11). The stress treatment was designed to mimic the acute stress components of shearing and consisted of 10 min of yarding, 10 min of transport in a stock trailer, 10 min of high water pressure hosing and then 3 hrs in a 4°C cold room. During these treatments, the control group (CTRL) remained in a paddock with as little human interaction as possible (ambient temperature of 6-12°C; Table 5.1). In this experiment the late-pregnancy treatment was commenced at D125 to allow completion of the stress treatment prior to the commencement of lambing.

Table 5.1 Minimum and maximum temperature and relative humidity during stress treatment and lambing periods. Retrieved from CustomWeather (Time and Date AS 1995–2017. All rights reserved).

Experiment	Stress treatment pregnancy timing		Prenatal treatment stress	Lambing
EXPERIMENT 1 - SHEARING AND HANDLING	MID	Date	7/08/14 - 14/08/14	29/09/14 - 17/10/14
		min temp (°C)	0	2
		max temp (°C)	15	24
		relative humidity (%)	63	56
EXPERIMENT 2 - COLD STRESS	LATE	Date	26/11/14-23/12/14	6/12/14 - 21/12/14 5/01/15 - 13/01/15
		min temp (°C)	10	8
		max temp (°C)	29	28
		relative humidity (%)	75	77
EXPERIMENT 3 - STRESSORS COMBINATION	MID	Date	15/07/15 - 5/08/15	14/09/15 - 24/09/15
		min temp (°C)	-5	0
		max temp (°C)	14	19
		relative humidity (%)	85	74
	LATE	Date	19/08/15 - 9/09/15	14/09/15 - 24/09/15
		min temp (°C)	-4	0
		max temp (°C)	18	19
		relative humidity (%)	76	74

5.2.4 Lambing

All live-born lambs born during the experiments were individually identified shortly after birth and included in the behavioural assessments. Cold challenge and thermoregulation assessment was conducted 4 hrs after birth. Weather data during lambing period are gathered in Table 5.1.

In *Experiment 1* of 30 ewes, 25 lambed during the experiment and a total of 35 lambs (16 singles, 16 twins and 3 triplets) were enrolled and two cases of dystocia were observed. In *Experiment 2*, of 61 ewes, 36 lambed during the experiment and a total of 38 lambs (30 singles and 8 twins) were enrolled and six cases of dystocia were observed. In *Experiment 3*, of 108 ewes, 60 lambed during the experiment and a total of 78 lambs (40 singles and 38 twins) were enrolled and one case of dystocia was observed. For all lambing events, in cases of observed dystocia, lambing was assisted and lambs were visually monitored after birth to confirm successful suckling of colostrum. Two lambs for *Experiment 1*, one for *Experiment 2* and two for *Experiment 3* with insufficient suckling activity were deemed to be too weak to undergo the cold challenge, and removed from the experiment. All five of those lambs received manual latching support, but died within the following 24 hrs.

5.2.4.1 Cold challenge

Following an initial behaviour test (data not presented here – Chapter 4 but of 10-15 minutes duration) lambs were placed into a cold challenge at 4 ˚C for 1h. They were transported to the temperature controlled room in a plastic carrier box to minimise human contact and immediately prior to being placed in the cold room, all lambs had their abdominal, inguinal and axillar areas manually wetted (tap water in half-filled 20 l bucket; temperature 8-10 ˚C) to ensure all lambs were equally wet, mimicking amniotic fluid cover immediately after birth. Lambs were then placed inside a temperature-controlled room (without fan-forced air movement) and held in a cradle to limit movement (Figure

5.1). The cradle was designed to support the lamb ventrally to maintain an upright position and consisted of a fabric mat suspended between two longitudinal metal bars, with four holes cut into the fabric to accommodate the legs. If required, the lamb was secured with a bandage around the shoulder girdle although we found that once placed into the cradle, lambs settled into the position and remained quiet.

During the cold challenge, thermograms were taken using an infrared thermography camera (ThermaCam T640, FLIR Systems AB, Danderyd, Sweden) every 10 min – T0, T10, T20, T30, T40, T50 and T60 (see the method described in Chapter 6).

To monitor for hypothermia, lambs' rectal temperatures were measured every 10 min with a digital thermometer (accuracy ± 0.1°C from 35-42°C; Flexible Rapid 10 Second Thermometer DT_K101A; Medshop Australia, Preston VIC) when thermograms were captured and lambs were removed if rectal temperature dropped to below 36.5°C at which time they were given warm milk. On the return from the cold room lambs were subjected again to a behaviour test prior to returning to their mothers, at which time ewe and lamb behaviour was monitored for 10 min to ensure the ewe-lamb bond was intact. In total, 18 lambs were removed prior to T60 under this protocol (for *Experiment 1*, 7 HA, 9 SH; for *Experiment 3*, 1 CTRL and 1 LATE; none for *Experiment 2*). For these lambs, data until the point of removal were included in the analysis.

Wool length classification ('short' < 3mm/'average' 3 – 6 mm/'long' > 6mm) and type of the coat ('hairy'/'wooly') were recorded for each lamb to evaluate potential differences in radiated temperature.

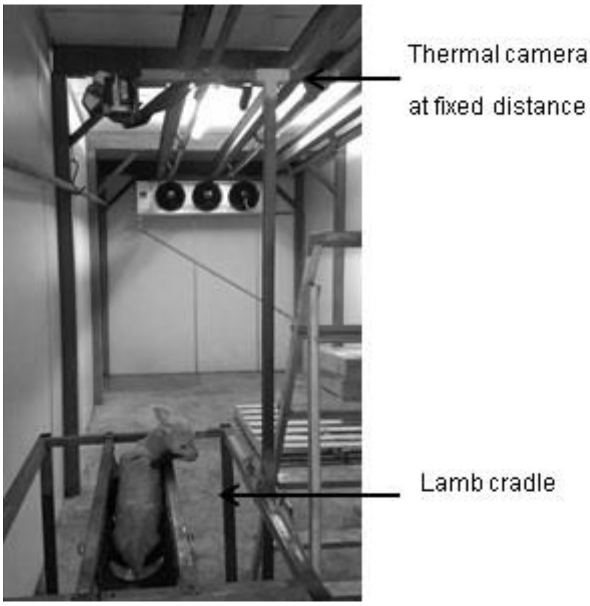
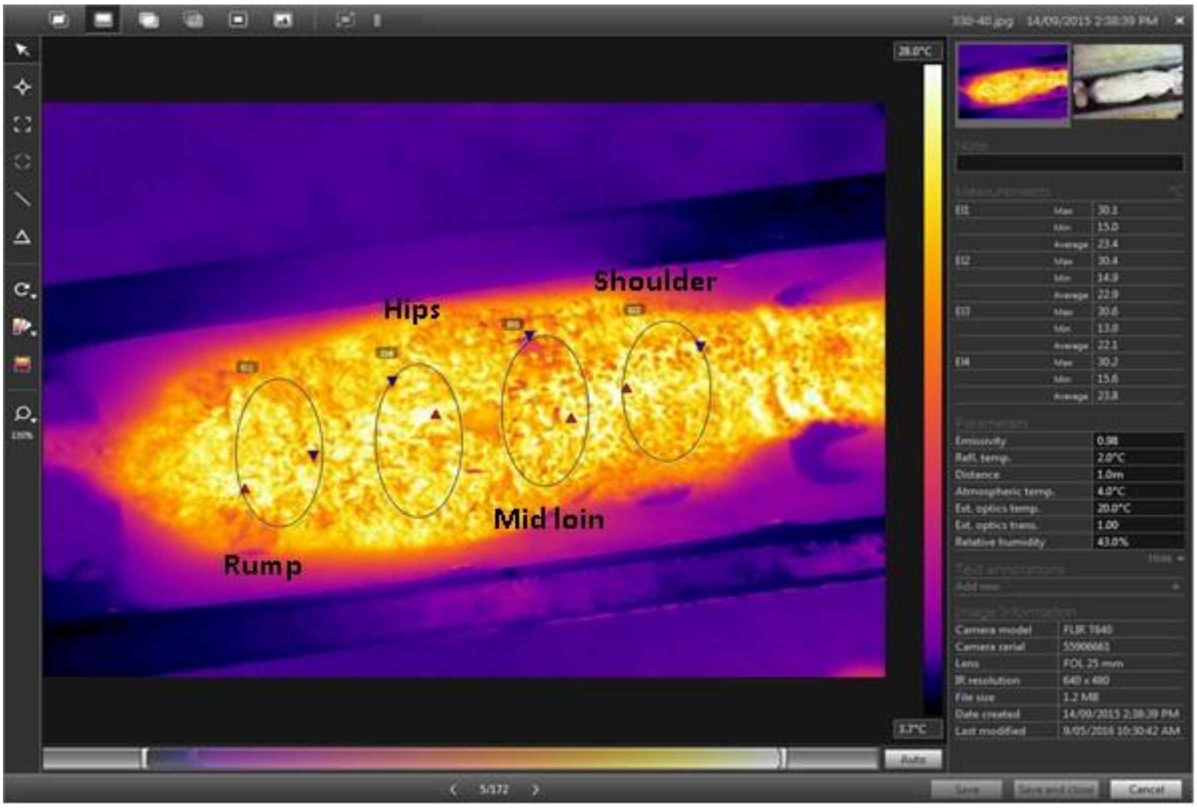


Figure 5.1 Cold room set up; cradle use during the cold challenge to restrain the lamb and the mount for the IRT camera 1 m above the back of the lamb.

5.2.4.2 Thermograms analysis

This methodology has been previously described by Labeur *et al.* (in press - Chapter 6). Thermograms were analysed using acquisition software (FLIR Tools, FLIR Systems AB). For each picture, calculated average and max temperature data were exported from the four fixed-size equidistant areas identified beforehand (shoulder, mid loin, hips and rump; Figure 5.2).



Measurements °C

El1	Max	30.1
	Min	15.0
	Average	23.4
El2	Max	30.4
	Min	14.9
	Average	22.9
El3	Max	30.6
	Min	13.0
	Average	22.1
El4	Max	30.2
	Min	15.6
	Average	23.8

Parameters

Emissivity	0.98
Refl. temp.	2.0°C
Distance	1.0m
Atmospheric temp.	4.0°C
Ext. optics temp.	20.0°C
Ext. optics trans.	1.00
Relative humidity	43.0%

Image information

Camera model	FLIR T640
Camera serial	55906661
Lens	FOL 25 mm
IR resolution	640 x 480
File size	1.2 MB
Date created	14/09/2015 2:38:39 PM
Last modified	9/05/2016 10:30:42 AM

Figure 5.2 Screenshot of FLIR Tool software, displaying the arrangement of the 4 zones across the back of the lamb, shoulder, mid loin, hips and rump. In the right panel, the values for each zone are displayed, minimum, maximum and average of which only the last 2 were recorded.

5.2.5 Statistical analysis

For each lamb the created variable Δ60-0 h represented the difference in body surface temperature between T60 and T0 and was used to assess the change over time. Because, for some lambs the cold challenge was terminated at T40 due to hypothermia (6 HA lambs, 7 SH lambs and 2 MID), an additional value Δ40-0 h was also calculated. Data was check for normality of distribution using a Shapiro-Wilk test. Most data were normally distributed and for non-normally distributed variables, a box cox procedure was used to determine the optimal transformation and reach normality. Non-parametric tests were used to compare groups were data could not be normalized. Body surface temperature on the whole body was analyzed using a repeated measures ANOVA mixed model R software (version 3.1.0, The R foundation for Statistical Computing), with treatment, location, time-point, wool length, litter size, sex and their interactions included as fixed effects and lamb ID as a random effect. Radiated body surface temperature was also analyzed at each measurement position using a repeated measures ANOVA mixed model, with treatment, time-point, sex, wool length, litter size and their interactions as fixed effects and lamb ID as a random effect. Data collected at the different zones was compared using an ANOVA mixed model with treatment, wool length, location, sex, litter size and their interactions as fixed effects and lamb ID as a random effect. When normally distributed, Δ60-0 h and Δ40-0 h were also analyzed using an ANOVA mixed model with treatment, location, wool length, sex and litter size and their interactions as fixed effects and lamb ID as a random effect.

In all models, dystocic lambs were included in the analysis as none of these lambs displayed altered thermoregulation.

All models were fitted with either body weight or girth circumference as a covariate to account for size variations, and these were removed when non-significant. Non-significant factors, such as wool length, coat type, sex, location- time and treatment-sex interactions were subsequently removed from the models.

Results are expressed as LSmeans ± standard deviation of the mean (S.D.). Results with an associated probability less or equal to 0.05 were considered significant and those above and less or equal to 0.09, a tendency.

5.3 Results

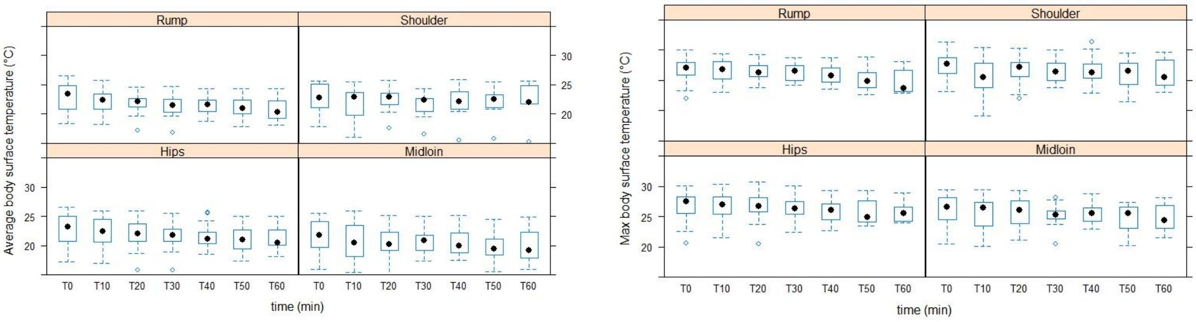
Experiment 1

Average and max body surface temperatures consistently decreased overtime in a similar manner at all four zones and whether treatment groups were combined or separate (Figure 5.3 &5.4). Lambs born to shorn ewes (SH) tended to have higher maximum surface temperatures than control lambs (HA) in the ‘Mid loin’ zone (P=0.0726; 26.24 vs 25.14°C).

A similar treatment effect was observed for Δ60-0 Max (P=0.0248) as well as a tendency for Δ40-0 Max (P=0.0715; Figure 5.5).

In average, average body surface temperature, for both treatment groups combined and averaged across timepoints, was higher at 'Hips' than at 'Shoulder' (P=0.0133) and 'Shoulder' average surface temperature also tended to be lower than 'Rump' (P=0.08) but was significantly higher than 'Mid loin' (P<0.001). 'Rump' body surface average temperature was higher than 'Mid loin' average temperature (P=<0.001). Maximum surface temperature was lower at 'Mid loin' than 'Hips', 'Rump' and 'Shoulder' (P<0.001) and 'Rump' was also lower than 'Shoulder' (P=0.0350).

For both average and max temperatures, across all time points and treatments, wool length significantly impacted body surface temperature, lambs with both short and medium length wool had significantly higher temperatures than long wool lambs (P<0.05; Table 5.2).



Average Body Surface Temperatures (a)

Max Body Surface Temperatures (b)

Figure 5.3 Average body surface temperature (a) and Max body surface temperature (b) profiles for 4 zones on lambs back (Shoulder, Mid loin, Hips and Rump) during a 1 hr cold challenge for lambs born to ewes shorn during mid-pregnancy (SH) and handled (HA).

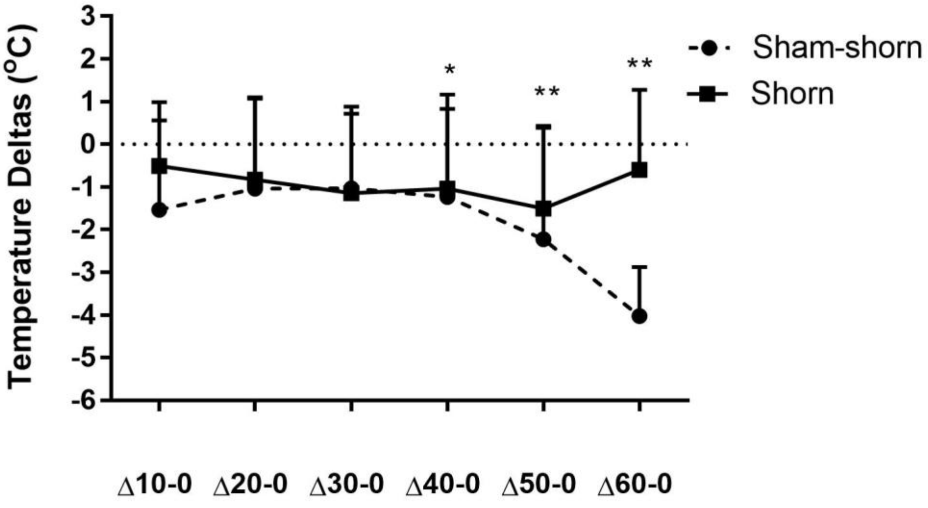


Figure 5.4 Max body surface temperature differences to baseline of cold challenged lambs for prenatally shorn and sham-shorn ewe. Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

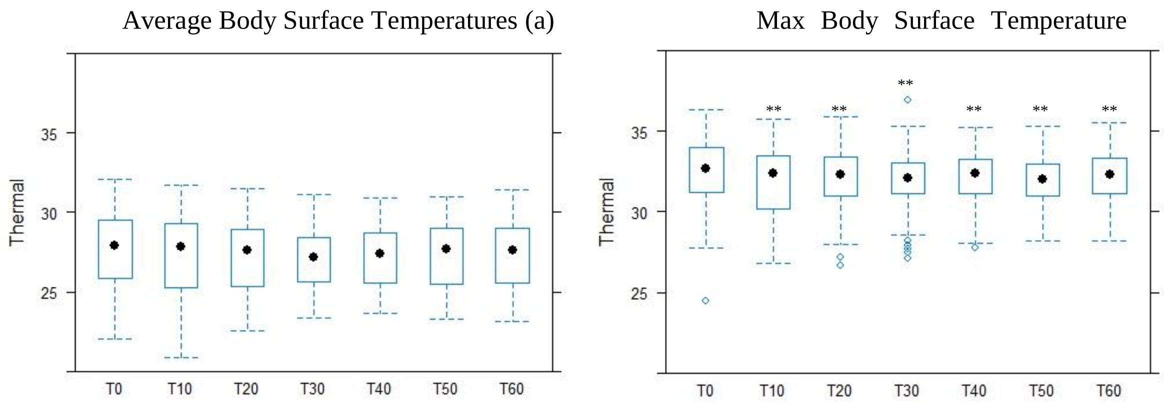
Table 5.2 Average and max lamb back skin temperatures across all timepoints, treatments and locations during a 1 hr cold challenge for lambs with short, medium and long wool ('short' < 3mm/'medium' 3 – 6 mm/'long' > 6mm)

	Average skin temperature (°C)	***Max skin temperature (°C)***
Short wool (<3mm)	*23.68 ± 0.50*	*26.86 ± 0.83*
Medium wool (3-6 mm)	*22.02 ± 0.94*	*26.08 ± 0.72*
Long wool (>6mm)	*18.45 ± 1.20*	*25.05 ± 0.85*

Experiment 2

For all four locations and treatments combined average back surface temperature was significantly higher at T0 than at T10 (P=0.0439). Similarly, max body surface temperature at T0 was significantly higher than temperature at all other time points (P<0.01). In lambs born to cold exposed ewes 'Rump' average surface temperature was significantly higher than 'Mid loin' and 'Shoulder' (respectively, 27.58±0.7250 vs 26.50±0.7320°C;

P<0.001 and 26.99±0.7608°C; P=0.049; Figure 5.6(a)). No significant treatment or wool length effects were observed (Table 5.3).


Average Body Surface Temperatures (a)

Max Body Surface Temperature

Figure 5.5 Average (a) and Maximum (b) body surface temperature profiles during a 1 hr cold challenge for lambs born to both ewes transported wetted and cold exposed and control ewes. ** represent P<0.05 for timepoints when compared with T0

Table 5.3 Average and max lamb back skin temperatures across all timepoints, treatments and locations during a 1 hr cold challenge for lambs with short, medium and long wool ('short' < 3mm/'long' > 6mm)

	***Average skin temperature (*°C)**	***Max skin temperature (*°C)**
Short wool	*26.73 ± 0.68*	*32.35 ± 0.32*
Long wool	*27.84±0.513*	*31.72±0.3993*

Experiment 3

At all timepoints and when treatments were combined, average body surface temperature was significantly higher at 'Hips' than 'Mid loin' (P<0.01) but was significantly lower than the temperature at 'Shoulder' (P=0.042; Figure 5.6). Temperature at 'Rump' was also significantly higher than 'Mid loin' (P<0.01) but tended to be lower than 'Shoulder' (P=0.06). There were similar results for max body surface temperatures (P<0.001 for all; Figure 5.7).

At ’Rump’, when averaged across timepoints, LATE lambs displayed significantly higher max surface temperature than MID ($P<0.05$) and was also higher than CTRL lambs ($P<0.05$). The difference Δ40-0 in average temperatures was significantly greater for MID lambs than LATE ($P=0.01$) and CTRL lambs ($P=0.0017$; Figure 5.8). Similar results occurred for Δ50-0 and Δ60-0.

No time effect was observed for max temperatures but average body surface temperature at T0 was significantly different from all the other time-points for both MID and CTRL groups ($P<0.05$).

Short wool lambs had significantly ($P<0.0001$) higher max body surface temperature than long wool lambs at ‘Shoulder’, ‘Rump’ and ‘Hips’ zones and higher average body surface than long wool at all 4 locations (Table 5-4). For ‘Rump’, ‘Mid loin’ and ‘Hips’ locations separately, single lambs had higher max body surface temperature than twin lambs ($P<0.001$).

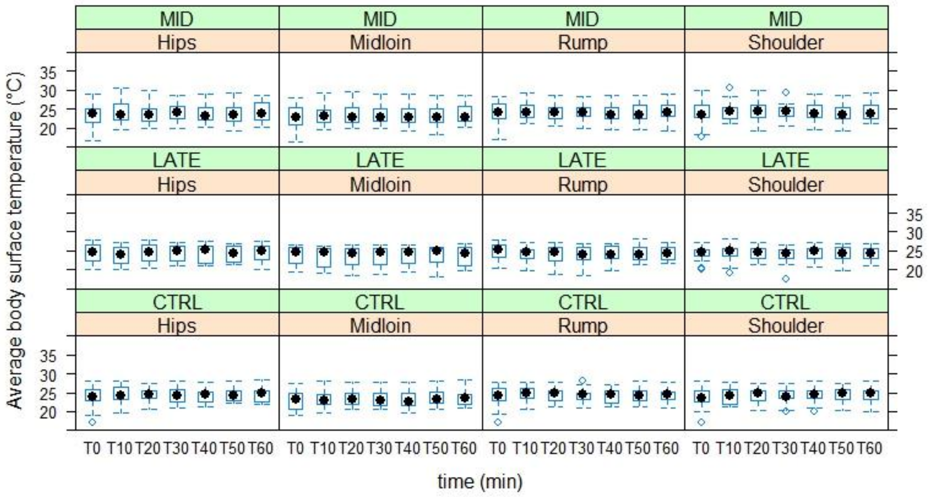


Figure 5.6 Average body surface temperature profiles for 4 zones on the back of the lamb (Shoulder, Mid loin, Hips and Rump) during a 1 hr cold challenge for lambs born to ewes yarded, transported and cold exposed during mid-pregnancy (MID), late-pregnancy (LATE) and control (control).

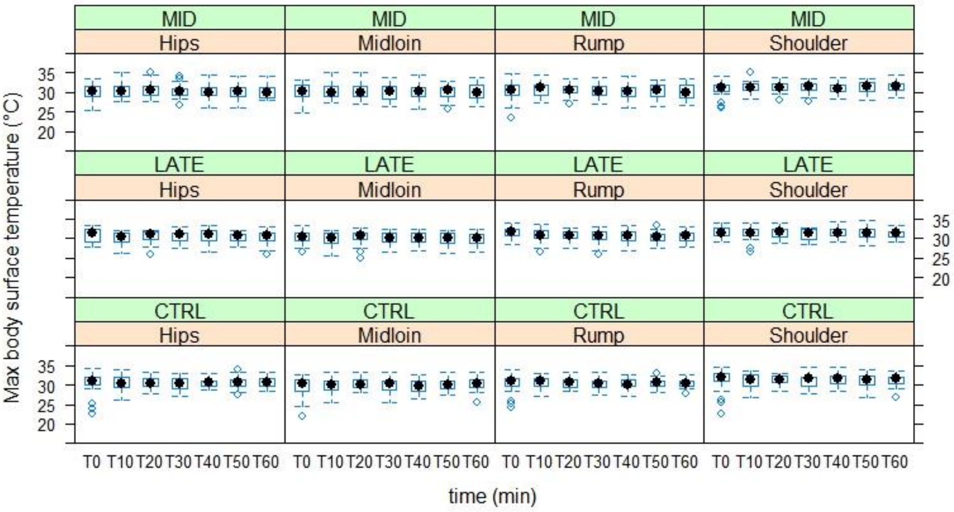


Figure 5.7 Maximum body surface temperature profiles for 4 zones the back of the lamb (Shoulder, Mid loin, Hips and Rump) during a 1 hr cold challenge for lambs born to ewes yarded, transported and cold exposed during mid-pregnancy (MID), late-pregnancy (LATE) and control

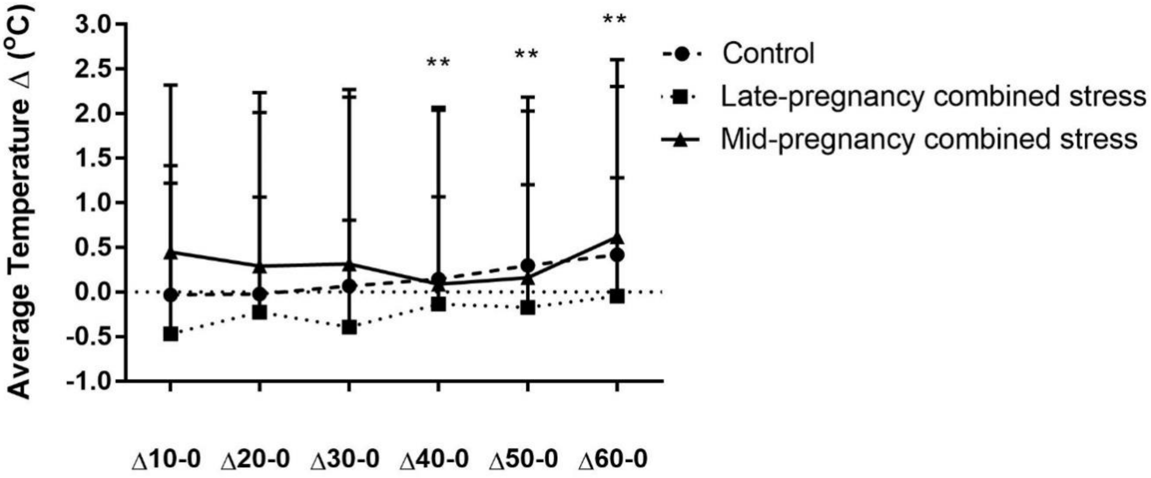


Figure 5.8 Average body surface temperature differences to baseline during a 1 hr cold challenge for lambs from ewes prenatally stressed during mid- or late-pregnancy or a control. Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

Table 5.4 Average and max lamb back skin temperatures across all time-points and treatments for all four locations ('Rump', 'Shoulder, 'Hips', Mid loin') during a 1 hr cold challenge for lambs with short and long wool. LS-Means for max temperatures are back-trasnformed.

Location	***Rump Skin Temperature (°C)***		***Shoulder Skin Temperature (°C)***		***Hips Skin Temperature (°C)***		***Mid loin Skin Temperature (°C)***
Value	***Average***	***Max***	***Average***	***Max***	***Average***	***Max***	***Average***
Short wool	*22.92 ± 0.35*	*29.88*	*23.12 ± 0.32*	*30.8*	*22.65 ± 0.37*	*29.7*	*21.69 ± 0.39*
Long wool	*24.93 ± 0.25*	*31.05*	*25.05 ± 0.23*	*31.73*	*25.07 ± 0.26*	*30.96*	*24.33 ± 0.28*

5.4 Discussion

The results of this study suggest similar effects of the shearing stressor and combined stressors on lamb body surface temperature patterns when compared to control lambs.

For both *Experiment* 1 and *2,* prenatal mild husbandry-like stressor and cold exposure resulted in lambs with increased body surface temperature when compared to control lambs. Furthermore, lambs born to stressed ewes displayed greater heat production than heat loss (Δ40-0 >0) and maintained their body surface temperature during the cold challenge than control lambs for which temperature dropped significantly over time. However, no difference was seen in *Experiment 2* between lambs born to ewes prenatally exposed to cold and the control lambs. This difference suggests that cold exposure might not be the sole contributor to the improved body surface temperature maintenance in the treatment lambs of *Experiments 1&3* or alternatively, that the ewes were not cold stressed and that the prenatal treatment did not produce an appropriate physiological response. This contrasts with the previous findings of Stott and Slee (1985) who noted a greater thermogenic response to a cold challenge of lambs born to ewes cold stressed during late-pregnancy (14 prior lambing event). In our study, improved body surface temperature maintenance resulted from a combination of both cold exposure and acute stressors, which aligns with observations by Symonds *et al.* (1992a) and Clarke *et al.* (1997). In their studies, the effect of chronic cold stress during the last month of pregnancy using winter shearing and the cold stress resulting from subsequent fleece removal was examined and they concluded that prenatally cold stress lambs displayed an enhanced thermogenic activity of the brown fat. Our protocol, was similar to these studies in that cold exposure was accompanied by an acute stress of shearing.

The different outcomes of the present research and previous studies could also be explained by a difference in the intensity and the duration of stress exposure. In our study, the stressors used aimed to represent a transient, short term and acute stressful experience which sheep would commonly experience during on-farm husbandry procedures and which have been previously experienced by these animals. The short acute cold exposure treatment we used contrasts with previously referenced studies, in which pregnant ewes were exposed to cold during a prolonged period, mimicking prolonged winter cold exposure and the stress treatment was considered chronic (Stott and Slee 1985; Symonds *et al.* 1992a; Clarke *et al.* 1997).

Previous studies focused only on the effects of cold stress, applied during the last month of pregnancy, associated with shearing or otherwise, while we observed a significant effect of the cold exposure during mid-pregnancy resulting from shearing and to a lesser extent to a combination of husbandry-like stressors and cold exposure designed to mimic shearing. In a previous paper, we have reported the effects of shearing and handling stress during late-pregnancy on lambs’ ability to maintain body surface temperature and observed higher body surface temperatures in lambs born to prenatally shorn ewes (Labeur *et al.* 2017) (Chapter 6). Some early studies focused on late-pregnancy bases on the hypothesis that cold treatment could lessen the foetal brown fat amount decline normally happening during that stage of development (Alexander 1978). This adaptation could be accounted for by either higher maternal and foetal glucose levels (Thompson *et al.* 1982) or by cold-induced vasoconstriction resulting in limited use of the brown fat reserves. However, shearing also produces a cascade of hormonal and metabolic adaptations in the ewe (Clarke *et al.* 1997) amongst which a rise in maternal cortisol levels (Hargreaves and Hutson 1990c; Mears *et al.* 1999), an increased maternal plasma non-esterified fatty acid (Elvidge and Coop 1974) and a long lasting elevation of the thyroid hormones T3 and T4 for both mid- and late-pregnancy shearing (Symonds *et al.* 1988a, 1989; Sherlock *et al.* 2003). It seems reasonable to assume that, the impact of shearing and cold exposure at different pregnancy stages would be mediated through those different mechanisms. In this case, mid-pregnancy shearing and cold stress might not affect the amount of brown fat deposited but could increase the foetal T3 and T4 levels and thus enhance brown fat activity. In contrast, the combination of stressors designed to mimic shearing during late-pregnancy, might affect maternal glucose levels and result in vasoconstriction, alleviating the brown fat reserves decline.

These suggestions must, be taken cautiously as the infrared thermography technology does not allow us to attribute the body surface temperature changes to a specific underlying mechanism; but only enables us to observe heat loss through the skin and body surface which is linked to heat production and thermoregulation. Future studies will be needed to investigate in parallel heat loss, hormonal levels and brown fat amounts to allow these to be linked.

Based on the pattern reported by Labeur *et al.* (2017) (Chapter 6), we used four fixed-size areas (shoulder, mid loin, hips and rump) spread along the lambs back. For all three experiments, there was a consistent trend for all these zones, but the most consistent, and always with the lowest temperatures were obtained at the 'Mid loin'. It has been hypothesized previously that, as most of the brown fat depots are located in the thoracic area of neonates (Symonds 2013), brown fat detection using methods such as infrared thermography, would be the most efficient around the shoulder area. However, the presence of significant brown fat depots in the peri-renal abdominal region of lambs might explain a greater sensitivity to thermogenesis changes at ‘Mid loin’ ((Labeur *et al.* 2017)– Chapter 6).

For both shearing and stressors combination experiments we noted significant effects of wool length which was expected as wool coat properties, type and length provide thermal insulation and have been shown to protect the lamb from cold as well as being linked to metabolic rate and consequently to core body temperature maintaining mechanisms (McCutcheon *et al.* 1983; Martin 1999; Allain *et al.* 2010). The insulative characteristics of the wool may explain why short wool length lambs displayed higher body surface temperature than long wool length lambs where a direct reading of the skin temperature might not be possible.

In all experiments the cold challenge protocol was similar and all lambs body surface temperature dropped within the first 10 minutes giving evidence of the initiation of a response. This is an important point as, it has been shown that cold stimulus and cutaneous cooling were essential triggers for the onset of non-shivering thermogenesis (Gunn *et al.* 1991).

In conclusion, we observed an effect of prenatal stress on newborn lamb ability to maintain body surface temperature when exposed to cold. Prenatal cold exposure combined with acute stressors in keeping with husbandry procedures impacted heat production in newborn lambs and resulted in difference in body surface temperature in response to a cold challenge when prenatal stress occurred during both mid- or late-pregnancy. The presence of effects at mid- and late-pregnancy stage suggests at least two alternate mechanisms triggered by maternal-pregnancy stress, one potentially involving thyroid hormones and regulating the activity of the brown fat and the other one potentially increasing the amount of brown fat in the newborn lamb.

Future research should involve longer cold challenges to ensure the onset of non-shivering thermogenesis as well as continuous rectal temperature recording to allow an understanding of the relationship between body surface temperature and thermogenesis.

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Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF ORIGINALITY

Chapter 5 - Impact of prenatal stress on lamb neonatal ability to maintain body surface temperature in cold conditions

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	**Page number/s**
All figures, diagrams and text	

Name of Candidate: Lea LABEUR

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Date

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21/9/2017_

Date

Principal Supervisor

Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

Chapter 5 - Impact of prenatal stress on lamb neonatal ability to maintain body surface temperature in cold conditions

We, the Research Master/PhD candidate and the candidate's Principal 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 in the *Statement of Originality*.

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	Alison Small	5%
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Date

Chapter 6: **Infrared thermal imaging as a method to evaluate heat loss in newborn lambs.**

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ABSTRACT

Thermal imaging technology has been identified as a potential method for non-invasive study of thermogenesis in the neonatal lamb. In comparison to measurement of the core body temperature, infrared thermography may observe thermal loss and thermogenesis linked to subcutaneous brown fat depots. This study aimed to identify a suitable method to measure heat loss in the neonatal lamb under a cold challenge. During late pregnancy (day 125), ewes were subjected to either shearing (n=15) or mock handling (sham-shorn for 2 min mimicking the shearing movements) (n=15). Previous studies have shown an increase in brown adipose tissue deposition in lambs born to ewes shorn during pregnancy and we hypothesized that the shearing treatment would impact thermoregulatory capacities in newborn lambs. Lambs born to control ewes (n=14; CONTROL) and shorn ewes (n=13; SHORN) were subjected to a cold challenge of 1h duration at 4 hrs after birth. During the cold challenge, thermography images were taken every 10 min, from above, at a fixed distance from the dorsal midline. On each image, four fixed-size areas were identified (shoulder, mid loin, hips and rump) and the average and maximum temperatures of each recorded. In all lambs, body surface temperature decreased over time. Overall the SHORN lambs appeared to maintain body surface temperature better than CONTROL lambs, while CONTROL lambs appeared to have higher core temperature. At 30 min post cold challenge SHORN lambs tended to have higher body surface temperatures than the CONTROL lambs ($P=0.0474$). Both average and maximum temperatures were highest at the hips. Average temperature was lowest at the shoulder ($P<0.05$), while maximum temperatures were lowest at both shoulder and rump ($P<0.005$). These results indicate that lambs born to shorn ewes maintained their radiated body surface temperature better than CONTROL lambs. In conjunction with core temperature changes under cold challenge, this insight will allow us to understand whether increased body surface temperature contributes to increased overall heat loss or whether increased body surface temperature is indeed a mechanism contributing to maintenance of core body temperature under cold challenge conditions. This study has confirmed the utility of infrared thermography images to capture and identify different levels of thermoregulatory capacity in newborn lambs.

Keywords: thermoregulation; cold challenge; sheep; neonate; brown fat; thermogenesis

Highlights

- Body surface temperature was higher at Hips zone over time when compared to other areas of the back.
- Body surface temperature decreased over time during a 1 hr cold challenge.
- While body surface temperatures decreased, rectal temperatures increased over the same time period.
- Lambs of shorn ewes maintained their skin temperature at a higher level than control lambs.

6.1 Introduction

Infrared thermography (IRT) has been shown to be a safe and non-invasive method for measuring and mapping the radiated heat loss at the body surface. In humans, Ng (2009) applied IRT to identify and visualize breast cancer, and Abbas and Leonhardt (2014) showed its use for neonatal monitoring. IRT also has a variety of practical applications in veterinary and animal science for detecting diseases (Poikalainen *et al.* 2012), lameness (Nikkhah *et al.* 2005), stress and more generally for assessing animal welfare (Schaefer *et al.* 1988; Stewart *et al.* 2005; 2009). In bats and voles, IRT has been used to study torpor and thermogenesis (Lancaster *et al.* 1997; Jackson *et al.* 2001). When compared to other methods such as calorimetry (Shuran and Nelson 1991; Adams *et al.* 2000) or standard equations, IRT has proven to be a very useful method to quantify heat loss.

Heat loss radiating through the body surface is an important factor in the etiology of hypothermia due to cold exposure in neonates. Brown fat depots in neonatal lambs contribute through non-shivering thermogenesis to the maintenance of homeostasis but the mechanisms are difficult to study in vivo (Hergenhan 2012). Indirect ways to assess non-shivering thermogenesis in lambs are the use of climate chambers to characterize lamb metabolic responses to the environment (Alexander and Peterson 1961), and measurements of lamb oxygen consumption in response to noradrenaline injections(Alexander and Williams 1968). Recently, McCoard *et al.* (2014) have described IRT continuous recording as a suitable and non-invasive method to study thermogenesis in the neonatal lamb. However, thermogenesis is traditionally assessed through core body temperature monitoring which does not specifically capture the infrared heat loss radiating from the skin of an animal, which can be measured as body surface temperature in the infrared frequency band of the light spectrum by IRT.

Late-pregnancy shearing (Symonds *et al.* 1992a) and cold stress (Stott and Slee 1985) have been shown to positively impact the deposition of brown adipose tissue (BAT) and thus the thermoregulatory capacity of the lambs. In the neonatal lamb, major BAT depots are located in the peri-renal abdominal and inguinal regions of lambs (Alexander and Bell 1975; Everett-Hincks and Duncan 2008). Subcutaneous fat in the pre-scapular and hind limb regions can also contain functional BAT, as shown for field voles by (Jackson *et al.* 2001), using IRT to identify regions of body with underlying BAT. Similarly, in lambs, infrared thermographs have been proposed as a method to identify the areas of heat production even though it might not provide measures of absolute temperature (Hergenhan 2012) or be sensitive enough to quantify BAT quantity (Jackson *et al.* 2001). We hypothesized that infrared thermographs taken during a cold challenge could be used to discriminate different levels of thermogenesis in newborn lambs developed as a result of different levels of ewe prenatal stress.

6.2 Materials and methods

All experimental procedures were approved by the CSIRO FD McMaster Laboratory, Chiswick, Animal Ethics Committee, Armidale, Australia (AEC No.14/17). Weather observations were obtained from CustomWeather (Time and Date AS 1995–2017. All rights reserved).

6.2.1 Animals and Treatment

Pregnant Merino multiparous ewes were shorn at day 125 of pregnancy (n=15; 13 single-bearing and 2 twin-bearing) while control ewes were sham-handled for 2 min alongside the shorn group (n=15; 13 single-bearing and 2 twin-bearing). Temperature at shearing was 9˚C (5/15˚C daily min/max temp; 88% humidity). Following shearing (D0), shorn ewes, but not control ewes, were wetted (water temperature 8 – 10 ˚C) using ceiling sprinklers for 30 min, 3 occasions over 7 days (D0, D3 [11˚C; 9/14˚C daily min/max; 99% humidity] &D7 [12˚C; 8/21˚C daily min/max; 58% humidity]) as a controlled cold stress. Animals were grazed *ad libitum* on improved native pasture, except during the treatment period of 15 days, when animals were kept indoor in group pens (3m^2/ewe) on slatted floor, separated according to treatment group. From 10 days before expected date of parturition the ewes were housed on straw bedding in indoor lambing pens at 3-4 ewes per pen (1.5 m^2 per ewe). During indoor periods animals were fed a 50:50 lucerne and oaten chaff mix supplemented with a 3:2 sheep pellets (based on wheat, millrun and lucerne; 17.5% protein, 2.5% fat, 17% crude fibre, 20% ADF, 34% NDF)/corn ration 200 g/day as required to maintain a body condition score of 3. Throughout the experiment all ewes maintained a BCS between 2.5 and 3.5 (scale 1-5). Ewes were visually checked at least every 30 min and video cameras were used to continuously monitor the lambing and bonding behaviours to ensure bonding was not subsequently altered with cold treatment. Each camera provided a view of the entire lambing pens. The cameras were connected to digital video recorders and footage captured using IVMS4200 software (Hangzhou Hikvision Digital Technology Co., Ltd). Gestation lengths were 139 – 152 days, and lambing took place between 1st October and 17th October 2014 at the CSIRO property Chiswick, Armidale, New South Wales, Australia. Average temperatures at lambing in the open-sided animal house were on average 13.5˚C (2˚C/24˚C min/max temperature; 59.5% humidity on average).

6.2.2 Lambs measurements

From 30 ewes, 25 lambed during the experimental period, and a total of 29 lambs were born (21 single births and 4 twin births) and subsequently enrolled in the trial. Four cases of dystocia were observed in the cohort in which case lambing was assisted. Successful latching and suckling of colostrum was visually monitored for each lamb. Two lambs with insufficient suckling activity were withdrawn from the experiment, and both died within the following 24hrs. At 4hrs of age, lamb birthweight, sex and girth circumference (measured with a soft measuring tape behind the front legs) were recorded. Immediately following these measurements, lambs (10 singles and 4 twins CONTROL lambs, born to control ewes; 9 singles and 4 twins SHORN lambs, born to shorn ewes) were wetted by brief immersion in cold water (8-10 ˚C) to ensure all lambs were equally wet as at this time after birth some lambs could be completely dry or still partially wet from amniotic fluid. Each lamb was then individually restrained in a lamb cradle (Figure 6.1) in a cold room at 4˚C for 1h (no use of fans for air movement). The cradle was designed to support the lamb ventrally to maintain in a stable upright position and consisted of a fabric mat suspended between two longitudinal metal bars, with four holes cut into the fabric to accommodate the legs. If required, the lamb was secured with a bandage around the shoulder girdle although we found that once placed into the cradle, lambs settled into the position and remained quiet.

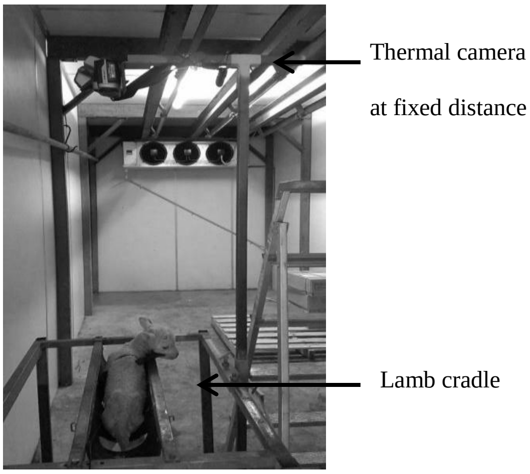
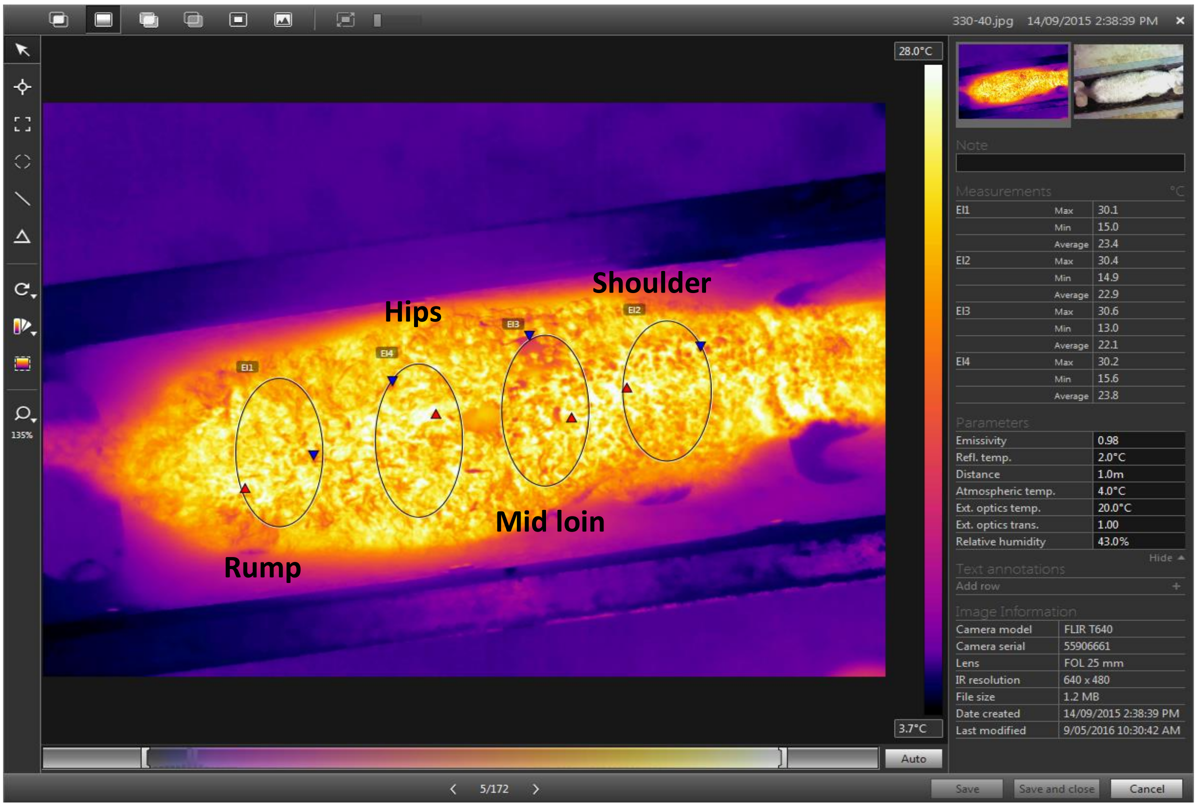


Figure 6.1 Cradle used during the cold challenge to restrain the lamb and the mount for the IRT camera 1 m above the back of the lamb.

Body surface heat map images of the back of the lambs were taken on entry into the cold room and at the following 10 min intervals (T0, T10, T20, T30, T40, T50 and T60) using an infrared thermography (IRT) camera (ThermaCam T640, FLIR Systems AB, Danderyd, Sweden). The IRT camera was mounted above the cradle at a fixed distance of 1 m from the dorsal surface of the lamb. Every 10 min when a thermal picture was taken, rectal temperature was measured using a digital rectal thermometer to monitor core body temperature. To prevent clinical hypothermia, lambs were removed from the cold room if their rectal temperature dropped below 36.5˚C, at which time they were placed in a warm environment and monitored until their body temperature had stabilized. Six lambs were removed prior to T60 under this protocol (4 SHORN and 2 CONTROL). Their data until the point of removal were included in the analysis. Wool length (< 3mm 'short'; 3 – 6 mm 'average'; > 6mm'long') and type of the coat ('hairy'; 'wooly') were recorded for each lamb to account for potential differences in radiated temperature.

6.2.3 Image analysis

Images were analyzed using acquisition software (FLIR Tools, FLIR Systems AB) and unfocussed images were rejected. For each picture of the lamb, four fixed-size equidistant areas were identified (shoulder, mid loin, hips and rump) (Figure 6.2). For each picture, the average and maximum temperatures of each of the locations calculated by the software were recorded. If a foreign object or an ear appeared across a zone maximum values only were recorded. In the acquisition software, the emissivity was set at 0.98 which is similar to human skin (Gerken 1996). Other parameters were set according to experimental conditions: distance was set at 1m (distance between the back surface and the camera, set to nearest full meter), atmospheric temperature at 4˚C and relative humidity as measured at 43%.



Measurements °C

El1	Max	30.1
	Min	15.0
	Average	23.4
El2	Max	30.4
	Min	14.9
	Average	22.9
El3	Max	30.6
	Min	13.0
	Average	22.1
El4	Max	30.2
	Min	15.6
	Average	23.8

Parameters

Emissivity	0.98
Refl. temp.	2.0°C
Distance	1.0m
Atmospheric temp.	4.0°C
Ext. optics temp.	20.0°C
Ext. optics trans.	1.00
Relative humidity	43.0%

Image Information

Camera model	FLIR T640
Camera serial	55906661
Lens	FOL 25 mm
IR resolution	640 x 480
File size	1.2 MB
Date created	14/09/2015 2:38:39 PM
Last modified	9/05/2016 10:30:42 AM

Figure 6.2 Screenshot of FLIR Tool software, displaying the arrangement of the 4 zones across the back of the lamb, shoulder, mid loin, hips and rump. In the right panel, the values for each zone are displayed, minimum, maximum and average of which only the last 2 only were recorded. Below are the details of the parameters set for each of the pictures analyzed.

6.2.4 Statistical analysis

For each lamb the created variable Δ60-0 h represented the difference in radiated temperature between T60 and T0 and was used to assess the change over time. Because, for some lambs the cold challenge was terminated at T40 due to hypothermia, an additional value Δ40-0 h was added to maximize the number of values included in the analysis (10 SHORN lambs and 13 CONTROL lambs). Radiated body temperature on the whole body was analyzed using a repeated measures ANOVA mixed model R software (version 3.1.0, The R foundation for Statistical Computing), with treatment, location, time-point, wool length, litter size, sex and their interactions as fixed effects and lamb ID as a random effect when data were normally distributed. Normality of the data was tested with a

Shapiro-Wilk test. Radiated body surface temperature was also analyzed at each location using a repeated measures ANOVA mixed model in, with treatment, time-point, sex, wool length, litter size and their interactions as fixed effects and lamb ID as a random effect. Comparison between locations was done using an ANOVA mixed model with treatment, wool length, location, sex, litter size and their interactions as fixed effects and lamb ID as a random effect when data were normally distributed. All models were fitted with either body weight or girth circumference as a covariate to account for area size variations, and these were removed when non-significant. Non-significant factors, such as wool length, coat type, sex, location-time interaction and treatment-sex interaction were subsequently removed from the models.

Rectal temperature at every time-point, Δ60-0 and Δ40-0 were analyzed independently using a mixed model in R with treatment and sex as fixed effects and lamb as a random effect. Rectal temperature was assessed over time using a repeated measure in a mixed model analysis in R.

Results are expressed as means ± standard deviation of the mean (S.D.). Results with an associated probability less or equal to 0.05 were considered significant and those above and less or equal to 0.09, a tendency.

6.3 Results

6.3.1 Rectal temperature

Most of the lambs (12/13 in SHORN and 12/15 in CONTROL groups) had a reduction in the rectal temperature of 0.2-2.5˚C between the baseline (T0) and the end of the cold challenge (T60), while 2 CONTROL lambs maintained rectal temperature and 2 lambs (1 CONTROL and 1 SHORN) had an increase in the rectal temperature of 0.2-0.6 ˚C. Six lambs (2 CONTROL and 4 SHORN) were removed from the cold room before the end of the cold challenge to prevent hypothermia. At T60, CONTROL lambs had a higher rectal temperature than SHORN lambs (P=0.0342; Figure 6.3), and there was a similar trend at T30 (P=0.0552). No treatment differences were observed when assessing the mean rectal temperature. CONTROL lambs appeared to enter the cool room with higher rectal temperatures than SHORN lambs (P=0.0624 at T0; +0.4 ˚C) and although not statistically significant seemed to maintain their rectal temperatures at slightly higher levels (Figure 6.3). In contrast, rectal temperatures measured before taking the lambs to the cold room differed only by 0.1 ˚C for both groups (39.24˚C for CONTROL and 39.33˚C for SHORN lambs).

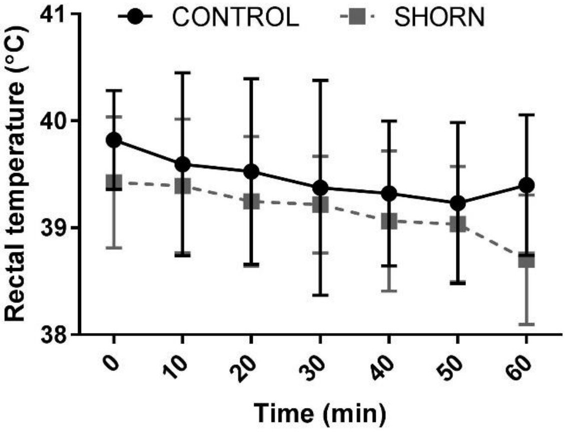


Figure 6.3 Rectal temperature over time of CONTROL and SHORN lambs. Values are means ± standard deviation. Asterisk denote tendency (0.05≤P<0.1) and double asterisk a significancy (P<0.05).

6.3.2 Thermal imaging

The pattern of heat loss at all 4 locations was consistent at each of the time-points and the observed body surface temperatures decreased over time (Figure 6.4). Within each treatment, on average values, body surface temperature was significantly lower at the ‘Shoulder’ ($P<0.05$) than at ‘Mid loin’, and temperature at ‘Hips’ was significantly higher than both ‘Mid loin’ and ‘Shoulder’ ($P<0.001$ for both). ‘Rump’ temperature was significantly lower than ‘Mid loin’ ($P<0.01$) and tended to be lower than ‘Hips’ temperature ($P=0.0587$). The maximum temperature pattern was slightly differently with the temperature at ‘Rump’ being significantly lower than at ‘Hips’ ($P<0.001$), as was the ‘Mid loin’ ($P<0.001$). The ‘Shoulder’ body surface temperature was significantly lower than the ‘Hips’ ($P<0.01$).

Overall, SHORN lambs displayed a narrower range of body surface temperature and a higher mean body surface temperature than CONTROL lambs across the entire 60 min observation period (Figure 6.5). At T30, SHORN lambs tended to have a warmer body surface temperature than CONTROL lambs ($P=0.0747$; 26.67±0.82˚C vs 23.96±0.62˚C; Figure 6.5).

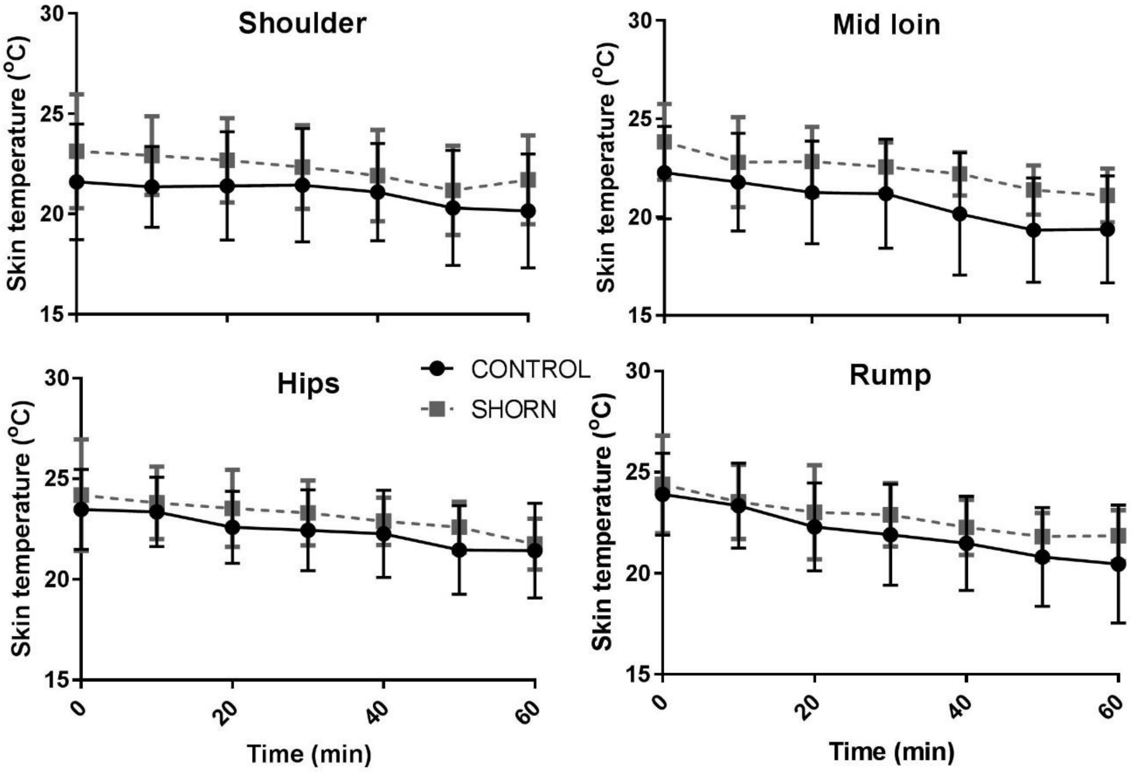


Figure 6.4 Average body surface temperature (°C) measure by infrared thermography at the four body locations: Shoulder, Mid loin, Hips and Rump across time for both treatment groups. Black circles represent CONTROL lambs; grey squares represent SHORN lambs. Values are means ± standard deviation.

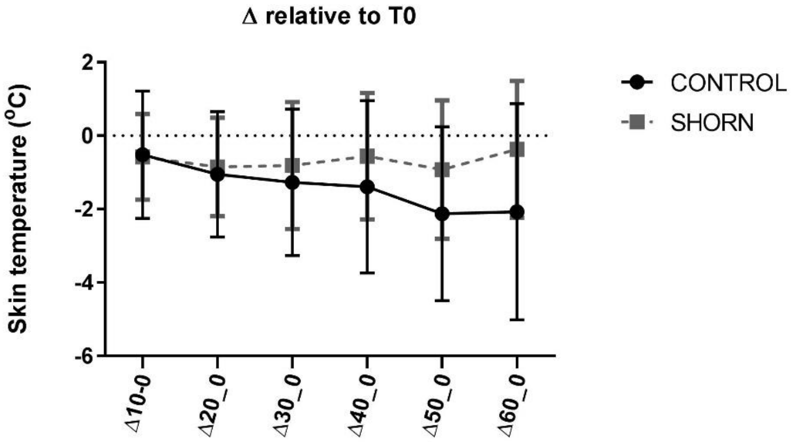


Figure 6.5 Change in body surface temperature over time relative to T0 across all locations. Black circles represent CONTROL lambs; grey squares represent SHORN lambs. Values are means ± standard deviation.

6.4 Discussion

In this study, we used IRT to investigate thermogenesis in neonatal lambs under cold challenge and the effect of prenatal treatment of ewes on thermogenesis in their lambs. To this end, we recorded core body temperatures using a rectal thermometer and used IRT in parallel to record body surface temperatures in four areas of the dorsal surface of the lamb. Although lambs were not shorn for this experiment due to animal ethics considerations, the hair cover of the skin was such that the skin was clearly visible and hence accessible for the infrared thermography readings. Measurements of maximum temperatures within a field were considered to relate to skin surface temperature. Statistical analysis showed that maximum temperature readings of the different zones behaved in the same way as temperature averaged across the zones, and wool length did not affect the results. Hence, we considered body surface temperatures indicative of skin temperatures. While treatment did not consistently affect all the body surface temperature measures in this study, we observed a trend in treatment group differences in core body temperatures which was different to that observed in the radiated temperature from the skin. Lambs from shorn ewes had higher core temperatures and lower body surface temperatures than lambs from control ewes. Body surface temperature was not recorded prior to commencement of the cold challenge, including wetting of the lambs with cold water, as lambs showed a range of degrees of wetness which would have likely influenced IRT recordings. It is conceivable that the physiological response to the wetting of the lambs was sufficiently fast to influence T0, which was recorded once lambs were installed in the cold room. However, core body temperatures measured before heading to the cold room did not differ between SHORN and CONTROL lambs. Previous studies have established factors such as breed, genetic markers, prenatal nutrition, litter size, sex and lamb birthweight impacted lambs core temperature (Samson and Slee 1981; Dwyer and Morgan

2006), however, sex, litter and birthweight weren’t significant factors in our study. Finally, it is also plausible the effect of treatment on T60 rectal temperature may be an artefact due to the reduced numbers of lambs after T40 when some lambs (2 CONTROL and 4 SHORN) were removed from the experiment.

Decreased body surface temperatures over time during cold challenges have been attributed to the vasoconstriction of the skin vessels and increased blood flow in BAT responsible of heat production (Alexander *et al.* 1972; 1973). While quantifying heat radiation from the body surface, which are indicative of skin temperature, we are not able at this stage to establish a direct link with the quantity and the activation of the BAT as previously suggested by Hergenhan (2012) in lambs and El Hadi *et al.* (2016) in humans.

Similarly to El Hadi *et al.* (2016) we used a fixed area for body surface temperature analysis. The four selected locations (shoulder, mid-back, hips and rump) behaved similarly for max and average surface temperature in each of the two treatment groups. For both the average and max surface temperatures, the ‘Hips’ area showed the highest temperatures while the lowest temperature was observed at ‘Rump’ in max surface temperature and at ‘Shoulder’ for average surface temperature. Previous studies have focused the analysis on the shoulder region as the literature indicates that BAT is predominantly deposited in the thoracic area in most mammals (Cannon *et al.* 1977; Symonds 2013). However, significant deposits of BAT are also found in the peri-renal abdominal area of lambs, so the lumbar region may also provide useful data, as shown in the current study which indicates that measurements at regions such as ‘Hips’ and ‘Mid loin’ may be more sensitive to changes in thermoregulatory capacity.

Previous studies found increased BAT depots in lambs resulting from late-pregnancy shearing and cold exposure (Alexander. 1978; Symonds *et al.* 1992a; Symonds and Lomax 1992). Our observation of lambs born to ewes shorn in late-pregnancy being better able to maintain their body surface temperature than CONTROL lambs from unshorn control ewes could suggest increased thermogenic abilities in the former group. On the other hand, body core temperatures appeared to be better maintained by CONTROL lambs than by those born to shorn ewes which could indicate a different underlying mechanism. However, the effects of late-pregnancy shearing on core temperature have to be treated carefully as in our study rectal temperatures stayed within physiological range, despite the 1hr cold challenge, whereas in previous studies Slee *et al.* (1980) had achieved greater declines with homeostasis not maintained. This could be a result of a considerable less acute cold challenge in the present study.

Thermoregulation is a very complex mechanism which is not fully understood and different physiological processes affect the ability of lambs to generate heat and maintain body temperature through BAT metabolism and non-shivering thermogenesis (Alexander 1978). Any observed decline in body temperature under cold challenge could be due to i) differences in shivering thermogenesis due to differences in energy reserves available for shivering; ii) differences in non-shivering/BAT thermogenesis; iii) differences in heat loss; iv) differences in metabolic rate. In the absence of IRT data, the observed decline in core body temperature, and the apparent, although not significant, difference in core body temperature between treatment groups could be due to any combination of these four mechanisms or treatment. The contrast in changes observed in lamb core temperature over time (2 lambs maintaining; 2 increasing and the rest decreasing) is an evidence of the complex mechanisms. Based on this current data we are not able to establish a direct link with quantity and activation of the BAT as previously suggested by Hergenhan (2012) in lambs and El Hadi *et al.* (2016) in humans, but we have shown that IRT could be a useful tool to quantify heat loss through the body surface and skin under cold challenge, and as such will be a valuable tool for further investigations. In our study, lambs presenting lower body surface temperature (CONTROL) tended to display higher rectal temperature which might be explained by the vasoconstriction resulting from the cold exposure, which is responsible for a reduced heat loss and in turn maintains core temperature higher. However, this study, while describing and using a practical application for lambs doesn’t allow to attribute the observed changes to a specific mechanism and further exploration is needed.

Taken together, the observed trends allow a cautious interpretation of being the result of the pre-natal treatment. Repeated experiments with large numbers of animals, and including baseline rectal temperature records before wetting of the lambs, will confirm whether this is the case.

Our current findings suggest that reduced heat loss through the skin and body surface might be the underlying mechanism for maintenance of higher core temperatures, although we need to reiterate that the reported results are not statistically significant and hence require further testing. Furthermore, cold exposure over a longer time will be required to establish whether core temperatures can be maintained for longer periods of time.

Future studies will also be needed to confirm whether observed treatment differences between lambs of shorn ewes and control ewes can be confirmed as significant. If this is the case, then IRT will be able to differentiate between the core temperature and the radiated heat loss. As such, IRT appears to be a useful tool to evaluate heat loss through the skin in neonatal lambs, and capable of quantifying this heat loss, and through this can help investigate how prenatal treatments can have an effect on the thermoregulation in neonatal lambs.

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STATEMENT OF ORIGINALITY

Chapter 6 - Infrared thermal imaging as a method to evaluate heat loss in newborn lambs.

We, the Research Master/PhD candidate and the candidate’s Principal Supervisor, certify that the following text, figures and diagrams are the candidate’s original work.

Type of work	**Page numbers**
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STATEMENT OF AUTHORS' CONTRIBUTION

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We, the Research Master/PhD candidate and the candidate's Principal 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 in the *Statement of Originality*.

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Chapter 7: **General Discussion and Conclusion**

In Australia and around the world, lamb survival represents a significant contributor to reproductive wastage. Despite constituting a welfare and economic concern for the sheep industries of many countries, the multi-factorial dimensions of the issue have meant that very little improvement has been made over decades.

In the past, pregnancy shearing has been used as a management tool to increase birthweights and survival (Dýrmundsson 1991; Morris *et al.* 2000a; Kenyon *et al.* 2003; Sherlock *et al.* 2003; Corner *et al.* 2006; Corner *et al.* 2007a; Sphor *et al.* 2011; De Barbieri *et al.* 2012, 2014). However, past studies have failed to replicate those effects using other stressors and/or identify the underlying mechanism (Roussel *et al.* 2004; Corner 2007b).

Furthermore, while reported improved neonatal vigour and survival following shearing, in other studies, prenatal stress including shearing have shown little effects on lamb behaviour after birth (Corner 2007b).

Finally, although no direct effects of shearing on lamb thermoregulatory capacities have been observed, prenatal cold stress and cold stress following shearing during pregnancy resulted in increased brown fat reserves, enhanced cold resistance and greater non-shivering thermogenesis, all key elements to an improved thermoregulatory capacity in newborn lambs (Stott and Slee 1985; Symonds *et al.* 1992a; Symonds and Lomax 1992).

The lack of effects of alternate stressors during pregnancy on lamb birthweight in previous studies led us to hypothesize that acute alternate stressors had to be combined with cold stress to impact foetal development in similar ways than shearing did.

In a series of 3 Experiments, this thesis has examined the effects of mild husbandry stressors such as yarding, handling and transport individually or combined and cold exposure during mid- and late-pregnancy on lamb vigour. The effects of these stressors on lamb vigour traits such as body dimensions, behavioural reactivity and thermoregulatory capacities were examined along with potential underlying mechanisms.

In the three studies, the stressors used were different in nature but not in magnitude as all stress events were transient, brief naturalistic and were applied during mid or late pregnancy. All stress treatments aimed at highlighting the effects of cold stress. The prenatal stressors for ewe treatments tested were:

- Handling (control) and shearing followed by 3 wetting (using sprinklers) occurrences (mid- and late-pregnancy),
- Short transport (control), short transport, individual wetting (using jet hose) and 3 hrs cold exposure, 3 times (late-pregnancy),
- Yarding, transport and cold exposure on 5 occasions (mid and late-pregnancy).

The results reported in chapter 3 suggest that cold exposure (cold exposure and/or cold exposure resulting from shearing), during both mid- and/or late-pregnancy impacts lamb body dimensions. The greatest effects on lamb size and weight were observed when shearing occurred during mid-pregnancy, which agrees with many previous observations (Corner *et al.* 2006; Corner *et al.* 2007a; deNicolo *et al.* 2008; De Barbieri *et al.* 2014). When we compared mid-pregnancy shearing to mid-pregnancy handling alone, highlighting the effects of cold exposure and lambs born to shorn lambs were heavier and bigger than control lambs. However, in the third experiment, exposure to a combination of husbandry-like stressors designed to mimic shearing (yarding, transport and cold exposure) during mid-pregnancy resulted in smaller and lighter lambs than control ewes or lambs from ewes exposed to this stress combination during late-pregnancy. These results support the hypothesis that husbandry-like stressors are not solely responsible for the increased birthweights observed in lambs born to shorn ewes (Corner *et al.* 2006; Corner *et al.* 2010) but the hypothesis that husbandry-like stressors combined with cold exposure could 'mimic' the effects of shearing was not validated.

In general, shearing and stress during pregnancy resulted in changes in maternal blood glucose concentrations, the direction of change being dependant on the stage of pregnancy. Stress resulting from shearing and sham-shearing in mid-pregnancy increased blood glucose level which aligns with previous studies reporting a hyperglycemia resulting from acute stress and increased blood glucose concentrations in shorn ewes compared to unshorn (Reid and Mills 1962; Symonds *et al.* 1988a; Clarke *et al.* 1997; Morris *et al.* 2000b). On the other hand, late-pregnancy shearing, sham-shearing and repeated cold exposure all resulted in a decrease in blood glucose level in pregnant ewes when levels were compared to baseline, before the first stress treatment occurrence. This contrasts to the observations of Clarke *et al.* (1997) who reported higher plasma glucose concentrations in shorn ewes between 126 and 140 days of gestation. Contrary to shearing (*Experiment 1*), the repeated occurrence of husbandry-like stressors and cold exposure (*Experiment 3*), designed to mimic shearing, appeared to have resulted in a reduction in maternal blood glucose levels for both mid- and late-pregnancy treatments. Unfortunately, our study did not help understanding the underlying mechanism of increased birthweight resulting from mid-pregnancy shearing. The mechanisms contributing to increases in lamb birthweight after shearing remain unknown, however, our results suggest different mechanisms involved in the effects of prenatal stressors

In chapter 4 the reduction reported in vocalisation and locomotor reactivity in lambs born to stressed ewes indicate that husbandry-like stressors and cold exposure implemented during mid and late pregnancy does impact on neonatal lamb behavioural development. In all three experiments, most of the lambs born to stressed ewes were significantly slower to reach the measured behaviours (bleat, stand and return to ewe) than lambs born to control ewes and this effect was particularly clear for latency to vocalize. Nevertheless, mid-pregnancy stressors combination (*Experiment 3*) resulted in lamb quicker to stand than control lambs and late-pregnancy shearing did not impact lamb behaviour as it did when occurring during mid-pregnancy. It appears that the timing (mid or late pregnancy) of the stressor changes the impact of the stress on lamb behaviours, but the effects were not consistent in this series of experiments.

The decreased reactivity we observed, is consistent with studies with rats which have suggested a neurotoxic effect of prenatal stress on foetal brain development (Fujioka *et al.* 1999; Weinstock 2008) and with sheep where decreased learning performance in a maze test, decreased locomotion in a 'human' test, increased pessimistic-like biased judgement and increased fear of humans (Coulon *et al.* 2011; Coulon *et al.* 2014; Coulon *et al.* 2015) have been reported. However, in both goats and sheep, husbandry-like stressors (transport and isolation) and shearing when applied once or occasionally, had a positive effect on offspring behaviour (Roussel *et al.* 2005; Corner *et al.* 2006; Roussel-Huchette *et al.* 2008; Banchero *et al.* 2010) and no previous studies had reported negative effects of husbandry-like prenatal stressors on neonatal behaviour.

Placental efficiency is considered, at this stage, the most likely candidate for in utero mechanisms affecting foetal brain development as, in past studies, placental insufficiency and hypoxemia have resulted in damaged and slowed neural connections and subsequently altered behaviour (Mallard et al. 1998; Rees et al. 1999; Mallard et al. 2000). When assessing the role of glucose levels in lambs in lamb reactivity, we observed high glucose extremes associated with longer latencies to bleat and return to the dam. More data are needed to support theses observation however, it is consistent with findings by Morton (2015) who observed delayed vocalisations with increased blood glucose values in new-born lambs.

In chapters 5&6 both mid- and late-pregnancy shearing and late-pregnancy stressors combination are reported to have resulted in cold-exposed neonatal lambs displaying higher body surface temperature and maintaining their body surface temperature better than control lambs. However, this effect was not apparent in the late-pregnancy cold exposure experiment which suggests that our prenatal treatments can affect developmental issues related to thermoregulations differently when occurring during mid- and late-pregnancy most likely through two separate mechanisms. Whether this is mediated by brown fat is not clear but it is possible that mid-pregnancy shearing and cold stress impacts the foetal T3 and T4 levels which in turn mediates the activity level of brown fat.

Our results also suggest that cold exposure is not the sole contributor to prenatal shearing or stressors combination effects on body surface temperature maintenance contrary to what had been previously postulated by Symonds *et al.* (1992a) and Clarke *et al.* (1997) who examined winter shearing effects on lamb thermoregulation.

It is important to note that interpretation of thermoregulation rests on the assumption that the body surface temperature (measured by infrared thermography) is correlated to heat production and thermoregulation. This needs further confirmation but the methodology has been able to discriminate between prenatal stress treatment effects.

Previously, Wassmuth *et al.* (2001) showed a link between thermoregulatory capacities and vigour with more vigorous lambs displaying a better ability to thermoregulate. In our study, while we did not analyse thermoregulation and behaviour data together, our results did not allow us to confirm this correlation. For most of the lambs which displayed increased body surface temperature during a cold challenge (lambs born to mid-pregnancy shorn ewes and ewes exposed to stressors combination during late-pregnancy) also displayed impaired reactivity and locomotor activity which we used as an indicator of vigour. However, while lambs born to mid-pregnancy stressed ewes also displayed apparent better thermoregulation, their behaviour seemed improved as evidenced by shorter latencies to stand when compared to control lambs. Nevertheless, we have to remain cautious with our conclusions as we used different vigour assessment than Wassmuth *et al.* (2001), who scored vigour according to latencies to reach early behavioural milestones whereas we measured latencies to bleat, stand and return to the ewe during a separation test 4hrs after birth. Dwyer and Bunger (2012) also reported that heavier lambs were quicker to suck after birth, however, our findings were too inconsistent to concur this observation.

In conclusion, cold exposure combined with husbandry-like stressors, impacted on various measures of lamb vigour, although such effects were not apparent from late-pregnancy cold stress treatments. This could have been due to the nature of the stressor but also to the duration and magnitude of the stress treatment and further studies to examine these variables in late pregnancy are needed. Further confirmation of links between lamb reactivity and subsequent survival are also required to determine the level of impact of these behavioural changes.

While the underlying mechanism by which prenatal shearing and stressors affect lamb birthweight remains unknown, we have eliminated the combination of cold exposure and husbandry-like stressors as an explanation for the positive effects of prenatal shearing. It seems that both behavioural development and brown fat metabolism were affected by both shearing and stressor combinations suggesting similar underlying mechanisms. If this is the case then, such stressors might be strategically used to improve lamb survival.

Unfortunately, at this stage, our study does not allow us to pick the "best" prenatal treatment to increase lamb survival as the effects on the different vigour traits measured seem inconsistent and the consequences of those on lamb survival are unknown. Nevertheless, it is conceivable that despite negatively impacting behavioural reactivity and locomotor activity or lamb body dimension (respectively mid-pregnancy shearing, and late-pregnancy stressors combination and mid-pregnancy stressors combination) those stressors could be used to improve thermoregulation which in turn might compensate for the loss in "survival chances".

POTENTIAL LIMITATIONS AND FUTURE RESEARCH

In order to complete the exploration of the effects of cold exposure, a mid-pregnancy cold exposure treatment could have been examined. However, the focus for the cold exposure experiment (*Experiment 2*) was originally to observe the effects on thermoregulatory capacities only and based on previous research we did not expect to see any effect when prenatal treatment occurred during mid-pregnancy. Furthermore, we would need to explore the effects of the ewes cold exposure to assess if this resulted in a physiological stress response. While we observed changes in blood and plasma glucose levels usually found in cold stressed sheep and shivering, standing and huddling, behaviours usually associated with cold exposure in sheep (Bøe 1990), our study lacked direct evidence of cold stress.

A more extensive investigation into the effects of prenatal stress on lamb hormonal profiles may provide insights into the altered physiological mechanisms. Although we did not measure cortisol levels in stressed ewes to assess the magnitude of the stress produced, glucose blood and plasma data suggests that all the stress treatments used were in the same physiological range.

While cortisol is known to be one of the hormones mediating the response of offspring to prenatal stress (Weinstock 1997), past research has not observed any effects of prenatal cortisol injections on lamb birthweight or behaviour (Corner *et al.* 2010). The role of noradrenaline which is a stress hormone as well as a hormone involved in the thermoregulatory metabolism could be further investigated using prenatal injections although appropriate physiological levels may be difficult to determine.

Finally, more work is needed on the impact of husbandry-like prenatal stress on ewe-lamb interactions. The ewe plays a crucial role in lamb survival and regardless of the lamb's abilities, further studies should also monitor the role of the ewe in initiating lamb responses. A more complex setting to investigate the impact of prenatal stress on lamb cognition, using for example maze tests, would provide added information to the reactivity and alertness observed in a separation test.

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