Metabolic maturity and vigour in neonatal lambs, and subsequent impacts on thermoregulation and survival

Kate Joanna Plush

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The School of Animal and Veterinary Sciences

The University of Adelaide

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Australia

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Abstract

Lamb mortality in Australia averages approximately 20%, representing a major constraint to the profitability of sheep enterprises and compromised animal welfare. Most postpartum lamb loss occurs within the first three days of life and is largely caused by starvation, exposure to cold conditions and mismothering from the ewe. In this thesis we developed an over-arching hypothesis that differences in metabolic or physiological maturity exist between lambs, and that these differences relate to early postnatal vigour and survival, particularly during exposure to cold conditions. To test this hypothesis, behaviour associated with initial vigour was quantified in breeds of sheep which differ widely in neonatal survival and more specifically, risk of hypothermia. Pre-suckling blood samples were collected from these animals in order to identify potential markers of maturity chosen to represent the hypothalamic- pituitary- adrenal (HPA) axis, renal function and energy metabolism. A number of metabolite and endocrine shifts were identified in those that were quicker to reach the udder of the ewe and begin sucking. Namely, creatine, non-esterified fatty acids, leptin and ghrelin concentrations were elevated, implying these individuals may be better able to regulate energy mobilisation soon after birth.

Lamb vigour was also strongly associated with rectal temperature at birth, indicating an association between maturity, post-natal behaviour and thermogenesis. A controlled water bath testing system was then used to experimentally induce mild hypothermia in the lambs, and metrics of thermoregulation included time taken for core body temperature to reach 35°C (cold resistance), and time to restore core temperature to 39°C (cold recovery). Lambs that were slow to stand and reach the udder had impaired cold resistance. None of the physiological measures (circulating metabolite and hormone concentrations at birth) were related to performance in the water bath. Somewhat surprisingly, those lambs identified as being more mature, as assessed by speed to perform peri-natal behaviours and physiological blood measures, experienced a delay in cold recovery when compared to those with lower vigour and maturity. We proposed that this may be due to a reduced ability to perform non-shivering thermogenesis in more mature individuals (as is observed to occur with age), but this need further exploration.

Given these strong relationships between maturity, peri-natal vigour and thermogenesis, an attempt to alter the metabolic maturity of newborn lambs was made. Peri-conception nutrition was shown previously to influence fetal HPA axis activation (responsible for the

maturation of a suite of fetal systems) hence differing nutritional treatments (0.7, 1.0 and 1.5 maintenance energy requirement) were applied to the ewes at this time. No effect of peri-conception nutritional manipulation on lamb survival was observed, but lambs from ewes fed a restricted diet around conception exhibited a decreased crown-rump length when compared to those from the high energy treatment. Whilst this had no effect on survival, under more inclement conditions this finding may increase risk of hypothermia through effects on surface area dependent heat loss.

In summary, the findings presented in this thesis provide strong evidence that the metabolic maturity of lambs at birth is related to initial vigour and thermoregulatory ability, two traits that are closely linked with survivability. The attempt to reduce lamb mortality through altering HPA axis activation by nutritional means had limited effects on lamb phenotype. Consequently, peri-conception nutrition failed to influence lamb survival. Future investigations should target other means by which metabolic maturity at birth can be enhanced in order to improve lamb survival.

Thesis Declaration

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Kate Plush

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Chapter One: Introduction

Background

Lamb survival is a key determinant of the profitability of sheep enterprises. Conservative estimates value peri-natal lamb losses at $56 million per annum (Sackett et al., 2006), with non-economic but increasingly important animal welfare considerations adding to the issue. Current knowledge of the factors contributing to peri-natal lamb losses and areas requiring further attention are briefly summarised in this literature review. Focus is given to managerial, maternal and direct lamb factors that influence survival. In depth analysis of the literature of primary interest to each series of experiments conducted within this thesis will be presented in more detail subsequently, in the form of thorough introductions to experimental chapters.

Lamb Mortality

In Australian flocks, which are predominated by the Merino breed, the average mortality rate is often estimated at being between 20 and 30% (Kilgour, 1992). A significant proportion of these deaths occur in the first days following birth, a time known as the peri-natal period (Arnold and Morgan, 1975). Approximately 5-10% of lambs are born dead, whilst 20% do not survive to day seven after birth (Brien et al., 2009, Hatcher et al., 2009). Mortality is only increased by a small percentage to day 30 (21% at lamb marking), highlighting the importance of this peri-natal period with regards to survival. Observed lamb mortality rates equate to approximately 10 to 20 million lamb deaths annually, with clear ramifications for both production and welfare. Little increase in survival rates has been witnessed in Australian flocks over the last 40 years, suggesting there is still considerable opportunity for improvement.

Impacts of lamb mortality

In lamb production, key determinants of profitability and efficiency are the prolificacy of the ewes, and the growth rate and survival of lambs. Direct effects of lamb loss include a reduction in the number of lambs sold and of ewe lambs available for flock replacement. Indirect costs involve wastage on reproductive expenditure such as ram purchase and

usage, artificial insemination and other emerging technologies such as embryo harvesting and juvenile *in vitro* embryo transfer (Cloete et al., 2005). Reduced lamb numbers directly influences the genetic pool available for selection, reducing both the selection differential and genetic gains. Additionally, in the wool sector, production is compromised when maternal nutrients are partitioned away from wool growth towards fetal development, reducing both wool quantity and quality. Thus, impacts of lamb mortality are far-reaching and justify research into methods aimed at improving survival.

One suggested means of improving the number of lambs weaned has been to increase the number of lambs born, or the prolificacy of the ewe. However, this method has raised significant ethical concerns. The ability of the ewe to provide adequate care for offspring decreases with increasing litter size (Dwyer and Lawrence, 1998, Hinch, 1989), and twin and multiple born lambs often display higher mortality rates when compared to singletons (Hinch et al., 1985). Thus, the production of increased number of lambs followed by high mortality rates is seen by many as being unacceptable from a welfare perspective (Nowak, 1996). Additionally, in extensive systems, care should be given to ensure that the animals possess behavioural and physiological traits that allow them to survive (Goddard et al., 2006). This is generally not witnessed in current farming practices in Australia as fine wool Merinos are widely farmed and have been shown to be inferior mothers when compared with other breed and is reflected in the higher mortality rates in fine-wool sheep (Alexander et al., 1990, Kuchel and Lindsay, 1999). It is unethical to farm sheep in a manner that results in increases in mortality (Martin and Kadokawa, 2006) thus methods to reduce mortality should be investigated.

Reasoning behind the ethical consideration of increased lamb mortality rates is, in most circumstances, the welfare of the lamb is compromised. In recent times, increased attention has been given the impact of mortality on animal welfare, with a significant proportion of lambs suffering as morbidity and/or mortality occurs. Trauma caused by the birth process, hunger, hypothermia, distress from maternal separation, and disease resulting from insufficient colostrum ingestion have all been identified as factors that impair the welfare of the neonatal lamb and it is also possible that emotional stress may be inflicted on the ewe at the time of lamb loss (Dwyer, 2008). Thus, lamb mortality has the potential to result in increased suffering for both lamb and ewe, reducing animal welfare within the sheep industry.

Causes of lamb mortality

Minor causes of lamb mortality are usually environmental in nature and generally account for less than 20% of the total deaths, however this figure can be significantly higher during catastrophic events (Haughey, 1983). These include, but are not limited to, mineral deficiencies, peri-natal infection and predation (Dennis, 1974). An important contributor to lamb mortality is the birthing process, with birth-related issues, termed dystocia, responsible for around 20% of the total mortality rate (Alexander et al., 1990). The three largest post-natal contributors to lamb mortality are starvation, mismothering and exposure (SME), together resulting in 65% of all peri-natal lamb deaths (Haughey, 1983). Techniques aimed at limiting mortality should target these three major causes.

Management and lamb survival

A fundamental technique to optimise lamb survival rates is to ensure correct farm management practises are in place. There are a number of management choices that can be employed, however the factor most cited as influencing survival in lambs is birth weight, through its influence on mortality from exposure and dystocia. Small lambs have a higher surface area to volume ratio and therefore lose larger amounts of heat to the environment increasing the risk of hypothermia. Large lambs, on the other hand, often encounter feto-pelvic disproportions during the birth process and can suffer from hypoxia and anoxia at this time (Alexander, 1974). Birth weight has been consistently shown to be related to survival by a curvilinear function, whereby lambs of intermediate weight experience the greatest survival rates (Atkins, 1980, Gardner et al., 2007, Malik et al., 1998, Mullaney, 1969). Thus it is crucial that ewe nutrition and management during gestation should focus on ensuring optimal lamb birth weights are achieved.

Ewe nutrition during gestation

Nutrition of the ewe during gestation has been the focus of a number of published reviews and so will only be briefly discussed here. Nutrition during mid-gestation is the time of maximal placental growth and therefore has significant effects on subsequent fetal growth. Later in gestation, fetal growth is at its highest and is influenced greatly by maternal nutrition during the last trimester. Generally speaking, increased levels of nutrition during these times increase birth weight whilst conversely; underfeeding causes growth retardation (Holst et al., 1986). This influence of ewe nutrition on birth weight was confirmed by results from the

‘Lifetime Wool Project’ which showed that the birth weight of individual lambs was related to the live weight profile of their mother during gestation (Oldham et al., 2011). Additionally, this investigation linked birth weight to lamb survival in a curvilinear fashion whereby lambs with both low and high birth weights suffered increased mortality rates.

There are a number of other ways in which maternal nutrition can affect lamb survival in addition to direct effects on lamb birth weight. Nutrition during gestation has shown to influence udder characteristics in the ewe. Restricting nutrition for as little as five days in the final stages of gestation retards mammogenesis, and colostrum production was reduced in these ewes (Mellor and Murray, 1985). Moreover, reducing ewe nutrition during gestation has also been shown to impair the level of maternal care given to offspring by the ewe. A 35% reduction in maternal feed intake reduced the amount of time a ewe spent grooming her lamb (important in facilitation of ewe-lamb bond; see later paragraphs) and increased the level of lamb-directed aggression (Dwyer et al., 2003). In this study, restricted ewes were also allocated a poorer maternal attachment score than well-fed ewes. Thus, ewe nutrition in mid to late gestation can exert effects on lamb survival both through influencing lamb birth weight, colostrum and milk production, as well as facilitating the ewe-lamb bond.

The influence of mid to late gestation nutrition is clear, but what is less apparent is the influence of nutrition around the time of conception on lamb survival. The emerging interest in the uterine environment in the initial stages of embryonic development and the idea of ‘fetal programming’ in human research (Barker, 1997), has led to a growing body of research in the influence of nutrition during this phase of gestation on offspring performance in livestock. Initial investigations in sheep have demonstrated that early gestation nutritional restriction results in conception rate differences, however little effects on birth weight are witnessed (Annett and Carson, 2006, Gardner et al., 2004, Oliver et al., 2005). Conversely, more recent investigations have shown nutrition during the early phase (first 30 days) of gestation alters fetal growth, with ewes restricted to 60% of maintenance requirements giving birth to lambs with heavier weights (Munoz et al., 2007). These lambs from restricted ewes tended to have increased immunoglobulin status (indicating a higher ingestion of colostrum) and exhibited increased survival rates compared with those from ewes fed at maintenance (100%) or a high plane (200%) of nutrition to day 39 of gestation. The finding that colostrum ingestion and survival was altered may suggest nutrition in this early stage has effects on other physiological processes within the lamb. Other investigations into the effects of peri-conception nutrition have identified shifts in the hypothalamic-pituitary-adrenal axis in the fetus (Bloomfield et al., 2004, Edwards and McMillen, 2002) which support the notion that nutrition during this time alters developmental physiology. The contrasting results

on birth weight and a lack of understanding of lamb physiology suggest the effects of nutrition around conception on fetal development and postnatal survival warrant further investigation in order to provide options for ewe management around mating to maximise lamb survival.

Genetic differences in lamb survival

The choice of livestock for production will have considerable ramifications for lamb survival as rates differ significantly between breeds, strains and even sire lines. With regard to sheep breed, there appears to be an association between heavily selected breeds (ie. Suffolk and Merino) and reduced survival, when compared to those with a lower selection intensity (ie. hill breeds and 'easy-care' sheep) (Dalton et al., 1980, Dwyer and Lawrence, 1998, Dwyer and Lawrence, 2005, Petersson and Danell, 1985, Slee et al., 1980). Why there is such a divergence in lamb survival across breeds is unclear but physiological changes in both the ewe and lamb that may be linked to production traits could be apparent. Cross breeding is one recognised method of improving lamb survival rates, as heterosis has been shown to reduce mortality (McGuirk et al., 1978, Wiener et al., 1983). This option however is not applicable to all types of sheep production. Wool-producing genotypes such as the Merino, for example, have a low heritability of lamb survival (0 to 0.1) (Brien et al., 2009, Hatcher et al., 2010, Safari et al., 2005), suggesting genetic gain will be slow. However selection for a composite trait that is influenced by survival may yield improved results. In South African Mutton Merinos, selection for multiple-rearing ability increases survival rate (77.4% in lambs from ewes selected for rearing ability versus 68.7% for those selected against). This was mainly attributed to an increased survival in multiple-born lambs (Cloete and Scholtz, 1998). These differences in lamb survival rates between breeds and lines offer options for sheep producers with regard to genetic improvement.

Controlling the paddock environment at lambing

There are a number of important features of the external environment in which a ewe gives birth that significantly influence lamb survival and these factors should receive managerial attention. Providing specific lambing paddocks that utilise the management techniques outlined below may aid in reducing lamb mortality figures.

The maternal offspring bond formed in the hours following birth is strongly linked to survival (see later paragraphs) and this bond is generally formed at the site on which the ewe gives

birth, known as the birth site. Ewes penned to the birth site for extended periods of time, or that are allowed to remain at the site for a desired length of time exhibit reduced separation levels and mortality when compared to those which are moved away from the area in which they gave birth (Putu et al., 1988). The results from this experimental induction of shepherding interference have been validated by farmer surveys indicating that 'easy care' or natural lambing systems in New Zealand that receive little disturbance successfully rear lambs in difficult environments (Fisher, 2003). Thus, disturbance around lambing should be minimised in order to facilitate a strong ewe-lamb bond.

Providing shelter from adverse weather conditions has beneficial consequences for lamb survival as it reduces the impact wind has in establishing hypothermia (Egan et al., 1972). Studies in fine-wool Merinos have shown that using grasses such as *Phalaris* hybrids reduced total lamb mortality by 10% in singletons and 32% in multiples (Alexander et al., 1980). Autopsies conducted on the dead lambs in this study revealed that of singles exposed to cold conditions, hypothermia as a direct cause of death was responsible for 18.4% of total deaths, whilst this figure decreased to 2.9% when shelter was provided. Similarly, hedging surrounding paddocks has reduced lamb mortality in the first 48 hours from 19% to 6%, a result attributed from a reduced wind speed in the protected paddocks (Egan et al., 1972). Ensuring that the lambing environment is protected from both human interference and inclement weather will assist in reducing mortality, thus paddock management is crucial for optimising lamb survival.

Maternal influence over lamb survival

Spontaneous maternal care does not occur in most mammalian species (Kendrick et al., 1997) with the majority having to undergo pregnancy to stimulate hormonal release in order to exhibit desirable behaviours. A dry or pregnant ewe is a social animal that experiences stress when isolated, is easily frightened, is indifferent or aggressive towards lambs and is highly gregarious. At parturition, there are a number of behavioural changes that occur aimed at establishing and maximising the maternal-offspring bond between ewe and lamb.

Desirable ewe behaviour at lambing

Around the time of parturition, extensively farmed ewes separate themselves from the flock, exhibit decreased fear responses, are attracted towards lambs and display reduced locomotive activity (Nowak, 1996). Desired behaviour exhibited by the ewe aids in the

formation of a strong ewe-lamb bond and includes isolation from flock (Alexander et al., 1990, Arnold and Morgan, 1975), grooming of the birth fluids (which also prevents heat loss, suffocation and encourages the lamb to stand) (Alexander et al., 1990, McGlone and Stobard, 1986), an increased time spent at the birth site (Cloete and Scholtz, 1998, Everett-Hincks et al., 2005), low-pitch, regular vocalisations from the ewe directed towards the lamb and active co-operation in feeding (Cloete et al., 2005, Nowak et al., 2000). The purpose of this bond is to ensure the ewe can distinguish and recognise her lamb from a foreign one, thus she is able to determine when the lamb is following and that she suckles only her own. The bond also assists the lamb in its ability to recognise its dam and facilitates following behaviour.

Mismothering

A failure to create this ewe-lamb bond often results in mismothering (Alexander, 1984), a term which refers to a number of negative behaviours directed by the ewe towards her lamb. Circling, backing and butting all prevent the lamb from sucking and thus increase the risk of lamb mortality (Alexander et al., 1990, Cloete and Scholtz, 1998). Abandonment, a situation in which the ewe fails to groom the lamb and leaves the birth site immediately after expulsion, may also arise. This commonly occurs after a long, painful parturition (Alexander, 1984). Mismothering is generally more common in primiparous ewes compared to multiparous and in breeds such as the Merino when compared with meat breeds (Alexander et al., 1990, Kuchel and Lindsay, 1999). In addition to the level of care given by the ewe, other maternal factors influence the survival of lambs. Udder characteristics and milk production have both been shown to impact upon lamb growth and survival (Jordan and Mayer, 1989, Snowder et al., 2001). The only possible factor that may be of similar or greater importance than the maternal environment is the direct effect of the lamb itself on survival.

Direct lamb influence over mortality

During the transition from intra- to extra-uterine life, a lamb has to undergo a number of obstacles to ensure survival. Firstly, a rapid temperature decline is witnessed whereby the lamb kept in the uterine environment at ewe body temperature is born into paddock conditions which are often inclement and can reach below 0°C. Coupled with the environmental conditions is the fact that the lamb's coat is wet with birth fluids, meaning heat loss is exacerbated (Alexander, 1962b). During this time, the lamb can be required to

increase its metabolism up to fifteen fold in order to prevent hypothermia (Alexander, 1962c, McCutcheon et al., 1981). Lambs are born with a well-developed thermoregulatory system when compared with other species (Alexander and McCance, 1958) and achieve this temperature homeostasis around birth through summit metabolic rate (Alexander, 1962c) using both shivering and non-shivering thermogeneisis. Whilst the amount of available energy present in the lamb at birth plays an important role in its ability to thermoregulate, other physiological mechanisms such as the ability to mobilise and utilise energy may also be a determinant, however reports on this are scarce. If the lamb is successful at maintaining its body temperature during this time, it then must go on to perform a number of key behaviours that are aimed at achieving enteral feeding.

There are a number of lamb behaviours observed soon after birth that have been shown to significantly influence survival as they assist in the formation of the ewe-lamb bond and are essential for the lamb to ingest colostrum. Immediately after birth, the lamb shows righting behaviours which are shortly followed by attempts to stand. After successfully standing, the lamb moves towards the ewe and after exploring the underneath of the ewes body, is eventually guided to the udder by thermotactile cues (Nowak and Poindron, 2006). The time taken for the lamb to perform these behaviours is generally within two hours (Nowak and Poindron, 2006), however there is large variation across individuals. A number of factors have been shown to influence the time taken for the lamb to successfully feed and will be explored in detail in a later chapter, but the underpinning explanation for this variation still remains unclear. At the point of enteral feeding, the lambs digestive system must shift from the utilisation of mostly carbohydrate rich diet metabolised *in utero*, to a colostrum and eventually milk diet high in fat (Greenwood et al., 2002). The ability of the lamb to perform this shift in energy utilisation is not well understood. As already mentioned, it is important for the ewe to remain at the birth site, however at some point the ewe must move on. Thus the lamb must be able to follow the ewe and discriminate her from others (Nowak and Poindron, 2006). All of these important lamb behaviours have been shown to exhibit relationships with lamb survival in some form or another and will be explored in detail subsequently.

Conclusion

The impact that lamb survival has on both production and welfare is widely recognised. Whilst simplified in the paragraphs above, the managerial, maternal and direct effects on lamb survival are often inter-related and some of these relationships will be discussed further in subsequent chapters. From the literature it is clear that there are a number of direct lamb

factors that are important for survival, and that these factors are often divergent across individuals, strains and breeds. What is less clear is why this divergence occurs. The aim of the experiments designed and carried out in the following chapters is to identify if the physiological, or metabolic maturity of the lamb around the time of birth can explain differences in these traits crucial for optimising survival. The following experiments were carried out in order to define the physiological maturity of lambs at birth with relation to postnatal behavioural progression, identify if a relationship between maturity at birth and a lamb's ability to thermoregulate exists and lastly, to identify if previously reported shifts in HPA axis activity during fetal development caused by peri-conception under-nutrition influence maturity, exerting effects on lamb vigour, thermoregulation and survival.

Chapter Two: Relationships between lamb vigour at birth and indications of metabolic maturity in the neonatal lamb

Introduction

Neonates are born with limited intrinsic energy supply and as such rely on acquiring additional sources, principally in the form of colostrum, to ensure survival. In altricial species, it is the mother and her level of care that ensures this survival, but it has been suggested that in precocious animals the role of the neonate is at least as important as that of its mother (Dwyer, 2003). Newborn lambs must display a number of key behaviours to ensure survival through maternal acceptance and colostrum and milk ingestion. These include standing soon after birth, sucking soon after standing, following the mother closely and moving to the mother if separated (Alexander, 1987). In Booroola Merino's, for every one minute increase in the time taken for the lamb to attempt to stand, stand or attempt to find the udder, survival decreases by approximately 1% (Owens et al., 1985). Why some lambs are better able to perform these important behaviours is not well understood, but the physiological maturity of the animal at birth may play a role. The physiological maturity of the animal can be defined as the ability to successfully adapt to the postnatal environment following birth, in terms of energy utilisation, oxygen metabolism and independent homeothermy. Understanding the variation in behaviour and maturity may provide alternate methods to increase lamb survival.

Outlined below are those factors that influence lamb behavioural progression. Specifically, the effects of the pre and post-natal environment, lamb phenotype and genetic background on lamb behaviour are explored. The physiological characteristics that define a neonate's maturity at birth are also examined and any links between this, peri-natal behaviour and vigour and survival are discussed.

Lamb behaviour immediately following birth

The effects of maternal nutritional supply to the fetus

Growth and organ maturation are highly dependent on the nutritional supply made available to the fetus. In mammals, the placenta is responsible for nutrient transfer between the mother and the developing fetus and associations between fetal growth and movement *in*

utero have been identified in humans (Bekedam et al., 1985), so it is reasonable to assume placental characteristics would have an influence over the development of systems that control postnatal behaviour. Placental efficiency (as defined as gram of lamb produced per gram of placenta) has been shown to be negatively related to the time taken for a lamb to stand (r^2 = 0.14), with lambs born from more efficient placentas standing quicker (Dwyer et al., 2005). Additionally, induced placental insufficiency from day 120 to 140 reduces neuro-developmental processes, such as myelination and growth of the cerebellum, that occur during late gestation (Mallard et al., 1998). The authors suggest that this would impact upon neural connectivity and may have functional consequences after birth. There are a number of established factors that affect the transfer capabilities of the placenta and their effects on postnatal behaviour are explored below.

The influence of ewe nutrition during gestation

It is well understood that nutrition during gestation can exert influences on the placenta, and subsequently fetal growth (Kelly, 1992). What is less understood is the effect that this nutritional alteration has on offspring behaviour. Whilst some investigations have shown no difference in lamb behaviour between low and high plane feeding throughout gestation (Arnold and Morgan, 1975), a moderate restriction in nutrition (80% of maintenance) during mid-gestation resulted in a tendency for lambs to spend more time standing in a 30 min period following birth, and also lambs that tended to attempt to stand, stand and attempt to suck quicker than those fed at 140% maintenance (Munoz et al., 2007). In contrast, reduction in ewe condition from conception to delivery had a negative effect on most lamb behaviours (Dwyer, 2003). These opposing results are most likely explained by the timing of restriction. Under-nutrition in mid gestation has been shown to reduce placental weight when measured in mid gestation (Clarke et al., 1998), but increases placental weight at term after feeding to meet maintenance requirements for the remainder of pregnancy (Heasman et al., 1998). This suggests that the placenta responds to early under-nutrition by a compensatory increase in weight, presumably as a means of protecting the fetus from poor nutrient supply. If chronic restriction occurs (for the total length of gestation), placental weight is decreased at both mid gestation and term (Osgerby et al., 2004). Ewe nutrition during gestation has a marked effect on placental development, with ramifications for peri-natal behaviour. The way in which nutrition exerts effects on behaviour is largely dependent on when restriction is imposed in gestation, and the length of time the dam is exposed to this restriction.

Litter size influences on lamb behaviour

Whilst placental size and transfer capability is increased when litter size is increased, this increase is not proportional to the increase in fetal number (Dwyer et al., 2005), resulting in placental insufficiency for multiple born lambs. The effects of litter size on lamb behaviour are varied, and whilst some identify no differences in peri-natal behaviour (Dwyer et al., 2001) others suggest multiple born lambs are at a significant disadvantage. Singletons and the first born of twins have been shown to be quicker to stand and suck than triplets or quads (Owens et al., 1985), and triplet born lambs were shown to display delayed behaviour when compared to singles and twins (Dwyer, 2003). The results of these last two investigations suggest that whilst singleton and twin born lambs exhibit a similar behavioural progression, any further increase in litter size (triplets or quads) is detrimental.

The effect of ewe parity

Early investigations into the effect of ewe parity on placental characteristics identified differences in morphology of the placenta. Ewe age (confounded with parity) was positively associated with cotyledon number and weight (Alexander, 1964). More recently, Dwyer *et al.* (2005) similarly showed an increase in cotyledon weight and an increase in placental weight and efficiency (as measured by weight of lamb produced by weight of placenta) with increased parity. The effect of ewe parity on lamb behaviour appears to be most pronounced in primiparous animals, as lambs born from these ewes are slower to attempt to stand (Owens et al., 1985), stand (Dwyer et al., 2005) and suck (Cloete et al., 2005, Cloete et al., 2002, Dwyer et al., 2005) compared to those from multiparous ewes. Cloete *et al.* (2005) also showed that this difference in lamb behaviour exhibited by lambs from maiden ewes remained even after adjustment for maternal behaviour, once again re-enforcing the effect of ewe parity on placental characteristics independent of post-natal environmental effects. A number of investigations support the notion that maiden ewes produce lambs that are at a disadvantage with respect to behavioural development when compared with those born to older ewes, and this could partially be explained by the immaturity of the uterine environment in these younger animals.

Impacts of gestation length and the birth process

There are two streams of thought as to the effect of gestation length on postnatal behaviour. Pre-term human infants have been shown to take longer to achieve nutritive sucking and exhibit shorter sucking bursts than those born later in gestation (Nyqvist et al., 1999),

suggesting that a reduction in gestation length retards postnatal behavioural progression. However, Dwyer et al. (1996) identified lambs with a reduced gestation length were quicker to progress behaviourally in an embryo transfer study. Blackface lambs displayed a shorter gestation than Suffolk's independent of the maternal breed and whilst Suffolk lambs experienced a longer gestation, they were slower to perform all postnatal behaviours. Results in sheep suggest that it is those lambs that experience a reduced gestation that are better able to perform key postnatal behaviours, but the direct effects of gestation length on lamb behaviour after birth clearly warrant further investigation.

It is well understood that the birth process can result in damage to the central nervous system of the lamb through trauma and/or hypoxia, with an increase in brain and spinal cord lesions witnessed with increasing labour duration (Haughey, 1980). Injury to the nervous system may affect the lamb's ability to perform key behaviours following birth, however the results of investigations into the effects of length of labour on behaviour are inconsistent. Whilst some have identified no relationship (Arnold and Morgan, 1975, Dwyer et al., 2005, Owens et al., 1985), others have shown clear links between parturition length and behaviour. An increase in birthing difficulty was shown to retard early lamb behaviours such as initial head shaking and ability to reach sternal recumbency, and time to stand and suck increased with increasing labour length (Dwyer et al., 1996). Additionally human assistance at lambing, indicative of a difficult birthing process, has shown to delay almost all postnatal behaviour (Dwyer, 2003, Dwyer and Lawrence, 1999, Dwyer et al., 2001). The delay in behavioural response in lambs that required assistance during labour continued past the peri-natal period, with these lambs showing decreased activity over the first three days of life. These results highlight the importance of the birth process on both peri-natal behaviour and longer term vigour. An increase in labour length results in damage to the central nervous system which is vital for co-ordination and thus behavioural progression in the lamb.

The influence of sex on early postnatal behaviour

It has been well documented that female lambs exhibit increased lamb survival rates when compared to their male counterparts (Brien et al., 2009, Hatcher et al., 2009, Sawalha et al., 2007), so the influence of sex on post-natal behaviour deserves attention. Indirectly, ram lambs have been shown to exhibit an increased length of labour (Cloete et al., 2002, Dwyer et al., 2003) which, for reasons outlined above, should subsequently affect the time taken for the lamb to stand and suck. Whilst it is logical to assume that the increased birth weight of ram lambs would most likely explain the delayed labour, birth weight is not the sole driver of

this extended parturition as sex effects remain after adjusting for weight (Dwyer, 2003). The authors suggests that sex differences in the lamb's ability to move into correct presentation before birth may exist, suggesting a link between fetal movement *in utero* and postnatal behaviour.

Results from investigations into the effects of lamb sex on behaviour are varied. Dwyer *et al.* (2003) noted that ram lambs from the Suffolk breed were behaviourally slower than ewe lambs and this same sex effect was witnessed in an ensuing investigation (Dwyer et al., 2005). Cloete *et al.* (2002), in contrast, showed that the observed increase in parturition length in males did not affect postnatal behaviour, supporting earlier reports (Dwyer and Lawrence, 1999, Dwyer and Lawrence, 2000). It appears that these contradictions cannot be easily explained by differences in experimental design (breed, lambing environment etc), and thus further investigation of the influence of sex on postnatal behaviour should occur before meaningful conclusions can be drawn. Additionally, mechanisms that drive this potential sex divergence should be explored.

Birth weight effects on lamb behaviour

Birth weight, in addition to being under some genetic influence, is indicative of the nutritional supply made available to the fetus during gestation. Restricted nutritional supply will result in impaired fetal growth and reduced birth weight, and may also impact upon the development of neurological pathways involved in the control of postnatal behaviour. Many studies have identified that it is smaller lambs that suffer a retarded behavioural progression. In the prolific Booroola Merino breed, an increase of 1kg in birth weight was shown to decrease the amount of time for the lamb to first attempt to stand by 3.3 min, successfully stand by 9.4 min, attempt to suck by 12.1 min and suck by 15.8 min (Owens et al., 1985). Subsequently, a similar effect of birth weight on behaviour has been observed in Suffolk lambs, and to a lesser degree in the Scottish Blackface breed (Dwyer, 2003). This behavioural retardation exhibited by smaller lambs may be explained by growth restriction *in utero,* in addition to thermoregulatory factors which are explored later. Contrasting this pre-natal influence, a heavier birth weight can also impede behavioural progress during the peri-natal stage. Lambs of a heavier birth weight are at increased risk of dystocia (Smith, 1977) as they are more likely to experience a longer labour (Dwyer, 2003). Parturition difficulty can result in hypoxia having damaging effects on the central nervous system as explored above. It may be expected therefore, that heavier lambs might be at a behavioural disadvantage as a consequence of this increased risk of extended labour.

Postnatal environmental influences on lamb behaviour

Behaviour is often thought of as a trait that is highly dependent on the environment that an individual experiences (Cloete et al., 2002). There are many factors that make up the environment into which a lamb is born and a few key aspects are highlighted below.

Effect of weather

Lambs are typically born during winter and spring months leaving them vulnerable to exposure from inclement weather. This exposure has been shown to affect behaviour both in the field (Slee and Springbett, 1986), and when induced experimentally in the newborn lamb (Alexander and Williams, 1966). Lambs that experience mild conditions after birth display a reduction in teat seeking behaviour when exposed to cold (Alexander and Williams, 1966). Depletion in energy reserves of approximately 20% resulted in no changes in behaviour in mild conditions, however these lambs showed a reduction in teat seeking behaviour when the coat was kept wet and the lamb was exposed to low air temperatures. The most marked effect was seen in hypothermic lambs, which did not stand or suck when their rectal temperatures were below 37°C. Failure for the lamb to reach the udder was associated with hypothermia in other field studies (Slee and Springbett, 1986) and lambs that were slower to perform key behaviours had reduced rectal temperatures (Dwyer and Morgan, 2006). These results suggest that cold conditions influence behaviour through a combination of discomfort, a depletion of energy reserves and hypothermia. Whilst these investigations highlight the impact of inclement weather on vigour, what is less understood is if a lamb's vigour is related to its ability to withstand cold. This is explored in subsequent chapters.

The effect of ewe behaviour

It is well documented that the environment provided by the ewe can affect postnatal lamb survival. Whist the most extreme example of this is maternal rejection resulting in lamb starvation, there is also evidence to suggest that the ewe has an effect on the behaviour exhibited by her lamb. A significant maternal permanent environmental effect of 0.17 has been shown for time taken for the lamb to suck (Cloete et al., 2002). Additionally, a difference in time from standing to sucking was identified between ewes selected for either good or poor rearing ability (Cloete and Scholtz, 1998). Ewes with superior maternal ability produced lambs with an increased rate of behavioural progression.

There are a number of important ewe behaviours that are implicated in lamb behavioural progress which may help to explain the observed differences mentioned above. It has been reported that grooming from the ewe is associated with a delay of greater than 10 min in the time taken for a lamb to stand and suck (Arnold and Morgan, 1975). Further investigation however showed little effect of ewe grooming on lamb behaviour (Dwyer and Lawrence, 1999, O'Connor and Lawrence, 1992). Only latency to stand was influenced by ewe grooming, but surprisingly length of grooming was associated with a delay in the behaviour (Dwyer and Lawrence, 1999) highlighting that increased grooming attention retarded the lamb's attempts to stand. These findings suggest that a quick succession from parturition to grooming from the ewe is required for optimal neonatal behaviour, but the intensity of grooming should not be at a level whereby it is hinders lamb progression. In addition to grooming, ewe vocalisations are associated with lamb behaviour. Ewe low-pitched bleats have been shown to be positively correlated with lamb bleats although the relationship with other behavioural measures was not examined (Dwyer et al., 2001). These investigations provide some support for the notion that the environment provided by the ewe can influence peri-natal lamb behaviour, but perhaps a greater influence is exerted by the genetics of the lamb.

Genetic effects on early postnatal behaviour

Clear breed differences in postnatal behaviour have been identified (O'Connor and Lawrence, 1992, Slee and Springbett, 1986) and much effort has been invested into determining why these breed differences exist (Dwyer et al., 1996, Dwyer, 2003, Dwyer et al., 2005, Dwyer and Lawrence, 1999, Dwyer and Lawrence, 2000, Dwyer et al., 1998, Dwyer and Morgan, 2006). Embryo transfer was employed to separate the breed effects on lamb behaviour from maternal environment both *in utero* and post natal. This allowed reciprocal transfers which identified that breed differences persist when ewe breed is altered (Dwyer et al., 1996). The breed divergence, in addition to observed selection line differences in sucking behaviour (Dwyer et al., 2001), suggests peri-natal lamb behaviour is under genetic control. Sire effects on lamb behaviour have also been identified, supporting the argument that behaviour is a genetically-controlled trait. The lamb's ability to stand, attempt to suck and successfully suck was shown to be affected by sire, and in Suffolk lambs there was a tendency for percentage of lambs sucking unaided to also be influenced by sire (Dwyer et al., 2005). Whilst sire effects have been identified for behaviour in other species and at later ages in sheep, this was the first study to indicate that peri-natal behaviour in sheep has a strong genetic component.

Behaviour is often difficult to measure because it requires intensive observations to be made on large numbers of animals often under trying conditions and over long periods of time. Few investigations therefore have adequate number of records for suitable genetic analysis resulting in scarce published estimates. One of the few studies suitable for such analysis estimated the heritability of initial lamb behaviour as being low (time to stand 0.10 ± 0.05 in SA Mutton Merinos and 0.22 ± 0.06 in Dormers, time to suck 0.08 ± 0.05 in SA Mutton Merino and 0.12 ± 0.05 in Dormers (Cloete et al., 2002)). Whilst these appear to be the only published genetic estimate of peri-natal lamb behaviour, the heritability of lamb vigour measures recorded at an older age has been estimated and is explored below.

Lamb vigour after birth

Peri-natal behaviour is difficult to measure as it requires intensive observation of the ewe around the time of lambing. This has resulted in attempts to quantify lamb vigour through subjective estimates often not measured directly after birth but rather around the time of tagging (within 12-36 hours of birth). Initial subjective measures of vigour (good, fair or poor) were proven to be inadequate in prolific Booroola Merino lambs presumably as they failed to successfully capture behaviour after birth (Owens et al., 1985). This has led to more detailed descriptions aimed at estimating lamb vigour (described in Table 2.1 (Brien et al., 2010)).

Table 2.1 Scoring system used to subjectively describe the vigour of the lamb at tagging.

Score	Description
0	Lamb still wet. New born- invalid record.
1	Constant struggle. Bleat in response to ewe. On release reaches ewe quickly and follows.
2	Regular struggle when held. Moves to the ewe on release. Bleating common.
3	Some struggle. Walking in direction of ewe bleats but no contact. May bleat.
4	Some struggle. Attempts to walk but aimless. No apparent response to ewe bleats.
5	Little movement when held. Lies on release.

The heritability of this vigour estimate was shown to be low to moderate (h^2 = 0.16 ± 0.02) suggesting some genetic gain could be made through selection (Brien et al., 2010). What is

not well understood is whether this subjective vigour score is directly related to key lamb behaviours witnessed soon after birth.

A more quantitative estimate of vigour was developed by Everett-Hincks et al. (2005) and involved the timing of lamb behaviours at the time of tagging. The behaviours of interest included time to stand, bleat, contact and follow dam and contact udder upon release from tagging. A number of variables have been shown to influence these timed behaviours and include age of lamb at tagging, the type of birth of the lamb and maternal behaviour (Everett-Hincks et al., 2005). Interestingly, unlike the behaviours recorded at birth, timed behaviours from tagging are not influenced by birth weight. They have, however been shown to be affected by pasture sward height at the time of parturition which may reflect differences in nutritional availability, and also the micro-environmental effect the pasture may create (Everett-Hincks et al., 2005). Heritability estimates for these behaviours performed upon release from tagging range from 0.09 - 0.16 (Brien et al., 2010) once again suggesting that there is some genetic component of lamb vigour. There is however little point in pursuing lamb vigour unless a clear relationship with survival can be established.

Links between early postnatal behaviour, vigour and survival

The ability of the lamb to stand and suck soon after birth can influence survival due to better nutrition and also through maternal acceptance. Many investigations have shown this relationship between behaviour and survival highlighting the importance of initial vigour. An early study showed a high proportion of lambs that failed to either contact the udder or took longer than average to do so, died after sucking (Arnold and Morgan, 1975). Whilst the authors showed no direct link between the length of time to stand or attempt to suck and survival could be established, this was demonstrated in ensuing studies. A reduction in the latency to perform the key behaviours was shown to result in increased survival rates (by between 0.2 and 0.9% dependent on the behaviour) (Owens et al., 1985). Additionally, lambs that died within three days of birth were shown to display a delay in time to reach knees, attempt to stand, stand and reach the udder when compared to those that survived the same period (Dwyer et al., 2001). When taken together, these results would suggest that the relationship between postnatal behaviour and survival is in reality not linear, but rather a threshold one. Lambs that fail to reach this threshold are at a greater risk of mortality and lambs that surpass it survive, however greater survival is not observed in lambs that at faster to display behavioural progression once above this threshold.

Whilst this research emphasises the phenotypic relationships between neonatal behaviours and survival, few investigations into the genetic relationships exist. The only published genetic investigation into peri-natal lamb behaviour did not explore correlations between behaviour and survival, but rather compared both the maternal and direct breeding values for behavioural traits between lambs that died and lambs that survived (Cloete et al., 2002). This analysis showed that lambs that died exhibited higher breeding values for parturition length (both direct and maternal), time taken from birth to standing and time taken from standing to sucking than those that survived. Indirect estimates of lamb vigour have also been shown to be genetically linked to survival. Although standard errors were high, subjective lamb vigour score was shown to exhibit a low but favourable genetic correlation with survival (-0.26 ± 0.21) (Brien et al., 2010). In this study, timed lamb vigour estimates were also shown to be related to survival, with time taken for the lamb to bleat showing the highest genetic correlation of -0.43 ± 0.32 with survival. Lambs that were quick to bleat were more likely to have increased survival in their offspring. This is promising as it suggests selection that takes into account behavioural traits may be a useful means of genetically improving lamb survival. Whilst this genetic link between behaviour and survival is important, understanding the relationship between behaviour and the 'metabolic maturity' of the lamb remains to be elucidated and should be considered.

'Metabolic maturity' of the neonate

'Metabolic or physiological maturity' of the newborn lamb has been implicated as an important contributor to peri-natal behaviour and lamb mortality (Thompson et al., 2006). The term 'physiological maturity' refers to the neonate's ability to adapt to the changes between intra-uterine and extra-uterine life. In particular it relates to the ability of the neonate to adapt to an abrupt change in energy metabolism from a diet high in amino acid and carbohydrate to one containing high fat and less carbohydrate (Greenwood et al., 2002). The neonate is also required to adjust to independent oxidative metabolism through lung expansion and pulmonary ventilation, as well as the autonomous regulation of homeothermy (explored in later chapters) (Bassett, 1989). Variation in blood metabolites and hormones may reflect differences in development or maturity having consequences for the successful adaptation to postnatal life (Leenhouwers et al., 2002b). Of the many physiological adaptations the neonate requires to make the transition from pre to post-natal life, perhaps the greatest is to achieve energy homeostasis. These metabolic adaptations are now considered.

Glucose metabolism in neonates

During the peri-natal period the fetus must shift from the passive placental supply of maternal glucose to independent regulation of its own energy supplies. This involves maintaining glucose supply through glycogenolysis and gluconeogenesis to ensure energy demands are met for temperature regulation and neural function. After birth, the neonate experiences profound hypoglycaemia (Girard et al., 1973) as maternal glucose is exhausted and a delay in glucose production ensues. It is essential that neonates begin endogenous glucose production, as even after enteral feeding, hypoglycaemia is commonly observed (Girard et al., 1992).

The most important stores of glycogen in the neonate are found in the liver and skeletal muscle. During the first day of life the maximal level to which these stores can be mobilised to glucose through glycogenolysis is 90% of liver and 60% or less of muscle glycogen (Mellor and Cockburn, 1986). The finding that growth retardation results in little difference in glycogen deposits at birth (Mellor and Cockburn, 1986) suggests differences in the maturity of glucose mobilisation in the neonate must be reflected through the endocrine control of glycogenolysis. Factors that control initial glycogenolysis are not fully understood but it is thought that glucagon and catecholamines play a role. In a number of species at birth, plasma glucagon increases rapidly whilst plasma insulin falls or remains at low concentrations, and this may be caused by the simultaneous rise in catecholamines (Girard et al., 1992, Sperling et al., 1984). This rise in catecholamines is triggered by both delivery (Hagnevik et al., 1984) and cord cutting (Padbury et al., 1985) and is thought to play a role in glycogenolysis both directly and/or indirectly (through stimulating the production of glucagon and inhibiting insulin). Whilst the mechanisms by which these hormones stimulate glycogenolysis are still unknown, preterm human infants exhibit lower levels of catecholamines at birth than those born at full term (Lagercrantz and Bistoletti, 1977) implicating their role in the neonatal maturity of glycogenolysis.

The stores of hepatic and muscle glycogen provide the supply of glucose (Liggins, 1994) however this initial glycogenolysis is only sufficient to provide about one third of the glucose requirement for the neonate, so rapid postnatal development of gluconeogenesis is required (Bassett, 1989). Gluconeogenesis occurs at very low levels in the fetus but develops quickly after birth (Townsend et al., 1989). Lactate, amino acids and glycerol are used as substrates in neonates for gluconeogenesis which occurs in the liver and kidney under the regulation of specific enzymes (Girard et al., 1992). The rate limiting enzyme for gluconeogenesis in the neonate is phospho*enol*pyruvate carboxykinase (PEPCK) (El Manoubi et al., 1983) and

evidence suggests its induction is mediated by both rises in cyclic adenosine monophosphate (cAMP) and glucagon and the fall in insulin (Girard et al., 1992) witnessed immediately after birth. In the premature rat, a delayed increase in cAMP as a result of a higher insulin/glucagon ratio and a delay in the appearance of PEPCK results in reduced gluconeogenesis (Fernandez et al., 1983). These same authors identified that gluconeogenesis only accounted for between 9 – 12% of glucose production in the neonate, and, as such, the hypoglycaemia witnessed after birth in term, and more so in preterm neonates, is largely due to insufficient glycogenolysis. This suggests that whilst gluconeogenesis is important, the onset of glycogenolysis is crucial to prevent hypoglycaemia in the neonate and thus regulators of this key process could be used to indicate physiological maturity at birth in the neonatal lamb.

Fat metabolism in neonates

Whilst fat metabolism is of importance in the neonate, it will not be the focus of this review as respiratory quotient estimates suggest that carbohydrate metabolism is of greater importance in the unfed newborn (Mellor and Cockburn, 1986). The low level of ketone bodies found in lambs at birth also suggest that fat is not a key energy source at this time (Girard et al., 1981). The definition of metabolic maturity at birth should examine the physiological environment of an individual before enteral feeding and so fat metabolism after colostrum/milk acquisition will not be explored. Additionally, lambs are born with limited white adipose fat stores , and whilst levels of brown fat are high they are primarily utilised for thermoregulation and will be examined in detail in a subsequent chapter.

Fat metabolism in the newborn lamb provides a energy source, is used as a substrate for non-shivering thermogenesis (Alexander, 1962a) and spares glucose for tissues that are solely dependent upon it (Girard et al., 1992). Lipolytic products do not cross the placenta from dam to fetus as readily as glucose and amino acids, and as a result lambs are born with limited fat deposits (Alexander and Bell, 1975). Triglycerol stores from brown adipose tissue, white adipose tissue and liver are rapidly metabolised to free fatty acids and glycerol and mobilised soon after birth (Girard et al., 1992). Lipolysis is controlled by hormone-sensitive lipase, and lipase activity is determined by catecholamines (lipolytic) and insulin (anti-lipolytic) (Girard et al., 1992). Due to the high catecholamine surge witnessed after birth and the maintenance of low insulin levels, the neonatal period is characterised by an increase in fatty acid availability (Girard et al., 1992).

Other hormonal control mechanisms of maturation in neonates

Towards the end of gestation, a fetal glucocorticoid surge is involved in the maturation of the fetus preparing it for extrauterine life (Gluckman et al., 1999). The role of cortisol in organ maturation is well understood (Liggins, 1994) and relationships between cortisol level and glycogen stores indicate a role for cortisol in neonatal energy regulation also (Leenhouwers et al., 2002b).

In an attempt to increase maturity in the lamb, ewes were injected with dexamethasone during late gestation and whilst no change in pre-suckling glucose, non-esterified fatty acid (NEFA), urea or leptin concentrations were observed, glucocorticoid treatment increased circulating ghrelin concentrations in male and singleton born lambs (Miller et al., 2009b). The authors concluded that whilst no differences in peri-natal behaviour or survival were witnessed in the glucocorticoid-treated animals, this increase in ghrelin increased the maturity of the lamb. Why this effect was only observed in singleton lambs is perplexing, and perhaps it could even be argued that there is a limited chance of observing immaturity in singles as they are less likely to suffer any uterine growth restriction. Regardless, ghrelin has previously been linked to gestational age in human infants (Farquhar et al., 2003) and it is implicated in energy balance in the neonate (Yokota, 2003), so this association of ghrelin with physiological maturity of lambs is logical.

Defining maturity in the neonate

In human investigations, the word prematurity can be defined by two distinct types of infants: those that are small as they were born early (preterm) and those that are small as they experienced *in utero* growth restriction (IUGR) (Berkowitz and Papiernik, 1993). Experiments aimed at defining maturity in the animal have utilised both and their main findings and validity are discussed below.

The incidence of preterm animals is low, so in order to induce a preterm infant experimentally, caesarean sections are required (Cooper, 1975). These neonates born via caesarean section experience the desired reduction in gestation length, however the birth process is artificial and as such they do not undergo ‘natural’ delivery. As mentioned above, ‘natural’ delivery is characterised by a peak in corticosteroids and catecholamines, both of which are essential for the physiological maturity of the neonate. Therefore, results from any investigation into maturity that utilise caesarean section should be viewed with caution due to the confounding of gestation length and delivery method. Nonetheless, experiments in

calves have shown that preterm animals delivered via caesarean display perturbed glucose production when compared to those born 'naturally' at full term (Steinhoff-Wagner et al., 2011). Preterm calves were unable to maintain plasma glucose levels in the first nine hours following birth and glucose levels were significantly lower at the end of the measurement period when compared to those born at term. This was explained by a reduction in gluconeogenesis in these preterm animals which the authors attribute to a reduced cortisol concentration at birth. As mentioned above, this reduction in cortisol concentration in the preterm animals may have occurred as they did not undergo a 'natural' delivery and, as such, IUGR may be a more powerful model for investigating maturity.

IUGR can occur in animals through both a reduced placental transport capability and a reduced maternal nutritional intake. In piglets, the 'runt' of the litter can often be diagnosed as IUGR as it has experienced a full gestation length but is often one third the weight of its littermates, displays perturbed organ weight ratios and is leaner (Cooper, 1975). IUGR is a little more difficult to define in animals that typically give birth to one or two offspring, such as the sheep, as there is little opportunity for comparison within litters, and across litter variation in birth weight is high and may also reflect genetic differences. Using birth weight as an indicator of metabolic maturity in such species is therefore questionable. Regardless, at birth, smaller lambs exhibit increased concentrations of urea nitrogen and somatotropin and decreased IGF-1 (Greenwood et al., 2002). It was implied that this increase in urea nitrogen reflected a reduction in maturity as it was assumed to indicate high levels of amino acid catabolism. A reduction in the maturation of the renal system was also implicated as the kidney is responsible for urea clearance after birth. The high somatatropin and low IGF-1 levels suggest 'metabolic maturity' as a transition from fetal to postnatal life is typically characterised by a decline in somatatropin and increase in IGF-1 (Gluckman et al., 1999).

Previous investigations have identified hormonal and metabolic shifts in animals defined as being less mature. However, models of maturity, namely prematurity and IGUR, may be ambiguous when used in the lamb, as explored above. Perhaps a novel technique to validate metabolic maturity at birth would take into account the behavioural progression of the neonate. Behaviour is controlled by a complex system of physiological events that we hypothesise would easily be affected by a disruption in metabolic maturity. Lambs that are slower to perform the key behaviours explored in previous paragraphs, such as the time taken for the lamb to stand and suck successfully, may be less mature physiologically, therefore may also be at an increased risk of mortality.

Links between maturity, behaviour and survival

As alluded to above, an increased 'metabolic maturity' is typified by an increased reliance on glucose and fatty acids coupled with a decline in the utilisation of protein sources (Greenwood et al., 2002). This shift in energy utilisation would presumably have ramifications for neural functioning and thus behaviour. Specifically, if glycogenolysis and gluconeogenesis are up-regulated in more mature individuals, an increased glucose supply would be available for the nervous system encouraging behavioural progression in these animals. The few published investigations attempting to link maturity through hormonal regulation of energy expenditure and behaviour neither fully support nor refute this hypothesis. Miller *et. al.* (2009) identified a negative relationship between ghrelin and gestation length, in which lambs grouped into a short gestation length category (< 146 days) exhibited the highest pre-suckling ghrelin concentrations. A tendency for lambs with lower circulating ghrelin concentrations to display longer standing times was also observed, suggesting that links between gestation length, behaviour and ghrelin concentrations warrant further investigation. Leptin concentrations were shown to be highest in short gestation lambs, and were also higher in lambs that took less time to seek the udder (Miller et al., 2009a). This heightened concentration of hormones implicated in energy expenditure in lambs with reduced gestation lengths and improved post-natal behaviours suggest a link between metabolic maturity and behaviour.

If increased neonatal maturity does result in increased behavioural progression (lambs that are faster to stand and suck), a subsequent influence on survival may be witnessed. Additionally, increasing maturity may influence survival through other mechanisms such as thermoregulatory ability. Early investigations into physiological maturity at birth were conducted in piglets in an effort to describe the biological background for genetic difference in piglet survival. Whilst no differences in glucose, fructose, albumin or estradiol were identified, average serum cortisol levels increased with increasing EBV for piglet survival (Leenhouwers et al., 2002a). Further studies in lambs identified that higher glucose and NEFA concentrations and lower BUN concentrations were associated with increased survival (Thompson et al., 2006), agreeing with previous hypotheses on maturity (Greenwood et al., 2002). The increase in glucose concentration was not explained by glycogen stores, but rather a down-regulation in the use of glucose to supply energy to muscle, suggesting increased efficiency or the use of another substrate. An alternate explanation is that these lambs were less able to clear glucose from the system, and this could be explained by differences in insulin and glucagon concentrations but these were not measured. Miller *et. al.* (2009) reported opposing results, with lambs with a decreased pre-

suckling glucose concentration exhibiting increased survival rates and this discrepancy was explained by the timing of blood collection. Samples were taken from the lambs 30 min post parturition and the authors suggested that lambs with increased glucose concentrations at this time experienced a delay in glucose utilisation translating to reduced maturity. The same investigation also showed a positive relationship between plasma ghrelin concentrations and survival, whereby more lambs with increased plasma ghrelin survived to 72 hours than those with average or low concentrations. Whilst there is increasing evidence linking metabolic maturity of the neonate (increased reliance on glucose and fats, and decreased reliance on amino acid for energy metabolism) and survival, there are few investigations into the relationships with behaviour.

Conclusions

Evidence linking peri-natal lamb behaviour and vigour to survival exists and thus should be explored as a potential method to reduce mortality. The behaviour of the lamb after birth is highly variable and some of this variation can be explained by genetics. Physiological differences between lambs may also help to explain this variation and it is logical to assume that 'metabolic maturity' at birth may influence behavioural progression. There is a clear requirement to classify maturity at birth in the lamb, identify the factors that affect it and investigate if relationships with behaviour and vigour exist. In the current investigation we aim to identify if hormones and metabolites previously implicated in 'metabolic maturity' show relationships with peri-natal lamb behaviour. We hypothesise that lambs that with enhanced behavioural progression and vigour following birth are born with increased 'metabolic maturity' as defined by endocrine and metabolite shifts, which may have consequences for postnatal lamb survival.

Methods

Animals

All experiments involving animals were carried out with approval from the University of Adelaide Animal Ethics Committee (S-2009-005). Two breeds of sheep were used for this investigation to maximise variation in the traits of interest. The Border Leicester and Merino breeds were selected as they have previously shown to differ in lamb survival rates (Fogarty, 2000), thus it was expected that they may also differ in crucial post-natal behaviour and lamb vigour. Multiparous ewes were chosen to eliminate any confounding of the lamb traits of interest with mismothering from the dam.

Management

In 2009, ewes were naturally mated on the properties from which they were sourced. Multiple sire mating was used and sire breed was the same as ewe breed. In 2010, ewes were artificially inseminated (AI) via laparoscopic technique with frozen semen from two Poll Dorset sires after synchronisation with progesterone-controlled internal drug release (CIDR) devices and injected with pregnant mare serum gonadatrophin (PMSG). In 2011, ewes were inseminated using the same sire breed as dam, and sires were selected from the Australian Sheep Co-operative Research Centre's Information Nucleus Flock (van der Werf et al., 2010) from estimated breeding values based on progeny performance for lamb survival and birth weight with the expectation of divergence in traits recorded. Sire solutions were calculated in ASREML (Gilmour et al., 2005) using an animal model with a maternal effect and the fixed effects of age of dam, sex, type of birth, flock, year, sire breed, sire type, dam breed and any significant two-way interactions. The dam permanent environmental effect was not significant, thus removed from the model. Covariates included day of birth, birth weight and birth wieght^2 (lamb survival only) and type of birth by birth weight interaction (lamb survival only). Merino sires were selected based on poor lamb survival and low birth weight whilst Border Leicester sires were chosen for increased survival and average birth weight to increase the chance of divergence in the traits of interest (Table 2.2).

Table 2.2 Estimated breeding values for lamb birth weight (BWT) and lamb survival to weaning (LSW), with (+BWT) and without (-BWT) adjusting for birth weight and the number of ewes that were inseminated for each sire.

Sire	Sire Breed	BWT	LSW (+bwt)	LSW (-bwt)	No of ewes inseminated
1	Merino	-0.1041	-0.03895	-0.02486	40
2	Merino	0.00586	-0.01194	-0.01416	48
3	Border Leicester	0.2542	0.01175	0.008434	20
4	Border Leicester	0.04967	0.01404	0.005558	20
5	Border Leicester	0.1612	-0.000812	0.003122	20

AI was conducted in four rounds over a two week period to facilitate a three to four week lambing. Each AI group contained an equal number of ewes from each breed and utilised semen from all sires. Ewes were scanned using ultrasonography at approximately day 50 to determine pregnancy status and litter size, and all non-pregnant ewes were removed from the study. Table 2.3 summarises the ewe management around conception across years.

Table 2.3 Ewe management at mating and pregnancy scan results conducted on day 50 for the three experimental years.

	2009		2010		2011	
Dam	Merino	Border Leicester	Merino	Border Leicester	Merino	Border Leicester
Sire	Merino	Border Leicester	Poll Dorset	Poll Dorset	Merino	Border Leicester
Mating	Natural		Artificial Insemination		Artificial Insemination	
Ewes pregnant	40	40	27	16	47	16
Expected lambs	66	66	30	22	71	27

All ewes were managed and run as a single flock after mating or insemination. At approximately day 145, the ewes were brought into a small paddock adjacent to the lambing shed to accustom them to human contact. Upon the birth of the first lamb, the ewes were drifted slowly into the lambing shed which contained four pens 10 m by 15 m in size. In 2009, each pen housed 20 ewes whilst in 2010 and 2011, due to the reduced conception

rate, pens housed between nine and 20 ewes. Additionally in the final two years, ewes were penned in their AI groups. Each pen contained feed (high energy pellet and lupins/peas), automated watering troughs and hay bedding. In 2009, ewes and lambs older than 48 hours were drifted to an outside paddock to reduce crowding in the pens and ewe interference with lambs. In subsequent years as pens housed fewer ewes, this occurred once all ewes in a single pen had lambed. Once the ewes and lambs had been moved to an outside paddock, daily inspection occurred to monitor mortality rates.

Measurements

The gestation length of each ewe was calculated in 2010 and 2011 by subtracting the insemination date from lambing date, however this was not possible in 2009 as ewes were naturally mated and the conception date was unknown.

Lambs were observed for a number of post-natal behaviours expected to indicate vigour. This was conducted by trained observers who gained view of all four pens via a raceway that stretched the length of the shed. Care was given to minimise disturbance whilst behaviour was being recorded. Ewes were observed from 06:00 until 20:00 daily and lambs born outside these hours were omitted from the investigation. Length of labour was defined as first appearance of the amniotic sac until the birth of the entire lamb. Any ewe in labour for more than three hours or where mal-presentation was observed was assisted, and this measure of parturition length was discarded from the results. Time to stand and suck were also recorded and defined as the length of time taken from birth until the lamb stood on all four legs for a period of no less than five seconds, and the time taken from birth until the lamb successfully received its first feed, respectively. In 2010 and 2011, time to first bleat, first stand attempt and first suck attempt were also recorded.

Additionally, in 2010 and 2011 a 5 mL pre-suckling blood sample was collected from the lambs. This was conducted after the lamb had stood but before it suckled and was performed by an experienced person to facilitate quick collection and thus reduce interference. Samples were generally collected within 30 min of birth and took less than two minutes to collect. The sample was taken via jugular venipuncture using a 22 gauge needle and collected into EDTA blood tubes. It was then analysed immediately for blood glucose (Hemocue Glucose 201+, Medipac Scientific, Australia) and centrifuged at 3000 rpm for five minutes. Plasma was frozen at -20°C whilst the trial was completed and then -80°C for

subsequent laboratory analysis. Table 2.4 shows the number of records collected over the three year experimental period.

Table 2.4 Number of records collected for behaviours and blood sampling at birth across three years for Border Leicester (BL), Merino (M), Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM) breeds.

	2009		2010		2011		
	BL	M	PDBL	PDM	BL	M	Total
Labour	19	23	8	19	9	31	109
Bleat	-	-	8	18	9	43	78
Stand attempt	-	-	9	19	9	43	80
Stand	24	26	10	18	9	41	128
Suck attempt	-	-	10	17	9	41	77
Suck	22	24	10	17	9	36	118
Blood collection	-	-	8	21	11	38	78

Within three hours of birth, lambs were tagged. At tagging the dam was identified, birth weight, type of birth, sex, and rectal temperature were recorded. A number of skeletal measurements were also collected including crown-rump length, thoracic circumference and metacarpal length (Figure 2.1). A birth coat score was allocated to each lamb with a score of one representing a lamb with a short coat and seven, a hairy lamb (Table 2.5).

a)  b)  c) 

Figure 2.1 Photos demonstrating measurement of a) crown rump length b) thoracic circumference and c) metacarpal length.

Table 2.5 Scoring system based on appearance of hairiness used to describe the birth coat of the lamb (Ponzoni et al., 1997).

Birth coat score	Description
1	Absolutely no halo hair. Short curly groups of fibres. No halo hairs visible when lamb is held up to light.
2	No halo hairs visible when lamb is viewed on ground. A very small number visible when lamb is held up to light.
3	A few halo hairs visible when lamb is on ground. Short curly fibres dominate appearance of animal.
4	Considerable number of halo hairs. Short curly fibres still easily visible.
5	Large number of halo hairs. Short curly fibres just visible through long fibres.
6	No short curly fibres visible. Long straight fibres only.
7	No short curly fibres visible. Very long straight fibres only.

A score of lamb vigour was recorded by a subjective scale based on how active and vocal the lamb was during and shortly after tagging (Table 2.6).

Table 2.6 Subjective scoring system used to describe the vigour of the lamb recorded at tagging (Brien et al., 2010).

Score	Description
0	Lamb still wet. New born- invalid record.
1	Constant struggle. Bleat in response to ewe. On release reaches ewe quickly and follows.
2	Regular struggle when held. Moves to the ewe on release. Bleating common.
3	Some struggle. Walking in direction of ewe bleats but no contact. May bleat.
4	Some struggle. Attempts to walk but aimless. No apparent response to ewe bleats.
5	Little movement when held. Lies on release.

Summary statistics of these phenotypic measurements are presented in Table 2.7. Ponderal index (PI) of the lamb was calculated using birth weight and crown rump measures. The formula used to calculate ponderal index was:

Ponderal index = birth weight (g) x 100 / crown-rump length (cm)

Table 2.7 Summary statistics of phenotypic measures from cross-bred (Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM)) and pure-bred (Border Leicester (BL) and Merino (M)) lambs recorded within three hours of birth.

Trait		n	Min	Max	SD
Birth weight (kg)					
Cross-bred					
	PDBL	24	3.0	7.3	1.2
	PDM	31	2.4	7.9	1.2
Pure-bred					
	BL	81	2.9	8.0	1.0
	M	137	1.3	8.1	1.2
Crown-rump length (cm)					
Cross-bred					
	PDBL	24	35.5	54.0	3.8
	PDM	31	36.5	54.5	3.7
Pure-bred					
	BL	81	33.0	55.5	4.3
	M	137	33.5	57.5	4.4
Thorax circumference (cm)					
Cross-bred					
	PDBL	24	31.0	50.0	2.4
	PDM	31	34.0	47.0	2.2
Pure-bred					
	BL	81	32.0	49.0	2.2
	M	137	28.0	53.0	2.3
Metacarpal length (cm)					
Cross-bred					
	PDBL	24	9.5	12.0	0.5
	PDM	31	9.5	12.5	0.5
Pure-bred					
	BL	81	8.5	12.0	0.8
	M	137	9.0	13.5	0.8
Birth coat score					
Cross-bred					
	PDBL	22	2	4	0.9
	PDM	31	2	6	1.1
Pure-bred					
	BL	74	1	6	1.4
	M	136	1	7	1.4
Rectal temperature (°C)					
Cross-bred					
	PDBL	22	28.6	40.2	0.5
	PDM	30	38.3	40.3	0.5
Pure-bred					
	BL	76	35.3	40.2	0.9
	M	136	34.0	40.3	1.2

Metabolite and hormone analysis

Most assays were performed in the Adelaide Research Assay Facility, School of Paediatrics and Reproductive Health, University of Adelaide by Dr. Micahel Boden, Dr. Anne MacPherson and Professor David Kennaway. The metabolites were assayed on a Roche Hitachi 912 Analyser. The quality control samples for the creatinine assay were 1.5 ± 0.12 mmol/L (CV = 8.3%) and 4.5 ± 0.4 mmol/L (CV = 8.0%). The quality control samples for the urea assay were 6.9 ± 0.7 mmol/L (CV = 9.6%) and 25.0 ± 1.0 mmol/L (CV = 4.1%).
The quality control samples for the NEFA assay (Lyphocheck Assay Chemistry Control Level 1 and Level 2; BioRad) were 1.6 ± 0.15 mmol/L (CV = 9.5%) and 0.8 ± 0.18 mmol/L (CV = 12.3%). Plasma samples were assayed for cortisol in duplicate by radioimmunoassay (Cat# IM1841, Immunotech, Prague, Czech Republic) according to the manufacturer's instructions using 50ul sample. The minimum detectable level was 20 nM. The intra-assay CV was less than 10%. The inter-assay CV was 6.4% at 148.8 nM (expected range 110-192 nM). Plasma samples were assayed for free triiodothyronine (fT_3) in duplicate by radioimmunoassay (Cat# IM1579, Immunotech, Prague, Czech Republic) according to the manufacturer's instructions using 25-100 uL sample. The minimum detectable level was 2.3 nM. The intra-assay CV was less than 10%. The inter-assay CV was 13.5% at 4.4 nM (expected range 3.0-5.1 nM). Plasma samples were assayed for adrenocorticotrophic hormone (ACTH) in duplicate by radioimmunoassay (Cat# 07-106102, MP Biomedicals Australasia, Seven Hills, NSW, Australia) according to the manufacturer's instructions using 100 uL sample. The minimum detectable level was 8 pg/mL. The intra-assay CV was less than 10%. The values of the low and high quality control samples supplied by the manufacturer for three separate kits were within the expected range.

Leptin and ghrelin analysis was conducted in the School of Animal Biology, The University of Western Australia by Mrs Margaret Blackberry. Plasma leptin was measured in duplicate by a double-antibody radioimmunoassay (Blache et al., 2000). All samples were processed in a single assay and the limit of detection was 0.05 ng/mL. The assay included six replicated of three control samples containing 0.63 ng/mL, 1.36 ng/mL and 2.58 ng/mL which were used to estimate the intra-assay CV of 7.7%, 4.1% and 1.9% respectively. Plasma ghrelin was measured in duplicate by a modified double-antibody radioimmunoassay (Miller et al., 2009b). All samples were processed in a single assay and the limit of detection was 39 pg/mL. The assay included six replicates of three control samples containing 92.6 pg/mL, 436.7 pg/mL and 2121 pg/mL which were used to estimate the intra-assay CV of 3.4%, 4.9% and 6.7% respectively.

Statistics

All traits were analysed using a general linear model conducted in ASREML (Gilmour et al., 2005) and a P value < 0.05 was deemed significant. As all breeds were not used across all years, confounding between sire breed and year was encountered in the analysis. Consequently, the pure-breds (M and BL) were analysed separately to the cross-bred (PDM and PDBL), thus direct statistical comparisons between these genotypes could not be made. All univariate analyses included the fixed effects of age of dam (2 to 7 years), sex (male or female), type of birth (single or multiple), breed (Merino or Border Leicester) and any significant two way interactions between these effects. The pure-bred analysis also contained the fixed effect of year (2009 and 2011). The covariates of birth weight and gestation length were included in the model where appropriate and allowed for regression analysis between these and other traits. All lamb behaviours were not normally distributed so were log-transformed for analysis and back-transformed for presentation of results. Significance levels obtained from these analyses will be presented in the Results section using the following scheme: P value < 0.1 is represented with †, P < 0.05 with *, P < 0.01 with ** and P < 0.001 ***.

Relationships between traits were examined using multivariate analyses conducted to estimate phenotypic correlations. These analyses included the fixed effects of age of dam (2 to 7 years), sex (male or female), type of birth (single or multiple), breed (Merino or Border Leicester) and any significant two way interactions between these effects. The multivariate analysis was run between five traits at any given time and estimated correlations were compared between analyses. Significance was determined if the correlation was greater than two times the standard error (Cloete et al., 2004).

Results

Gestation length

Singleton lambs experienced a longer gestation than multiples in the pure-bred lambs (147.8 ± 0.3 and 147.1 ± 0.2 respectively; P < 0.05), however the effect of type of birth on gestation length was reversed when birth weight was included in the statistical model. Age of dam had a significant effect on gestation length for both cross-bred and pure-bred lambs (Figure 2.2). Lambs from four year old ewes were born sooner (day 145.2 ± 0.9) compared to all other age groups (day 147.6 ± 0.7) in the cross-breeds (P < 0.001) whilst pure-bred lambs from three year old ewes exhibited a shorter gestation length of 144.8 ± 1.2 days and those from seven year old ewes were delayed (149.4 ± 0.4 days (P < 0.001)) with other age groups being intermediate.

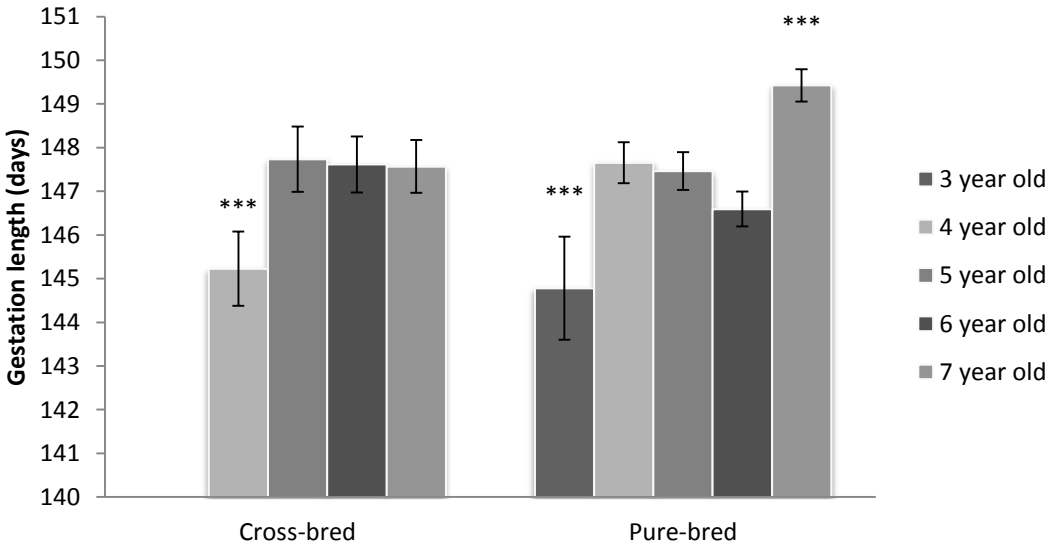

Figure 2.2 Mean ± SEM gestation length (days) for increasing age of dam (3-7 year olds) for both cross-bred (Merino x Poll Dorset and Border Leicester x Poll Dorset) and pure-bred (Merino and Border Leicester) lambs (* represents significant difference (P < 0.001) within genotype).**

Lamb breed also influenced gestation length (P < 0.001) with the cross-bred PDM lambs exhibiting a longer gestation length (149.2 ± 0.2 days) when compared with the PDBL cross-bred lambs (145.5 ± 0.4 days; Figure 2.3). This was mirrored in the pure-bred lambs (M 149.6 ± 0.2 days and BL 144.0 ± 0.4 days).

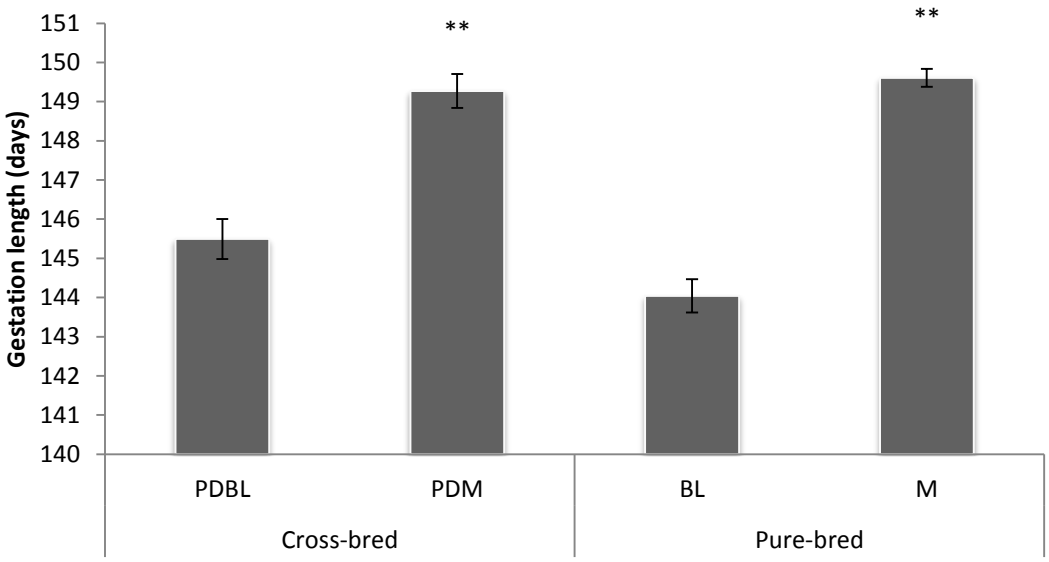

Figure 2.3 Mean ± SEM gestation length (days) for both cross-bred (Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM)) and pure-bred (Border Leicester (BL) and Merino (M)) lambs (represents significant difference (P < 0.01) within genotype).**

Lamb phenotype

Birth weight was affected by lamb breed (Table 2.8), with pure-bred BL lambs weighing more than their M counterparts ($P < 0.001$). This divergence disappeared in the cross-bred lambs. Lamb shape was also heavily influenced by lamb breed, with crown rump length decreased in the BL lambs for both cross-breds ($P < 0.01$) and pure-breds ($P < 0.001$). Metacarpal length was also decreased in BL's in the cross-breds ($P < 0.001$) and pure-breds ($P < 0.001$). Pure-bred BL lambs were hairier compared with M lambs ($P < 0.01$), however this difference was not witnessed in the cross-breds.

Table 2.8 Mean (± SEM) phenotypic measurements from cross-bred (Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM)) and pure-bred (Border Leicester (BL) and Merino (M)) lambs recorded within three hours of birth.

Trait		Mean	SEM	P-value
Birth weight (kg)				
Cross-bred				
	PDBL	5.4	0.2	0.544
	PDM	5.2	0.2	
Pure-bred				
	BL	5.7	0.1	***
	M	4.6	0.1	
Crown-rump length (cm)				
Cross-bred				
	PDBL	44.0	0.8	***
	PDM	47.6	0.7	
Pure-bred				
	BL	43.3	0.5	***
	M	46.5	0.4	
Thorax circumference (cm)				
Cross-bred				
	PDBL	42.1	0.5	0.699
	PDM	42.1	0.4	
Pure-bred				
	BL	41.0	0.2	†
	M	40.5	0.2	
Metacarpal length (cm)				
Cross-bred				
	PDBL	10.7	0.1	***
	PDM	11.2	0.1	
Pure-bred				
	BL	10.1	0.1	***
	M	11.5	0.1	
Birth coat score				
Cross-bred				
	PDBL	3.0	0.2	0.746
	PDM	2.8	0.2	
Pure-bred				
	BL	2.7	0.2	**
	M	2.2	0.1	

Ponderal index (PI) was influenced by gestation length in the pure-bred lambs, with a decrease in 0.12 for every increase of one day ($P < 0.001$). A trend ($P = 0.10$) for a similar relationship was observed in the cross-bred lambs however significance was not established. Lamb breed also affected PI, with BL lambs exhibiting increased ratios in both the cross-breds (PDBL 6.3 ± 0.3 and PDM 4.9 ± 0.3; $P < 0.05$) and pure-breds (BL 6.0 ± 0.2 and M 4.9 ± 0.1; $P < 0.001$).

Length of parturition

Heavier birth weights were significantly associated with increased parturition length in the pure-bred lambs ($P < 0.05$) but this relationship disappeared when examined in the cross-breds. Gestation length exhibited a positive relationship with parturition length in the cross-bred lambs only ($P < 0.01$). This relationship became weaker, but still remained significant after birth weight was fitted as a covariate. In the cross-breds, PDBL lambs were born significantly faster than PDM lambs (14.0 ± 14.1 min and 61.7 ± 9.6 min respectively; $P = 0.01$). No significant breed differences were observed between the purebreds.

Peri-natal lamb behaviour

There was a tendency for time taken for the lamb to bleat following birth to be increased with a longer gestation length ($\log_{10}$ regression co-efficient 0.37, $P < 0.1$) which remained after adjustment for birth weight. This effect of gestation length however was not seen on any other behavioural measures. The effect of birth weight on lamb behaviours was varied (Table 2.9). The only behaviour affected by birth weight in the cross-bred lambs was time to suck, and this was reduced in heavier lambs ($P < 0.01$). Time to suck also tended to be reduced in heavier lambs in the pure-breds ($P = 0.1$), and additionally time taken to stand was reduced by increased birth weight in this genotype ($P < 0.05$). However, lighter lambs were quicker to attempt to stand in the pure-breds ($P < 0.05$). In cross-bred lambs, initial behaviours were unaffected by breed but latency to stand, attempt to suck and successfully suck were reduced in the PDBL lambs when compared to the M's ($P < 0.05$). Whilst there was a tendency for differences in most behaviour, the only measure that differed in the pure-bred lambs was time taken to perform a suck attempt, whereby the BL lambs were faster to exhibit this behaviour ($P < 0.05$).

Table 2.9 Time for lamb to perform peri-natal behaviour (mean ± SEM) for cross-bred (Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM)) and pure-bred (Border Leicester (BL) and Merino (M)) lambs, with no covariate and with the inclusion of birth weight as a covariate in the statistical model ([a] and [b] represents significance (P < 0.05) of birth weight in cross-breds and pure-breds respectively).

		Time to perform behaviour (min)					
		Cross-bred		P-value	Pure-bred		P-value
		PDBL	PDM		BL	M	
No covariate							
	Bleat	2.5 ± 3.5	4.0 ± 2.4	0.379	2.6 ± 8.2	3.1 ± 4.2	0.977
	Stand attempt	5.4 ± 1.5	4.5 ± 1.0	0.497	13.6 ± 5.3	8.0 ± 2.7	†
	Stand	12.8 ± 3.9	15.8 ± 2.9	**	18.5 ± 5.5	39.5 ± 4.5	†
	Suck attempt	25.2 ± 7.3	32.6 ± 5.7	***	38.8 ± 14.1	54.9 ± 7.4	*
	Suck	42.9 ± 12.3	57.9 ± 9.7	*	53.0 ± 11.7	81.6 ± 9.5	0.174
Birth weight covariate							
	Bleat	2.5 ± 4.1	4.0 ± 2.4	0.387	2.4 ± 8.0	3.0 ± 4.1	0.94
	Stand attempt [a]	5.6 ± 1.7	4.5 ± 1.1	0.512	12.5 ± 5.0	7.8 ± 2.6	†
	Stand [a]	13.9 ± 3.8	16.0 ± 3.7	**	18.7 ± 6.0	39.4 ± 4.7	†
	Suck attempt	25.7 ± 10.0	32.8 ± 7.7	***	39.1 ± 14.4	55.0 ± 7.5	†
	Suck [b]	48.4 ± 11.6	60.2 ± 8.7	*	53.0 ± 11.7	81.6 ± 9.5	0.218

In the pure-bred lambs, females tended to stand faster than males (23.8 ± 5.4 min and 30.2 ± 5.0 min respectively; P = 0.12) and they were also quicker to attempt to suck (44.5 ± 10.8 min and 54.9 ± 9.8 min respectively; P < 0.05). There was no effect of age of dam or type of birth on any of the peri-natal behaviours recorded in both the pure-bred and cross-bred lambs. All of the behaviours recorded exhibited significant positive phenotypic correlations with one another (Table 2.10). Thus, the quicker the lamb was to bleat, the quicker it attempted to stand, successfully stood, attempted to suck and successfully sucked.

Table 2.10 Phenotypic correlations (± SE) between lamb behaviours observed after birth.*

	Bleat	Stand attempt	Stand	Suck attempt
Stand attempt	***0.53 ± 0.11***			
Stand	***0.35 ± 0.15***	***0.70 ± 0.08***		
Suck attempt	***0.36 ± 0.13***	***0.41 ± 0.12***	***0.75 ± 0.06***	
Suck	***0.35 ± 0.14***	***0.38 ± 0.12***	***0.68 ± 0.12***	***0.78 ± 0.05***

*Significant correlations (ie. value greater than two times the standard error) are shown in bold and italics.

Subjective lamb vigour

Vigour score was affected by birth weight in the pure-breds, with heavier lambs displaying increased vigour (P < 0.001). For every one kg increase in birth weight, the lambs were more vigorous receiving a 0.19 reduction in score. This influence of birth weight was not witnessed in the cross-breds. Age of dam also influenced vigour in the pure-breds (Figure 2.4), with lambs from five year old ewes displaying the highest vigour and thus obtaining the lowest score (1.4 ± 0.1; P < 0.05).

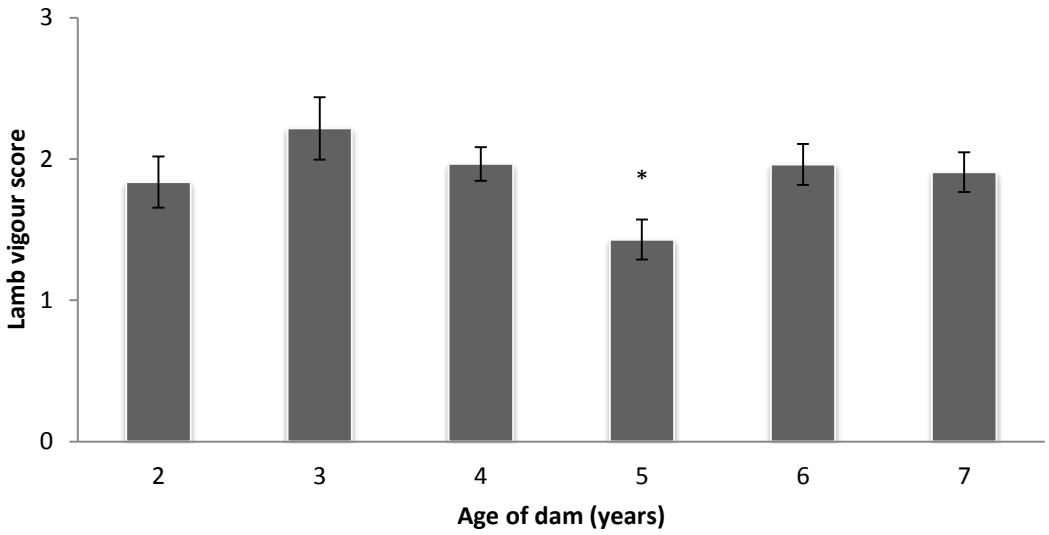

Figure 2.4 Subjective lamb vigour score (mean ± SEM) for increasing age of dam (years) in pure-bred (Border Leicester and Merino) lambs (* represents significant difference (P < 0.05)).

There was a tendency (P = 0.061) for male lambs to display poorer vigour than females (2.2 ± 0.1 and 2.0 ± 0.2 respectively) in the cross-bred lambs, and this obtained significance (P < 0.01) in the pure-bred lambs with males receiving a higher score and thus being less vigorous (2.2 ± 0.1) than females (1.6 ± 0.1). Relationships between lamb vigour score and behaviour were also identified. In both the cross-bred and pure-bred lambs vigour was positively related to the $\log_{10}$ time taken to stand (cross-bred 0.2; P < 0.05 and pure-bred 0.06; P < 0.05) and $\log_{10}$ time to perform a suck attempt (cross-bred 0.06; P < 0.05 and pure-bred 0.31; P < 0.001). The other behaviours however were not related to lamb vigour.

Pre-suckling lamb physiology

Blood metabolites

For every one day increase in gestation length, blood glucose levels were increased by 0.28 mmol/L (P < 0.05). Glucose levels were affected by type of birth (Table 2.11), with singleton lambs displaying higher pre-suckling levels than twins (5.10 ± 0.41 mmol/L and 4.35 ± 0.31 mmol/L respectively; P = 0.05). No behaviours recorded were related to blood glucose concentrations.

Table 2.11 Pre-sucking lamb metabolite concentrations (mean ± SEM) for fixed effects of age of dam, sex, type of birth and breed (BL- Border Leicester, M- Merino, PDBL- Poll Dorset Border Leicester cross and PDM- Poll Dorset Merino cross).

Fixed effects		Glucose (mmol/L)		NEFA (mEq/L)		BUN (mmol/L)		Creatinine (mmol/L)	
		Mean±SEM	Sig	Mean±SEM	Sig	Mean±SEM	Sig	Mean±SEM	Sig
Age of dam	3	3.1 ± 1.8	NS	NA	†	6.7 ± 3.3	NS	0.21 ± 0.05	NS
	4	4.0 ± 0.5		1.2 ± 0.1		6.7 ± 1.1		0.13 ± 0.01	
	5	5.0 ± 0.5		0.5 ± 0.2		7.8 ± 1.0		0.14 ± 0.01	
	6	4.8 ± 0.5		1.2 ± 0.1		9.8 ± 1.0		0.15 ± 0.01	
	7	4.9 ± 0.5		1.0 ± 0.1		8.0 ± 1.2		0.12 ± 0.02	
Sex	Male	4.8 ± 0.3	NS	1.0 ± 0.1	NS	8.4 ± 0.6	*	0.14 ± 0.01	NS
	Female	4.5 ± 0.4		1.0 ± 0.1		7.6 ± 0.6		0.13 ± 0.01	
Type of birth	Single	5.1 ± 0.4	*	1.1 ± 0.1	NS	8.1 ± 0.7	NS	0.15 ± 0.01	*
	Multiple	4.4 ± 0.3		1.0 ± 0.1		7.9 ± 0.7		0.13 ± 0.01	
Breed	BL	4.5 ± 0.6	NS	NA	NS	7.2 ± 1.3	NS	0.13 ± 0.02	NS
	M	4.6 ± 0.4		NA		7.0 ± 0.7		0.15 ± 0.01	
	PDBL	4.8 ± 0.7		1.2 ± 0.1		8.0 ± 1.5		0.12 ± 0.02	
	PDM	4.7 ± 0.5		0.9 ± 0.1		10.1 ± 1.1		0.15 ± 0.01	

There was a tendency (P = 0.08) for lambs from five year old ewes to exhibit lower pre-suckling NEFA levels (0.54 ± 0.19 mEq/L) when compared to other ages (1.14 ± 0.13 mEq/L). A positive association between NEFA and length of labour was observed (0.35 ± 0.16). NEFA concentrations were also related to lamb behaviour. Negative correlations were observed with attempt to suck and suck, thus lambs that performed these behaviours faster showed increased NEFA concentrations. (Table 2.12).

Table 2.12 Phenotypic correlations (± SE) between lamb pre-sucking plasma metabolite concentration and peri-natal behaviour.*

	Time taken for lamb to perform behaviour				
	Bleat	Stand attempt	Stand	Suck attempt	Suck
Glucose	-0.00 ± 0.16	0.14 ± 0.15	-0.02 ± 0.16	-0.23 ± 0.16	-0.14 ± 0.16
NEFA	-0.33 ± 0.18	-0.38 ± 0.23	0.03 ± 0.27	***-0.82 ± 0.07***	***-0.72 ± 0.10***
BUN	***-0.41 ± 0.16***	***-0.47 ± 0.13***	-0.21 ± 0.16	0.30 ± 0.18	0.06 ± 0.20
Creatinine	-0.01 ± 0.15	-0.00 ± 0.17	-0.16 ± 0.17	***-0.39 ± 0.16***	***-0.38 ± 0.16***

*Significant correlations (ie. value greater than two times the standard error) are shown in bold and italics.

Male lambs tended to display higher BUN levels than their female counterparts (8.40 ± 0.40 mmol/L and 7.55 ± 0.63 mmol/L respectively; P = 0.05). Negative relationships were observed between BUN and gestation length (0.39 ± 0.15) and initial lamb behaviour (bleat and stand attempt; Table 2.12). Type of birth differences in plasma creatinine levels were observed with singles (0.15 ± 0.01 mmol/L) presenting higher levels than twins (0.13 ± 0.01 mmol/L, P < 0.05). Creatinine was also strongly related to gestation length, with lambs with shorter gestation displaying higher levels than those born later (-0.90 ± 0.04 mmol/L). There was a negative relationship between creatinine and sucking behaviour observed (Table 2.12). Additionally, lamb shape was shown to be associated with creatinine levels, as crown rump length was positively correlated with creatinine (0.42 ± 0.17).

Hormones

ACTH levels were unaffected by any of the examined fixed effects and showed only a moderate negative correlation with gestation length (-0.48 ± 0.16) and length of labour (-0.41 ± 0.14). Cortisol levels were affected by lamb breed (Figure 2.5). PDM lambs exhibited higher pre-suckling cortisol levels than PDBL's, whilst BL lambs displayed higher concentrations than M lambs (P < 0.05).

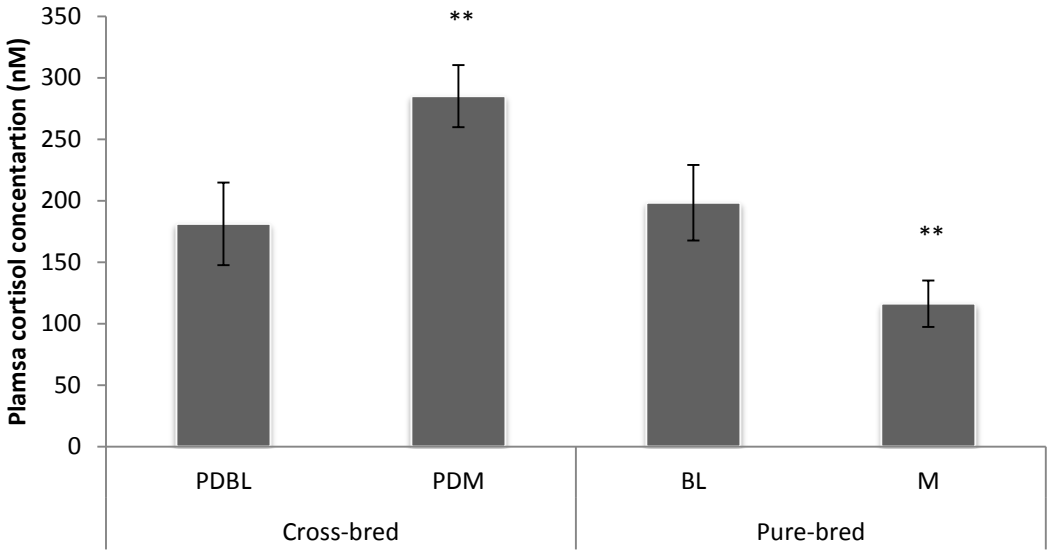

Figure 2.5 Mean (± SEM) plasma cortisol concentration (nM) for both cross-bred (Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM)) and pure-bred (Border Leicester (BL) and Merino (M)) lambs (* represents significant difference (P < 0.01) within genotype).

The only behaviour that exhibited a relationship with cortisol was the lambs attempt at standing (Table 2.13). ACTH and cortisol levels were positively correlated with one another (0.62 ± 0.08). Both ACTH and cortisol levels were also positively associated with BUN (0.39 ± 0.12 and 0.46 ± 0.12 respectively).

Table 2.13 Phenotypic correlations (± SE) between lamb pre-sucking plasma hormone concentration and peri-natal behaviour.*

	Time taken for lamb to perform behaviour				
	Bleat	Stand attempt	Stand	Suck attempt	Suck
Cortisol	-0.15 ± 0.17	***-0.44 ± 0.13***	-0.27 ± 0.16	-0.04 ± 0.18	0.03 ± 0.19
ACTH	-0.25 ± 0.16	-0.27 ± 0.15	-0.06 ± 0.17	0.05 ± 0.18	0.14 ± 0.19
Ghrelin	-0.18 ± 0.15	-0.05 ± 0.16	-0.20 ± 0.17	***-0.46 ± 0.14***	-0.33 ± 0.16
Leptin	-0.14 ± 0.21	0.00 ± 0.21	0.17 ± 0.23	***-0.47 ± 0.18***	-0.10 ± 0.22

*Significant correlations (ie. value greater than two times the standard error) are shown in bold and italics.

Ghrelin levels were unaffected by the fixed effects examined, but displayed negative relationships with feeding behaviours (Table 2.13). Ghrelin concentrations were also correlated with glucose concentrations (0.45 ± 0.14). There was a tendency for pre-suckling leptin levels to be affected by type of birth with singles (0.98 ± 0.05) displaying higher circulating plasma levels than twins (0.81 ± 0.05; P = 0.10). Increased leptin levels were associated with decreased cortisol and ACTH levels (correlations -0.35 ± 0.13 and -0.43 ± 0.14 respectively). The only behaviour that was correlated with circulating leptin was attempt to suck (Table 2.13).

Discussion

The results presented from this investigation suggest that improved metabolic maturity in the neonate is typified by a reduction in gestation length which is also associated with increased plasma creatinine levels, and increased fat mobilisation following birth as identified by increased pre-suckling plasma NEFA concentrations. Increased plasma leptin and ghrelin levels also appear to aid in the definition of maturity. Lambs with heightened concentrations of these metabolites and hormones are indeed quicker to progress to sucking following birth, suggesting that metabolic maturity and post-natal behaviour in the lamb are related.

Gestation length

Labour initiation has been shown to be under the control of the adrenal cortex in the fetus (Bassett and Thornburn, 1969) and so it is the lamb rather than the ewe that determines gestation length in sheep (Dwyer et al., 1996). This would suggest that fetuses with a fast-tracked development of the hypothalamic-pituitary-adrenal (HPA) system would display a reduction in gestation length. The finding that multiple born lambs experience a longer gestation when adjusted for birth weight agrees with previous investigations (Brown, 2007, Fogarty et al., 2005) and implies that these animals are less developmentally mature than singleton born lambs. The pre-partum cortisol surge has previously been shown to be delayed when measured in individual twin lambs when compared to singletons (Edwards and McMillen, 2002). Multiple born lambs experience *in utero* growth restriction (Dwyer et al., 2005) even during the initial stages of fetal development which disrupts the development of the HPA axis (Edwards and McMillen, 2002). Parturition is observed to occur sooner for twin born lambs unadjusted for weight as cumulatively, twin lambs produce significantly higher levels of cortisol than singletons initiating labour earlier in gestation. The age of the dam has been shown to exert influences on gestation length, with lambs from ewes of intermediate age experiencing a reduced gestation than those from both younger and older ewes (Forbes, 1967). This reduction of gestation length is commonly explained by an increased placental efficiency and fetal growth trajectory (Dwyer et al., 1996) and may also indicate an increased metabolic maturity in the lamb. Whilst lambs from three (cross-bred) and four (pure-bred) year old ewes were shown to display this reduction in gestation length compared with older ewes, there were insufficient numbers in younger age groups to draw conclusions similar to those reported previously. Both breed differences in pure-breds (Glimp, 1971) and sire breed differences in cross-breds (Fogarty et al., 2005) in gestation length have been identified and agree with the present findings. Whilst sex exerted no influence on gestation length, type of birth, age of dam and lamb breed were all

determinants of gestation length, and these effects may be explained by fetal nutrition and development, and thus maturation of the HPA axis.

Ponderal index

Ponderal index (PI), a measure of a neonate's weight for its height, is a reasonable predictor of intra-uterine growth restriction (IUGR) in human infants (Fay et al., 1991). In sheep, PI has been shown to be positively correlated (r = 0.53) with placental weight (Dwyer et al., 2005) which implies it may also be a good predictor of growth restriction in lambs. The observed reduction in PI with increased gestation length suggests that animals with increased gestation have experienced IUGR. This agrees with the explanations offered above that conclude growth restriction alters the HPA axis resulting in a delay in parturition (Edwards and McMillen, 2002). Interestingly, PI did not differ between singleton and multiple born lambs in the present study which means there is little evidence of growth restriction in twin and triplets or PI was a poor predictor. One of the few investigations into PI in lambs did identify that an increased litter size results in a decreased PI (Dwyer et al., 2005). The disparity between these results and those obtained in the present study are difficult to explain, but may be due to absence of early parity ewes in the present study which would exacerbate IUGR in multiple born lambs (Dwyer et al., 2005). The breed differences in PI observed are consistent with the identified differences in gestation length; that is breeds with a shorter gestation length also exhibit higher PI. Both the decreased gestation length and increased PI in the BL lambs (cross-bred and pure-bred) suggests that this breed may experience an increased maturation of the HPA axis associated with reduced IUGR.

Parturition length

The length of parturition experienced by the lamb impacts on survival both directly, through dystocia, as well as indirectly, through secondary effects of hypoxic damage to the central nervous system (Haughey, 1980). The positive relationship between birth weight and length of parturition observed in the pure-bred lambs has been reported previously (Dwyer, 2003), however the same effect was not seen in the cross-bred lambs. This may be explained by a reduction in the variation of birth weight in these cross-breds, which would decrease the likelihood of detecting any relationship with parturition length in the limited numbers of animals used in this investigation. The positive relationship between gestation length and parturition length observed in the cross-bred lambs independent of birth weight was an interesting finding. Previously, when effects have been shown to influence length of

parturition, the correct positioning of the fetus at the time of birth, or uterine behaviour, was offered as an explanation (Dwyer, 2003). This reasoning may also explain the present result. The findings discussed above suggest an increase in gestation length is indicative of lambs that have experienced IUGR, a delay in HPA axis activation and thus reduced physiologically maturity. This immaturity in the lambs may affect their ability to move themselves into an acceptable position that allows for a prompt delivery. Human IUGR fetuses have been shown to display less unstimulated and stimulated activity *in utero* throughout gestation when compared to those that experience normal growth (Vindla et al., 1999). The notion of effects on the uterine behaviour in fetal lambs is intriguing and warrants further investigation.

Significant breed differences in labour length were identified in the cross-bred lambs which were absent in the pure-breds. Whilst no statistical comparisons could be made between the pure-bred and cross-bred lambs due to confounding between genotype and year, it appears that the use of a PD sire increased the birth weight of the M lambs in the cross-breds relative to the pure-breds. This increase does not appear to be evident in the BL lambs. Ewe breed did not differ when the PD sires were used which would suggest ewe weight, and potentially pelvic size did not change (although not measured). Thus, the increase in birth weight with no corresponding increase in ewe size leading to a feto-pelvic disproportion may have contributed to this increased labour length observed in the cross-bred PDM lambs. Feto-pelvic disproportions have been identified in breed comparisons previously and used to explain differences in parturition length (Cloete et al., 1998).

Postnatal lamb behaviour

The only behaviour that was influenced by gestation length was time to bleat. This suggests that there is little direct effect of gestation length on postnatal lamb behaviour. However indirectly, in both previous findings (Dwyer et al., 1996) and those reported presently, breeds that experience a shorter gestation are quicker to progress behaviourally. In both the cross-bred and pure-bred lambs BL experienced a shorter gestation and were generally quicker to perform the recorded behaviours than their M counterparts. Thus within a breed, gestation length appears to have little influence on lamb behaviours, however across breeds, those with a reduced gestation display improved behavioural progression.

Two breeds of ewe were utilised in the current experiment in order to increase variation in lamb neonatal behavioural progression. However, this assumes that different breeds follow

the same behavioural patterns following birth and this may be untrue. One way to avoid such an assumption would have been to use a single breed, however this would have required significantly higher numbers of animals. Given the intensive nature of the investigation, large animal numbers would have been impractical. It has been demonstrated previously that M lambs are quicker to stand than BL lambs (Alexander et al., 1990). Similarly, M lambs have been shown to display a higher proportion of lambs standing after one hour when compared with BL's (Slee and Springbett, 1986). It is interesting to note however that both of these previous investigations did identify that BL outperformed M in other behavioural aspects (ability to follow ewe (Alexander et al., 1990) and proportion of lambs to reach the udder in one hour (Slee and Springbett, 1986)) which agree with the present findings that BL lambs tended to be at a behavioural advantage when compared with M in both the pure-bred and cross-bred analysis. The only behaviour M lambs tended to perform quicker was first stand attempt, and this was most likely explained by the fact that M lambs were lighter than BL. In the across breed analysis also, birth weight was shown to have a significant positive effect on first stand attempt. This may be explained by the birth weight effect on parturition length. If lighter lambs experience a shorter parturition, birth trauma would be reduced and thus lighter lambs may exhibit faster initial behavioural progression. Similar results have been reported previously, with birthing difficulty being associated with a delay in head shaking and ability to attempt to stand (Dwyer et al., 1996). Additionally, birth difficulty has been shown to explain a higher proportion of the variance in initial behaviour than those performed later (Dwyer, 2003). All other behaviours for which the effect of birth weight was significant were negatively (favourably) influenced, that is, heavier lambs were quicker to stand and suck. This is consistent with previous findings and reflects the increased energy availability and thermoregulatory ability in these heavier lambs (Dwyer, 2003, Owens et al., 1985).

One of the biggest limiting factors of this experimental design was the confounding between lamb breed and year caused by the inclusion of Poll Dorset sires in one of the three experimental years. The obvious ways in which this could have been avoided were to have only used the pure-bred lambs, or to have included the cross-bred genotypes across all years. Whilst a direct statistical comparison between the cross-bred and pure-bred lambs could not be made in this experimental design, generally cross-breds were quicker to perform almost all of the behaviours examined. There is little published evidence that heterosis influences peri-natal lamb behaviour, with the only investigation identifying that pure-bred Texel lambs required increased sucking assistance when compared with cross-bred Mule x Texel (Dwyer and Bünger, 2012). When measured at an older age, heterosis also appears to increase vigour, with cross-bred weaner lambs being more vocal and active in tests used to determine emotional reactivity than pure-breds (Boissy et al., 2005). Cross-

bred lambs experience decreased peri-natal and post-natal mortality when compared with pure-breds (Fogarty, 2000) suggesting that heterosis also increases lamb survival. In fact, lamb survival has been shown to be increased by 7% through direct heterosis (Mortimer and Atkins, 1997). The effect of heterosis on lamb behaviour and vigour as a means of explaining this observed increase in survival should be explored further.

All of the behaviours recorded in this investigation exhibited a moderate to strong correlation with one another. That is, if a lamb was quick to perform a given behaviour, generally it was also quick to perform other behaviours. The strength of the reported relationships is unique as previous reports have identified little to no correlations between postnatal behaviours. In Merino lambs, no relationship could be established between time from birth to standing and time from standing to drinking (Arnold and Morgan, 1975). Similarly, correlations were not significant between the interval from expulsion to standing and standing to sucking for Finnsheep and Suffolk breeds, but a moderate correlation (0.48) was reported for Romanov lambs (Fahmy et al., 1997). The disagreement between the present study and the findings reported previously may be explained by the definition of behavioural traits. Behaviours in the present investigation were defined from birth, whilst others report length from last recorded behaviour. Additionally, the present analysis was conducted across breeds whilst previous reported were only analysed within breed. Interestingly, the association between any two behaviours was stronger when the behaviours were closer in the expected progressive order. For example, the relationship between time taken to perform a suck attempt and suck was stronger than the relationship between time taken to bleat and suck. This would largely be due to the increase in the level of variation for every behavioural measure between the two traits of interest. These findings suggest that postnatal behaviours are strongly related to one another in across-breed analysis, and that these relationships are stronger when the behaviours are closer in progressive order.

It should be mentioned that ewes in this investigation had no experience with indoor lambing as all had previously reared lambs under extensive conditions and this may have impacted on the lamb behaviours under investigation. Indoor lambing results in a much higher stocking density than observed under paddock conditions, and an increased stocking density has shown to increase the occurrence of ewe-lamb separation caused by interference by other peri-parturient ewes (Winfield, 1970). This separation could increase the time the lamb takes to reach the behavioural milestones recorded. Conversely, providing a more sheltered environment would reduce the risk of hypothermia in the lambs as protection from rain and wind would limit wind chill. Hypothermia has been shown to reduce lamb vigour after birth (Alexander and Williams, 1966, Slee and Springbett, 1986), which may have improved the

timed lamb behaviours measured. Regardless, the time taken for the lambs born into shed conditions in the present investigation to reach the udder and feed roughly agree with those reported under extensive conditions (Slee and Springbett, 1986).

Lamb vigour after birth

The ability to subjectively quantify lamb vigour is of great importance as post-natal behaviours are extremely difficult to measure. The score used in the present investigation was easily allocated (the lamb could only receive one of five possible scores) and the lamb could be recorded for vigour within 12 hours of birth at tagging. These factors make this vigour score attractive as it does not require complex, time consuming measures of lamb behaviour. However, recording such a score is meaningless if relationships with quantitative measures of vigour cannot be established. The subjective vigour score used in the current investigation was increased in heavier lambs, which is consistent with the intensive behavioural measures recorded and reported above. Female lambs were allocated a superior vigour score than males, which once again agrees with the present behavioural results, in addition to those reported previously (Dwyer, 2003, Dwyer et al., 2005). Most importantly, significant positive relationships were observed between postnatal lamb behaviour and the subjective lamb vigour score. Thus, lambs that were slower to progress after birth were allocated a poorer vigour score. These results suggest both indirectly and directly that the lamb vigour score utilised in the present investigation is related to initial behavioural progression, and this verifies the use of such a score to subjectively quantify lamb vigour. Previous analysis has identified that there is a genetic component to this score (h^2 = 0.16) and although standard errors were high, a negative genetic association between this score and lamb survival was identified (Brien et al., 2010). The fact that this subjective vigour score is heritable, related to postnatal behaviour and genetically linked to lamb survival is of interest and suggests it may be of benefit as a means to select for reduced lamb mortality rates.

Blood metabolites in the neonatal lamb

The result that lamb circulating blood glucose concentration measured shortly after birth was positively associated with gestation length was interesting and unexpected. It was originally hypothesised that physiologically more mature lambs (those that experienced a reduced gestation length, as explained above) would be better able to initiate glycogenesis and gluconeogenesis, thus display increased circulating glucose concentrations after birth,

however the reverse was identified. Experimentally-induced growth restriction via caruncle-ectomy has previously been shown to increase circulating glucose concentrations in the newborn lamb (Mellor and Pearson, 1977). This was explained by hypoxaemic effects on catecholamine production as hypoxia has been shown to result in increased plasma epinephrine in fetal lambs (Cohen et al., 1982). In the current investigation, gestation length exerted a positive influence on birth weight, which subsequently increased length of labour. Thus, the increase in parturition length in heavier lambs may have led to hypoxia, increasing catecholamine levels (although not measured) and subsequently glucose concentrations.

Whilst the definition of maturity includes an increased reliance on glucose, there is little evidence to suggest that glucose levels at birth are indicative of metabolic maturity in the neonate. In the present investigation, no relationship could be established between blood glucose concentration and any of the behaviours recorded, consistent with previous findings that investigated postnatal vigour (Miller et al., 2009a). In a growth restriction model of maturity in the lamb, no difference in circulating glucose concentrations was identified between small and large lambs (Greenwood et al., 2002). Similarly, no difference in glucose measured at birth was witnessed between term and pre-term dairy cattle, however preterm calves were unable to maintain glucose concentrations when fasted over the first 24 hours of life (Steinhoff-Wagner et al., 2011). Combined, these results suggest that glucose level at birth alone is not a suitable marker for neonatal maturity. In order to identify the maturity of glucose metabolism and its influence on postnatal behaviour, circulating glucose concentrations over time or hormonal regulation of glycogenolysis and gluconeogenesis should have been targeted. Future investigations should explore the relationships between epinephrine, glucagon and insulin and lamb vigour. The conclusion that birth glucose concentrations are not indicative of maturity may also help to explain the inconsistency in reported links between glucose at birth and lamb survival, (Duyne et al., 1960, Miller et al., 2009a, Thompson et al., 2006) and why fatty acid concentration may provide an improved marker for maturity and survival (Duyne et al., 1960).

Feeding behaviours were negatively associated with NEFA concentrations at birth, thus lambs that were quicker to progress behaviourally exhibited elevated NEFA levels. If behavioural progression is indicative of a lamb's physiological maturity, this result supports others. Pre-term calves have been shown to exhibit reduced NEFA concentrations when compared to those born at full term (Steinhoff-Wagner et al., 2011). The elevated fatty acid concentration witnessed both in the present findings and elsewhere is most likely due to an increased ability to hydrolyse triglycerides, as was observed in pre-term human infants (Behrman et al., 1976). With regard to survival, lambs from a selection line that experienced

increased survival rates were shown to have elevated NEFA concentrations when measured 60 minutes after birth (Thompson et al., 2006). These findings are in agreement with the original definition of maturity that states the initiation of fat metabolism is crucial for successful adaptation to postnatal life. Why fatty acid concentration is a better marker for maturity than glucose is interesting as the release of both metabolites is regulated via the sympathetic nervous system by catecholamines, glucagon and insulin. Duyne (1960), when reporting similar findings, offered the explanation that as the effect of epinephrine on glucose is far greater than that on free fatty acids, nor-epinephrine is more likely to be responsible for the elevated free fatty acid concentrations observed after birth. However, investigations in human neonates suggest the sensitivity of lipolysis to nor-epinephrine is limited initially and increases over time (Wolf et al., 1974). Perhaps a more logical explanation involves the timing of blood sample collection and maternal energy supply. Glucose and amino acids are known to readily cross the placenta from dam to fetus (Herrera and Amusquivar, 2000). In the current investigation blood samples were collected from lambs between standing and sucking and this occurred within 30 minutes of birth. Therefore, glucose concentrations at this time may represent maternal supply as well as other glucose homeostasis mechanisms within the lamb. Perhaps glucose homeostasis should have been monitored over a longer period of time following birth. Fatty acids, on the other hand, do not cross the placenta as readily (Herrera and Amusquivar, 2000), and as such circulating concentrations may be more indicative of neonatal metabolism since the maternal effect is lessened. The present results and those reported previously highlight the importance of NEFA in defining maturity in the neonate, which in turn shows associations with postnatal lamb vigour and survival.

The moderate negative relationship between gestation length and BUN concentration was unanticipated. The definition of maturity includes a reduction in reliance on protein sources after birth. Thus by our reasoning, more mature individuals should have experienced a reduced gestation length and a reduction in BUN concentrations. Instead, lambs with reduced BUN levels were those born with longer gestation lengths. Additionally, correlations between BUN and postnatal behaviour were inconsistent, with initial behaviours being performed quicker by lambs with highest BUN concentrations, but little to no relationships identified with subsequent sucking behaviour. Supporting these inconsistencies are the findings of Steinhoff-Wagner et al. (2011) who failed to identify any difference in urea level between pre-term and full-term calves. However, they did report an increase in BUN in the pre-term calves on day two suggesting these immature neonates had failed to shift to carbohydrate and fat metabolism at this later stage. A growth-restriction model of immaturity in lambs did identify reduced urea levels in low birth weight lambs, however sampling occurred two hours after birth (Greenwood et al., 2002). This would suggest that pre-

suckling (within 30 minutes of birth) analysis of BUN does not allow the lamb adequate time to perform the shift in energy homeostasis that defines maturity. Thus, future investigations into metabolic maturity in the lamb should sample for protein metabolism over longer time periods relative to birth.

Creatinine levels are elevated at birth as the neonate is born with leaky tubular and vascular structures of the kidney (Matos et al., 1998), thus creatinine is re-absorbed into the blood rather than being excreted (Guignard and Drukker, 1999). In humans, creatinine levels are higher in pre-term than term infants and this is commonly explained by a reduced maturation of the renal system (Finney et al., 2000). Our results support those reported in human neonates as creatinine levels were highest in lambs with a reduced gestation length. The associations between creatinine levels at birth and behavioural progression were varied, but generally, increased creatinine levels were found in lambs that were quicker to perform postnatal behaviours. This supports our hypothesis that lambs born earlier in gestation are more developmentally mature and display advanced behavioural response. Thus, the increased creatinine levels observed in lambs that were quicker to perform sucking behaviours are largely explained by the reduction in gestation length. This finding suggests that creatinine levels at birth may indirectly indicate maturity in the neonate through the influence of gestation length on maturity. Previous investigations have identified that the renal system of the fetal lamb is able to perform basic functional capabilities after day 70 of gestation (Berry et al., 1995) thus lambs with increased creatinine levels at birth may not be at a physiological disadvantage. Combined, these findings imply creatinine level is a strong predictor of gestation length, and through this association with gestation length, creatinine concentrations at birth are a suitable indicator or metabolic maturity in the neonatal lamb.

Plasma hormone levels in the neonatal lamb

A delay in the maturation of the HPA axis has previously been used to define maturity due to the large range of processes under its control important for the transition from fetal to neonatal life (maturation of organs, initiation of labour, influence on energy metabolism). In foals defined as being immature, ACTH levels have been shown to be elevated and cortisol levels reduced which suggests a reduction in the responsiveness of the adrenal cortex (Rossdale et al., 1984). Similarly, preterm calves were shown to display increased cortisol concentrations at birth when compared to those born at term (Steinhoff-Wagner et al., 2011). Both ACTH and cortisol were measured in the current investigation with the hope of identifying this previously reported shift in HPA axis responsiveness. However, little to no

relationship between either hormone and any lamb behaviour observed was established. The failure to identify a HPA axis shifts in immature neonates is not unique in lambs, as corticosteroid concentration has been shown not to differ after growth restriction (Mellor and Pearson, 1977) and in a breed (Suffolk) that has previously been defined as immature at birth (Dwyer and Morgan, 2006). This would suggest that circulating concentrations of ACTH and cortisol alone are not indicative of maturity in the lamb, and perhaps an imposed challenge that tests the responsiveness of the HPA axis is required. Again in foals it was shown that an ACTH challenge resulted in a reduced response in premature foals when compared with those that experienced a full gestation (Silver et al., 1984). Whilst it is accepted that immature neonates display a reduced sensitivity of the adrenal cortex to ACTH release resulting in reduced cortisol levels, measuring these two hormones alone does not appear to add to the definition of maturity in the lamb. Future investigations should concentrate on identifying if differences in HPA axis responsiveness to challenge exist between those that differ in maturity.

Ghrelin is a stimulator of growth-hormone (Kojima et al., 1999) and has been linked to gestational age (Farquhar et al., 2003), and birth weight and length (Kitamura et al., 2003) in human infants. Despite these previous findings, no relationship could be established between gestation length, birth weight or litter size in the present study, which is in agreement with Miller *et al.* (2009). Combined, these results suggest ghrelin may have less of a role in growth promotion in the lamb but may be of greater significance in preparing the fetus for the extra-uterine environment. Miller et al. (2009) suggested the importance of investigating the relationship between ghrelin and cortisol concentrations in the neonate to determine its role in fetal maturation, parturition and thermoregulation. However, no significant correlation between the two hormones was found in the present investigation. There was a positive association identified with glucose levels, potentially indicating its role in energy metabolism in the neonate. Ghrelin was targeted in the present investigation as it has previously been linked to lamb survival, with increased neonatal ghrelin concentrations tending to be associated with improved survival to 72 hours (Miller et al., 2009a). This study failed to link ghrelin concentrations with most postnatal behaviours which contrasts those reported presently. Negative associations between feeding behaviour and pre-sucking ghrelin concentrations identified may reflect ghrelin's role in appetite regulation. In neonatal rats, fasting has been shown to decrease ghrelin levels in the gut but increase them in plasma and the authors concluded that ghrelin in the blood stimulates appetite in order to increase milk intake (Hayashida et al., 2002). Thus, lambs that displayed elevated ghrelin levels at birth may have experienced a greater sucking drive, were quicker to reach the udder and achieve enteral feeding.

Leptin is a fat signalling hormone that reduces appetite and increases energy expenditure, may be involved in organ maturation in late gestation (Henson and Castracane, 2006), and has been shown to be influenced by gestational age, birth weight and sex in human infants (Yokota, 2003). In the present investigation none of these factors were shown to display a significant relationship with leptin, suggesting leptin levels are not influenced by the same effects in neonatal lambs as in humans. This disparity may be explained by the differing metabolic requirements between human infants and lambs. One of the biggest post-natal challenges inflicted on the lamb is thermoregulation and there is some evidence to suggest that leptin is involved in thermogenesis. Treating lambs with exogenous leptin has been shown to increase colonic temperature when compared with vehicle treated controls (Mostyn et al., 2000). Thus, in the lamb, circulating leptin levels may relate to thermogenic capacity. The only behaviour to exhibit an association with leptin was time taken for the lamb to perform a suck attempt. Interestingly, this was the only behaviour reported to be related to leptin concentrations in a previous investigation (Miller et al., 2009a). The authors explained the impact of leptin on this one behaviour as an indication of increased energy reserves leading to increased thermoregulatory ability rather than increased suckling drive, as time to suck remained uninfluenced. However, increased energy levels and ability to thermoregulate should, in theory, influence all postnatal behaviours. Why this is the only behaviour that shows any relationship with leptin is perplexing and should be investigated further. A single behaviour was shown to exhibit a relationship with pre-suckling leptin levels, but perhaps of greater importance is the link between leptin and thermogenesis in the neonatal lamb and this is explored in a subsequent chapter.

Whilst not established in the present investigation, elevated levels of both leptin and ghrelin have previously been identified in lambs born with shorter gestation lengths (Miller et al., 2009a). The authors suggest that this increases in leptin and ghrelin is indicative of an increased in metabolic and endocrine maturation and this agrees with the present notion that lambs experiencing a shorter gestation are physiologically more mature at birth. Why no relationship could be established between these two hormones and gestation length in the present investigation is most likely explained by the scale of the experiment. Standard errors on correlations with gestation length were high, thus larger investigations examining the relationship between leptin and ghrelin and gestation length are essential to confirm previous findings.

Conclusion

Using behavioural progression as a model of metabolic maturity in the neonatal lamb appears valid as hormonal and metabolic differences were identified in those animals that differed in vigour following birth. From this investigation it can be concluded that length of gestation may be related to metabolic maturity at birth, with lambs experiencing a fast-tracked fetal maturation experiencing a reduced gestation length. Plasma creatinine levels are an adequate predictor of gestation length, thus indirectly fetal maturation, and show links with lamb behaviour. These lambs with improved maturity at birth are typified by a shift in energy regulation, whereby fat metabolism is of increased importance, as indicated by elevated levels of plasma NEFA in lambs that were quicker to suck following birth. Levels of glucose and BUN during this initial stage do not appear to add to the definition of metabolic maturity in the lamb. Results from others suggest that a reduction in the sensitivity of the adrenal cortex is observed in immature neonates as shown by an increase in ACTH levels but a decrease in cortisol, however this was not observed in the lamb. Pre-suckling plasma ghrelin and leptin concentrations are related to feeding behaviours in the lamb and may also be implicated in thermoregulation, but further investigations are warranted into these hormonal markers of maturity.

Chapter Three: Thermoregulation in the newborn lamb and links with peri-natal behaviour and metabolism

Introduction

The starvation/mismothering/exposure (SME) complex is often cited as being one of the leading causes of lamb mortality (Cloete et al., 1993, Cloete and Scholtz, 1998, Haughey, 1991). However, the proportion of mortality attributed directly to exposure is highly variable and can range from less than two to over 90% (reviewed by Hinch, 2008). This variability is most likely due to environmental conditions, largely weather which is comprised of temperature, precipitation and wind velocity. However, other effects such as the integrated nature of the three causes (starvation, exposure and mismothering), the environmental effects outside of weather such as the maternal environment, and the lamb's ability to thermoregulate directly, may also be implicated. To reduce mortality from exposure, thermogenesis and factors that influence a lamb's ability to thermoregulate need to be understood. Consequently, this review considers the mechanisms operating to achieve thermoregulation in the newborn lamb. Factors that may influence heat production in the neonatal lamb are highlighted, with a focus on cold resistance.

Defining mortality by exposure

'Exposure' is termed lethal hypothermia with minimal depletion of body reserves whilst 'starvation' is defined as the exhaustion of body reserves in the absence of hypothermia (McCutcheon et al., 1981). Despite this distinction, there is a strong likelihood that both are physiologically linked (Figure 3.1). For example, one consequence of starvation in the lamb is a decrease in its ability to maintain a high metabolic rate, making it more susceptible to hypothermia (Dwyer, 2008, McCutcheon et al., 1981). Similarly, cooling suppresses some body functions and, as such, chilled lambs may be deterred from sucking (Alexander and McCance, 1958, Dwyer, 2008).

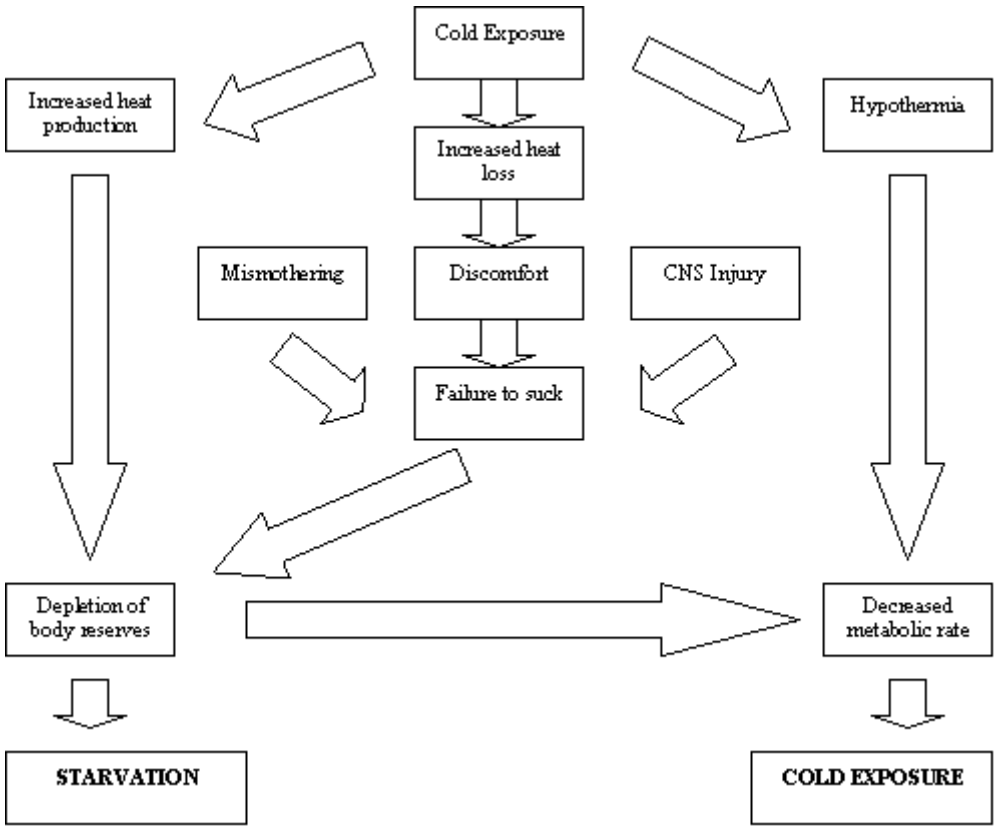

Figure 3.1 Interactions between starvation and exposure in the newborn lamb (McCutcheon et al., 1981).

There are two prominent periods when lambs are most susceptible to hypothermia. From birth to five hours post-partum, excessive heat loss contributes to the condition, whilst from 12 to 36 hours post-partum, a depletion of energy reserves depresses heat production increasing the risk of hypothermia (Eales et al., 1982). Therefore, for a lamb to be viable it must be able to maintain its body temperature immediately after birth and possess sufficient energy reserves to continue this maintenance (Alexander and McCance, 1958, Stott and Slee, 1985). Lambs are born with body reserves that are able to sustain life for approximately three to five days, a period which is reduced in cold conditions (McCutcheon et al., 1981). This explains why most deaths from starvation/exposure occur in the peri-natal period.

Determining a discrete cause of death (starvation, mismothering or exposure) is difficult due to the interactions described above. As a result, early investigations into causes of mortality often grouped starvation, mismothering, and exposure into what was termed the 'SME complex'. Probable events leading to this diagnosis included prenatal impairment, poor maternal-offspring behaviour, management-induced mismothering, misadventure, poor milk

supply, teat and udder abnormalities, and cold-induced starvation (Haughey, 1991). The cause was assigned if the lamb had been born alive, and there was no clear evidence of predation or disease. The SME classification is still used on-farm, however experimentally, autopsy has allowed for a more specific diagnosis of mortality. The following information is used to diagnose mortality from exposure using autopsy. Failure to feed will result in decreased energy availability for thermoregulation leading to secondary hypothermia; however primary hypothermia (exposure) occurs in lambs when milk is present in the abomasum (Haughey, 1991). This can be both in the presence and absence of complete brown fat metabolism. Additionally, lambs dying from exposure will present with yellow, subcutaneous oedema at the hind leg (Holst, 2004).

Thermogenesis in the neonate

Thermogenesis is the production of heat and is of great importance in lambs at risk of exposure. The ability to maintain body temperature is a major determinant of lamb survival (Alexander and McCance, 1958, Stott and Slee, 1985). A strong contrast in ambient temperatures is seen around the time of birth, with post-partum temperatures being significantly colder than those experienced *in utero*. In most species, a resistance to cooling can take time to develop, however sheep are thought to have a well-developed thermoregulatory mechanism at the time of birth (Alexander and McCance, 1958), with lambs being able to maintain a rectal temperature within the normal range in ambient temperatures of 0°C and below (Alexander, 1961b). The only improvement with age appears to be in the growth of fleece and a reduction in the surface area to volume ratio.

Immediately after birth, the rectal temperature of a lamb is either equal to or greater than its dam, which may be explained by either a higher maternal uterine than rectal temperature, a higher fetal metabolic rate or an increased heat production within the lamb whilst temperature homeostasis develops (Alexander and McCance, 1958). The temperature then declines to between 32 - 35 °C (Alexander and McCance, 1958), at which point the lamb may have to increase its heat production by up to 15 times to compensate for heat lost to the environment (McCutcheon et al., 1981). Subsequently, the temperature rises at a steady rate to 38 - 41°C within several hours of birth (Alexander and McCance, 1958). Some lambs, however, do not recover from the postpartum decrease in temperature and may continue to decrease as much as 11°C in 30 minutes (Alexander and McCance, 1958). This occurs when the maximal sustainable metabolic rate is surpassed by the rate at which heat is being lost to the environment, causing a decrease in deep body temperature (McCutcheon et al.,

1981). Generally, when a lamb's deep body temperature falls below 30ºC, hypothermia becomes too difficult to overcome and death is probable (McCutcheon et al., 1981).

Summit Metabolic Rate

Cold-induced summit metabolic rate (SMR) can be defined as the highest level of heat production obtainable at normal body temperature, without voluntary muscle activity (Alexander, 1962c). An estimate of summit metabolism can be made by measuring average heat production during exposure to conditions that result in a small, controllable fall in rectal temperature over a 20 minute period. Average summit metabolism in lambs has been estimated at approximately 70 kJ kg^{-1} hr^{-1} using this method (Alexander, 1962c). At rectal temperatures below 36ºC, summit metabolism is directly proportional to rectal temperature. Above 36°C however, SMR falls below expected rates meaning that at 'normal' temperatures, summit metabolism is unpredictable (Alexander, 1962c). SMR does not increase with age or nutritional status, but rather has a tendency to decrease as the lamb gets older and is independent of pre-natal nutrition, litter size and birthcoat (Alexander, 1962c). Half of SMR is achieved from shivering whilst the other half is derived from non-shivering thermogenesis (Slee et al., 1987, Stott and Slee, 1985)

Non-Shivering Thermogenesis

Non-shivering thermogenesis (NST) is a facultative method of rapid heat production in response to cold conditions (and over-feeding) (Bianco and Silva, 1987a). In the new-born lamb, NST is essential for maintaining body temperature after birth. The percentage contribution of NST to total thermogenesis is approximately 50% at birth, but decreases in lambs with age, so that by 32 days of age it makes no contribution (Gemmel et al., 1972). Brown adipose tissue (BAT) was traditionally thought to be the site of action and main energy reserve for NST for two reasons. Firstly, the decline in NST with age occurs at the same rate as the degradation of BAT, and secondly because an increase in deep body temperature around particular organs is apparent when the lamb is exposed to cold which corresponds in location to the position of BAT (Alexander et al., 1970). It is now commonly accepted that NST occurs in this tissue.

Brown Adipose Tissue

Metabolic studies have shown that energy production from protein utilisation is not sufficient to maintain life and it was suggested that the control of protein as an energy source is not developed in the lamb until three days post-partum (Alexander, 1962a). Additionally, this study demonstrated that glycogen and fat reserves were depleted in starved lambs suggesting that carbohydrates and lipids are the main fuel source for metabolism (Alexander, 1962a). Estimates of respiration quotient (R.Q.) indicate that the energy source utilised for the high metabolic rates of neonatal lambs after birth is mainly fat, which accounts for approximately 2 - 3% of total body weight at birth (Alexander, 1962b, Alexander, 1962a).

BAT is the principle site of NST in the newborn lamb (Alexander and Williams, 1968) and is primarily found in the thorax region of other species (Cannon et al., 1977), but is more commonly found in the peri-renal areas of the lamb (Gemmel et al., 1972). Not only does this tissue contribute to heat production, but it also acts as a major energy reserve for other metabolic processes (Stott and Slee, 1985), making up a significant proportion of the 17-42 x 10^5 joules reserves found in new born lambs (Alexander, 1962b). Levels of BAT can differ between animals (8 - 24 g) and can also differ in triglyceride content (0.40 - 0.80 mg) (Cannon et al., 1977). BAT is brown in colour due to its high cytochrome *c* content, and contains many densely-packed mitochondria and smaller fat vacuoles (Hahn and Novak, 1975). The mitochondria found in this tissue differ from that found in white adipose tissue as the cristae (inner membranes of the mitochondria) are more numerous in number and more tightly packed (Hahn and Novak, 1975). These mitochondria are situated closely to the fat vacuoles, a position well-suited for rapid oxidation of fat (Hull, 1966). The tissue has a high requirement for oxygen (Hull, 1966) which is maintained by its vast vascular network (Trayhurn, 1993). This network also acts to dissipate the heat created around the body to the organs.

Brown adipose tissue metabolism

Literature related to brown fat metabolism is voluminous (Himms-Hagen, 1985, Himms-Hagen, 1989, Nicholls, 1983, Nicholls and Locke, 1984, Smith et al., 2004) and is briefly summarised here. Heat production is a consequence of all metabolic reactions, however in BAT heat is the primary product and function of the tissue (Trayhurn, 1993). The mitochondria present in BAT are unique in that the proton conductance mechanism allows protons to run back along the gradient without producing ATP, thus becoming reversibly

uncoupled. This facilitates oxidation independent of the need to phosphorylate ADP (reviewed by Himms-Hagen 1985). The process allows the energy normally captured as ATP to be dissipated as heat. This mechanism is controlled by a 32 - kD protein known as un-coupling protein (UCP1), or thermogenin. UCP1 is unique to BAT and is found in the inner membrane of the mitochondria. UCP1 facilitates the return of electrons actively pumped from the cell by the electron transport chain, a process known as un-coupled oxidative phosphorylation. The levels of UCP1 determine the capacity of BAT for heat production.

BAT thermogenesis is controlled by the sympathetic nervous system (reviewed by Himms-Hagen 1985). When unstimulated, the action of UCP1 is blocked by cytosolic puride nucleotides, but when nor-epinephrine binds to β-adrenergic receptors located in the plasma membrane, a cascade of lipolysis follows, activating UCP1. This eventuates in mitochondrial respiration being uncoupled from ATP production resulting in the release of energy as heat (Cannon and Nedergaard, 2011). This and other endocrine control mechanisms of BAT metabolism will be explored in later paragraphs.

In vivo methods used to quantify thermogenesis in the lamb

In order to identify factors that influence thermogenesis, methods for quantifying thermogenic capacity in the lamb are required. Many *in vitro* methods can be employed to measure heat production in BAT and these include guanosine 5'-diphospate (GDP) binding and UCP1 levels (Himms-Hagen, 1985). However, such methods only quantify NST, and as mentioned above, shivering contributes approximately half of overall thermogenesis. Additionally, as these methods are performed *in vitro*, the animal must be sacrificed in order to collect tissue for analysis. Several methods for quantifying thermogenesis in live animals have been employed and are outlined below.

Nor-epinephrine challenge

As already mentioned, nor-epinephrine causes the activation of UCP1 in BAT, resulting in an increase in heat production from this tissue. Thus, measuring a lamb's response in oxygen consumption and rectal temperature after injection of nor-epinephrine has been used to estimate NST capacity. Early investigations into summit metabolic rate in the lamb utilised nor-epinephrine, in combination with drugs that cause muscle paralysis to ameliorate shivering in an attempt to quantify the contribution of NST to overall thermogenesis

(Alexander and Williams, 1968). This method has been used to characterise the decline in NST with age (Thompson and Jenkinson, 1969) and to identity other phenotypic and genetic factors important for NST in the lamb (Simpson and Slee, 1988, Slee and Simpson, 1991, Slee et al., 1987).

Climate chamber

The development of a chamber which allowed precise control over moisture, wind and temperature greatly facilitated studies of thermogenesis (Alexander, 1961a). The chamber was designed to mimic the wet, windy and cold conditions witnessed around the time of birth. Testing involved the lamb being dampened to simulate the fetal fluids present on the coat following birth. Wind was created in the chamber by a fan, with velocity controlled via a butterfly valve. The internal temperature of the chamber was maintained by a surrounding jacket filled with solution and was controlled by a thermostat which regulated the influx of either cold or warmed solution into the jacket dependent on the desired temperature. The chamber also contained a spirometer allowing for the estimation of oxygen consumption via indirect calorimetry. This climate chambers design and use was instrumental in characterising the lamb's metabolic response to environmental conditions (Alexander, 1961a, Alexander, 1961b, Alexander, 1962b, Alexander, 1962c, Alexander and McCance, 1958, Alexander and Williams, 1962),.

Cold challenge

To examine thermogenesis in the lamb, Slee (1981) suggested that a test be controllable, repeatable, economic, simple, and allow easy application to large numbers of animals. Initially, exposing the lambs to a constant water temperature (between 15°C and 20°C) was explored but the animal response proved to be too variable between animals and breeds thus was deemed unsuitable (Slee et al., 1980). Another method involved progressively reducing the temperature of a water bath in which the lamb was suitably restrained in a standing position with the water at neck level (Slee et al., 1980). Cold resistance was defined as the time taken for a lamb's rectal temperature to reach 35°C by cooling the water from 37°C to 12°C. Whilst this test was not as controlled and did not represent environmental conditions as accurately as the climate chamber described above, it was 95% repeatable within animals. Not only were breed differences apparent in the water bath test (Slee, 1981), but test results correlated strongly with field data on the frequency of hypothermia and rectal temperature one hour postpartum (Table 3.1).

Table 3.1 Breed differences in rectal temperature one hour after birth, percentage of lambs exhibiting hypothermia in the field, and water bath test results exhibiting the relationship between the three measures (Slee et al., 1980).

Breed	Rectal temp (ºC) 1 h after birth	Lambs hypothermic (%)	Water bath test (min)
Cheviot	39.6	9.1	98
Scottish Blackface	39.6	0.16	87
Boreray Blackface	39.5	0	55
Welsh	39.3	8.5	89
Oxford	39.2	0	79
Soay	39.2	14.8	36
Southdown	37.6	47.1	51
Border Leicester	37.4	36.4	80
Merino	34.7	68.8	45
Finnish Landrace	32.8	84.6	38

That thermogenesis in the neonatal lamb is under some genetic control is implied by the large breed differences in water bath test results, neonatal rectal temperatures and frequency of hypothermia. Selection for divergent water bath test performance also produced differences in sire lines for response to nor-epinephrine (Slee and Simpson, 1991), implying a genetic component to NST.

Factors that influence thermogenesis in the lamb

Heritability of cold resistance

The finding that cold resistance differs between breeds and sire lines implies that the trait is at least partially genetically-determined. Preliminary estimates in Scottish Blackface lambs indicated that cold resistance was approximately 30% heritable (Slee and Stott, 1986). A subsequent investigation in the Merino breed established that the heritability of cold resistance was significantly higher than that reported in the Scottish Blackface (0.70 ± 0.25) (Slee et al., 1991). This discrepancy in estimates would most likely be explained by differing environments in which these breeds are farmed. Subsequent to these quantitative genetic investigations, work in New Zealand has focused on molecular techniques aimed at elucidating the genetic basis of cold resistance.

Genetic markers

Adrenergic receptors are G-protein coupled receptors that specifically bind endogenous ligands (the catecholamines epinephrine and nor-epinephrine) resulting in lipolysis and the activation on UCP1 in BAT. Polymorphic variation at the β_3-adrenergic receptor (ADRB3) locus has been suggested as a gene marker for thermogenesis in lambs (Forrest et al., 2007). ADRB3 is primarily located on the surface of adipocytes and is responsible for the thermogenic effect of high catecholamine concentrations as receptor stimulation results in NST and lipolysis in brown and white adipose tissue (Forrest et al., 2007). From a total of eight alleles, two have been shown to reduce the risk of cold-related mortality, whilst three others tended to be associated with increased cold-related mortality (Forrest et al., 2007). Of the three alleles with increased risk, allele D demonstrated the strongest association. A sequence variation within exon-1 of this allele predicts two amino acid substitutions, occurring in positions considered to be involved in ligand binding, which may in turn affect receptor function (Forrest et al., 2007). Additional to cold resistance, ADRB3 has been shown to be linked to birth weight, growth rate and carcass composition, supporting the hypothesis that the receptor is implicated in energy homeostasis (Forrest et al., 2003). This gene marker is currently being trialled in larger populations across New Zealand, and may prove to be of importance in reducing lamb mortality through improvements in cold resistance.

Lamb phenotype

Several non-genetic aspects of lamb phenotype have been shown to be related to cold resistance as measured by the water bath test. Whilst lamb age at testing has been identified as influencing cold resistance (a small but significant decline in resistance was observed with increasing age) (Stott and Slee, 1987), other investigations have shown this not to be the case (Samson and Slee, 1981, Slee et al., 1991). Weather conditions on the day of testing appear to exert no influence on results (Slee et al., 1991), however day of testing, when ranging over months, was significant (Samson and Slee, 1981). Lamb sex (Samson and Slee, 1981) and age of dam (Slee et al., 1991) do not affect the trait. Interestingly, litter size has been shown to influence cold resistance (Samson and Slee, 1981) but this may be explained by differences in birth weight.

The phenotypic traits exerting the largest effects on cold resistance are those related to weight, coat and skin properties. Birth weight was shown to exert a positive influence on resistance to cooling in the lamb when measured across a range of breeds and explained

part of the variation in observed breed differences (Samson and Slee, 1981). It was presumed that the influence of birth weight on cold resistance could be explained by both a reduction in the surface area relative to mass in heavier lambs, and that these heavier lambs would have access to greater energy reserves. This effect of birth weight was also identified in the Merino breed, in which a positive phenotypic correlation with cold resistance (0.40 ± 0.06) was reported (Slee et al., 1991).

Birth coat has been shown to influence lamb survival in the field and it was stipulated that this was due to insulation from cold (Purser and Karam, 1967). Merino lambs display a significant phenotypic correlation between coat depth and cold resistance (0.24 ± 0.06) (Slee et al., 1991), however this was shown not be the case in an across breed analysis involving ten breeds of sheep (Samson and Slee, 1981). The latter study did report a significant positive relationship with wool sample weight and resistance to cooling in Blackface and Cheviot lambs. Lastly, coat grade (measured on a scale of one to five: fine to hairy) was also shown to influence cold resistance in a positive manor (0.20 ± 0.06) (Slee et al., 1991). These findings imply that coat properties may be of importance within breed, but across breed, are less important.

Skin thickness, measured across five sites on the lamb using calipers, has also been shown to influence cold resistance in within-breed analyses across a number of breeds (Samson and Slee, 1981) and in the Merino (Slee et al., 1991). Lambs with a greater skin thickness exhibit increased resistance to cooling and this is thought to be attributed to greater subcutaneous fat deposition. Whilst minimal in lambs (Alexander, 1978), variation in subcutaneous fat must exist and this would have implications for insulation from cold and available energy for increased metabolism. However, Slee et. al. (1991) argued that lambs have no subcutaneous fat, and thus the insulative properties are due to the blood vessels being located further from the temperatures external to the lamb. Even after adjustment for weight, skin and coat properties, significant breed differences persist (Samson and Slee, 1981) suggesting there are underlying physiological changes that allow some breeds to perform better under cold challenge.

Metabolic maturity at birth, behaviour and cold resistance

It has been argued that cold resistance may be an indicator trait for increased fitness or early lamb vigour (Slee et al., 1991), and if this is the case, lambs that display increased vigour and cold resistance may be more physiologically mature at birth. However, few

investigations have been aimed at linking these factors together in lambs. In an attempt to explain the thermogenic differences between two lines selected for or against cold resistance in lambs, NST was estimated by response to injection of nor-epinephrine (Slee et al., 1987). The authors concluded that whilst genetic variation in NST does exist, it does not explain divergences in cold resistance. This interesting finding implicates other metabolic processes in the lamb's ability to withstand cold. In another livestock species, calves have been shown to display variations in survival, maturity at birth (as measured by plasma hormone and metabolite concentration) and thermogenic response to nor-epinephrine challenge (Carstens et al., 1997) warranting research in this area in sheep. In order to determine if maturity may influence thermogenesis, the hormonal control of metabolism in this tissue must first be understood.

As with other physiological processes, there is a large discrepancy between thermoregulation *in utero* and that in post-natal life. Thermoregulation is minimal in the fetus, and in fact inhibition of this process predominates at this time (Sawa et al., 1991). At birth, the lamb experiences a rapid decline in ambient temperature and consequently the ability to maintain body temperature around this peri-natal period is of great importance. Since heat production is regulated through a number of metabolic processes, it is logical to suggest that differences in physiological maturity at birth may impact upon thermogenesis in the lamb. To produce heat through shivering, stored glycogen in muscle tissue must be released. Thus, the lamb's ability to thermoregulate through mechanical means is dependent on glycogenolysis (explored in a previous chapter). BAT also has a very high demand for glucose, with uptake stimulated by nor-epinephrine under cold stress (Cannon and Nedergaard, 2011). It could be assumed that the function of glucose in BAT is as a direct thermogenic energy source, but estimates of glucose as an oxidative substrate are small, suggesting that it may be of greater importance elsewhere. One suggested function of glucose in NST is not dissimilar to that witnessed in skeletal muscle during exercise. In the absence of sufficient levels of mitochondrial ATP in BAT, glycolysis produces ATP by substrate level phosphorylation, and the resulting lactate transported back to the liver for reconversion to glucose (Himms-Hagen, 1989). Glucose has also been implicated in lipogenesis (Himms-Hagen, 1989), and lipolysis (Cannon and Nedergaard, 2011). Whilst its exact role in the metabolic functioning of BAT remains to be elucidated, it is clear that the tissue utilises glucose. Consequently, insulin is involved in the regulation of BAT metabolism.

Fatty acids serve as the major substrate for oxidative phosphorylation in BAT and are also implicated in the uncoupling process as they interact directly with UCP1 (Girard et al., 1992).

Lipid regulation in BAT is mediated predominantly by nor-epinephrine (Girard et al., 1992) through the sympathetic nervous system (Duyne et al., 1960), and occurs directly through secretions from the sympathetic nerves rather than circulating catecholamine's as higher blood infusion levels of nor-epinephrine are required in order to stimulate NST than that observed during cold exposure (Himms-Hagen, 1984). Nor-epinephrine interactions with adrenergic receptors, most importantly β-adrenergic receptors, result in a number of metabolic events which eventuate in increased lipolysis and fatty acid oxidation (Himms-Hagen, 1985) and reduced lipogenesis (Himms-Hagen, 1989) leading to the production of heat. Specifically, after nor-epinephrine is released via the sympathetic nervous system and binds to adrenergic receptors on brown adipocytes, adenylate cylase is activated, increasing levels of cytosolic cAMP. This in turn activates protein kinases resulting in the release from triglycerides of free fatty acids which are the substrate for uncoupled oxidation and thus thermogenesis (Cannon and Nedergaard, 2004).

At birth, the neonate experiences a surge in thyroid stimulating hormone (TSH) levels caused by umbilical cord cutting (Sack et al., 1976). This results in increases in both triiodothyronine (T_3) concentrations (Erenberg et al., 1974) and NST in BAT. The interactions between thyroid hormones and thermogenesis are complex (Girard et al., 1992), however the identification of the type II iodothyronine 5'deiodinase enzyme in BAT and its role in thyroid hormone conversion appears to be of great importance. As reviewed by Silva (1995), increases in cold exposure increase sympathetic activity, subsequently increasing heat production in BAT. Declines in ambient temperature also result an increase in thyroid hormone concentrations which act to increase overall metabolism. The type II iodothyronine 5'deiodinase enzyme found in BAT tissue is responsible for the conversion of thyroxine (T_4) to T_3, the more metabolically-active of the two thyroid hormones. Both increases of nor-epinephrine and cold exposure have been shown to stimulate the activity of type II iodothyronine 5'deiodinase enzyme (Silva and Larsen, 1983), increasing the conversion of T_4 to T_3 and subsequently, metabolic rate. Whilst an overall increase in metabolic rate would increase metabolism in BAT and thus NST, what is less clear is the direct effect of the increased levels of T_3 in BAT (Silva, 1995). BAT appears to be highly sensitive to T_3 as it contains nuclear T_3 receptors comparable in number to those found in the liver and pituitary (Bianco and Silva, 1987b). Thyroidectomised rats have been shown to display a significant reduction in basal UCP levels, and rapidly become hypothermic under cold conditions (Bianco and Silva, 1987a). Similarly, thyroidectomised lambs have a lower colonic temperature and increased incidence of shivering thermogenesis (Schermer et al., 1996), implicating the role of T_3 in the transcription of UCP1 gene. So, in addition to the overall

effect of thyroid hormones on metabolism. T_3 may be of importance to other cellular processes in BAT.

Given the information summarised above, the hormones of greatest importance for BAT metabolism and thus thermogenesis in the neonatal lamb are nor-epinephrine, insulin and thyroid hormones. These hormones are also important for energy homeostasis and this has been discussed in a preceding chapter. Whilst nor-epinephrine exerts the largest single effect on NST, it is the direct transmission of this catecholamine by sympathetic nerves, rather than circulating levels, that influence BAT metabolism, thus few investigations have targeted plasma concentrations in an effort to identify thermogenic maturity. Additionally, whilst insulin is of importance in regulating the role of glucose in BAT, it has a range of actions outside thermogenesis and thus is often difficult to link directly to BAT metabolism. Thyroid concentrations are commonly the most cited method of indirect thermogenic measure *in vivo*. Specifically, measuring levels of T_4 and T_3, and indeed the ratio of the two can be suggestive of type II iodothyronine 5'deiodinase enzyme, and BAT, activity. Thus efforts to quantify thermogenic maturity *in vivo* often target circulating thyroid hormone concentrations.

Evidence linking maturity of the neonate at birth and homeothermy exists in the lamb. As discussed in a previous chapter, the birth process results in a number of hormonal shifts that prepare the fetus for extra-uterine life. It has been suggested that animals born via caesarean section do not undergo this cascade of endocrine changes and may be less physiologically-mature. Lambs born by 'natural' delivery display increased thermoregulatory ability as measured by colonic temperature after birth, circulating thyroid hormone concentrations, and levels of nor-epinephrine, UCP and GDP binding in BAT (Clarke et al., 1997b). Similarly, premature lambs induced through glucocorticoid administration exhibit reduced thermogenic capacity when compared to those born at term (Alexander et al., 1973b). These results suggest that metabolically-immature lambs are less able to regulate homeothermy following birth.

As discussed in the preceding chapter, postnatal behaviour may be linked to metabolic maturity of the lamb, and there is also some evidence to suggest that there are additional relationships with thermoregulatory ability. Birth coat characteristics have been shown to be related to cold resistance as animals with hairier coats exhibit a genetic relationship with peak rectal temperature (Slee et al., 1991). The authors also mention that these hairier lambs displayed increased struggling movements in the cold resistance test and stipulated that a genetic relationship between coat grade, cold resistance and lamb vigour exists.

However, no measures of early postnatal behaviour were made in this investigation. Subsequently, Dwyer and Morgan (2006) identified that lambs that were slower to progress in behaviour following birth displayed lower rectal temperatures, confirming this link between vigour and thermoregulation. Additionally, thyroid concentrations tended to be higher (T_4) or were significantly higher (T_3) in a breed of lamb (Scottish Blackface) that displayed increased vigour after birth. It was concluded that lambs exhibiting slower behavioural progression also experience difficulty maintaining body temperature. Whilst the authors suggest the increases in thermoregulatory response may be due in an increase in activity, they also discuss differences in physiology between the lambs and implicate metabolic maturity. Discussions on the relationship between postnatal behaviour, metabolic maturity and thermoregulation appear commonly in the literature, however experiments specifically designed to elucidate these associations are scarce.

Conclusions

The risk of mortality is increased when a lamb's ability to thermoregulate is reduced. Whilst the effects of adverse weather are not consistent from year to year, inclement conditions can have devastating effects. Moreover, the lamb's ability to withstand cold may impact upon other processes such as ability to gain additional energy, representing its overall viability. Thermogenesis, and specifically NST, is the mechanism that allows lambs to withstand cold and resistance to cooling can be measured simply through the water bath test. Clear breed differences exist in cold resistance as measured by this test, and whilst some phenotypic traits help to explain some of this variation, genetic divergence in resistance to cooling may be due to differences in metabolic processes. Metabolic maturity at birth may be implicated in the lamb's ability to maintain core body temperature in the neonatal period. The aim of the following experiment is to identify if cold resistance is dependent on the lamb's metabolic state at birth as measured by both physiological and behavioural techniques, and to monitor the lamb's metabolic response to cooling using the water bath test.

Method

Animals

All experiments involving animals were carried out with approval from the University of Adelaide Animal Ethics Committee (S-2009-005). Two breeds of sheep were selected from the work of Slee *et. al.* (1980) that showed clear breed divergence in cold resistance (Table 3.2). In this previous work, cold resistance was measured using time taken for lamb rectal temperature to reach 35°C whilst submerged in a water bath with a gradual water temperature decrease from 37°C to 15°C over a one hour period.

Table 3.2 Resistance to induced hypothermia by water bath emersion for various breeds of lambs (Slee et al., 1980).

Breed	n	Water bath cold resistance (min)
Cheviot	35	98
Welsh	21	89
Scottish Blackface	33	87
Border Leicester	23	80
Oxford	21	79
Boreray Blackface	25	55
Southdown	26	51
Merino	21	45
Finnish Landrace	23	38
Soay	38	36

Due to availability of sheep breeds within Australia, the Border Leicester and Merino breeds were chosen and forty pregnant ewes from each were sourced. Border Leicester ewes were brought from Inverbrackie stud located in the Adelaide Hills, South Australia whilst the Merino ewes came from a dual purpose flock at Turretfield Research Centre in Rosedale, South Australia.

Management

This experiment was conducted concurrently with that described in Chapter 2. Information on management of ewes at mating, during pregnancy and of ewes and lambs at lambing is outlined in Methods section of that chapter.

Measurements

Blood samples and behaviour measures were collected at birth. Approximately three hours after birth lambs were tagged for individual identification. At this time, birth weight, type of birth, sex, birth coat score, rectal temperature, vigour score, crown rump length, thoracic circumference and metacarpal length was recorded. A detailed description of these measures is outlined in the Methods section of Chapter Two.

Ponderal index was calculated in a similar manner to that reported in the previous chapter. A rough surface area for the body of each lamb was calculated from the crown rump length and thoracic circumference using the following formula:

$$\text{Surface area} = 2\ (pi \cdot ½ \cdot \text{thorax}^2\ (\text{cm})) + (2\ pi \cdot ½ \cdot \text{thorax}^2\ (\text{cm})) \times \text{crown rump (cm)}$$

Treatment

Approximately 24 hours after birth lambs were subjected to the cold water bath test. All lambs expected to undergo the test were catheterised in the morning and returned to their pen until required to reduce handling time. A catheter was introduced into the jugular vein after local anaesthetic administration using 18 gauge catheter placement units and polyethylene tubing with an internal diameter of 0.8 mm. The line was flushed with heparinised saline and held in place using Leukoplast adhesive tape (Smith & Nephew, Australia). Lambs were tested in the order they were born the previous day in an attempt to standardise time between birth and testing.

The cold water bath test was based on the methods described by Slee *et al.* (1980). The water bath was constructed of stainless steel and measured 1 m by 1 m by 0.5 m in size. Water was heated via an element inside the bath and cooled by an external chiller that was connected to the bath by insulated rubber piping (Simms Refrigeration, Australia). These pipes also acted to circulate the water in order to ensure temperature uniformity across the bath. The temperature of the bath was between 36°C and 37°C at the beginning of the test, and was slowly cooled to 15°C over a one hour period (Table 3.3). A linear reduction of temperature was observed in the bath, thus an increase in time of one minute resulted in a 0.36 ± 0.08 °C reduction in water temperature. When the temperature of the bath reached 15°C, the chiller would shut off and the temperature in the bath would remain constant until the completion of the test.

Table 3.3 Mean water bath temperature and standard error of the measure taken at five minute intervals.

	Time from start of cooling (min)												
	0	5	10	15	20	25	30	35	40	45	50	55	60
Temp (°C)	35.9	33.3	31.1	29.0	27.1	25.3	23.8	22.2	20.7	19.3	17.8	16.9	15.6
SEM	0.5	0.9	1.0	1.3	1.3	1.4	1.3	1.1	1.0	0.8	0.8	0.4	0.3

Before testing, an initial 5 mL blood sample was collected from the jugular catheter into an EDTA blood tube and rectal temperature was recorded. Lambs were restrained by a harness made from shade cloth that was permeable to water and submerged in the bath. The head was kept out of the water by a sling placed under the chin of the animal. A thermometer probe was placed 5 cm into the rectum of the lamb and this was connected to Labchart 7.0 via an eight channel PowerLab (Ad Instruments, Australia). Rectal temperature was monitored and recorded continually. A mask connected to Labchart 7.0 via a Gas Analyser and the PowerLab was placed over the lamb's muzzle. The respiration rate, flow and carbon dioxide and oxygen concentration of expired air were measured using this technique for two minutes every 15 minutes during the test. Blood samples were also collected at these time points.

The time from the beginning of the test to when the lamb's rectal temperature fell to 35°C was recorded and termed 'cold resistance'. At this point the lamb was removed from the bath and towel dried. Drying time was standardised to 30 seconds and one towel per lamb was used to absorb excess moisture. The number of tests conducted for each year is presented in Table 3.4 and was largely determined by the ability to impregnate ewes across years.

Table 3.4 Number of cold resistance records collected over experimental three years for Border Leicester and Merino lambs.

	Number of Records		
Breed	2009	2010	2011
Poll Dorset Border Leicester		9	
Poll Dorset Merino		25	
Border Leicester	22		13
Merino	18		28
Total	40	36	41

The lamb was then restrained by an assistant in a rewarming box. This box was maintained between 26°C and 29°C, a temperature deemed thermo-neutral for a lamb (Alexander et al.,

1973a). Rectal temperature was once again continually monitored and the time taken for the lamb to plateau within the expected range (38.5°C to 39.5°C) was recorded and termed 'cold recovery'. At this point the lamb was returned to its dam. Occasionally (approximately 10% incidence), mismothering by the dam after the test was observed and was thought to be attributed to olfactory confusion. The rejection of tested lambs was avoided by drying the lamb off with an unused towel rubbed on the back end of its dam and penning the dam in close proximity to her lamb after the test for a period of 24 hours.

After collection, blood samples were analysed for glucose (Hemocue Glucose 201+, Medipac Scientific, Australia) and then spun for plasma which was then stored at -20°C. Subsequently, plasma samples were analysed for free T_3, cortisol, blood urea nitrogen (BUN) and non-esterified fatty acids (NEFA). The assays were performed in the Adelaide Research Assay Facility, School of Paediatrics and Reproductive Health, University of Adelaide by Dr. Michael Boden, Dr. Anne MacPherson and Professor David Kennaway. The metabolites were assayed on a Roche Hitachi 912 Analyser. The quality control samples for the BUN assay were 6.9 ± 0.7 mmol/L (CV = 9.6%) and 25.0 ± 1.0 mmol/L (CV = 4.1%). The quality control samples for the NEFA assay (Lyphocheck Assay Chemistry Control Level 1 and Level 2; BioRad) were 1.6 ± 0.15 mmol/L (CV = 9.5%) and 0.8 ± 0.18 mmol/L (CV = 12.3%). Plasma samples were assayed for cortisol in duplicate by radioimmunoassay (Cat# IM1841, Immunotech, Prague, Czech Republic) according to the manufacturer's instructions using 50ul sample. The minimum detectable level was 20nM. The intra-assay CV was less than 10%. The inter-assay CV was 6.4% at 148.8 nM (expected range 110-192nM). Plasma samples were assayed for free triiodothyronine (fT_3) in duplicate by radioimmunoassay (Cat# IM1579, Immunotech, Prague, Czech Republic) according to the manufacturer's instructions using 25-100ul sample. The minimum detectable level was 2.3nM. The intra-assay CV was less than 10%. The inter-assay CV was 13.5% at 4.4 nM (expected range 3.0-5.1nM).

Leptin analysis was conducted in the School of Animal Biology, The University of Western Australia by Ms Margaret Blackberry. Plasma leptin was measured in duplicate by a double-antibody radioimmunoassay (Blache et al., 2000). All samples were processed in a single assay and the limit of detection was 0.05 ng/mL. The assay included six replicated of three control samples containing 0.63 ng/mL, 1.36 ng/mL and 2.58 ng/mL which were used to estimate the intra-assay CV of 7.7%, 4.1% and 1.9% respectively.

Statistics

All traits were analysed using a general linear model conducted in ASREML (Gilmour et al., 2005) and a P value < 0.05 was deemed significant. As all breeds were not used across all years, confounding between sire breed and year was encountered in the analysis. Consequently, the pure-breds (M and BL) were analysed separately to the cross-bred (PDM and PDBL), thus direct statistical comparisons between these genotypes could not be made. All univariate analyses included the fixed effects of age of dam (2 to 7 years), sex (male or female), type of birth (single or multiple), breed (M and BL, or PDM and PDBL) and any significant two way interactions between these effects. The pure-bred analysis also contained the fixed effect of year (2009 and 2011). The covariates of birth weight and gestation length were included in the model where appropriate and allowed for regression analysis between these and other traits examined. The effect of lamb behaviour on thermoregulation indicators was also examined using a general linear model. Lambs were graded according to behavioural progression (slow (bottom 25%), medium (25-50%) or fast (top 25%) and this grade, along with year (2009, 2010 and 2011), age of dam (2 to 7 years), sex (male or female), type of birth (single or multiple), and ewe breed (M and BL) were fitted as fixed effects in the model.

Multivariate analysis was conducted to estimate correlations between lamb phenotype, behaviour, blood parameters and thermoregulatory indicators, and included the fixed effects of age of dam (2 to 7 years), sex (male or female), type of birth (single or multiple), breed (M and BL, or PDM and PDBL) and any significant two way interactions between these effects. Birth weight was included as a covariate for the analysis between shape measures and thermoregulatory indicators only. The multivariate analysis was run between five traits at any given time and estimated correlations were compared between analyses. Significance was determined if the correlation was greater than two times the standard error (Cloete et al., 2004).

Blood metabolite and hormonal concentrations collected during the water bath test and at recovery were analysed using a general linear model with the fixed effects of age of dam (2 to 7 years), sex (male or female), type of birth (single or multiple), breed (M and BL, or PDM and PDBL), time (0, 15, 30, 45, 60 and recovery) and any significant two way interactions between these effects. Regression analysis of the samples collected during the bath also occurred to determine if profiles differed with lamb breed. The regression model contained the fixed effects of age of dam (2 to 7 years), sex (male or female), type of birth (single or

multiple), breed (M and BL, or PDM and PDBL), and time (both as a linear and quadratic term) and any significant two way interactions between these effects.

Significance levels obtained from these analyses will be presented in the Results section using the following scheme: P value < 0.1 is represented with †, P < 0.05 with *, P < 0.01 with ** and P < 0.001 ***.

Results

Rectal temperature at birth

Birth weight was shown to be related to rectal temperature in the pure-bred lambs (Figure 3.2). For every 1 kg increase in weight, lambs exhibited a 0.25°C increase in temperature ($P < 0.001$). This effect was not witnessed in the cross-bred lambs. There was a tendency for male pure-bred lambs to display a lower rectal temperature than their female counterparts (males 38.9 ± 0.1 °C, females 39.2 ± 0.1 °C; $P = 0.06$). Once again, this was not observed in cross-breds. No other fixed effects examined were shown to influence rectal temperature at birth.

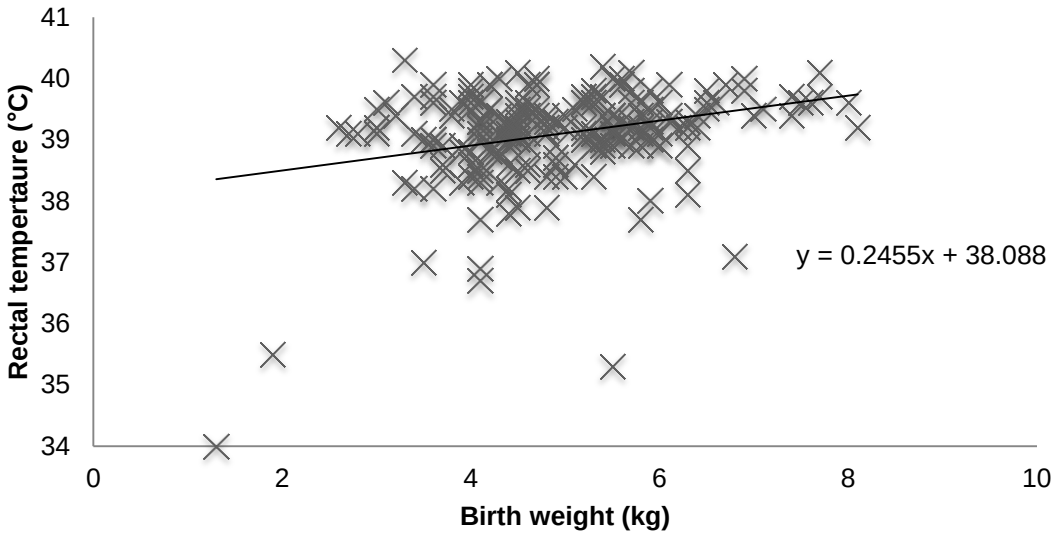

Figure 3.2 Influence of birth weight (kg) on rectal temperature (°C) measured within 3 hours of birth in pure-bred lambs.

Surface area of the lamb

Birth weight significantly influenced the surface area of the lamb in both cross-breds and pure-breds ($P < 0.001$). For every 1 kg increase in birth weight, the surface area was increased by 276 cm^2. The remaining fixed effects were adjusted for birth weight in order to estimate a surface area to volume ratio. There was a tendency for female lambs to exhibit an increase in surface area to volume ratio compared with males in the pure-bred genotype (2115 ± 22 cm^2 and 2071 ± 22 cm^2 respectively; $P < 0.10$), however this was not observed in

the cross-bred lambs. Surface area adjusted for birth weight was also shown to be affected by lamb breed in both cross-bred and pure-bred lambs (Figure 3.3). PDM and M lambs had higher surface areas when adjusted for birth weight than PDBL and BL's (2299 ± 35 cm^2 and 2155 ± 21 cm^2 for PDM and M respectively vs 2152 ±41 cm^2 and 2052 ± 26 cm^2 for PDBL and BL respectively; $P < 0.05$).

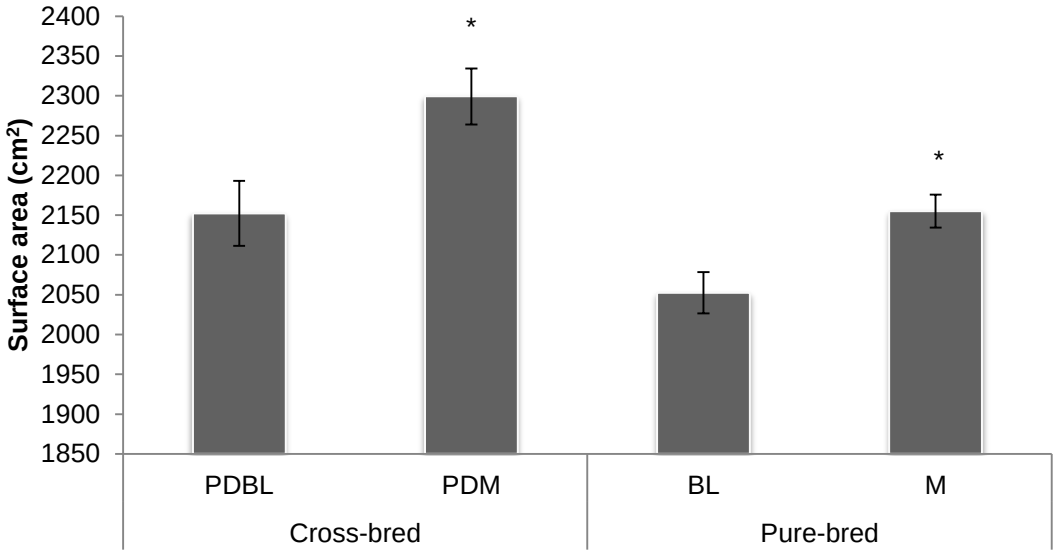

Figure 3.3 Mean (± SEM) surface area (cm2) adjusted for birth weight (kg) for cross-bred (Poll Dorset x Border Leicester (PDBL) and Poll Dorset x Merino (PDM)) and pure-bred (Border Leicester (BL) and Merino (M)) lambs (* represents significant difference (P < 0.05) within genotype).

Cold resistance

Cold resistance (as measured by time taken for the lamb to reach 35°C whilst immersed in the water bath) was significantly influenced by birth weight. Cross-bred lambs had a 3.2 min greater cold resistance for every 1 kg increase in birth weight ($P < 0.001$). In the pure-breds a similar effect of birth weight was observed, with an increase of 4.2 min for every 1 kg increase in birth weight ($P < 0.001$). There was no effect of sex on cold resistance when birth weight was excluded from the model, however after adjusting for birth weight, females outperformed males ($P < 0.01$) in the pure-bred but not cross-bred lambs (Table 3.5). The only effect of type of birth on cold resistance was observed in the cross-breds. Singletons exhibited increased resistance compared with multiples ($P = 0.001$), however this was largely explained by the difference in birth weight as the type of birth effect was removed

after birth weight was included in the statistical model. Cold resistance was significantly affected by lamb breed. In the cross-breds, PDBL lambs were more cold resistant than PDM's both without ($P < 0.05$) and with ($P < 0.10$) the inclusion of birth weight in the statistical model. A similar result was observed in the pure-bred lambs without the inclusion of birth weight ($P < 0.01$), however when adjusted for birth weight M lambs were more cold resistant than BL's ($P < 0.05$).

Table 3.5 Influence of fixed effects (mean ± SEM) on time for the lamb's rectal temperature to reach 35°C (cold resistance (min)) in a water bath progressively cooled from 37°C to 15°C over a one hour time period, without and with fitting birth weight as a covariate in the statistical model.

		Cross-breds						Pure-breds					
		Cold resistance (min) *No covariate*			Cold resistance (min) *Birth weight covariate*			Cold resistance (min) *No covariate*			Cold resistance (min) *Birth weight covariate*		
Fixed effect		Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value
Age of dam	2			0.131			0.264	56.4	2.7	0.665	55.9	2.4	0.777
	3							54.8	4.2		51.4	3.9	
	4	68.0	3.2		65.7	3.0		56.6	2.2		55.8	2.0	
	5	59.7	2.9		58.1	2.7		57.0	2.2		57.3	2.0	
	6	63.0	2.2		63.1	2.0		55.2	2.7		56.1	2.4	
	7	58.9	2.2		58.5	2.0		58.2	2.6		56.8	2.4	
Sex	Male	66.6	1.6	0.243	64.1	1.9	0.377	55.2	1.4	0.202	52.9	1.4	**
	Female	59.7	2.2		57.5	1.6		58.0	1.7		59.3	1.5	
Type of birth	Single	66.6	1.8	***	64.1	1.5	0.128	56.9	2.0	0.960	55.0	1.9	0.456
	Multiple	56.2	1.7		57.5	2.0		56.3	1.3		56.4	1.1	
Breed	PDBL	69.2	2.3	**	66.4	2.4	†						
	PDM	57.3	1.4		57.8	1.3							
	BL							58.3	1.5	***	55.9	1.5	*
	M							53.8	1.4		56.0	1.4	

Recovery after cold resistance test

Birth weight, age of dam, sex of lamb, type of birth, and breed were all shown to have no effect on the recovery rate (time taken to reach pre-bath rectal temperature) of lambs after the cold resistance test.

Relationship between lamb phenotype and thermoregulation

Rectal temperature measured at birth showed a positive relationship with cold resistance (0.56 ± 0.10) but this relationship could not be established between rectal temperature and cold recovery (0.02 ± 0.16). Similarly, no association was observed between cold resistance and cold recovery (0.03 ± 0.11). Little relationship existed between any of the lamb phenotypic measures and rectal temperature measured at birth. Only metacarpal length was shown to exhibit a slight positive correlation with rectal temperature (Table 3.6). When birth weight was fitted as a covariate, correlations between lamb phenotype and rectal temperature were negative. At a given birth weight, lambs that exhibited a smaller thoracic circumference displayed increased temperatures at birth (Table 3.6). There was a positive association between cold resistance and birth weight, crown rump length, thorax circumference, metacarpal length and surface area, thus the larger the lamb the higher the cold resistance. However, all of these relationships disappeared when birth weight was included in the model. The only measure of shape that was shown to influence cold recovery was thorax circumference, and this was significant when adjusted for birth weight (Table 3.6).

Table 3.6 Phenotypic correlations (± SE) between lamb thermoregulatory ability indicators (rectal temperature measured at birth, cold resistance and cold recovery) and phenotype (birth weight, birth coat score, crown rump length, thorax circumference, metacarpal length and surface area) without (-bw) and with (+bw) the inclusion of birth weight as a covariate.*

	Thermoregulation indicators (-bw)			Thermoregulation indicators (+bw)		
	Rectal temperature	Cold resistance	Cold recovery	Rectal temperature	Cold resistance	Cold recovery
Birth weight	0.18 ± 0.09	***0.45 ± 0.09***	0.05 ± 0.12			
Birth coat score	0.09 ± 0.08	0.16 ± 0.11	-0.04 ± 0.13			
Crown rump length	0.06 ± 0.04	***0.37 ± 0.09***	-0.06 ± 0.11	-0.26 ± 0.14	0.16 ± 0.12	-0.10 ± 0.12
Thorax circumference	0.07 ± 0.02	***0.38 ± 0.09***	-0.12 ± 0.11	***-0.41 ± 0.13***	0.04 ± 0.12	***-0.25 ± 0.11***
Metacarpal length	***0.16 ± 0.07***	***0.31 ± 0.10***	-0.07 ± 0.12	-0.20 ± 0.15	0.01 ± 0.12	-0.10 ± 0.12
Surface area	0.08 ± 0.08	***0.39 ± 0.09***	-0.08 ± 0.11	-0.04 ± 0.16	0.19 ± 0.14	-0.20 ± 0.13

*Significant correlations (ie. value greater than two times the standard error) are shown in bold and italics.

Physiological profile of lambs under cold exposure

Respiration rate decreased over time, so that the last measure represented a 22% and 16% reduction of the initial measure for the cross-breds and pure-breds respectively ($P < 0.001$; Table 3.7). Metabolic rate as estimated by VCO_2 increased to 45 min for both cross-bred and pure-bred lambs ($P < 0.001$). Whilst VCO_2 begun to decline so that the 60 min and 15 min measure where not different from one another in the cross-breds, insufficient data at this time point for the pure-bred lambs meant means could not be predicted.

Table 3.7 Physiological parameters over time for lambs immersed in a water bath cooled from 37°C to 15°C over a one hour time period. Mean values (± SEM), along with significance level (P-value) are presented for number of breaths per minute, metabolic rate (VCO_2), circulating blood urea nitrogen (BUN), non-esterified fatty acids (NEFA), cortisol, free T_3 (fT_3) and leptin concentrations*.

	Cross-bred lambs Time (min)							Pure-bred lambs Time (min)						
	0	15	30	45	60	R	P-value	0	15	30	45	60	R	P-value
Metabolism														
Respiration rate (min^{-1})	86.3 ±3.0^{a}	88.6 ±2.9^{a}	87.1 ±2.9^{a}	76.6 ±2.9^{b}	69.7 ±3.4^{c}	-	***	87.5 ±2.9^{a}	88.8 ±2.9^{a}	81.4 ±2.9^{b}	77.7 ±3.3^{bc}	73.4 ±7.3^{c}	-	***
VCO_2 ($mL/kg^{0.75}/min$)	18.8 ±4.6^{a}	36.1 ±4.5^{b}	50.1 ±4.6^{c}	46.4 ±4.6^{c}	42.9 ±5.7^{bc}	-	***	25.4 ±4.5^{a}	33.5 ±4.6^{bc}	39.9 ±4.5^{c}	46.2 ±5.1^{c}	-	-	***
Metabolites														
Glucose (mmol/L)	6.5 ±0.3^{a}	7.1 ±0.3^{b}	8.0 ±0.3^{c}	9.3 ±0.3^{d}	9.6 ±0.4^{d}	7.3 ±0.3^{b}	***	6.2 ±0.2^{a}	6.9 ±0.2^{b}	8.0 ±0.2^{c}	8.8 ±0.2^{d}	8.9 ±0.3^{d}	6.8 ±0.3^{ab}	***
BUN (nmol/L)	14.9 ±0.8^{a}	15.0 ±0.8^{a}	14.7 ±0.7^{a}	15.1 ±0.8^{a}	15.3 ±0.8^{a}	15.9 ±0.8^{a}	ns	12.4 ±0.6^{a}	12.3 ±0.6^{a}	12.4 ±0.6^{a}	12.6 ±0.6^{a}	13.7 ±0.6^{b}	13.7 ±0.6^{b}	*
NEFA (mEq/L)	2.22 ±0.3^{a}	2.39 ±0.3^{a}	2.27 ±0.3^{a}	2.25 ±0.3^{a}	2.28 ±0.3^{a}	4.06 ±0.3^{b}	***	1.76 ±0.1	1.63 ±0.1	1.67 ±0.1	1.86 ±0.1	1.78 ±0.2	-	ns
Hormones														
Cortisol (nM)	72.1 ±12.3^{a}	110.0 ±12.3^{b}	195.5 ±12.4^{c}	257.7 ±12.4^{d}	283.0 ±15.1^{d}	218.0 ±12.8^{c}	***	59.4 ±6.6^{a}	139.1 ±9.4^{b}	196.3 ±9.4^{c}	218.9 ±7.1^{d}	271.1 ±14.5^{e}	156.7 ±8.7^{b}	***
fT_3 (nM)	41.1 ±2.2^{a}	42.6 ±2.2^{a}	39.3 ±2.3^{a}	39.5 ±2.3^{a}	41.5 ±2.7^{a}	64.0 ±2.3^{b}	***	28.6 ±1.3^{a}	28.6 ±1.7^{a}	29.7 ±1.7^{a}	28.9 ±1.3^{a}	27.8 ±2.5^{a}	41.6 ±1.6^{b}	NS
Leptin (ng/mL)	1.30±0 .04	1.32±0 .04	1.23±0 .04	1.22±0 .04	1.21±0 .05	1.21±0 .04	***	1.26±0 .04	1.26±0 .04	1.26±0 .04	1.19±0 .04	1.22±0 .05	1.10±0 .04	***

* Means within a row with differing superscripts are significantly different from one another ($P < 0.05$).

The regression analysis of blood samples collected during the water bath test identified a significant quadratic association for blood glucose over time in both the cross-bred and pure-bred lambs (Figure 3.4; $P < 0.05$). In the cross-breds, both PDBL and PDM lambs had not reached their predicted maximal blood glucose concentrations at the final blood sample, but PDBL lambs exhibited significantly higher levels than PDM's at this point (11.0 mmol/L and 8.9 mmol/L respectively). In pure-bred lambs BL took longer to reach their peak glucose level (50 min) than M's (40 min). This peak value was also higher for the BL lambs than M's, (9.4 BL vs 7.7 mmol/L respectively).

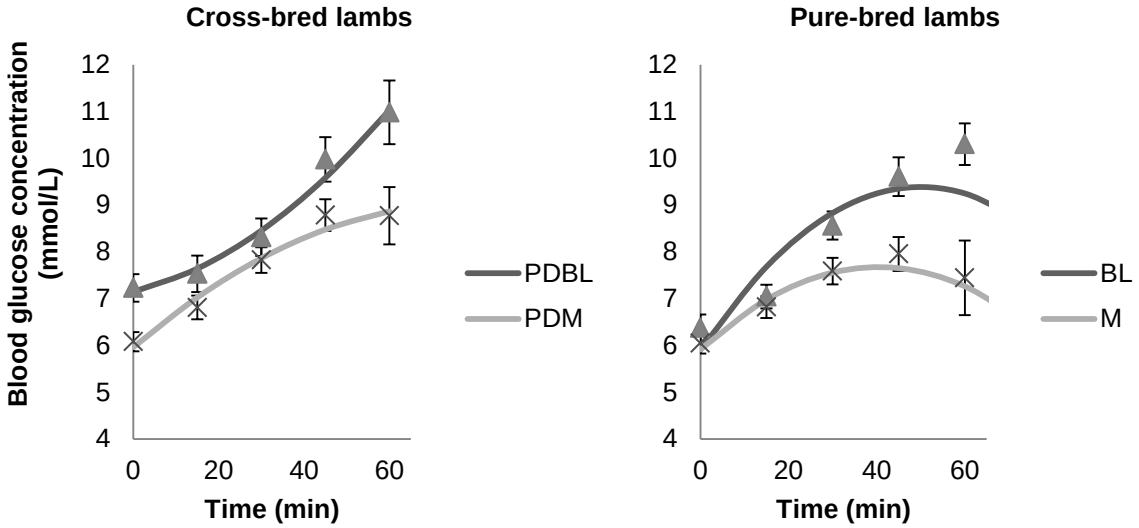

Figure 3.4 Mean ± SEM blood glucose concentration (mmol/L) over time (min) during the water bath test for cross-bred (Poll Dorset Border Leicester (PDBL) and Poll Dorset Merino (PDM) and pure-bred (Border Leicester (BL) and Merino (M)) lambs. The relationship between concentrations over time was determined using coefficients from the regression analysis and the best prediction of this relationship (linear or quadratic) is displayed on the figure.

BUN concentration did not differ across breeds over time whilst under cold stress in cross-bred lambs, however in the pure-breds, BUN increased over time in a quadratic manner and this relationship was different for BL and M's (Figure 3.5). At 60 min, BL lambs recorded higher BUN levels (15.6 ± 0.8 nmol/L) than M's (12.4 nmol/L; $P < 0.05$).

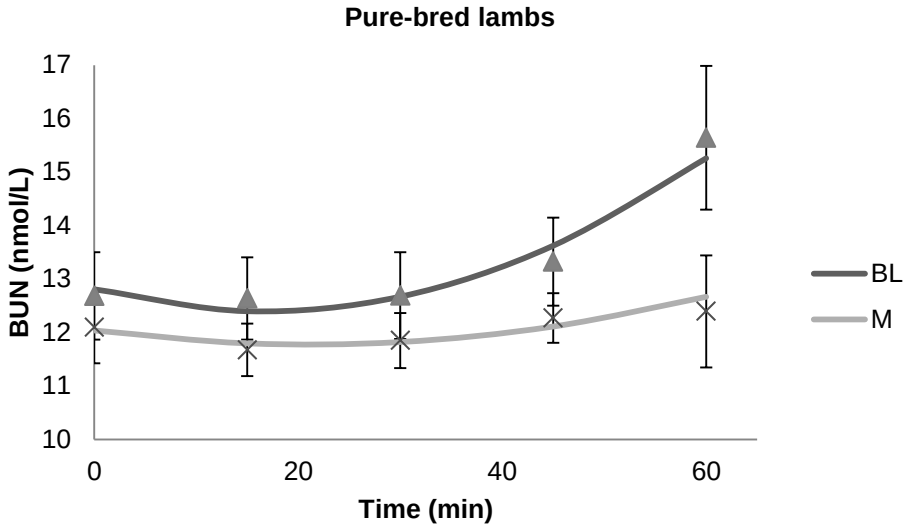

Figure 3.5 Mean ± SEM blood urea nitrogen (BUN; mmol) over time (min) during the water bath test for pure-bred (Border Leicester (BL) and Merino (M)) lambs. The relationship between concentrations over time was determined using coefficients from the regression analysis and the best prediction of this relationship (linear or quadratic) is displayed on the figure.

Breed had no effect on the rate of increase in plasma cortisol concentration in the pure-bred lambs. However, cortisol concentrations increased at a greater rate in PDM than PDBL (Figure 3.6). Whilst not significantly different before 30 min, PDM lambs displayed higher cortisol levels at 45 min (282.1 ± 18.7 nM) and 60 min (298.3 ± 25.1 nM) than PDBL's (45 min 210.5 ± 14.8 nM and 60 min 238.7 ± 16.0 nM; $P < 0.05$).

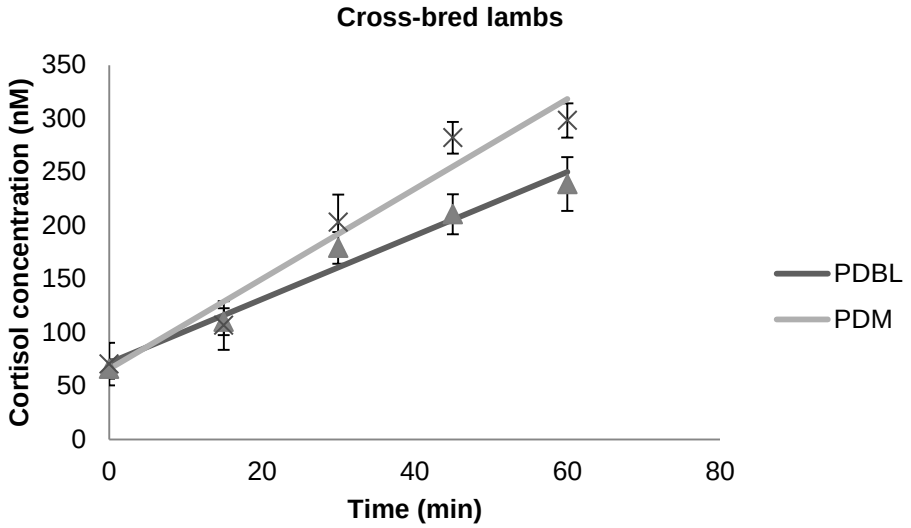

Figure 3.6 Mean ± SEM cortisol (nM) over time (min) during water bath test for cross-bred (Poll Dorset Border Leicester (PDBL) and Poll Dorset Merino (PDM)) lambs. The relationship between concentrations over time was determined using coefficients from the regression analysis and the best prediction of this relationship (linear or quadratic) is displayed on the figure.

Relationships between behaviour, maturity at birth and thermoregulation

Lamb vigour score was the only behaviour to show a significant relationship with rectal temperature at birth, with lambs that were more vigorous exhibiting higher temperatures (Table 3.8). No significant correlations between any of the behaviour measures and cold resistance could be established. Cold recovery was negatively associated with sucking behaviour thus lambs slower to perform these behaviours were also slower to recover from cold (Table 3.8).

Table 3.8 Phenotypic correlations (± SE) between indicators of lamb thermoregulatory ability (rectal temperature measured at birth, cold resistance and cold recovery) and behaviour around birth (time taken for the lamb to bleat, attempt to stand, stand, attempt to suck and subjective lamb vigour score with a score of one being very vigorous and five being a lamb with poor vigour).*

	Thermoregulatory ability indicators		
	Rectal temperature	Cold resistance	Cold recovery
Bleat	0.10 ±0.08	0.03 ± 0.18	-0.10 ± 0.19
Stand attempt	0.22 ± 0.16	0.42 ± 0.22	-0.16 ± 0.08
Stand	0.17 ± 0.14	0.30 ± 0.17	-0.18 ± 0.16
Suck attempt	0.12 ± 0.09	-0.08 ± 0.20	***-0.56 ± 0.12***
Suck	0.13 ± 0.15	-0.07 ± 0.16	***-0.33 ± 0.12***
Lamb vigour score	***-0.28 ± 0.08***	-0.18 ± 0.12	-0.10 ± 0.12

*Significant correlations (ie. value greater than two times the standard error) are shown in bold and italics.

When lambs were graded into categories based on their behavioural performance after birth (Table 3.9), there was a tendency for those that were slow to stand and suck to display lower rectal temperatures when compared to those that were quickest to perform the same behaviours ($P < 0.1$). Similarly, lambs that were slow to suck tended to display reduced cold resistance compared with those that were average or fast to reach the udder ($P < 0.1$). No relationship between lamb behaviours and cold recovery were identified.

Table 3.9 Mean ± SEM thermoregulatory indicators (rectal temperature measured at birth, cold resistance and cold recovery) for lambs that were classed as slow (bottom 25%), medium (middle 50%) and fast (top 25%) to first stand and suck after birth.

		Temperature (°C)		P value	Cold resistance (min)		P value	Cold recovery (min)		P value
		Mean	SEM		Mean	SEM		Mean	SEM	
Time taken to stand				†			0.179			0.920
	Slow	39.2	0.3		53.7	2.2		34.6	1.9	
	Medium	39.3	0.2		58.9	1.4		36.3	1.2	
	Fast	39.5	0.3		57.3	2.1		34.2	2.0	
Time taken to suck				†			†			0.959
	Slow	39.2	0.3		51.6	2.1		33.6	1.7	
	Medium	39.4	0.2		59.0	1.3		36.3	1.4	
	Fast	39.4	0.3		59.6	2.1		35.5	2.0	

Almost all metabolite and hormone concentrations were negatively related to rectal temperature measured at birth (Table 3.10). Thus, lambs with increased rectal temperatures displayed decreased pre-suckling circulating levels of plasma BUN, ACTH, cortisol, ghrelin and leptin. Cold resistance was positively associated with increased ghrelin levels at birth. Cold recovery was positively correlated with glucose, NEFA, creatinine, ghrelin and leptin concentrations at birth (Table 3.10).

Table 3.10 Phenotypic correlations (± SE) between lamb thermoregulatory ability indicators (rectal temperature measured at birth, cold resistance and cold recovery) and plasma metabolite and circulating hormone concentrations at birth (glucose, non-esterified fatty acids (NEFA), blood urea nitrogen (BUN), creatinine, adrenocorticotrophic hormone (ACTH), cortisol, ghrelin and leptin).*

	Thermoregulatory ability indicators		
	Rectal temperature	Cold resistance	Cold recovery
Glucose	-0.06 ± 0.12	-0.08 ± 0.16	***0.41 ± 0.14***
NEFA	***-0.34 ± 0.16***	-0.13 ± 0.24	***0.52 ± 0.18***
BUN	***-0.39 ± 0.15***	0.04 ± 0.17	-0.22 ± 0.18
Creatinine	***-0.22 ± 0.10***	-0.24 ± 0.17	***0.68 ± 0.12***
ACTH	***-0.39 ± 0.13***	0.31 ± 0.16	-0.07 ± 0.19
Cortisol	***-0.37 ± 0.14***	0.08 ± 0.17	-0.13 ± 0.18
Ghrelin	***-0.33 ± 0.16***	***0.49 ± 0.13***	***0.34 ± 0.16***
Leptin	***-0.51 ± 0.16***	0.37 ± 0.23	***0.66 ± 0.15***

*Significant correlations (ie. value greater than two times the standard error) are shown in bold and italics.

Discussion

This investigation was designed to examine the phenotypic influences on thermoregulation in the lamb, and to better understand the physiological parameters and processes of importance when under cold stress. Females were identified as having improved thermoregulatory response when compared with males. Breed differences in cold resistance were confirmed. The biggest single phenotypic influence on thermoregulation in the lamb was birth weight. Lambs that were slower to stand and reach the udder tended to be at a thermoregulatory disadvantage, as indicated by rectal temperature after birth and cold resistance, when compared to those that progressed at a faster rate. No relationships between cold resistance and the lamb's physiological maturity at birth could be established, however lambs classed as having improved maturity took a greater amount of time to recover after cold exposure. These results have helped to provide a better understanding of thermoregulation in the lamb, and support the notion that physiological maturity at birth, along with peri-natal lamb behaviour, are linked to thermoregulation.

Thermoregulation in pure-bred lambs

The observed influence of birth weight on both temperature at birth and cold resistance is not unique (Dwyer and Morgan, 2006, Samson and Slee, 1981, Slee et al., 1991) and is due to a combination of the following factors. Metabolism has been shown to be influenced by weight, with summit metabolism per unit of surface area increasing with increasing weight (Alexander, 1962c), meaning heavier lambs are better able to maintain increased metabolism under conditions of high heat loss. This increased ability to maintain summit metabolism is because of increased body reserves in heavier lambs. Lambs weighing less than 2.5 kg have reduced fat and energy levels when compared to those greater than 4.5 kg (Greenwood et al., 1998). In addition to the improvement in metabolism, heavier lambs exhibit a reduction in surface area relative to volume, thus heat loss would be minimised in these animals compared with those which were lighter. The improvement in thermoregulatory ability with increased birth weight may also be due to differences in brown fat metabolism and thus NST. Thyroid hormone levels have been shown to be greater in heavier lambs around the time of birth (Dwyer and Morgan, 2006) and this may be due to increased brown adipose tissue (BAT) activity. Investigations into effects of ewe nutrition during gestation have confirmed this association with heavier lambs from well-fed ewes exhibiting increased un-coupling protein (UCP) and guanosine 5'-

diphospate (GDP) levels in BAT compared to lighter lambs from under-fed ewes (Budge et al., 2000). Combined, these findings suggest lighter lambs are at a thermoregulatory disadvantage, being less able to maintain rectal temperatures after birth and during a cold challenge.

Whilst effects of birth weight on temperature after birth and cold resistance are clear, it is perhaps not surprising that birth weight showed little influence over recovery rate after cold exposure. As already discussed, under times of cold stress, larger lambs are at a considerable advantage. This advantage is still apparent with regards to metabolic capability and available energy reserves during recovery from cold, with larger lambs being expected to be able to maintain or increase thermogenesis in order to reinstate temperature homeostasis at a faster rate. A considerable disadvantage that these heavier lambs encounter is with regards to heat convection. When lambs were placed in the thermo-neutral environment, air temperature would have been significantly higher than that of the fleece and skin of the lamb. Heavier lambs would have a larger volume relative to surface area, thus warming through convective means would have been decreased when compared to smaller, lighter animals. The competing effects between the increased metabolism of heavier lambs and the reduction in warming through convection may have acted to cancel one another out, resulting in the absence of any relationship between weight and cold recovery time.

Without adjusting for birth weight, the present findings and those reported previously (Samson and Slee, 1981) indicate little difference in cold resistance between male and female lambs. However, when adjusted for birth weight, females were better able to regulate their temperature compared with males, demonstrating both increased rectal temperatures at birth and cold resistance. These results were unexpected as the female lambs also demonstrated a higher surface area relative to birth weight when compared with males, which would act to increase heat loss. This would suggest there are differences in metabolism between genders. Early reports on the effects of sex on thermoregulation were inconsistent (Himms-Hagen, 1985). However in more recent times, the body of literature that supports females having a higher thermogenic capacity than males is increasing. In rats, increases in BAT as a percentage of total body weight, (Justo et al., 2005), oxygen consumption, UCP levels, adrenergic receptor sensitivity (Rodríguez-Cuenca et al., 2002), II iodothyronine 5'deiodinase activity (Gabaldon et al., 1995) and changes in mitochondrial subpopulations (Justo et al., 2005) have been reported in females. This

ability of females to outperform males with regards to thermoregulation would have consequences for survival, and not surprisingly females have been reported to display reduced mortality than males (Hatcher et al., 2009, Sawalha et al., 2007).

Breed differences in cold resistance have been reported previously (Slee et al., 1980) as the trait is reported to be under genetic control (Slee et al., 1991, Slee and Springbett, 1986). The unadjusted cold resistance of BL lambs being greater than M's was anticipated as this breed divergence has been reported elsewhere (Samson and Slee, 1981, Slee et al., 1980) and was the basis of breed selection for these experiments. What was unforeseen was that after accounting for breed differences in birth weight, M lambs were, albeit only slightly, more cold resistant than BL lambs. The finding was in contrast to Samson and Slee (1981), who showed that even after including birth weight in the statistical model, BL lambs outperformed those from the M breed. This previous investigation did report a temperature-transformed cold resistance which involved accounting for the reduction in severity of cooling experienced by those lambs with reduced cold resistance (lambs that became hypothermic before the completion of the test would not have experienced the lower temperature ranges). The transformation resulted in the exacerbation of breed differences, which may explain why cold resistance still differed after the adjustment for birth weight. Additionally, Samson and Slee (1981) used the Tasmanian Merino, a strain used for fine-wool production and generally thought to have low 'fitness'. Thus, the finding that at a given birth weight the M lambs were more cold resistant than BL lambs in the present investigation would most likely be due to the fact that cold resistance was not adjusted for temperatures experienced and that a 'hardier' South Australian Merino strain was used.

Thermoregulation in cross-bred lambs

Few fixed effects were shown to influence any of the thermoregulation traits in the cross-bred lambs. Why the birth weight and sex effects were not observed in the cross-bred lambs is intriguing, but may be explained by the range of rectal temperatures observed. The lowest temperature recorded for pure-bred lambs was 34.0°C, whilst cross-breds did not measure below 38°C. The reduction in variation of temperature in the cross-breds (most likely explained by the sharing of a common sire breed) would reduce the likelihood of detecting whether birth weight or sex significantly influenced the trait with the small number of animals used.

Whilst a direct, statistical comparison between the cross-bred and pure-bred lambs could not be made as all genotypes were not utilised across all years, it would appear that heterosis increased cold resistance. Thermoregulatory ability increased in both BL and M breeds when a Poll Dorset sire was used compared with using the same sire breed as the maternal breed. Hybrid vigour is known to increase many important lamb production traits such as growth (Cloete et al., 2007) and survival (Gama et al., 1991), so an increase in cold resistance from cross breeding was not unexpected.

Across-breed analysis of thermoregulation

Few significant correlations were identified between weight and shape measures and rectal temperature when recorded at birth across the genotypes. It was expected, given the impact of surface area on thermoregulation, that larger, heavier lambs would display increases in temperature after birth. A positive phenotypic correlation between birth weight and rectal temperature ($r = 0.54$) has been reported previously (Alexander and McCance, 1958). The discrepancy would be due to the range of birth weights observed in the present study. Alexander and McCance (1958), when reporting their positive correlation between birth weight and rectal temperature, stated it was strongest in twin lambs and at weights less than 4 kg. Only 45 of the 274 lambs analysed in this study weighed less than 4 kg, thus the likelihood of detecting any relationship would be decreased. The current investigation was also conducted within a shed which reduced the probability of colder temperatures and eliminated wind and rain from the environment. Additionally, the analysis was conducted across two pure-bred and two cross-bred genotypes. The result may signify that across breeds, birth weight is not important when maintaining rectal temperature after birth. This notion is supported by Slee et. al. (1980) who showed that Scottish Blackface lambs registered higher temperatures one hour after birth when compared with Border Leicesters, despite weighing over 1 kg lighter. Thus, relationships between birth weight and rectal temperature after birth disappear when birth weights are above average, conditions are mild and across breed analysis occurs.

Another way in which to interpret these results is that there is truly no relationship between lamb weight and shape and rectal temperature when measured so close to

parturition. Dwyer and Morgan (2001) did show that lighter lambs exhibited decreased temperatures one hour following birth, but this only just achieved statistical significance ($P = 0.045$). The impact of birth weight on temperature was larger when measured at 24 ($P < 0.001$) and 72 ($P = 0.016$) hours after birth. These results, in part, support the current findings that weight and shape exhibited less of an influence over temperature measured around birth, but did impact upon cold resistance measured at approximately 24 hours of age. In agreement with the within-breed analysis, a significant positive relationship between birth weight and cold resistance was reported across-breed, thus heavier lambs displayed increased thermoregulatory ability. This finding supports previous results in which a similar relationship was reported in an analysis containing ten sheep breeds (Samson and Slee, 1981). Similar positive correlations were reported for shape measures, whereby larger lambs with increased crown rump lengths, increased thoracic circumferences, longer legs, and increased surface areas were more cold resistant. However, when birth weight was fitted as a covariate in the analysis, all these associations disappeared. It can be concluded that cold resistance in the lamb can be largely explained by weight alone and that shape is of less importance.

Another intriguing result was that no relationship between birth coat score and any thermoregulatory trait could be established. This may be due to the fact that the measure of birth coat used was a seven point score, thus it was a subjective estimate of the coat of the lamb. More objective coat measures have been investigated and linked to thermoregulatory ability, with coat depth being related to metabolic rate required to maintain rectal temperature under cold stress (McCutcheon et al., 1983). Whilst such objective measures may be more accurate, subjective coat estimates have been previously shown to be sufficient. In Merino lambs allocated a coat grade of fine, medium or hairy, hairier coats were shown to be advantageous with regards to heat conservation when compared to finer coated lambs (Alexander, 1962b). Thus, the discrepancy in results must not be due to the coat measure itself. It is more likely that the way in which the data was analysed is responsible for the lack of association, with the present analysis including lambs from a range of genotypes rather than from just one breed or strain. Samson and Slee (1981) identified that coat properties such as coat depth are only important within a breed, and across breed are of little importance.

Metabolic responses to lambs whilst under cold stress

Results from the metabolic analysis show that as cooling progressed, the lambs respiration rate declined and carbon dioxide production, as measured by VCO_2 increased, which suggests an increased metabolic rate and is in agreement with previous reports (Alexander, 1961b, Mercer et al., 1979). Blood glucose levels were shown to increase as time under cold challenge increased and rectal temperatures declined. Respiratory quotient (R.Q.) estimates in lambs under summit metabolism, the maximal metabolic response to cold challenge, report R.Q. values of around 0.9 suggesting carbohydrate metabolism is most important at this time (Alexander and Williams, 1968), agreeing with the present findings. Additionally, the R.Q. of lambs under cold conditions has been estimated at 0.93, which again suggests carbohydrates are the most important substrate for thermogenesis (Mellor and Cockburn, 1986).

The metabolic analysis supports the reported breed divergence in cold resistance and provides insight into why these genetic differences were observed. In pure-bred lambs, glucose levels were significantly higher and took longer to decline in the BL's when compared with M lambs, providing this breed with increased substrates for thermoregulation, thus they were able to resist hypothermia for longer. A similar finding was observed in the cross-bred lambs, whereby PDBL exhibited increased glucose levels, however in this genotype, levels were still rising at the final blood sampling time point. The fact that blood glucose levels had not begun to decline after an hour of cold stress, and that mean levels were higher at a given time in the cross-bred lambs may help to explain why cold resistance was increased by hybrid vigour when compared with a pure genotype.

Breed differences in circulating glucose levels were apparent, however explanation of these results is difficult. Tissue samples were not collected from lambs so it is unclear if these breed divergences were due to an increase in glycogen stores or efficiency in the mobilisation of these stores. PDM lambs had higher plasma cortisol concentrations but lower blood glucose levels than PDBL lambs. There was no difference in cortisol concentrations between breeds in the pure-bred lambs. Combined, these results suggest that the differences in glucose concentration cannot be explained by altered HPA axis activity or glucocorticoid secretion. It has been suggested however, that increases in glucose during thermoregulation in the lamb are due to hepatic glycogenolysis (Clarke et al., 1994) thus production of glucose in

the liver would most likely explain the observed breed differences. In addition to this, perhaps sucking behaviour differences between the breeds identified in the previous chapter can help to explain the glucose profile differences. Both BL and PDBL lambs were quicker to perform post-natal behaviours and although not measured, this may have led to increased colostrum ingestion over the first 24 hours. Increased colostrum levels have been associated with increased rectal temperature, and increases in circulating glucose levels of up to 43% after cold exposure (Hamadeh et al., 2000). Additionally, colostrum ingestion increases summit metabolic rate in lambs after birth, and R.Q. estimates attribute this increased metabolism to the carbohydrate component in colostrum (Eales and Small, 1981). To be able to support this notion, further investigation into links between lamb behaviour after birth, colostrum intake in the first 24 hours, and cold resistance needs to occur.

Given that NST results in the production of glycerol and NEFA from triglycerides in BAT (Dawkins and Hull, 1964), plasma NEFA levels were measured in the lambs whilst under cold stress. Whilst in the cold water bath, NEFA levels did not differ over time for both pure and cross-bred lambs. However, the sample collected after the lamb had recovered from hypothermia was significantly higher than those collected in the water bath. Little or no increases in NEFA concentrations during cold stress have been reported previously (Alexander and Williams, 1968, Wrutniak and Cabello, 1989) and this is explained by the fact that the majority of free fatty acids produced during lipolysis in BAT are oxidised and only a small amount is released into circulation (Dawkins and Hull, 1964). The lack of increasing plasma NEFA concentration with time does not suggest that fat metabolism is not occurring, but rather products of lipolysis are being used *in situ* during NST. R.Q. values of 0.95 and 0.85 have been reported in the newborn lamb representing a carbohydrate and lipid usage ratio of 83:17 and 50:50 respectively, suggesting that whilst not at the rates of carbohydrate metabolism, fat is still an important fuel source during times of cold stress (Mellor and Cockburn, 1986). During mild conditions however, Alexander and Williams (1968) have suggested that fat metabolism is of greater importance to NST, and this would explain the rise in plasma NEFA observed in the recovery sample collected from the cross-bred lambs (recovery sample not analysed in the pure-bred lambs) once temperature homeostasis had been restored under thermo-neutral conditions.

Decreases in ambient temperature increase metabolism in BAT, increasing type II iodothyronine 5'deiodinase enzyme activity, and thus the conversion of T_4 to T_3

(Silva, 1995). However the reported finding that T_3 concentrations did not rise during cold exposure is consistent with previous reports in both calves (Carstens et al., 1997) and lambs (Clarke et al., 1997a, Clarke et al., 1997b, Clarke and Symonds, 1998). Wrutniak and Cabello (1989) did report an increase in T_3 levels in lambs, though this was only observed after two to three hours of constant exposure to 4°C ambient temperature. Cold exposure results in rapid activation of the hypothalamic-pituitary thyroid axis, increasing levels of thyroid-stimulating hormone (TSH), which after a lag of a few hours, is proceeded by increases in thyroid hormone levels (Hefco et al., 1975). This lag between TSH and thyroid hormone level production would explain why levels of T_3 remained unchanged, but were shown to have risen in the recovery sample collected approximately two hours after the commencement of the water bath test.

Leptin plays a role in energy homeostasis and is secreted by both white and brown adipose tissue (Trayhurn, 1993), although it appears that brown adipocytes themselves are not involved in leptin expression but rather white adipocytes present in BAT are responsible for the hormones release (Cinti et al., 1997). Increased leptin levels result in increased glucose utilisation and lipolysis in adipocytes (Siegrist-Kaiser et al., 1997). In obese rats, leptin administration positively regulates sympathetic regulation of BAT (Collins et al., 1996), and a similar result has been reported in lambs with an increased ability to maintain core temperature after leptin administration reported (Mostyn et al., 2002). In the present investigation, circulating leptin levels decreased as cold exposure increased and rectal temperature fell, and this is consistent with findings in humans (Ricci et al., 2000) and rats (Abelenda et al., 2003, Puerta et al., 2002). Cold exposure results in the sympathetic release of nor-epinephrine which binds to β-adrenergic receptors resulting in lipolysis and the activation of UCP1. The decline in leptin when cold exposure occurs can be explained by this catecholamine release, as increased nor-epinephrine concentrations inhibits leptin secretion in adipose tissue (Trayhurn et al., 1995). Catecholamines have been shown to reduce leptin release by 50% (Scriba et al., 2000) and reduce leptin gene expression to 20% (Kosaki et al., 1996) in adipocytes *in vitro*. Thus whilst NST is up-regulated by leptin, leptin levels are reduced under cold conditions by increased nor-epinephrine secretion.

Links between lamb behaviour and thermoregulation

Lambs with reduced behavioural competency display lower rectal temperatures (Slee and Springbett, 1986), so similar results were expected in the present investigation. Indeed, a significant negative correlation between lamb vigour score and rectal temperature was identified signifying more vigorous lambs recorded higher temperatures after birth. However, given the measures were collected at the same time and the vigour measure was made based on how much the lamb moved about when restrained, the improvements in rectal temperature were most likely caused by mechanical means ie. temperature may have been increased by the fact the lambs moved more. No significant correlations could be established between rectal temperature and behaviour (suggesting the relationship between the two traits is non-linear), however when lambs were graded according to behavioural progression (fast, moderate or slow) following birth, tendencies for lambs with reduced post-natal vigour to display reduced rectal temperatures was established. These results are consistent with others who report that lambs that are slowest to stand and suck, with durations exceeding one and two hours respectively, exhibit lower temperatures one hour after parturition (Dwyer and Morgan, 2006). There are a number of mechanisms that may be responsible for the findings here and elsewhere, and these include a reduction in convective heat loss to the ground in lambs that are quick to stand, and additional energy reserves for thermogenesis provided by colostrum obtainment in lambs that are quick to suck. Additionally, the muscle activity of lambs standing and sucking quickly may have generated heat increasing body temperatures compared with those that were slow to progress behaviourally. Finally, lambs that were slow to perform initial behaviours may have been less physiologically mature, thus struggling to maintain thermogenesis during these initial stages and this will be explored in subsequent paragraphs.

Whilst it has been stipulated that initial lamb behaviours and thermogenic capacity are related, this is the first investigation that has been designed to identify if this is the case. Indirectly, lamb breeds that are quicker to stand and suck have also been shown to display increased T_3 and T_4 levels in the days following birth (Dwyer and Morgan, 2006). The correlation analysis in the present study failed to establish any linear relationship between behaviour traits and cold resistance. However, the reported tendency for lambs that were classified as slow (in the bottom quartile) to stand (and in a similar manner although not statistically significant, those slow to suck) to display reduced cold resistance provides confirmation that the two key

processes in lambs are linked. Whilst the relationship between behaviour and cold resistance is not linear (as lambs become increasingly quicker to perform behaviours a similar increase in cold resistance is observed), lambs that are slowest to progress behaviourally struggle to perform when under cold stress suggesting the relationship may be a threshold one. The largest predisposing factor previously linked to poor lamb vigour that would also influence cold resistance is decreased birth weight. Lambs that are lighter at birth display an increase in latency to stand and suck (Dwyer, 2003, Owens et al., 1985). This lighter weight would increase the surface area to volume ratio exacerbating heat loss, and also reduce available energy stores for thermoregulation. Lambs that were slower to perform key behaviours may also be at a considerable disadvantage at obtaining energy from colostrum. Links between time taken to first suck and IgG levels at day 2 (indicating colostrum ingestion) have been reported in foals (Ousey et al., 2004) suggesting neonates that are slow at reaching the udder ingest less colostrum over the first few days of life. This would exacerbate the already decreased energy levels available for thermogenesis in light lambs.

Metabolic maturity of the lamb around birth and relationships with thermogenesis

Given the complexity of thermogenesis and the processes involved in maintaining core body temperature under times of stress, it appeared logical to make the assumption that lambs born with increased physiological maturity would be better able to cope under inclement conditions than those whose key metabolic systems were still under development. The results reported presently do not entirely support this hypothesis. No link between most of the metabolites and hormones investigated and cold resistance could be established. The only exception was a significant positive correlation between cold resistance and pre-suckling plasma ghrelin levels. Given the lack of relationship between any of the maturity parameters and cold resistance it can be concluded that so called maturity at birth does not influence the lamb's ability to resist body cooling when tested at 24 hours of age. Perhaps there are too many environmental factors that occurred between the blood sample collection 30 minutes after birth and the cold water bath test at one day of age. A key influence may be the ingestion of additional energy in the form of colostrum. Given cold resistance was positively correlated with ghrelin concentrations, and in fact ghrelin concentrations were also shown to be positively associated with feeding

behaviours in the preceding chapter, this is the most likely explanation. Lambs were not starved prior to water bath testing thus milk ingestion was not standardised across lambs. To be able to conclusively state whether or not metabolic maturity at birth does influence thermoregulation, lambs should be tested for cold resistance closer to birth or standardised for energy consumption.

Cold recovery has been shown both in the present investigation and those reported previously to be unaffected by any common factors such as birth weight, birth type (single, twin), sex, and is not related in any way to the lambs ability to withstand cooling. Variation still exists in the trait, so metabolic differences may be a plausible explanation. A pivotal finding of the present investigation was that most of the maturity markers at birth exhibited positive relationships with cold recovery. Lambs with increased blood glucose and plasma NEFA levels (and decreased BUN although not significant), increased plasma creatinine, and increased plasma ghrelin and leptin levels, took longer to recover after a cold challenge. This finding is in complete contrast to what was originally hypothesised as lambs defined as being more 'metabolically mature' at birth were expected to display improved thermoregulation but were in fact disadvantaged when recovering from cold. Whilst initially difficult to explain, the definition of what constitutes maturity in the present investigation may be responsible for this result. In the previous chapter, lambs were classed as having improved physiological maturity based on fetal maturation, energy mobilisation after birth and behavioural progression. Lambs that were faster to mobilise energy provided substrates for the vast number of physiological processes within the body that require it for functioning. Skeletal and neural tissues are not exempt from a high energy requirement, and indeed links between maturity markers and behavioural progression were identified. Cold recovery was tested when the lambs were one day of age, after the severe metabolic challenge of the water bath test. Perhaps lambs that were classed as being more mature were so effective at mobilising energy reserves around the peri-parturient period that after being more active in the hours following birth, combined with a cold challenge that would dramatically deplete energy stores, there was little left to be directed towards thermoregulation.

During the recovery period, lambs were observed to shiver only initially, suggesting NST was the primary means of heat production during this cold recovery time. Perhaps in agreement with the chosen maturity markers and the finding that those lambs classified as having improved maturity struggle with regards to NST is the fact

that the ability to perform NST declines with age. Initially, NST accounts for approximately 50% of thermoregulatory ability, with the remaining 50% achieved through shivering. The contribution of NST to overall thermogenesis declines as the lamb matures with age, so that the lamb is solely reliant on shivering by three weeks of age, even when conditions experienced are mild (Alexander and Williams, 1968). Thus thermoregulation via NST is decreased as the lamb matures with age. If our classification of improved maturity at birth is similar to that which occurs with age in the lamb, the more mature individuals would be less reliant on NST as a method of stabilising body temperature. If the ability to achieve NST is reduced in more mature individuals, this may help to explain why time taken to recover was increased in so-called more mature lambs. This hypothesis that more mature individuals at birth rely more on shivering than NST requires further exploration.

Conclusions

Birth weight was shown to be the largest regulator of cold resistance, females displayed improved thermoregulation over males, and carbohydrate metabolism is of greatest importance in neonatal lambs exposed to cold stress. There are strong links between physiological maturity at birth, behaviour directly following birth and thermoregulation in the newborn lamb. Slow behavioural progression from birth to standing and sucking is associated with decreased rectal temperatures after birth in addition to a poorer ability to withstand cooling at one day of age. Lambs that are classified as being more mature based on metabolite and hormonal markers such as creatinine, NEFA, ghrelin and leptin are not at an advantage with regards to cold resistance at one day of age. However, improved maturity resulted in a decreased ability to recover after a cold challenge. It is proposed that this is due to a reduced ability to perform NST. Given these findings, methods aimed at manipulating metabolic maturity should be explored.

Chapter Four: Impact of peri-conception nutrition on post natal survival of lambs

Introduction

Background

Offspring mortality represents a significant reproductive loss to livestock industries. Whilst there are many suggested means of improving the immediate postnatal survival of lambs, the influence of maternal nutrition has long been shown to be of great importance. Recently, results from the Lifetime Wool project have re-enforced this knowledge, identifying that ewes kept in healthy condition (condition score three) throughout pregnancy exhibit the highest progeny survival (Ferguson et al., 2011).

Obviously birth weight is a prime candidate linking ewe nutrition to lamb survival but recent interest in fetal 'programming', in which restricted intrauterine growth and subsequent low birth weight can influence lifetime health and survival, has raised the possibility that birth weight alone may not be the only driver of survival. The so-called 'Barker hypothesis' relating intrauterine growth to the development of various metabolic syndromes, has led to an explosion of interest in fetal 'programming' in which the establishment of organs and systems are influenced by the uterine environment. Many of these programming events are epigenetic in nature; that is, occurring by environmental modifications to gene expression. A special case of this programming applies in the peri-conception period, when the nutritional requirements of the conceptus are minimal, but metabolic activity is high (Robinson et al., 1999). This review examines the evidence that fetal 'programming' initiated as early as the peri-conception period, influences metabolic and physiological events with potential impacts on neonatal maturity and lamb survival.

The hypothesis that peri-conception nutrition could influence fetal growth and development was formed based on three previous occurrences, both *in vitro* and *in vivo,* that highlighted the importance of diet and early embryonic environment during this early phase of gestation.

The Dutch winter famine

A unique opportunity to study the impacts of nutritional deprivation at various stages of human pregnancy was presented by the so-called Dutch Winter Famine which took place between 1944 to 1945 during World War 2. The study was unique in that the population under study had previously been well-nourished, the period of deprivation was relatively short, and the subjects had extensive and accurate medical data records (Roseboom et al., 2001). The most severe phase of the famine was from December to April and resulted in the adult rations consisting of between 400 and 800 calories per day. However the food situation improved rapidly and by June caloric intake was back to over 2000. This allowed the impact of various maternal deprivation times of pregnancy in humans, including peri-conception and early gestation.

Birth weight was shown to be influenced by the famine only to those exposed during the last trimester of pregnancy (Stein and Susser, 1975). However placenta weight, but not offspring birth weight, increased in women who experienced the famine during early gestation (Lumey, 1998). The offspring from those women who experienced nutritional restriction during early gestation exhibited an increased risk of obesity later in life (Ravelli et al., 1999). There was also evidence of an increased risk of coronary heart disease in early restricted fetuses and overall these people rated their health as being poorer (Roseboom et al., 2001). Mortality to the age of 50 was higher in those exposed to the famine during early (11.5%), mid (11.2%) and late (14.6%) gestation when compared to those conceived subsequently (Roseboom et al., 2001).

These results illustrate that whilst birth weight was reduced only when exposure occurred during late gestation, early gestation nutrition affected placenta weight and adult health without impacting upon birth weight. This suggests that adaptations may occur to sustain fetal growth but with as yet unexplained consequences for other metabolic and physiological processes.

The large offspring phenomenon

Manipulation of an embryo during the pre-implantation phase has effects on fetal growth which can consequently result in significantly larger offspring (Walker et al., 1996). This phenomenon was first observed after nuclear transfer studies (Willadsen

et al., 1991) but has also been identified after embryonic *in vitro* culture (Behboodi et al., 1995), asynchronous embryo transfer (Wilmut and Sales, 1981) and maternal progesterone treatment (Kleemann et al., 1994). It appears that reproductive technologies involving exposure of the embryo to *ex vivo* conditions adversely influences its growth and development, through mechanisms as yet undetermined. In addition to increased offspring weights, increased abortion rates, increased gestation length, physical abnormalities and increased peri-natal mortality can be observed after early embryo manipulation (Walker et al., 1996).

It is unclear whether the explanation for the accelerated growth patterns is the same for all the above mentioned reproductive technologies, however all involve an altered environment for the embryo prior to genomic transcription suggesting a potential role for epigenetic factors (Walker et al., 1996). Embryonic manipulation coincides with the de-methylation of some imprinted genes, with the most likely affected candidates being insulin-like growth factors (IGF's) as they play a role in placental and fetal development (Hiendleder et al., 2006).

The Barker hypothesis

The Barker hypothesis, now more commonly referred to as the 'fetal origins of adult disease' hypothesis, states that intrauterine nutritional deprivation can result in fetal adaptation which permanently reprograms key organ systems (Barker, 1997). Fetal growth trajectory is altered by metabolic adaptations in response to environmental cues and has negative implications for the health of the offspring.

Support for this hypothesis has been provided by associations between low birth weight and metabolic syndromes such as obesity (Seidman et al., 1991), non-insulin-dependent diabetes (Lithell et al., 1996), stroke (Martyn et al., 1996), hypertension (Curhan et al., 1996), coronary heart disease (Rich-Edwards et al., 1997) and renal disease (Lackland et al., 2000).

Nutrition around conception can exert effects on the early developing embryo

Investigations have highlighted that nutrient restriction prior to mating can influence subsequent embryonic development. *In vitro* blastocyst formation was shown to be reduced when donor ewes were fed 60% ME requirements for eight weeks prior to

ovulation (Borowczyk et al., 2006). Furthermore, a previous investigation concluded embryo growth rate was reduced by lowering maternal food intake 14 days before mating (Rhind et al., 1989). However, when the nutrient restriction was continued past conception, a higher number of good-quality embryos were found in super-ovulated ewes fed at 1.5 and 0.5 maintenance energy requirements compared to those fed *ad libitum* (Lozano et al., 2003). These results suggest restriction prior to mating exerts negative effects on the embryo, but following conception restriction may be beneficial.

Whilst most studies of peri-conception nutrition have concentrated on energy supply, protein levels have also been shown to influence embryo growth. Studies in rats have highlighted that a low protein (6% casein) diet during the pre-implantation phase reduces cell number in the early conceptus, first in the inner cell mass (ICM) and later in both the ICM and trophectoderm (Kwong et al., 2000). This reduction in cell number was due to a slower rate of proliferation rather than an increase in apoptosis. Additionally, protein restriction has shown to induce a mild hyperglycaemia in the dam and this was implicated as the cause of the reduced pre-implantation cell proliferation and reduced ICM and trophectoderm cell numbers. Alternatively, or additionally, amino acid depletion may have contributed to reduced cell proliferation.

These demonstrated influences of peri-conception nutrition on embryonic growth have important ramifications for the management of animals around mating, and may be explained by the effects that restricted nutrition around the time on conception exudes on the developing placenta.

Peri-conception nutrition influences the embryo via placental development

Maternal and fetal exchange of respiratory gases, nutrients and waste products occurs through the placenta, thus it is estimated that two thirds of the variation observed in birth weight in offspring is accounted for by placental weight in the ewe (Mellor, 1983). The placenta is formed from trophectoderm cells during early pregnancy. It has been suggested that altered growth or function of the placenta during this time may explain the influence of nutrition on fetal development (Kind et al., 2006, Robinson et al., 1999).

Evidence that this is the case was provided when an increase in the number of trophectoderm cells in blastocysts from superovulated ewes fed 0.5M for 18 days pre-, and six days post-ovulation was observed (Kakar et al., 2005), resulting in a shift in the ratio of trophectoderm to inner cell mass (ICM). The crucial time point that resulted in this shift appeared to be from ovulation until the day of blastocyst formation. The authors proposed that this increase in trophectoderm cell number from restricted animals may have occurred due to increased feto-maternal contact or enhanced signalling between the conceptus and mother during nutritional deprivation. It was also suggested that the signalling mechanisms that influenced blastocyst development in response to dietary intake were either IGF-1 or progesterone, both of which are altered in concentration during nutrition treatment (McEvoy et al., 1995, McGuire et al., 1992) and affect embryo quality (Kleemann et al., 1994, Sirisathien et al., 2003).

It should not be assumed that the effect of nutrition on placental development is similar for single- and multiple-bearing animals. Maternal nutrient restriction during the peri-conception period, when measured at day 56, resulted in a disruption of the relationship between maternal weight gain and utero-placental growth in singletons but in twins the relationship was reversed (MacLaughlin et al., 2005). This suggests that the nutritional demand of the fetus(es) on the ewe can influence the effects that restriction of nutrition has on placental development. In addition to these gross placental measures, peri-conception nutrient restriction has been shown to directly impact upon maternal and fetal exchange as blood O_2 and pH were reduced when measured in sheep fetuses from ewes nutritionally restricted to achieve a 10-15% reduction in weight 61 days prior and 30 days post artificial insemination (Oliver et al., 2005). This suggests a restriction of nutrition results in a limitation in placental function, with specific reductions in gas exchange between the maternal tissue and fetus.

Given that the trophectoderm cells differentiate not long after conception, it is reasonable to assume that nutrition during this time may influence placental development. This has been shown to be true by the above mentioned investigations, and as the placental impacts on fetal development have long been established, it would be logical to hypothesise that peri-conception nutrition would influence growth of the fetus.

Fetal growth is altered by restriction of peri-conception nutrition

Weight and Shape

Rationally, if embryonic and placental development is altered by peri-conception nutrition, fetal size and shape may also, in turn, be affected. Whilst there is little evidence that fetal weight is changed, it appears that morphology differs between those fetuses that are restricted around conception and those that are not.. Munoz et al. (2007) reported peri-conception nutritional restriction resulted in fetuses which displayed smaller cranial and abdominal diameters measured at day 57. This difference, however disappeared by mid pregnancy, suggesting that the fetus has the ability for compensatory growth after early nutritional insult. This compensatory growth may explain why other investigations have failed to witness the decrease in abdominal measures when measured later in gestation (Quigley et al., 2005).

Organ and Muscle Development

In addition to the impact of peri-conception nutrition on overall fetal size and shape, organ weights can be influenced during this early stage of development. Ewes fed to induce a reduction in body weight of 10-15% from 61 days before until 30 days after artificial insemination produced fetuses with increased liver and heart weight relative to fetal weight (Oliver et al., 2005). Whilst this clear difference in organ weight has been identified, nutrient restriction from 60 days prior to 8 days post conception had no effect on fat development and deposition, with peri-renal adipose tissue weight and peri-renal adipose tissue relative to fetal weight measured at day 143 of gestation showing no difference with nutrition (Budge et al., 2004).

Restricting nutrition around conception has shown to impact upon myogenesis, as muscle fibre development occurs during early pregnancy (Robinson et al., 1999). Overgrowth after *in vitro* embryo culture produced an increase in muscle mass, primary and secondary muscle fibre area, and secondary to primary muscle fibre ratio after culture and subsequent transfer when compared to control animals (Maxfield et al., 1998). Restriction from 18 days before until 6 days after ovulation resulted in day 75 fetuses with lower secondary and thus lower total muscle fibre types in the semitendinosus muscle compared to those that were fed at high requirements for the same period (Quigley et al., 2005). The authors suggest that the decreased concentrations of oviductal IGF-1 reported by Kakar (2003) resulted in the

delayed fetal thus myogenic development. These observed differences in muscle development produced by decreased IGF-1 concentrations may not only have an impact on postnatal survival but may also influence the physiology of muscle metabolism and economically-important carcase traits such as meat yield.

Not only is muscle development altered by peri-conception nutrition, but there is evidence that other physiological systems are affected. An extended period of nutrient and protein restriction has been shown to result in vascular dysfunction (Nishina et al., 2003). In this study ewes were fed either a reduced energy or protein diet commencing 12 days prior to conception and concluding at 70 days of gestation. Fetuses were collected at day 70 and although no difference in body or organ weights were apparent, an imbalance in vasodilators and vasoconstrictors was observed in energy-constrained animals. The authors stipulate that this would have consequences for cardiovascular disorders such as hypertension and coronary heart disease in later life.

Peri-conception nutrition has been implicated as having a role in the aetiology of diabetes with a 78% increase in fetal plasma and 47% increase in maternal plasma taurine witnessed at 119 days gestation when ewe nutrition was restricted from -61 days to 30 days after mating (Oliver et al., 2001). It was suggested by the authors that this increase in taurine may influence maturation of the pancreas and subsequently showed that fetal insulin response to glucose was increased highlighting the importance of nutrition during this stage on the development of insulin secretion. This is an important concept as it may relate to early survival via effects on energy metabolism in the newborn. Energy metabolism is of great importance in key processes such as thermoregulation and initial vigour in neonatal lambs.

Altered nutrient supply during the peri-conception period can also affect the fetal reproductive system with restriction from day 0 to 30 of gestation resulting in significantly more primordial follicles and a reduction in developed primary and pre-antral follicles measured at day 110, independent of significant effects on ovarian weight or morphology (Rae et al., 2001). This suggests that under nutrition can affect follicular development even before ovarian differentiation without impacting upon mass.

Eventual outcomes of peri-conception nutrition on offspring

Gestation length

Prior to birth, a surge in fetal cortisol occurs, which is critical for the maturation of many organ systems and the initiation of parturition (Bloomfield et al., 2003). The first investigation into the effects of peri-conception nutrition on the hypothalamo-pituitary adrenal axis (HPA) identified that ACTH concentrations in late gestation were increased in nutritionally restricted twin lamb fetuses (Edwards and McMillen, 2002). Subsequently, a study involving nutrient restriction (to cause a 15% loss in maternal body weight) from 60 before until 30 days after conception showed that restricted ewes gave birth earlier (139 days) than *ad libitum* controls (146 days) (Bloomfield et al., 2003). Fetal plasma cortisol concentration increased earlier and ACTH concentration was higher in the restricted fetuses, suggesting an accelerated fetal HPA axis maturation.

While these results were supported by later studies in both sheep (Oliver et al., 2005) and humans (Rayco-Solon et al., 2005) contrasting results were obtained by Munoz et al (2007) who showed that gestation length was shorter for ewes fed at 2 ME requirements when compared to those restricted to 0.6 ME requirements for the first 39 days of pregnancy. These contrasting results may reflect timing of restriction, as the latter was only applied subsequent to conception. It seems reasonable on the basis of results to date to conclude that peri-conception nutrition significantly alters the development of the HPA axis in the fetus with significant ramifications for gestation length. What is less clear is if this shift in HPA axis activity indicates differences in neonatal maturity.

Birth weight, shape and growth rate

Birth weight and body shape of the late-gestation fetus have obvious ramifications for neonatal survival. Birth weight is related to survival rate via a polynomic curve (Purser and Young, 1959) with reduced survival at low weights explained by increased susceptibility of death from exposure, and high birth weight lambs being at increased risk of mortality from dystocia. The shape of the neonate also has implications for survival in a similar process to those outlined for birth weight.

Munoz et. al. (2007) showed an increase in birth weight of lambs from ewes restricted nutritionally from day 0-39 of gestation. Lambs from ewes fed at 60% maintenance were 330g heavier than those fed at 100% maintenance. This observed difference in early growth may be explained by the inverse relationship between nutrition and progesterone. Investigations in sheep showed that circulating progesterone concentrations were consistently higher in sheep fed 0.25 ME requirements when compared to those fed at maintenance and double maintenance requirements during oestrous (Williams and Cumming, 1982). Additionally, serum progesterone concentrations in the ovarian vein have been shown to be highest in ewes fed low energy diet and lowest in ewes given *ad libitum* access to feed (Lozano et al., 2003). This increased level of circulating progesterone in the first days after conception has been shown to induce changes in embryo development and increase fetal growth when measured at day 74 (Kleemann et al., 1994).

Whilst a previous investigation identified that nutritional restriction over the first 30 days of gestation tended to result in an increase in total lamb birth weight per ewe mated, this was explained by an increase in conception rate and not an increase in birth weight (Annett and Carson, 2006). As birth weight remained unaffected, the authors concluded that nutrition in the first month of gestation does not impact on growth potential of the fetus and nutrient transfer capacity of the placenta. This agreed with previous reports in which no effect of reduced nutrition during early gestation was seen on birth weight (Gardner et al., 2004, Oliver et al., 2005, Hernandez et al., 2009). Todd et al., (2009) also showed no difference in birth weight but did identify a level of nutrition by type of birth interaction at ten months of age, whereby singleton lambs from restricted ewes weighed more than unrestricted lambs but this difference was not witnessed in twins. The effect of peri-conception nutrition on growth rate was also reported by Munoz *et al* (2007), who showed that lambs from restricted and overfed ewes exhibited greater growth rates to six weeks of age than maintenance fed lambs. This finding however, only carried through to weaning for the lambs from overfed ewes.

In addition to the observed results on growth rate, morphology appears to be influence by peri-conception nutrition. Whilst no effect of early pregnancy nutrition was observed for head length, crown rump length and thoracic circumference, lambs from ewes fed 0.6 ME requirements from the first thirty days of pregnancy had shorter hind legs than those from ewes fed 2.0 ME requirements (Annett and Carson, 2006). This suggests that long bone development is affected by early nutrition levels,

the timing of which coincides with the development and differentiation of the limb bud. Munoz et. al. (2007) also showed early pregnancy nutrition affects limb length, however this study identified that both low and high early pregnancy nutrition resulted had an effect. Such changes in body shape may have important ramifications for lamb survival if surface area to volume ratio is adversely altered.

Vigour and behaviour

Although studies of the effects of peri-conception nutrition on metabolic and endocrine systems are becoming more numerous, only recently has the behaviour of the offspring been investigated. Immediate postnatal behaviour of offspring is crucial to survival as it facilitates the bond with the dam, increases colostrum ingestion and reduces predation risk. Whilst no difference in immediate post natal behaviours have been observed when energy was restricted around conception (Hernandez et al., 2009, Munoz et al., 2007), peri-conception cobalt deficiency resulted in lambs that were less active and spent less time interacting with their dam, which would be detrimental to the development of the ewe-lamb bond (Mitchell et al., 2007). The authors of this study explained this delayed behaviour may be explained by alterations in the activity of methionine synthase for which cobalt is an essential co-factor, and associated effects on the methylation of genes involved in behaviour regulation. Tested at an older age, lambs from ewes restricted to achieve a 10-15% reduction in body weight from 60 days before until 30 days after mating have been shown to be slower in approaching a human measured in an arena test (Hernendez et al., 2007) and display fewer escape attempts when isolated from flockmates (Hernandez et al., 2010). Whilst this increase in aversion to human handling might not affect postnatal survival, obvious on-farm consequences for ease of handling of these animals would be of importance. The idea that complex traits such as behaviour may be programmed during early embryonic development is intriguing and worthy of further investigation.

Health and survival

The fact that peri-conception nutrition may influence metabolism and health, as suggested by the Dutch winter famine, has resulted in a number of investigations into effects on cardiovascular function, glucose metabolism and immunity, all of which may influence neonatal survival. The finding that cardiovascular disease experienced

during later life in offspring exposed to under-nutrition during the first trimester (MacLaughlin and McMillen, 2007) re-enforces previous research efforts in which the effect of peri-conception nutrition on hypertension was investigated. Reduced protein levels both around the time of conception (Langley-Evans et al., 1996) and during the pre-implantation (Kwong et al., 2000) phase have resulted in an increase in systolic blood pressure in rat pups. Subsequent investigations in sheep have shown peri-implantation restriction results in increased pulse pressure, reduced rate pressure product and a leftward shift in baroflex function curve (Gardner et al., 2004).

Investigation into the effects of peri-conception nutrition on glucose homeostasis identified that lambs from undernourished ewes exhibited a 10% increase in the area under the plasma glucose/time curve, a reduced early insulin response and a decreased insulin to glucose ratio when measured at ten months of age (Todd et al., 2009). These differences however, were not witnessed when measured at four months of age. The authors stipulate that this altered glucose tolerance late in life was due to a reduced number of mature β-cells in the pancreas. These results suggest that glucose tolerance is affected by altered nutrition before and during early pregnancy which may have consequences for the lifetime health of the animal, but effects on early postnatal metabolism may be negligible, if any at all.

In addition to effects on glucose homeostasis, nutrition during early pregnancy has been shown to influence both naturally-acquired passive immunity and thermoregulatory ability, both of which would have enormous ramifications for neonatal survival. Lambs from restricted ewes (0.6 ME requirements from day 0 to day 39) tended to have higher immune status as measured by zinc turbidity units (reflecting the amount of immunity ingested from the colostrum by the lamb) and T_4 levels and significantly higher free T_3 levels than those fed at maintenance or above (Munoz et al., 2007). This led to a tendency for these lambs from ewes restricted during early pregnancy to exhibit reduced mortality when compared to medium or high levels of nutrition. This is the first study to identify a link between peri-conception nutrition and postnatal survival, with previous reports reporting no treatment effect (Annett and Carson, 2006).

Conclusions

Many of the reported impacts of peri-conception nutrition on offspring birth weight, shape, health and metabolism are inconsistent, but there is sufficient evidence to support the notion that peri-conception nutrition may influence several key parameters known to be associated with survival of the neonate. As shifts in the HPA axis of fetal lambs have been reported, metabolic maturity at birth may be influenced which, given the findings of previous chapters, may impact upon postnatal behaviour and thermoregulation. Knowledge of the impact of nutrition on early 'programming' of the fetus through effects on hormones and epigenetic modifications will undoubtedly grow over the next decade or so. In this investigation however, the aim is to identify whether differences in parameters important for post natal survival exist between ewes fed at different nutritional levels around the peri-conception period, and if changes exist, identify whether these changes are great enough to influence lamb survival.

Method

Animals

All experiments involving animals were carried out with approval from the University of Adelaide Animal Ethics Committee (S-025-2008). The animals belonged to the dual-purpose flock housed at Turretfield Research Centre, Rosedale South Australia which experiences a Mediterranean climate with an annual rainfall of 468mm. This flock was established in 1996 with the objective of improving both fleece and meat quality using a performance index and visual assessment. A total of 450 Merino ewes with an average live weight of 59.7 ± 0.47 kg were used for the experimental treatments, and 68 single and 64 multiple bearing ewes lambed from the H treatment group, 60 single and 46 multiple bearing ewes from the M treatment and 51 single and 61 multiple bearing ewes from the L treatment.

Treatment

The animals grazed on principally subterranean clover together with volunteer grasses, including annual ryegrass and barley grass prior to the treatment being imposed. The three nutritional treatments involved feeding the animals at 0.7 maintenance energy requirements (M), 1.0 M and 1.5 M (ARC, 1980) and feed constituents for 1.0 M diet are outlined in Table 4.1. Levels of feeding were adjusted according to treatment. The nutritional treatment was imposed on animals 17 days before and concluded 6 days after artificial insemination. After the conclusion of the treatment all groups were run as a single flock until lambing, and fed to maintain a body condition score of 3.0 – 3.5 (where 1 represents an extremely emaciated animal, whilst 5 represents an exceptionally obese animal).

Table 4.1 Ingredients and amount (kg) used to formulate diet for maintenance energy requirement with metabolisable energy (ME), crude protein (CP), acid detergent fibre (ADF) and dry matter (DM) (Pullman and Hughes, 1980).

Ingredient	Amount (kg)	ME	CP (%)	ADF	DM (%)
Grass, clover, hay	0.5	8.5	8.1	330	85
Peas	0.15	13.4	23.1	80	90
Barley	0.35	13.7	12.2	53	90

Management

The ewes were synchronised using progesterone sponges (Chronogest, Australia) inserted intra-vaginally and removed 12 days later, two days prior to laparoscopic insemination. The two replicate groups were inseminated on subsequent days. On day 50, ewes were scanned via ultrasonography to determine pregnancy status and litter size. All non-pregnant ewes were removed from the study. On day 125, the pregnant ewe flock was weighed, ewes were separated into their treatment groups and grazed in paddocks of similar pasture quality and availability, and with similar access to shelter. A subset containing ten twin and eight single bearing ewes from each treatment group were housed in an additional paddock and run as a single flock with similar pasture quality to the larger flocks. After lambing had commenced, each day, any ewe that had not lambed by 15:00 h in the subset group was drifted off into an adjacent paddock.

Measurements

Ewes were weighed before being allocated to a treatment (day -75 relative to conception), prior to the application of the treatment (day -20), at artificial insemination (day 0), at the conclusion of the treatment (day 15), at day 90 and at day 125 of gestation. Within twelve hours of birth lambs were pedigreed, sexed, weighed and litter size was recorded. An estimated age of the lamb was given according to the wetness of coat (Table 4.2).

Table 4.2 Estimation of lamb age based on coat wetness and lamb vigour.

Score	Description
0	Wet- limited membrane breakage on feet (< 1 hour- new born)
1	Has walked- still wet and at birth site (1- 4 hours)
2	Dry- difficult to catch and follows mother (> 4 hours)

Rectal temperature was measured using a digital clinical thermometer, lamb shape (crown-rump, metacarpal and thoracic length), subjective vigour score and birth coat score was recorded using the methods outlined in Chapter 2.

In addition to the subjective vigour score, lambs were also timed for a number of behaviours after release from the tagger. The lamb was placed on the ground after all other measures were recorded and at this time a stopwatch was started.

Latencies for the lamb to bleat, stand, contact the dam and follow the dam were measured. Due to time constraints, any of these behaviours that took longer than three minutes were not recorded.

Nine sets of twins (n = 18; three sets of twins from each nutritional treatment) were randomly chosen to receive an implant (DST- micro T temperature logger, Star-Oddi, Iceland) that logged core body temperature every 10 min continually for a period of five days. The devices were inserted the day of lambing after local anaesthetic (2 mL Lignocaine 20 w Adrenaline, Independent Veterinary Supplies, Australia) subcutaneously in the inguinal region of the lamb and held in place using a single suture. Additional to these measurements, the subset group were weighed, measured for rectal temperature and a 5 mL blood sample was collected on days one, three and five into lithium heparin blood tubes. Before bleeding both ewes and lambs were herded into a smaller yard where upon the ewes udder was covered for a period of no less than two hours. The blood samples were analysed for glucose levels immediately after collection using a glucometer (Hemocue Glucose 201+, Medipac Scientific, Australia). The blood was then spun and plasma was stored at -20ºC.

The date of lamb death and lamb survival was noted for all treatment groups. Survival was divided into three intervals which included birth to three days of age (0-3), birth to seven days of age (0-7) and birth to marking (approximately 35 days of age; 0-M). An autopsy was carried out on all lamb deaths to determine the cause of death (Holst, 2004).

On day 5, the lambs from the subset group (n = 72) were killed by lethal injection of pentobarbitone sodium (1 ml/ 2kg body weight Lethabarb, Virbac, Australia). Tissues collected and weighed from each lamb included adrenals, kidneys, fat (both white and brown adipose tissue), brain liver, lung, spleen, heart, thymus, and muscle (quadricep, tricep, longissimus, semitendinosis). Within an hour of death, tissues were placed in RNALater® (Applied Biosystems, Australia) for storage at -20ºC.

Statistics

All analyses were conducted using Genstat for Windows 11th Edition (Payne et al., 2008) and a P-value < 0.05 was deemed significant. All data that was not normally

distributed were transformed with the appropriate transformation, and back transformed for presentation of results. All traits were analysed using a linear mixed model (LMM), with the exception of binomial traits (conception rate and lamb survival), which were analysed using a generalised linear mixed model with binomial distribution and a logit-link function. Where the analyses did not include a random term, a general linear model (GLM) was used. Along with main effects, any significant first order interactions were included in the models. Analysis type and model terms can be found in Table 4.3.

Significance levels obtained from these analyses will be presented in the Results section using the following scheme: P value < 0.1 is represented with †, P < 0.05 with *, P < 0.01 with ** and P < 0.001 ***.

Table 4.3 Statistical models used for analysis of all traits recorded, where x indicates inclusion in the model.

Trait	Analysis	Covariates					Random Effect	Fixed Effects				
		Group	Date of birth	Lamb age	Birth weight	Birth weight^2	Sire group	Nutrition	Age of dam	Sex	Birth type	Maternal score
Ewe weight and condition	GLM							x	x			
Conception rate	GLMM							x	x			
Gestation length	GLM	x						x	x	x	x	
Litter size	LMM	x						x	x			
Birth weight	LMM	x	x	x			x	x	x	x	x	
Birth coat	LMM		x	x			x	x	x	x	x	
Skeletal measures	LMM		x	x	x		x	x	x	x	x	
Temperature	LMM		x	x	x		x	x	x	x	x	
Lamb vigour	LMM		x	x	x		x	x	x	x	x	
Timed lamb behaviours	LMM		x	x	x		x	x	x	x	x	x
Weight over 5 days	GLM		x					nested within day	x	x	x	
Temperature over 5 days	GLM		x					nested within day	x	x	x	
Glucose over 5 days	GLM		x					nested within day	x	x		
Lamb survival	GLMM	x	x	x	x	x	x	x	x	x	x	

Experimental Schedule

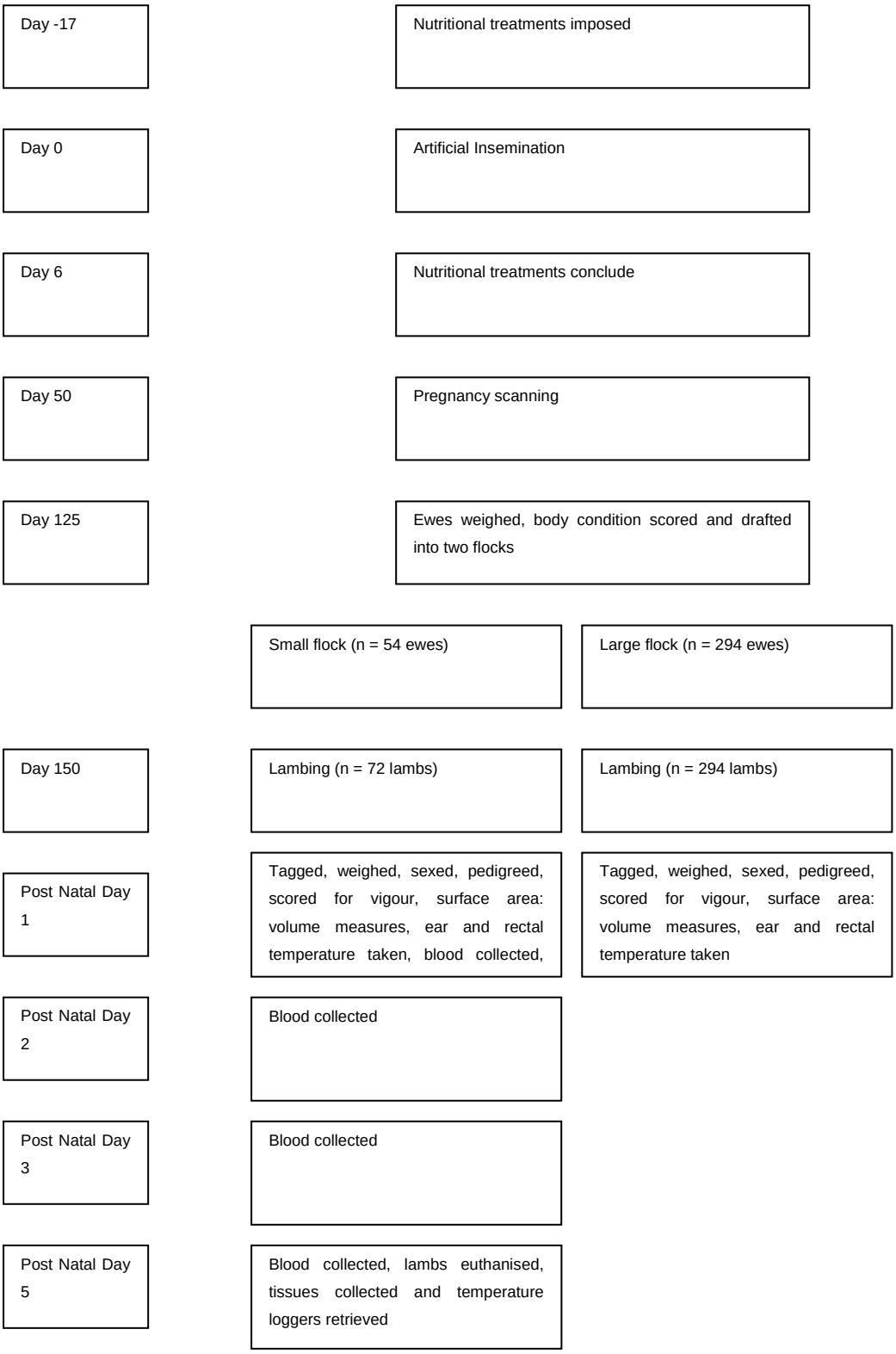

Figure 4.1 Experimental schedule identifying flock structure, timing of nutritional treatments and recorded traits.

Results

Ewe weights and condition scores

All ewes followed the same pattern in body weight change throughout gestation. Ewes decreased in weight from -20 days to artificial insemination and then gained weight up until the last measurement at day 127 (Figure 4.2). There was no difference in ewe body weight prior to, or at the commencement of, the nutritional treatment. However, ewes fed at 1.5 M were heavier at artificial insemination in the middle of the treatment (62.07 ± 0.59 kg) as well as 15 days after insemination at the end of the treatment (68.46 ± 0.64 kg) when compared to those being fed at 1.0 M (day 0: 59.32 ± 0.59, day 15: 66.28 ± 0.63 kg) and 0.7 M (day 0: 59.16 ± 0.60, day 15: 65.98 ± 0.65 kg; $P < 0.01$). By day 75, ewes fed at 1.0 M and 0.7 M no longer differed in weight from 1.5 M ewes and this continued until the last recorded measure.

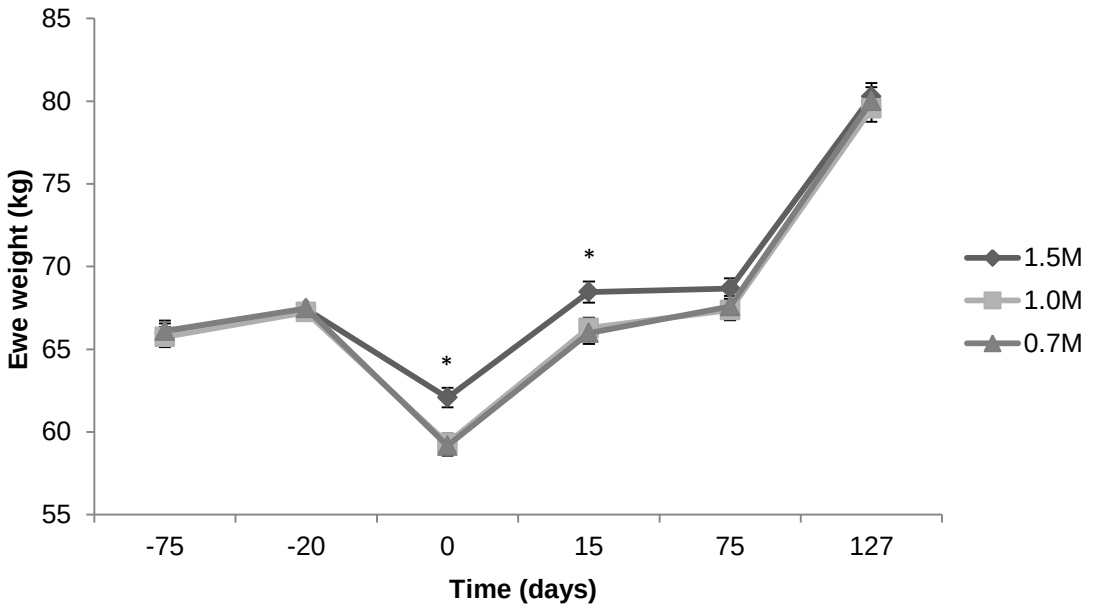

Figure 4.2 Body weight (mean ± SEM) changes over time for ewes fed 0.7, 1.0 and 1.5 maintenance energy requirements (M) from days -17 to +6 around insemination (* represents significant difference ($P < 0.05$)). Day 0 marks artificial insemination.

Reproduction

Pregnancy status

When pregnancy scan status was analysed there was no difference between 0.7 M and 1.0 M ewes, however there was a trend (P = 0.05) for ewes belonging to the 1.5 M treatment to have a higher percentage of positive scans. Ewes being fed above maintenance averaged a pregnancy rate of 64% whilst those ewes being fed at or below maintenance averaged 54% and 52% respectively.

Gestation length

The average overall gestation length was 149.3 ± 0.1 days. Nutrition did not influence gestation length but there was a trend for increasing age of dam to result in increasing gestation length (P = 0.1). Type of birth was highly significant with singles experiencing a longer gestation (149.9 ±0.2 days) compared to multiples (149.0 ± 0.1 days; P < 0.001).

Litter Size

Litter size at birth remained unaffected by nutrition, but age of dam was shown to exert a significant effect. Six- year old ewes had a smaller litter size (1.39 ± 0.13) when compared with all other age groups (Figure 4.3; P < 0.05).

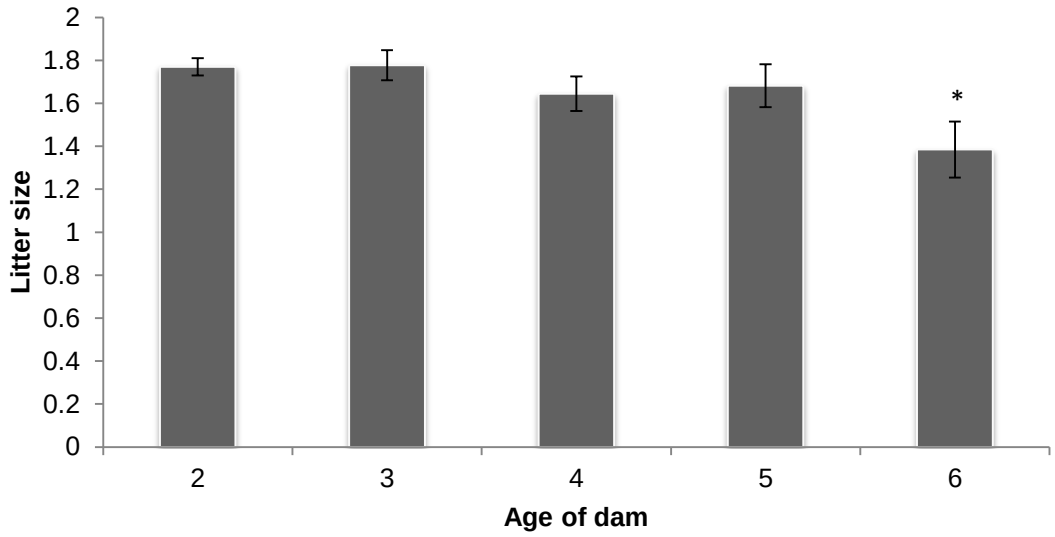

Figure 4.3 The influence of age of dam on litter size (mean ± SEM) recorded on day of birth (* represents significant difference (P < 0.05)).

Lamb weights and size

Nutrition of the ewe had no impact on birth weight of the lamb. Birth weight was affected by sex, with male lambs weighing approximately 10% more than females. Additionally, twins weighed only 85% of single lambs. Birth weight was significantly ($P < 0.001$) affected by gestation length with a longer gestation resulting in a heavier lamb (Figure 4.4). For every day increase in gestation length, lambs gained an additional 0.21 ± 0.03 kg *in utero*. Weaning weight was also unaffected by nutrition, but was increased in male and single lambs ($P < 0.001$).

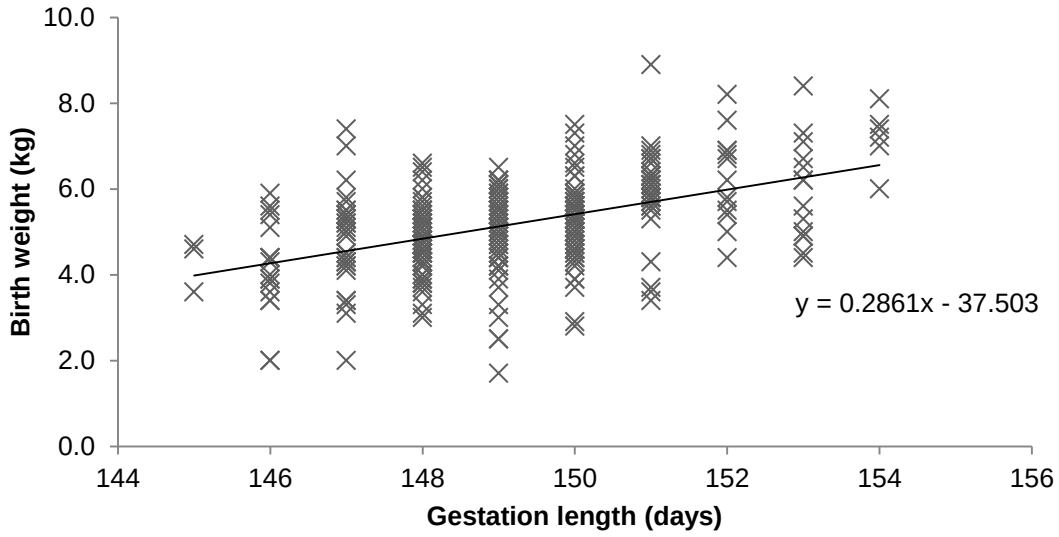

Figure 4.4 The relationship between gestation length (days) and birth weight (kg) of lambs measured within 12 hours of birth.

A relationship between age of dam and birth weight was found, with lamb weight increasing with ewe age (Table 4.4). Lambs born to two-year old ewes weighed 15% less than those born to five and six-year olds.

Table 4.4 The effect of age of dam on lamb birth weight (mean ± SEM; where [a] denotes significant (P < 0.05) difference from [b]).

Age of Dam	Birth weight (kg)
2	4.4 ± 0.23[a]
3	4.82 ± 0.24[ab]
4	4.78 ± 0.23[ab]
5	5.18 ± 0.26[b]
6	5.09 ± 0.27[b]

Metacarpal length was affected by age of dam, sex of lamb and type of birth but when birth weight was fitted as a covariate in the model, sex was the only factor with an influence. Male lambs had a greater metacarpal length (11.27 ± 0.1 cm) than females (11.10 ± 0.1 cm; P < 0.01) at the same weight. Sex and type of birth significantly influenced thoracic circumference prior to birth weight being fitted, however the inclusion of birth weight removed any significant effects. Both metacarpal length and thoracic circumference were not affected by the nutritional treatment. The crown- rump length of the lamb was influenced by birth weight, sex, type of birth and nutrition, however when adjusted for birth weight, only nutrition

attained significance (Figure 4.5). Thus when all lambs were brought to the same birth weight, those from ewes fed 1.5 M were longer (50.8 ± 0.59 cm) than lambs born to the restricted group (0.7 M: 49.4 ± 0.55 cm; P < 0.05).

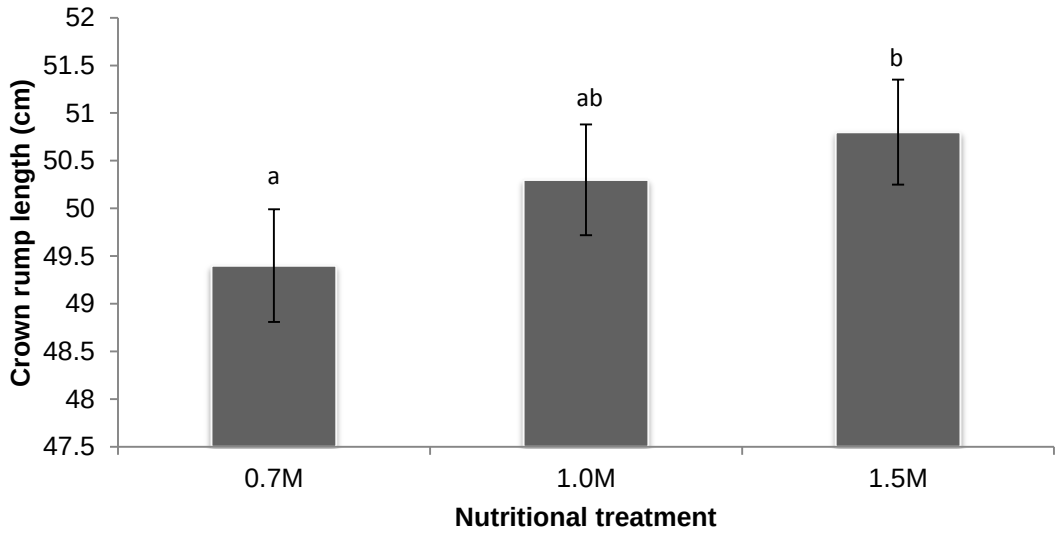

Figure 4.5 Crown- rump length (cm) for lambs born to ewes fed at 0.7, 1.0 and 1.5 maintenance energy requirements (M) from days -17 to +6 of insemination ([a] represents significant (P < 0.05) difference from [b]).

Rectal temperature

Estimated lamb age at tagging affected rectal temperature (Figure 4.6). Lambs with an estimated age of less than one hour had a lower rectal temperature (38.5 ± 0.34 ºC) compared to lambs one to four hours of age (39.5 ± 0.07 ºC), which were in turn higher than those greater than four hours old (39.04 ± 0.06 ºC; P < 0.01).

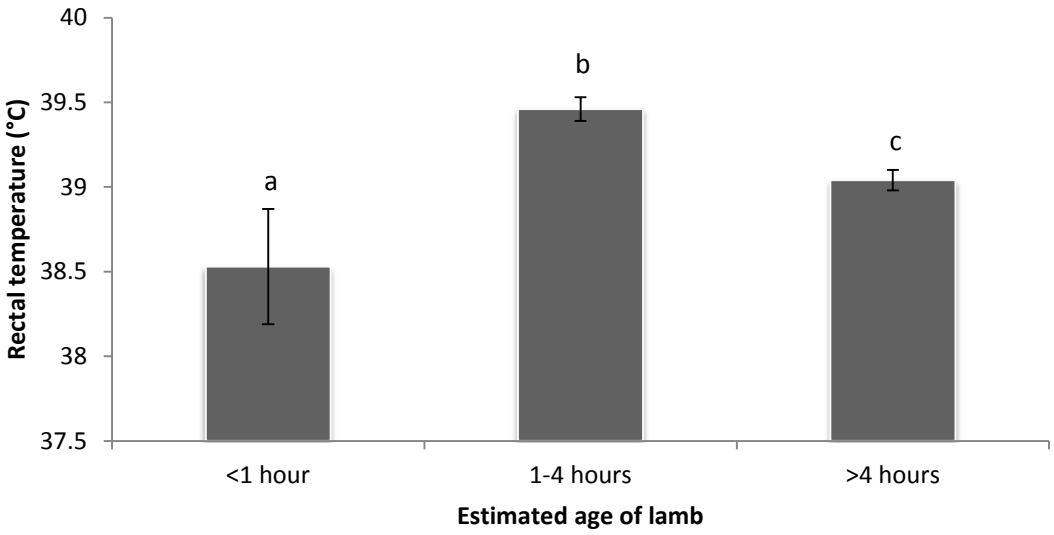

Figure 4.6 Rectal temperature (°C) for lambs with an estimate age of less than one hour, one to four hours and greater than four hours of age at tagging (^{a}, ^{b}, ^{c} denotes significant (P < 0.01) difference).

Birth coat score

Female lambs received a higher birth coat score and thus were hairier than males (2.37 ± 0.28 and 2.13 ± 0.27 respectively; P < 0.05). Nutrition alone did not impact on birth coat. There was a significant interaction between sex and nutrition, with female lambs receiving a higher score than males in the high nutrition group only (Figure 4.7). Additionally, twins were hairier when compared to singles (2.38 ± 0.28 and 2.11 ± 0.27 respectively; P < 0.05).

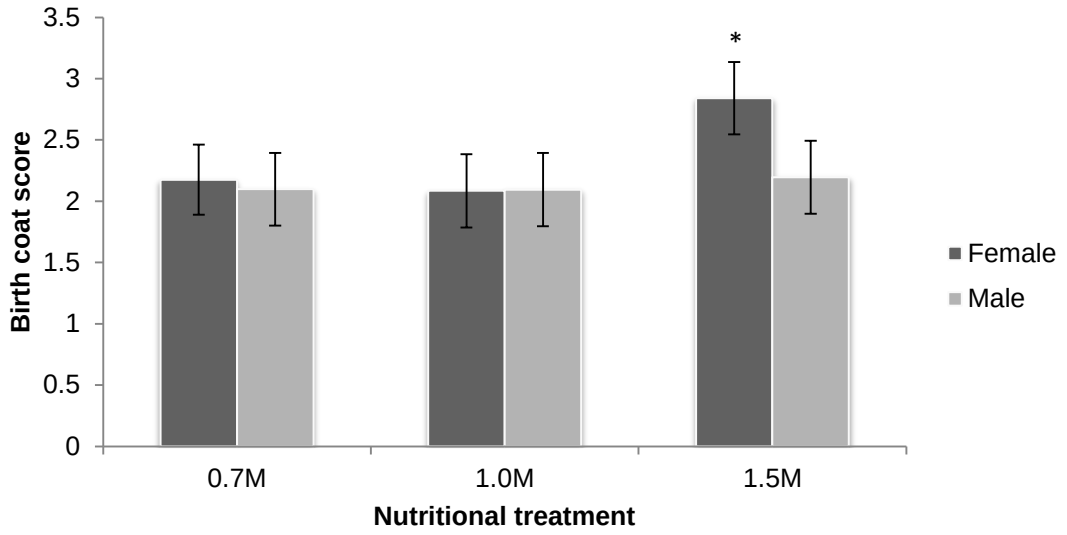

Figure 4.7 Average birth coat score recorded at birth for male and female lambs fed at 0.7, 1.0 and 1.5 maintenance energy requirements (M) from days -17 to +6 around insemination (* represents significant difference (P < 0.05)).

Lamb vigour

Subjective lamb vigour score

Male lambs were allocated a higher average vigour score than their female counterparts, ie. they were less vigorous (2.45 ± 0.17 versus 2.23 ± 0.17 respectively; P < 0.05). Furthermore, lambs tagged at a younger age were less vigorous than older lambs (Figure 4.8).

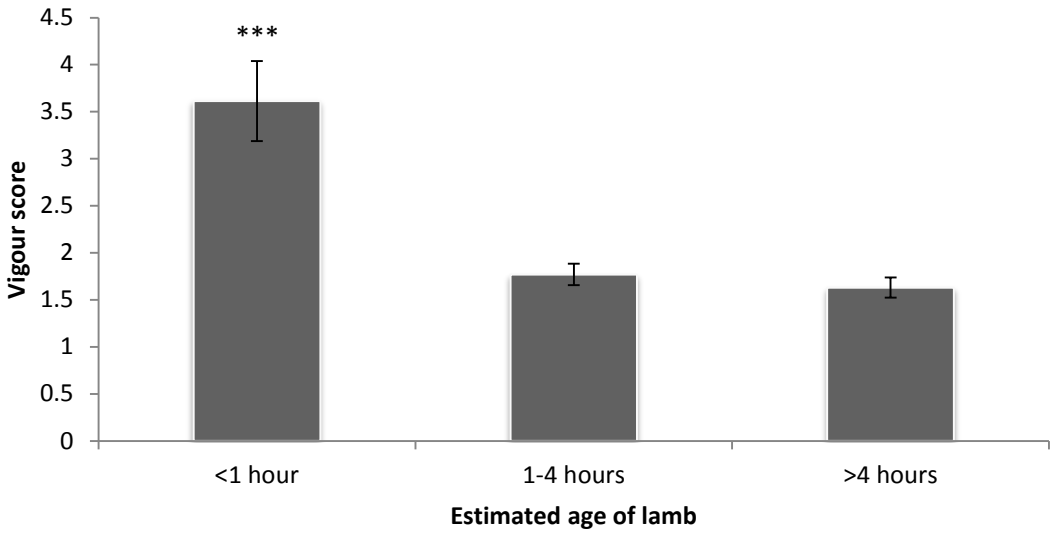

Figure 4.8 Average vigour score (mean ± SEM), where 1 represents high whilst 5 represents low vigour, for lambs tagged at less than one hour old, one to four hours old and greater than four hours old (* represents significant difference (P < 0.001)).

Timed lamb behaviour measures

Maternal behaviour score (MBS) exhibited the biggest influence over all the timed vigour measures recorded. All behaviours increased in latency for increasing (or poorer) MBS (Table 4.5).

Table 4.5 Time taken for the lamb to bleat, stand, contact and follow a dam (mean ± SEM) for ewes with differing maternal behaviour scores (MBS) ([a,b,c,] represents significant difference (P < 0.05) from other MBS categories).

	Latency to perform behaviour (sec)			
MBS	Bleat	Stand	Contact Dam	Follow Dam
1	1.2 ± 7.0[a]	23.2 ± 11.7[a]	9.8 ± 11.9[a]	49.4 ± 8.2[a]
2	2.9 ± 7.3[a]	29.1 ± 12.0[ab]	16.7 ± 12.1[a]	52.2 ± 6.2[a]
3	8.8 ± 7.5[ab]	37.2 ± 12.5[ab]	51.6 ± 12.2[b]	70.2 ± 7.7[b]
4	15.2 ± 7.5[ab]	47.0 ± 12.5[ab]	103.3 ± 16.4[c]	106.1 ± 26.9[c]
5	25.2 ± 10.5[b]	52.6 ± 18.6[b]	-	-

Age of dam also affected the timed measures, however after MBS was fitted to the model, it was only significant for time taken for the lamb to follow. The slowest lambs

to follow were from two year old ewes (106.8 ± 11.06 sec) in comparison to all other age groups (60.2 ± 11.9 sec; P < 0.05). Additionally, type of birth remained significant for time to contact and follow dam (Figure 4.9), with single lambs performing these behaviours faster (contact: 36 ± 4.8 and follow: 55.3 ± 9.1) than multiples (contact: 56.2 ± 4 and follow: 81.1 ± 8.8; P < 0.05).

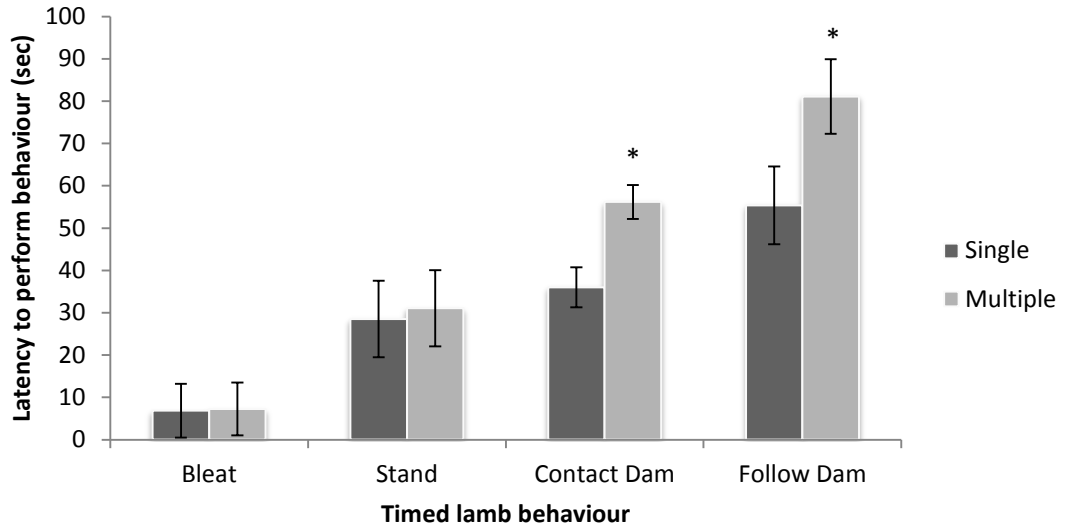

Figure 4.9 Observed differences in latency (mean ± SEM) for the lamb to bleat, stand, contact and follow dam (sec) upon release from tagging between single and multiple born lambs (* denotes significant difference (P < 0.05).

Lamb temperatures, weights and blood glucose over the first five days

Rectal temperature did not vary over day one, three or five and was not influenced by age of dam, sex, type of birth or nutrition over these time points. Weight increased over time but there were no differences at any given time point between nutritional treatments. Similarly, level of nutrition did not affect blood glucose concentrations however, type of birth was significant for blood glucose over time (Figure 4.10). Measured at day one, singleton lambs exhibited a higher glucose concentration than twins (singles: 6.13 ± 0.32 and twins: 4.85 ± 0.21 mmol/L; P < 0.01). This difference disappeared by day three but by day five, twin lambs had increased glucose concentrations above singles (singles: 5.74 ± 0.32 and twins: 6.51 ± 0.23 mmol/L; P < 0.01). Single lamb blood glucose concentration remained constant to day 5 whilst twins increased over time.

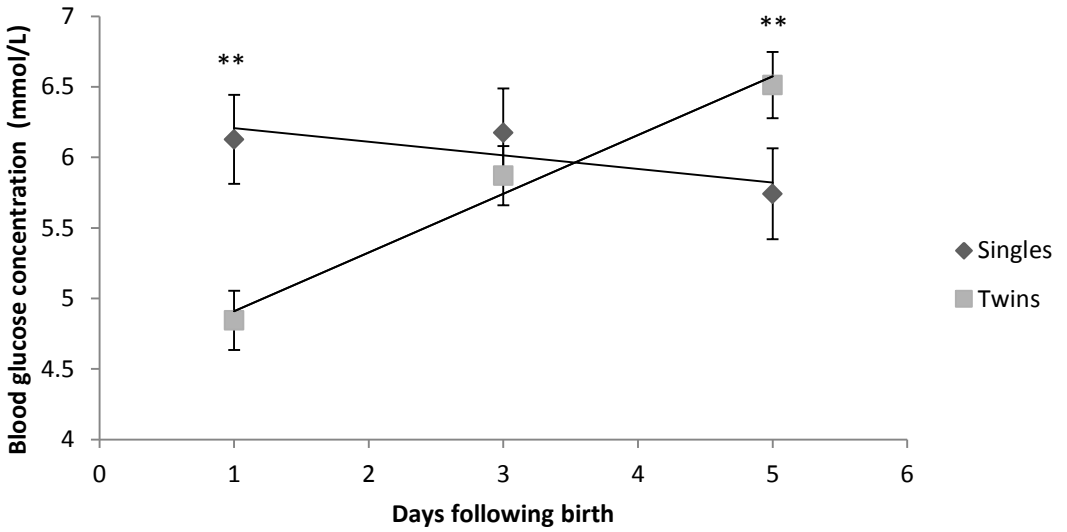

Figure 4.10 Blood glucose concentrations (mean ± SEM) measured on days one, three and five for single and twin born lambs (where ** represents P < 0.01).

Organ weights

There was no difference in relative (measured as a percentage of total body weight) organ weights between the three nutritional treatments when measured at day five. Absolute thymus and ovary weight were the only two organs shown to be affected by the nutritional treatments, and these were increased in the lambs from ewes fed 1.5M ($P < 0.05$).

Lamb survival

The overall survival rate for lambs born in the present study was 87%. The effect of birth weight and birth weight^2 was significant for survival measured at all intervals (Table 4.6). Age of dam, sex of the lamb, type of birth and nutritional group had no influence on survival.

Table 4.6 The significance level of fixed effects used to estimate lamb survival measured at either 3 days (0-3), 7 days (0-7) or marking (0-Marking).

	Age when survival analysed		
Fixed effect	0-3 days	0-7 days	0-Marking
Birth weight	***	***	***
Birth weight²	**	**	**
Age at tagging	†	***	**
Day of birth	NS	*	*
Age of dam	NS	NS	NS
Sex	NS	NS	NS
Type of birth	NS	NS	NS
Nutrition	NS	NS	NS

The age of the lamb when tagged influenced survival (Table 4.6 and Figure 4.11). Lambs tagged immediately after birth exhibited lower survival rates than those tagged later (greater than one hour after birth).

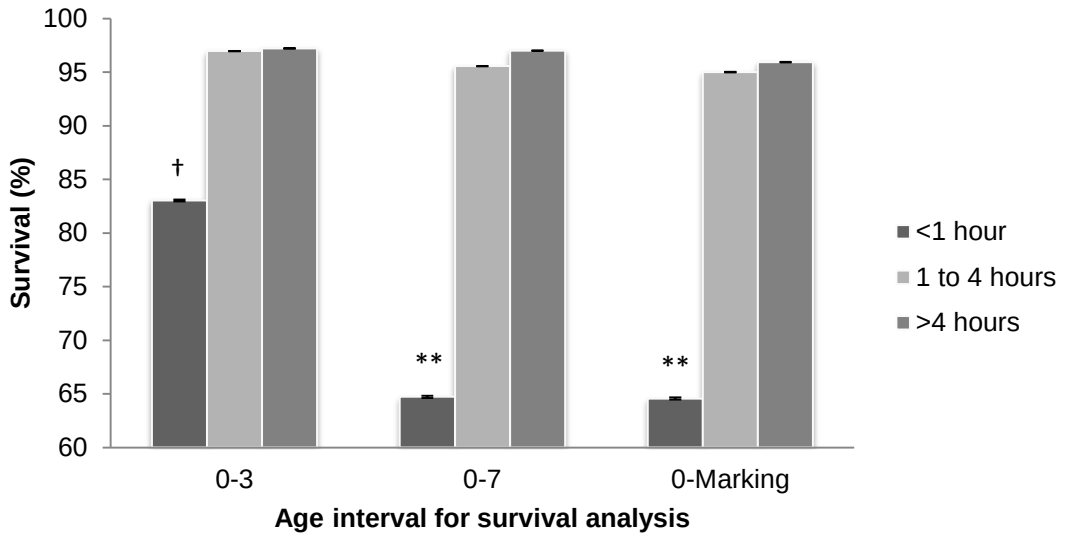

Figure 4.11 Survival of lambs (mean ± SEM) measured at either 3 days (0-3), 7 days (0-7) or marking (0-Marking) for lambs tagged at less than one hour (<1 hour), one to four hours (1 to 4 hours) and greater than four hours of age (>4 hours), († denotes trend (P < 0.1) and ** denotes significant difference (P < 0.01) within interval analysis.

Causes of mortality

A total of 37 lambs died from the total of 275 births, giving an overall mortality rate of 13%. The majority of lambs were diagnosed as having died from either dystocia or starvation (Table 4.7). There were a high proportion of lambs classified as dying *in utero* or from deformity. Due to the low number of lamb deaths, the effect of nutrition or any other factors on cause of death could not be estimated.

Table 4.7 Cause of death determined by autopsy (Holst, 2004) for lambs and the percentage that this cause contributes to overall mortality.

Cause of lamb mortality	Number of animals	Percentage (%)
Dystocia	12	32.4
Starvation	11	29.7
Exposure	2	5.4
Predation	1	2.7
Deformity	4	10.8
Dead *in utero*	4	10.8
Undiagnosed	3	8.1
Total	37	100

Plasma and tissue sample analysis

Little treatment effects were observed on any of the traits examined in this investigation, and subsequently no difference in lamb survival was identified. Plasma and tissue samples collected from autopsies were not pursued further.

Discussion

Results from this experiment have identified restriction of nutrition during the peri-conception period does not impact on offspring survival, most likely explained by the fact that no treatment effects were exerted on behaviour or other phenotypic traits of importance. Restriction of nutrition around conception did result in a decreased lamb length after adjustment for birth weight, which may have ramifications for thermoregulation and survival, although this was not realised in the present investigation. Lamb vigour measured recorded around tagging were shown to be influenced by estimated lamb age, behaviour of the ewe and if the lamb was a single or multiple.

Ewe live weights throughout nutritional treatment

Confounding between nutritional treatment regime and subsequent weight gain represents a major flaw in this experimental design. After the treatments had been implemented, ewes were returned to paddocks where effects on ewe weight disappeared by mid gestation. So in addition to altered nutritional availability during the treatment period, the treatments differed in weight gain (ie. ewes on maintenance or restricted diets undertook a period of increased feed intake). This could have been avoided if the animals were individually housed and fed identical diets after the treatment concluded maintaining the weight divergence. However, as the main measure was lamb survival, high animal numbers were required making this difficult to implement. Another design error was the separation of the ewes into treatment groups at day 125 of gestation. Whilst efforts were made to ensure paddocks were similar, this would have resulted in further confounding between nutritional treatment and paddock environment in late gestation. This could have been circumvented if ewes were randomly allocated to paddocks with regards to treatment group.

The objective of this experiment was to determine the effects of ewe nutrition during the peri-conception period (days -17 to +6 of conception). Nutritional treatments were designed to achieve weight loss, maintenance and gain in the ewe over the experimental period. Unfortunately the treatments imposed resulted in all treatment groups losing weight over the 17 days leading to artificial insemination. Ewes fed at 1.5 M should have put gained weight whilst those fed at 1.0 M and 0.7 M should have maintained and lost weight respectively. It appears that the nutritional quality of paddocks the ewes grazed on was over estimated and it wasn't detected until they

were brought in for insemination. This could have been avoided by weighing the ewes at more frequent intervals during the treatment period, as well as more accurately measuring ewe feed intake, paddock feed availability and herbage quality.

Despite all ewes losing weight, at insemination ewes fed 1.5 M were heavier. There was however no difference in weight between the 1.0 M and 0.7 M groups and this may be explained by the diets not being divergent enough for such a short treatment period. Edwards et at., (2005) used a longer period of nutritional treatment (-60 to +7) and showed that whilst ewes fed at 1.0 M were heavier at the conclusion of the treatment period than those fed at 0.7 M, after two weeks the groups were not different, which is in agreement with the results observed here. Previous investigations have used alternate approaches to impose nutritional treatments in which weight differences were achieved. A variation to feeding a diet estimated to provide each sheep within a group a certain level of energy is to feed diets that result in a specific weight reduction (10-15%) over time (Hernandez et al., 2009, Oliver et al., 2001, Rumball et al., 2009, Todd et al., 2009), and this approach may have been more suitable for such a short treatment period. However, this requires housing animals individually which would not have been possible in the present study as large numbers of animals were necessary in order to measure lamb survival.

The ewes did begin gaining weight after insemination. Whilst at the end of the treatment ewes from the 1.5 M treatment were heavier, there was no difference between the two subsequent treatments and as such interpretations of differing results between 1.0 M and 0.7 M should be taken with caution. There may however, be physiological effects as a result of the treatments that are independent of weight. Differences in live weight were not large or apparent at conception, nevertheless treatment comparisons are valid in that real nutritional treatments were imposed and it cannot be discounted that they induced significant physiological and metabolic changes within the ewe that could be passed on to offspring. Indeed there is evidence, as reviewed earlier in this chapter, that live weight independent effects occur from such treatments.

Conception rate and gestation length of the ewe

Whether a ewe is pregnant at scanning is a function of conception rate and early embryo loss. Previously, low levels of nutrition have been identified as being of

highest benefit to oocyte quality. More oocytes were classified as grade one (high quality) in ewes fed 0.5 M compared with *ad libitum* access to feed (Lozano et al., 2003). The underfed ewes also exhibited greater cleavage rates in the oocytes. This suggests that restriction of dietary energy prior to mating increases oocyte quality and thus it would be expected, increases conception rate, however, results from the same study showed oocytes from 0.5 M ewes tended to have the lowest fertilisation rate after mating. Additionally, ewes fed at 1.5 M had the highest percentage of good quality embryos (eight cells or more at time of measurement and acceptable morphology) when measured at day four compared to 0.5M and *ad libitum* ewes. Embryo survival was also shown to be reduced in ewes fed low levels of nutrition after mating (Rhind et al., 1989). These last results are in agreement with the present finding that 1.5 M ewes tended to have increased positive pregnancy scan results when compared to ewes fed at decreased dietary energy levels.

As identified and discussed in Chapter 2, lambs with reduced gestation may experience a fast-tracked maturation *in utero,* and are therefore better able to signal parturition at an earlier fetal age. Ewes nutritionally restricted around conception have been shown previously to experience a gestation length six days shorter than control animals (Bloomfield et al., 2003). The authors explained this finding in a subsequent investigation that identified accelerated HPA axis maturation in the fetuses from restricted ewes (Bloomfield et al., 2004). This altered maturation formed the basis for the nutritional manipulations in the present investigation, but no difference in gestation length was observed, which would suggest the treatments applied failed to alter fetal maturation. This disparity could be explained by the length and severity of nutritional treatment, with Bloomfield et al., (2003) using a greater period of restriction (-60 to +30 days) and a higher level of dietary restriction (to cause a 15% loss in weight) than those inflicted in the present study. Munoz, et al., (2007) also showed no difference in gestation length from ewes fed 0.6 M and 1.0 M maintenance from 0 to 39 days, in accord with the present results. It would appear that the dietary treatments imposed were too short in length, were imposed at the incorrect stage of gestation or may have not provided adequate nutritional restriction to result in the shifts in fetal maturation reported previously.

Shape and morphology of the lamb

The finding that birth weight remained unaffected by peri-conception nutrition is not unique (Annett and Carson, 2006, Bloomfield et al., 2004, Gardner et al., 2004, Hernandez et al., 2009, Lassoued et al., 2004, Todd et al., 2009). It is thought that physiological changes due to altered nutritional levels occur in the fetus independent of birth weight. However, previous investigations have identified a link between weights at older ages and nutrition in contrast with the absence of differences in weaning weights in the present experiment. Todd et al. (2009) showed in singleton lambs, restricted nutrition resulted in higher live weights measured when weaned at ten months of age. Once again, this disparity may be explained by the severity and length of nutritional insult. Whilst not observed presently, the difference in mature weight independent of birth weight is of interest not only in production animals for maximising growth rates, but also in human health with increasing awareness in the phenomenon of 'fetal origins of adult disease' with specific reference to obesity.

Clear genetic and phenotypic correlations between birth weight and gestation length have been established across a wide range of livestock species including beef cattle (Bourdon and Brinks, 1982, Reynolds et al., 1980), dairy cattle (Davis et al., 1954, DeFries et al., 1959), pigs (Omtvedt et al., 1965, Rydhmer et al., 2008) goats (Mellado et al., 2000) and alpacas (Davis et al., 1997). In sheep, regression analysis has shown an increase of 0.9 kg/day (Fogarty et al., 2005) which is far greater than observed results in the present study. This would in part be explained by genetic effects, as the crossbred lambs investigated in the previous study may display an increased growth trajectory when compared to purebred Merino used presently.

Investigations into the effects of peri-conception nutrition on bone development and growth have shown varied results when measured both *in utero* and on subsequent offspring. Whist fetuses have been shown to display decreased girth circumference after restriction (Oliver et al., 2005), this difference has shown to disappear when measured later in gestation and after birth (Munoz et al., 2007), which is in agreement with the present study. The fact that thoracic circumferences are similar across nutritional treatments when measured in older fetuses and post-natally suggests fetal compensatory growth has occurred. Hind limb length has shown to be altered by early gestation nutrition and was explained by the fact that the timing of treatment coincided with limb bud development (Annett and Carson, 2006, Munoz et

al., 2007), however both studies failed to show an increase in forelimb length supporting present results in which metacarpal length remained unaffected.

Whilst differences in embryonic length have been previously identified (Parr et al., 1982), the finding that crown rump length increased with increasing nutrition when measured at term is unique and has not been reported in early pregnancy nutrition investigations elsewhere. This may be explained by the influence of peri-conception nutrition on levels of IGF-1. Kakar (2003) showed oviductal concentrations of IGF-1 were lower in ewes fed a restricted diet around conception and crown rump length has been shown to exhibit a positive relationship with IGF-1 in humans (Ashton et al., 1985) and in cattle (Hiendleder et al., 2006). Interestingly, Brien *et. al.* (2010) identified a link between crown rump length and lamb survival to weaning. When adjusted for birth weight (as was done presently) a negative genetic (-0.54) correlation was found, translating to longer lambs displaying decreased survival. It is presumed that this increase in survival from lambs with shorter crown rump lengths could be explained by a reduction in the surface area to volume ratio and thus a reduction in heat loss. As weather conditions were mild throughout the experiment and death from exposure was minimal, the lambs may not have received adequate challenge to show divergence in survival. This shape difference in the lambs is also of interest in human health as long, thin offspring, known as the 'thrifty phenotype', have increased risk of hypertension (Barker et al., 1992) and insulin resistance (Phillips et al., 1994) in later life.

Thermoregulation in the lamb and associated parameters

It is becoming clear that rectal temperature measured in the newborn lamb has strong phenotypic and genetic links with lamb survival (Brien et al., 2010). Rectal temperature of the lamb was shown to be dependent on age at time of measure and this result is similar to previous findings. Alexander and McCance (1958) identified that immediately following birth, the rectal temperature of a lamb is higher than the ewe however it soon decreases to below that of its mother. This low temperature was witnessed in animals whose rectal temperature was recorded an estimated one hour of within birth and is explained by rapid heat loss due to a cooler external environment and the wetness of the coat from birth fluids. The fall in rectal temperature from that above the ewe to well below also explains the high variation in this measure. After this initial fall there is a subsequent rise due to an increase in

heat production, known as summit metabolic rate (Alexander, 1962c), after which time a steady state of around 39°C is achieved. It should be noted that lamb age in the present study was a subjective estimation based on coat wetness, however agreement with previous thermogenic results appear to, in part, validate this measure.

Birth coat score was measured in the present investigation as it has been shown to have both phenotypic (Purser and Karam, 1967) and genetic (Brien et al., 2009) associations with lamb survival, especially in the first days after birth. Lambs with fine birth coats suffer in harsh conditions presumably due to loss of body heat and because of energy depletion in attempts to maintain core body temperature (Purser and Karam, 1967). Clear sex differences in the hairiness of newborn lambs have previously been identified, with females receiving higher scores than males (Schinckel, 1955). This may be explained by the lower secondary to primary follicle ratio witnessed in females (Butler, 1981) which would give an appearance of hairiness. The finding that females were hairier than males in the high nutrition group only was unanticipated given these past results. This observation was also unexpected as nutrition has only shown to influence follicle formation during the mid to late stages of gestation (Hutchison and Mellor, 1983) as this is when initiation occurs (Hardy and Lyne, 1956). As increasing fetal number and subsequent litter size is in effect a nutritional restriction throughout gestation, it is perhaps not surprising that twin lambs were hairier than singles. This is in agreement with previous results whereby an increase in density of primary follicles measured at day 140 in the fetus (Revell et al., 2002) and a reduction in number of secondary follicles at birth (Doney and Smith, 1964) was observed, which has also shown to persist through to later life (Butler, 1981).

Postnatal behaviour in the lamb

The present study is one of few attempting to relate peri-conception nutrition to early postnatal behaviours. A reduction in latency to perform key behaviours after birth such as to stand and suck has been shown to increase lamb survival rates (Owens et al., 1985) and thus are of great interest. Performing these behaviours soon after birth provides much needed energy to the lamb in the form of colostrum and reduces maternal rejection by improving the ewe-lamb bond (Alexander, 1987). In an attempt to collectively describe these important behaviours without laborious observations, a

number of vigour estimates were measured on the lamb at tagging. No vigour estimate was influenced by level of nutrition which is consistent with previous investigations (Hernandez et al., 2009, Munoz et al., 2007). The observed sex difference in the subjective vigour measurement is agreeable with earlier reports, in which ram lambs in another heavily selected breed (Suffolk) were slower to progress to standing and sucking than ewe lambs (Dwyer, 2003). The fact that males are less vigorous when measured by the vigour score here and in intensive neonatal observations elsewhere may validate this subjective estimation. The finding that vigour score was lower in lambs tagged at less than one hour of age supports previous findings whereby time to stand after tagging was reduced in younger lambs (Everett-Hincks et al., 2005). It would be expected that strength and available energy would be increased in older lambs when compared to those born closer to tagging.

This above mentioned study also produced results which partly support the association between the observed timed lamb behaviours and maternal behaviour score (MBS). Whilst Everett-Hincks *et. al*. (2005) showed no relationship between the other timed behaviours, time taken to stand and to contact dam after tagging was reduced in lambs from ewes with favourable MBS's. The increase in time to stand in lambs from ewes with poor MBS may be explained by the lamb's tendency to display the prey response of feigning death. It was observed that if a ewe was out of sight and earshot of the lamb upon release from the tagger, the lamb's response was to freeze, and often remained in this lifeless position until the ewe returned. This behaviour would also explain the delay in latency to bleat. The proximity of the ewe to her lambs would have an obvious influence on contact time, which would consequently affect follow time. Age of the dam and type of birth of the lamb also influenced the timed lamb behaviours and the observed results are in agreement with preceding investigations (Dwyer, 2003). The behavioural delay seen in lambs from primiparous ewes and increased litter sizes may be explained by both pre and post-natal factors. It has previously been shown that maternal restriction of fetal growth occurs in first parity ewes as placental weight, cotyledon weight, placental efficiency and eventual birth weight of the lamb is reduced in these animals (Dwyer et al., 2005). Similarly, whilst placental efficiency is increased for increasing litter size, this increase is not proportional for placental weight and cotyledon number (Dwyer et al., 2005). This reduction in placental efficiency for nulliparous ewes and reduction in placental and cotyledon weight available to each fetus when litter size is increased would reduce nutritive transport to the fetus, which may result in physiological changes outside the obvious reduction in birth weight. In rats, neuro-motor

retardation was witnessed in pups born to malnourished mothers when post-natal behaviours such as horizontal movement, righting, head lifting, grooming and standing were measured (Simonson et al., 1969). In addition to these pre-natal influences, there are key post-natal environmental effects which may influence the timed behaviours measured at tagging. An inexperienced mother may be more likely to walk away from the tagger without her lamb than those with more maternal experience thus increasing follow time in those lambs born to primiparous dams. This decrease in maternal ability of first parity ewes has been explored previously (Alexander et al., 1993, Dwyer et al., 1998). In litters with more than one lamb, the dam may only contact one lamb and wait for this lamb to follow, leaving other sibling(s) behind resulting in an increase the timed behaviours for multiples. Indeed, high percentages of separation have been witnessed in Merino ewes with multiple lambs (Alexander et al., 1983).

Glucose metabolism in the lamb over the first days

The differing relationship between glucose concentrations over the first five days for single and twin born lambs is intriguing. As previously mentioned, whilst placental weight, number and weight of cotyledons and placental efficiency is increased in ewes carrying twin lambs, this increase is not proportional resulting in a reduction in the transfer of nutrients for increased litter sizes (Dwyer et al., 2005) An increase in litter size has been associated with a decrease in a number of metabolites measured at birth, including glucose, which can be attributed to this placental insufficiency (Stafford et al., 2007) and explains the observed results on the first day. However, on the third day twin lambs showed similar concentrations but by the fifth had exceeded singles in blood glucose concentrations. Previous investigations have identified that twins do exhibit lower glucose at birth but this difference disappears by day seven and subsequent measures (Jaquiery et al., 2011). Further investigation into this divergence in glucose in the first few days of life is warranted.

Lamb survival

The observed lamb survival rate was considerably higher than the estimated average of 80% for the Australian sheep flock (Kilgour, 1992). This was most likely due to the mild weather conditions at the research centre throughout the lambing period. Given the high survival rates, it is probable the 319 lambs utilised for the analysis were too

few in order to identify significant treatment effects. Regardless, the finding that peri-conception nutrition had no consequence for survival agrees with earlier investigations (Annett and Carson, 2006), but contrasts those reported by Munoz *et al.* (2007) who showed that lambs from ewes fed at 60% maintenance showed an increased survival rate at weaning compared with those fed at 100% and 200% maintenance. The authors suggest this increase in survival would most likely be explained by the increased gestation length, increased birth weight, trend for increased immune status and T_4 leve,l and increased free T_3 level in the restricted lambs. This disagreement with present results may be explained by the unexpected weight changes observed in the ewes during the treatment period. Munoz et al. (2007) showed a weight gain in those fed a high energy diet, a slight loss in the medium and a significant loss of weight in the low energy animals throughout the treatment period. The ewes in the present study all lost weight to insemination, and all gained weight in the later stages of the treatment period. Additionally, weight gain differed, which may have removed any treatment effects.

A unique finding of this investigation is that survival was influenced by estimated age at tagging, with those tagged closer to birth demonstrating significantly lower survival rates. An explanation for this observation may be that tagging the lamb closer to birth may interfere with the crucial ewe-lamb bond that is formed in the first few hours following parturition. Time spent at the birth site is of great importance in the formation of this bond (Nowak and Poindron, 2006) and the strength of this bond may be reduced if the ewe is frightened off the site by the human tagger, leading to increased levels of mismothering. A more plausible explanation however may be that estimated age of tagging is confounded with lamb rejection. If a lamb is rejected by its mother following parturition it will not be groomed and will display a damper coat. Thus, a rejected lamb will receive a lower lamb age score and will have a higher probability of mortality from starvation. The mortality of lambs estimated at being less than one hour of age at tagging was 17% from birth to day three and then 18% from day three to day seven. This supports the latter explanation as death from starvation has been shown to occur between 16 hours and five days following birth (Alexander, 1962a).

Observed causes of lamb mortality in the current study appeared to be within the range of previous findings however large variation in autopsy results was reported (as reviewed by Hinch, 2008). This significant range is largely explained by environmental differences experienced across farms, studies and locations. For

example, mortality from exposure can range from 1.2% to well over 90% and is highly dependent on weather conditions experienced by the lamb soon after birth, collectively described as wind chill index (Donnelly, 1984). Previous methods used to standardise environmental conditions such as weather include exposing lambs to known temperatures (Stott and Slee, 1985), climate controlled chambers (Alexander, 1962b) and cold water baths (Slee et al., 1980). As mortality from exposure was in the lower end of the expected range, such a treatment would have been beneficial, especially to test whether lambs from the restricted group with decreased crown rump length displayed increased thermoregulatory capabilities.

Conclusion

In conclusion, while there is some evidence from the literature that maternal nutrition in the peri-conception period can influence the physiological and metabolic 'programming' of the resulting offspring, with potential consequences for lamb survival, our experiment showed few effects on the lamb phenotype and survival. The design of this investigation may be responsible as compensatory weight gain after treatment imposition was observed in the ewes, and lamb numbers may have been too low to detect significant differences given the high survival rates observed. It is also tempting to conclude that this may reflect insufficient treatment severity as evidenced by the small effect on ewe live weight, but this should be tempered by the relatively large differences in nutrition that were imposed and that other reports of live weight-independent 'programming' exists. Moreover, we did record a significant reduction in the crown-rump length of restricted lambs. Changes in lamb shape caused by nutrition around the peri-conception period may influence lamb survival through effects on thermoregulation (surface-area dependent heat loss), although this was not reported as conditions were mild. Future work should focus on testing the ability of lambs from more severe peri-conception nutritional treatments to withstand environmental challenges in addition to exploration of other methods aimed at increasing maturity in the neonatal lamb.

Despite being a significant contributor to reproductive wastage and an area of animal welfare concern within sheep industries, little improvement in lamb survival rates have been reported within Australia in recent times. This is most likely explained by the fact that survival is largely determined by environmental conditions, and that these conditions are multi-factorial, in that the conditions experienced by the lamb include the environment provided by the ewe, the producer, the weather and so on. Additionally, further complications are realised as the environment a lamb must endure is often unique across locations and years, with what is of benefit in one situation potentially being of little benefit subsequently. Given that most lamb loss occurs in the first few days following birth, so that the chance of survival significantly improves with age, it is logical to target improvements in maturity at birth which may enhance ability to withstand the highly variable environmental conditions experienced by lambs.

Improving the physiological maturity of the neonate at birth may aid in the successful transition from pre to post-natal life. Indeed, endocrine shifts have been reported in piglets differing in genetic merit for survival, with increased cortisol levels established in those with high breeding values for survival (Leenhouwers et al., 2002a). Similarly, lambs from a selection line with improved survival differed in metabolic profiles, with increased glucose and NEFA and decreased BUN concentrations identified in the selection line that demonstrated high survival (Thompson et al., 2006). Both investigations speculated that maturity was improved in the line with reduced mortality, however few targeted investigations aimed at defining maturity in the neonate have been published. Even fewer that aim to identify how this maturity aids in survival exist, or target improvements in maturity through experimental manipulation and as a result, the experiments contained within this thesis were carried out to address these gaps in knowledge.

In order to successfully define maturity, a model that captured both immature individuals as well as those with improved maturity was required. Previous models of immaturity, such as IGUR and prematurity, were deemed inappropriate for use in the lamb (discussed previously), thus the unique model that was chosen for use in this series of experiments was behavioural progression as any physiological disruption

caused by an unsuccessful transition to extra-uterine life would easy influence neural regulation of behaviour. In agreement with our chosen definition of maturity, the other models discussed also result in impaired behavioural regulation in the human fetus and neonate (Als et al., 1988, Arduini et al., 1989, Leijon et al., 1980). There are many physiological processes that contribute to the survival of lambs and perhaps of greatest importance prior to birth is the ability to successfully signal the dam that the developmental stage for extra-uterine life has been reached, and following birth, is the ability to independently regulate oxidative metabolism, energy homeostasis and thermoregulation. Any definition of maturity during this time should account for all of these processes.

Results from findings reported in Chapter 2 aid the definition of metabolic maturity in the neonatal lamb. Increased plasma creatinine and NEFA levels, as well as increases in circulating plasma ghrelin and leptin concentrations approximately 30 minutes after birth were identified in those individuals that were classified as being more mature through examining gestation length and post-natal behaviours. Creatinine was chosen as levels have been shown to be elevated in premature human infants (Finney et al., 2000), and as expected results were similar in lambs, with increased creatinine levels in lambs born after a shorter gestation length. Given that the majority of lambs were born within a 144 – 153 day gestation range however, no lambs could be classified as premature, thus it is put forward that those lambs born with increased creatinine levels were more developmentally mature at an earlier fetal age and better able to signal birth through increased HPA axis activity. After birth, a negative association between sucking behaviours and creatinine levels were observed which, in addition to links with gestation length, confirmed the use of creatinine in any maturity definition. Circulating plasma NEFA levels were also strongly related to sucking behaviours in the lamb, with those displaying increased NEFA after birth reaching the udder and sucking sooner than those with lower levels. These results are in support of earlier maturity definitions which suggest that fat and carbohydrate in place of protein reliance soon after birth are an indication of successful transition to extra-uterine life (Greenwood et al., 2002). Glucose and BUN levels were not shown to differ with maturity in the lamb and this was most likely due to the fact that samples were collected at 30 minutes following birth. Samples taken so soon after parturition may reflect maternal nutrient supplies and additionally, did not allow enough time for the neonate to down-regulate protein metabolism.

The HPA axis is involved in fetal maturation of many physiological systems, however cortisol and ACTH levels were unaffected by maturity using the behavioural model explored, and this is not dissimilar to previous findings in the lamb (Dwyer and Morgan, 2006, Mellor and Pearson, 1977). What remains to be determined is if HPA responsiveness to challenge differs with metabolic maturity and this should be explored further in neonatal lambs. A challenge was imposed on the lambs in the present series of experiments at one day of age using the water bath test, however samples were only analysed for cortisol levels. Perhaps further analysis of samples for ACTH should occur. Using a more recognised test, such as corticotrophin-releasing hormone (CRH) or ACTH administration should also be explored. Hormones that did demonstrate relationships with maturity were ghrelin and leptin, both of which have been implicated in maturity previously due to their roles in energy metabolism (Miller et al., 2009a). An interesting finding was that both hormones were only shown to be related to sucking behaviours, as were the metabolites, creatinine and NEFA, discussed above. This would suggest that complex behaviours involving a higher level of co-ordination may be more sensitive and appropriate for detecting differences in metabolic maturity in the lamb. Nonetheless, the current model outlined in Chapter 2 was successful at determining the significance of metabolites creatinine and NEFA, and hormones leptin and ghrelin in neonatal maturation.

Whilst many have speculated that metabolic maturity at birth may impact thermoregulation in the neonatal lamb, experiments conducted in Chapter 3 were specifically designed to determine if this was in fact accurate. Measurements that are indirect thermoregulatory indicators such as rectal temperature, or thyroid hormones levels have been linked to physiological status of newborn lambs previously (Dwyer and Morgan, 2006). The present investigation however was unique in that a cold stress, the water bath test, was used to quantify the lamb's ability to resist chilling and to restore temperature homeostasis after being chilled. Links between post-natal behaviour and rectal temperature soon after birth confirmed earlier findings with slow lambs recording lower temperatures. A novel finding was that behaviour after birth tended to influence cold resistance at one day of age, with those being classed as slow to suck being less able to resist chilling during the water bath test. However this influence of behaviour did not extend to ability to recover after the chilling event. Correlation analysis, where behavioural progression was fitted as a continuous variable, demonstrated no linear relationship between behaviour and cold resistance, though enhanced sucking behaviour was associated with a poorer ability to recover after cold exposure. Physiological measures identified in Chapter 2 were also higher

in those lambs poorer at cold recovery. Thus taken together, an enhanced maturity at birth inhibits recovery after chilling. We have postulated that this is due to a reduced ability to perform NST as lambs were only observed to shiver initially, thus core temperature increases must have been achieved through NST. We arrived at this conclusion as when a lamb matures with age, ability to perform NST declines and is replaced by the mechanical action of shivering. The thyroid hormones used as an indicator of NST, T_3 and T_4, were not investigated in the blood samples collected from lambs at birth and perhaps if analysed, may have helped support or counter this suggestion. Further investigation into the effects of maturity at birth on NST are therefore warranted and should utilise the nor-epinephrine challenge (Alexander and Williams, 1968) as response to this test should signify the non-shivering component of overall thermogenesis. Through results reported in Chapters 2 and 3 it can be concluded that there are irrefutable links between lamb behaviour, metabolic maturity at birth and thermoregulation.

As maturity is linked with behaviour of the lamb and ability to maintain temperature homeostasis, the experiment reported in Chapter 4 was designed influence maturity at birth and examine effects on overall lamb survival. Nutrition during the peri-conception period was chosen as a treatment during this time as it has shown to influence HPA axis development in the fetus (Edwards and McMillen, 2002, MacLaughlin and McMillen, 2007, McMillen et al., 2004), which would have consequences for fetal maturation as glucocorticoids prepare a range of systems for impending birth. Disappointingly, providing ewes with a restricted or above maintenance energy requitement for the 23 days around conception resulted in little effect on almost all phenotypic traits recorded, no influence on lamb vigour and as a result, peri-conception nutrition was shown not to impact upon lamb survival. The easy reasoning for this treatment failure is that the weight pattern followed by the ewes during the nutritional regime was not as expected. All ewes lost weight despite two treatments receiving maintenance levels or above, no difference in weight was witnessed between those that were restricted and those on a maintenance provision, and treatments differed in post-treatment weight gain. Perhaps the length of treatment was not suffice for weight divergence, and pasture quality was underestimated. Nonetheless, severe nutritional treatment was imposed on ewes around conception with no outcome for postnatal lamb survival. The only influence peri-conception nutrition exerted was a reduced crown rump length in lambs when ewes were restricted to 0.7 M compared with those provided with 1.5 M. Although not encountered, we have discussed that this may impact survival through surface area-

dependent heat loss under more inclement conditions. Ideally, a cold challenge such as the water bath test imposed in Chapter 3 should have been included in trial design, however survival was the trait of interest, thus no additional manipulation that could influence survival was employed. There were associations between lamb weight and shape and cold resistance identified in Chapter 3. This would support our suggestion that peri-conception nutrition may influence survival through impacts on lamb shape, however further experimentation that directly examines effects of nutrition around conception on thermoregulation is required to determine if this is in fact the case.

Blood and tissue samples collected from lambs in Chapter 3 were not analysed further, and it is therefore difficult to definitively state whether any of the maturity markers identified in Chapter 2 were altered by nutrition level around conception. The decision not to pursue further analysis was reached after contemplation of the following factors. Vigour score was shown to be associated with post-natal behaviours in Chapter 2, and this did not differ with peri-conception nutritional manipulation. Moreover, there was also no effect of nutrition on gestation length which, coupled with post-natal behaviour was discussed previously as being important in any definition of lamb maturity at birth. Finally, the nutritional manipulations made around conception failed to result in any impact for lamb survival. So whilst direct measures of metabolic maturity were not made, there is sufficient indirect evidence to argue that peri-conception nutritional manipulations made 16 days before and completing 7 days after artificial insemination did not result in alterations in maturity in the neonatal lamb.

This demands discussion on the success of other targeted methods utilised to alter maturation around birth with the goal of improvements in lamb survival. Selection lines have been shown to display increased metabolic maturity markers and exploiting these breeds or strains commercially may be one way to improve maturity in the lamb. A line selected for increased staple strength was shown to display increased survival, and when examined further, lambs from this line showed a reduction in gestation length and elevated glucose and NEFA levels coupled with a decrease in BUN level (Thompson et al., 2006). Dwyer and Morgan (2006) showed that lambs from the Blackface breed displayed a shorter gestation length, improved post-natal behaviour and thermoregulatory ability as measured by thyroid hormone levels in addition to rectal temperature when compared with Suffolk lambs, which the authors stipulated that when combined, translated to improved neonatal maturation.

The peri-conception nutritional treatments outlined in Chapter 4 were imposed to induce previously reported shifts in the HPA axis of the fetus, but another more direct way in which a similar outcome could be achieved is to treat ewes directly with glucocorticoids. Single dose, ewe dexamethasone administration in late gestation has shown to be ineffective at increasing physiological maturity, with little effect of treatment on metabolic and hormonal parameters, lamb behaviour or survival (Miller et al., 2009b). Perhaps the timing or single dose administration of glucocorticoid described in the publication above was not suffice to induce maturational changes in the fetus, and increased treatment regimes should be explored with care given to limit the effects of glucocortioid administration on birth weight and the induction of premature parturition. This may be achieved through physiological rather than pharmacological dosage rates similar to which occur naturally in the near-term fetus, with evidence that this results in morphological and functional maturation in the lung, morphological and enzymatic maturation of the small gut, increased accumulation of liver glycogen, increased glomelular filtration rates in the kidney and stimulates the production of anti-oxidants (reviewed by Liggins, 1994). Additionally, glucocorticoid treatment may influence thermogenesis with increases in thyroid hormone and UCP1 levels reported after fetal infusion of cortisol (Mostyn et al., 2003). Given this large body of evidence in support of cortisol-regulated fetal maturation, altering late gestation fetal HPA axis activity either directly through glucocorticoid administration or indirectly by nutritional or other manipulation should be exploited further.

To conclude, there are strong links between metabolic maturity at birth, key post-natal behaviours and thermoregulation in the lamb. The series of experiments conducted within this thesis provide the first evidence that maturity can be defined by examining a few key circulating physiological indicators in lambs that vary with post-natal behavioural progression, that lambs with increased maturity are better able to regulate core temperature after birth, but subsequently may struggle to re-instate temperature homeostasis after chilling. Given these findings efforts were made to improve metabolic maturity of the lamb at birth. Altering ewe nutrition around conception failed to result in maturational changes great enough to exert influence over lamb survival. Further investigation into alternate methods targeted at improvements in lamb maturation should occur as benefits in lamb behaviour and thermoregulation will impact lamb survival.

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