Investigating the Impact of Prenatal Stress on the Development of the Offspring of Sheep

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Declaration

I declare that I composed the present thesis. The work is my own and any assistance has been acknowledged. This work has not been submitted for any other degree or professional qualification.

Leonor Valente



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Glossary

11ß-HSD 11 beta-hydroxysteroid dehydrogenase

APP Acute phase proteins

APR Acute phase response

ADG average daily gain

ACTH Adrenocorticotropic hormone

AGD Anogenital distance

AVP Arginine vasopressin

BLA Basolateral nucleus of the amygdala

BCS Body condition score

BSA Bovine serum albumin

cDNA complementary deoxyribonucleic acid

CeA Central nucleus of the amygdala

CI confidence interval

CS Conditioned stimulus

CA1 Cornu ammonis 1

CRH Corticotrophin-releasing hormone

CpG ilands Cytosine bases adjacent to guanine bases ilands

DM Dry matter

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

GCM Glucocorticoid metabolite

GR Glucocorticoid receptor

Hp Haptoglobin

HCA Hydrocortisone-acetate

HPA Hypothalamic-pituitary-adrenal

IgG Immunoglobulin

IL Interleukin

LPS Lipopolysaccharide

LTP Long-term potentiation

MRT Mean retention time

MR Mineralocorticoid receptor

UCP1 Mitochondrial uncoupling protein

mRNA messenger ribonucleic acid

NGFI-A Nerve growth factor-inducible protein A

Nr3C1 Glucocorticoid receptor gene

Nutritional restriction NR

pPVN Parvocellular paraventricular nucleus

PBS Phosphate buffered saline

PNS prenatal stress

PI Ponderal index

RT-qPCR.Quantitative real-time polymerase chain reaction

REML Restricted maximum likelihood procedure

SAA Serum amyloid A

BOHB ß-hydroxybutyrate

TS Threatening stimulus

T_4 Thyroxine

T_3 Triiodothyronine

TNF- α Tumor necrosis factor alpha

US Unconditional stimulus

Abstract

Studies across different species have demonstrated that maternal stress during pregnancy can affect offspring development and welfare, both at the physiological and behavioural level. Throughout pregnancy, domestic livestock may experience various stressors that can inhibit health and productivity, and consequently impact on the development of the offspring. Several of these relate to the gestational social environment, a subject that has not received much attention from a prenatal stress perspective. In the UK, approximately half of ewes are housed prior to lambing. As a consequence, factors such as social instability, high stocking density, dog exposure and unpredictable feeding schedule have been identified as potential stressors for housed sheep. Some of these impose a degree of competition for resources while others expose the pregnant ewes to an uncertain and unpredictable environment. This project aimed to investigate the potential effect of these aspects of ewe husbandry when applied during pregnancy on lamb behaviour and welfare.

The first experiment investigated the effects of the legally maximum stocking density and social mixing during pregnancy on the offspring. The second experiment looked at the effects of three different levels of maternal stress on the offspring, which included a higher stress environment where ewes were exposed to a dog, the feeding schedule was unpredictable and stocking density was the maximum allowed legally; a medium stress environment where there was no dog exposure and feeding schedule was predictable but stocking density was kept the same; and a lower stress environment with lower stocking density, *ad libitum* feed and no dog exposure. Lambs were followed from birth to slaughter and the effects of these potential maternal stressors on lamb behaviour and factors related to survival, immunity, growth, and epigenetic regulation of neuroendocrine stress axis function were investigated.

The maternal stressors had very subtle impact on lamb behaviour and growth but birth weight and immunity were not affected. However, effects of prenatal treatment were found on brain morphology and factors regulating stress axis

activity. Therefore there seems to be no major implications for lamb welfare of these maternal stressors. Hence, from a farm management perspective this study suggests that, in general, current ewe management practices in the UK are not detrimental for lamb welfare.

Lay summary

Studies across different species have demonstrated that maternal stress during pregnancy can affect offspring development and have detrimental effects on offspring welfare. In livestock, maternal stress may be experienced as a consequence of normal husbandry conditions. In the UK, approximately half of ewes will be housed prior to lambing and, as a consequence, may be exposed to stressors related to this practice such as social mixing, low space allowance, competition for feed and exposure to dogs.

Two experiments were conducted to assess the impact of sheep husbandry practices in the UK during pregnancy on lamb welfare. The first experiment investigated the effect of a high stocking density and social instability during late pregnancy on the behavioural, physiological, growth and epigenetic and genetic outcomes of lambs. The second experiment investigated the effects of an unpredictable feeding schedule and dog exposure on the behavioural, physiological and growth outcomes of lambs. The offspring of these ewes were monitored from birth to slaughter age.

The results from this study suggest that current husbandry practices on farms in the UK have potential effects on lamb behaviour and growth but do not affect factors of vital importance for lamb survival during the perinatal period.

Conference Proceedings

Valente L, Yusof N, Rutherford R, Stevenson C, Donbavand J, Sinclair K and Dwyer D. (2015). Effect of prenatal stress on twin lambs' development. **Oral communication.** ISAE Regional Meeting, Cork, Ireland, November 2015.

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CHAPTER 1

Literature review

1.1 INTRODUCTION

Prenatal stress (PNS) may be defined as factors with a biological impact on pregnant mothers that consequently influence the development of the offspring (Braastad 1998). The literature on PNS is vast and there are numerous studies focusing on the distinct and long-lasting effects that this type of stress can have on the offspring. Evidence shows that PNS can alter stress-coping ability and behaviour in aversive situations. Further, alterations in exploratory, social, sexual and maternal behaviour and impaired learning ability and motor development have been reported (Braastad 1998). The majority of the research has been conducted on rats, although there is also some focus around humans and very little work done on non-human primates (Kofman 2002).The effects of PNS on farm animals (i.e. livestock) only have only started to be studied more recently (Merlot et al. 2013).

The negative effects of PNS on the offspring might occur as pathologies due to malfunction of a system; however, in other cases they may represent early life adaptation/trade-offs or long-term adaptive strategy that went wrong. From a behavioural-evolutionary ecology perspective, it has been proposed that communicating the presence of external stressors to the fetus could have an adaptive role (Gluckman et al. 2005). The current adaptive theories propose that the effects of adverse conditions may benefit the offspring either in the short (developmental constraints) or in the long term (predictive adaptive responses) in the expectation of future adaptive advantages (Berghänel et al. 2016). For instance, female lizards regularly exposed to the scent of a predator gave birth to heavier, longer tailed and highly predator odour sensitive offspring (Shine and Downes, 1999). These modifications, or programming during the fetal phase of life, plausibly reduce the offspring's vulnerability to predators, and hence may be interpreted as an adaptive maternal manipulation of the postnatal phenotype. These effects benefit the offspring in the long term but at the expense of other more quality-related features (e.g. skill acquisition or immune function) which benefits accumulate with increasing lifespan (Metcalfe & Monaghan 2001, Belsky et al. 2015). When the benefits of PNS are seen in the short term, offspring is predicted to reduce its investment in development, particularly growth, and accelerate

reproductive development increasing the chances for adequate maturation and the opportunity for reproduction (Coall & Chisholm 2003).

The adaptive role of mother-fetus communication can be lost if there is an incongruence between the predicted and the actual postnatal environment. In this case, the characteristics of the offspring will not match the environment and this programming can be maladaptive (Gluckman et al. 2005). For instance, an adaptive increase in vigilance in offspring may become maladaptive when it causes a disabling level of phobia in some individuals (Glover 2011). Additionally, stress during the fetal period increases the susceptibility of the offspring to different pathologies (e.g. obesity, cognitive impairment and altered emotionality) (Brunton 2010), ultimately impacting on animal welfare. With the variety of functions and systems that have been reported to be affected by PNS (e.g. behaviour, brain, reproductive, neuroendocrine, autonomic, immune function) (Kaiser & Sachser 2005), and given that a large number of the outcomes may become maladaptive for the offspring in a captive environment, it becomes essential to have a clearer idea of what the exact consequences are in terms of welfare.

One of the fields that have received increased attention is the consequences of PNS on the regulation of the hypothalamic-pituitary-adrenal (HPA) axis in the offspring. One of the roles of the HPA axis is to modulate the stress response in reaction to stressors. Its correct functioning via negative feedback also ensures that the cascade of events that prepare the body for the “fight or flight” response is restrained when the stressor no longer poses a threat. In the rodent literature, offspring born to PNS mothers frequently show hyperactivity of the HPA axis and prolonged stress responses (Koehl et al. 2001). Such hyper responsiveness could represent an adaptive trait for an animal living in the wild (e.g. increased awareness of the environment in order to increase the chances to escape predators) but this may not be the case in captive environments. Farm animals often encounter sources of stress (handling, restraint, transportation, cold/heat exposure, etc.) throughout their reproductive cycle and if PNS influences the offspring in a comparable way as mentioned above then this can have negative welfare implications. Thus understanding the mechanisms underlying fetal

adaptations to PNS may provide key insights to improve production management conditions in order to minimize states of suffering.

1.2 THE HPA AXIS AND THE STRESS RESPONSE

A stressor can be defined as an actual or perceived threat to the homeostasis of an individual (Schneiderman et al. 2005). In either case, when in the presence of a stressor the organism responds by mounting a biological response in order to restore homeostasis (Moberg 2000). For this, the central nervous system coordinates a biological response which can include a combination of behavioural, autonomic and neuroendocrine responses (Moberg 2000).

The initial response to stress is produced by the autonomic nervous system (Moberg 2000). The sympathetic nervous system produces the catecholamines adrenaline and noradrenaline (Moberg 1985). The physiological effects of an increased circulation of adrenaline include increased heart rate, blood pressure and respiration rate (Tortora & Grabowski 2003), increased blood flow to the brain, heart and skeletal muscles and a decrease in clotting time (von Holst 1998). Additionally, adrenaline triggers the release of glucose and lipids from temporary storage sites in the body into circulation (e.g. liver, skeletal muscle, adipose tissue) (von Holst 1998). These nutrients supply energy to all parts of the body, preparing the animal to quickly react to a stressor (Tortora & Grabowski 2003).

When a challenge to homeostasis persists, the body may adapt to react to the stressor by activating the neuroendocrine system. The predominant neuroendocrine response to a stressor is secretion of glucocorticoids into the systemic circulation by activation of the HPA axis (Brunton & Russell 2008). Multiple rostral (limbic and prefrontal cortical regions) and caudal (brainstem relays, such as the noradrenergic nucleus tractus solitarius and serotonergic raphe neurons) neural pathways, which process primarily emotional or physical stressors, respectively, integrate the information about the stimulus and transmit it to the parvocellular paraventricular nucleus (pPVN) of the hypothalamus (Ulrich-Lai & Herman 2009). Following this, corticotrophin-

releasing hormone (CRH) and arginine vasopressin (AVP) neurons release these peptides from the pPVN, stimulating the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH then acts upon the adrenal cortex, stimulating the production and release of glucocorticoids (corticosterone in rodents and cortisol in other mammals) into circulation. The role of glucocorticoids, together with adrenaline and noradrenaline, is to prepare the body for the “flight or fight response” both from a physiological and behavioural perspective. Their systemic actions include facilitation of sympathetic activation; carbohydrate, protein and fat metabolism, regulation of blood glucose (Dallman et al. 1999); and modulation of the immune system response (Pruett 2001).

Under basal conditions, HPA axis function is controlled through a negative feedback mechanism that allows the axis to regulate itself. Glucocorticoids act at the suprahypothalamic limbic centres (hippocampus), the hypothalamus, and the pituitary gland. They play a key role in the regulation of basal activity of the HPA axis and on the termination of the stress response (Bradbury et al. 1993). By binding to specific receptors: glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (de Kloet et al. 1998); glucocorticoids stimulate the hippocampus to decrease neuronal activity in the parvocellular division of the PVN and inhibiting glucocorticoid secretion (Jacobson & Sapolsky 1991). Additionally, they also act on the hypothalamus PVN CRH neurons (Di et al. 2003) consequently decreasing the release of CRH from the hypothalamus and of ACTH from the pituitary. Both GR and MR receptors are expressed in the brain, with GR being more widespread but enriched in the cornu ammonis 1 (CA1) and dentate gyrus hippocampal subfields, PVN and pituitary gland. MR is mainly found in the limbic system, especially in the hippocampal subfields (Joëls 2008) but also present in the anterior pituitary (Reul & Kloet 1985). MR have higher affinity to glucocorticoids causing them to be extensively bound in basal conditions, and they are thought to be important in ambient glucocorticoid signaling processes (e.g., controlling basal secretion across the circadian cycle) (Dallman et al. 1989). GR is bound when there is high circulation of glucocorticoids such as in stressful situations. Given this binding profile, the

majority of the negative feedback mechanisms are mediated by GR (de Kloet et al. 1998).

In contrast to the hippocampus, amygdala GR activation is thought to be involved in feedforward excitation of the HPA axis. Stimulation of amygdala neurons promotes glucocorticoid synthesis and their release into the systemic circulation (Van de Kar & Blair 1999). Both GR and MR are expressed in the amygdala and glucocorticoids increase expression of CRH in the central nucleus of the amygdala (CeA) and potentiate autonomic responses to chronic stressors (Smith & Vale 2006).

In situations where secretion of glucocorticoids is maintained at a high level (e.g. due to chronic stress), there is a possibility for pathophysiological conditions to occur due to impaired negative feedback (McEwen 2007). Consequences of long-term activation of the HPA axis include depletion of energy stores, lack of immune responses and suppression of anabolic processes which can damage the organism's body (Kofman 2002).

1.3 PNS EFFECTS ON HPA AXIS AND BEHAVIOUR

Previous studies have repeatedly associated maternal stress with altered HPA responsiveness to stress in the offspring. The HPA axis is particularly sensitive to glucocorticoids during development (Gould et al., 1991; Gould and Tanapat, 1999; Welberg and Seckl, 2001) and the perinatal period is a time of particular plasticity at the brain level (Welberg and Seckl, 2001). This leaves brain systems susceptible to remodelling and modification (Brunton, 2010).

Rodents

In rat offspring, PNS usually results in increased or extended duration of the HPA response to stress. For example, results from studies where different maternal stressors have been used show elevated levels of basal plasma cortiscosterone (restraint - Koehl et al., 1997 and 1999; daily handling and saline injection third week of gestation - Ward, 2000) and in response to stress (restraint on the third week of gestation - Henry et al., 1994; social

defeat -Brunton & Russell, 2010). However, the effects of PNS on basal activity of the HPA axis seem to vary depending on the stressor used, the gestation period in which it was applied and the sex and age of the offspring. Indeed, prenatally stressed female rats have higher corticosterone levels throughout the diurnal cycle, whereas males only show increased levels at the end of the light phase (Koehl et al., 1999). However, other studies fail to report any effects of PNS on basal ACTH and corticosterone secretion in males (Brunton & Russell, 2010; Henry et al., 1994). In guinea pigs, prenatally stressed males display elevated basal cortisol when maternal stress was applied on gestational days 50-52 but not when it was applied at 60-62 (Kapoor & Matthews 2005), whereas in females no effects of PNS (same stressor and schedule) on basal cortisol secretion were observed (Kapoor & Matthews 2008).

An important component of a stressor is the perception and control that the threatened individual has of the threat. Hence, in PNS it is important to consider the mother's perception of the stressor. A good example of this was described by Takahashi et al. (1988) who showed that exposing pregnant rats to inescapable compared to escapable electric shocks had different outcomes on the offspring. Offspring from mothers that lacked control over the stressor exhibited higher plasma concentrations of ACTH and corticosterone.

Together with the observed alterations at the HPA axis level, systems and functions that are known to be influenced by HPA axis activity have also been shown to be altered. Indeed, experiments conducted in rodents have emphasized the consequences of maternal stress on offspring emotionality (Thompson 1957, Weinstock et al. 1992, Fujioka et al. 2001), immune system (Vanbesien-Mailliot et al. 2007) sexual behaviour (Velazquez-Moctezuma et al. 1993, Marchlewska-Koj et al. 2003) and learning ability (Lordi et al. 1997).

It is very difficult to interpret results from studies that have used different stressors, in different species and at different stages of pregnancy. However, in the rodent literature, the results show greater consistency when considering the effects of PNS on the HPA axis response to acute stress

rather than its basal activity. In general, PNS offspring have exaggerated and prolonged HPA axis responses to stress in adulthood suggesting impaired negative feedback (Brunton & Russell 2011). Additionally, in rodent studies where offspring gender was taken into account, the HPA axis of female offspring appears to be more susceptible to PNS programming than that of males' (Koehl et al. 1999, McCormick et al. 2000, Weinstock 2007, García-Cáceres et al. 2010).

Farm animals

Despite clear evidence for the effects of PNS on the HPA axis, rodent data cannot be simply transposed to farm animals due to important species differences. Rodents are altricial species, which means that they give birth to very immature offspring with a relatively immature state of brain development at birth. In these species, a critical phase of HPA axis development occurs after birth (Sapolsky & Meaney 1986). In contrast, farm animals give birth to precocious offspring which are relatively mature and mobile from the moment of birth. In precocious species, the major part of neuroendocrine development, particularly of the HPA axis, occurs while inside the uterus (Dobbing & Sands 1979). Hence, stress applied during pregnancy has the potential to have more important effects in later life in precocious animals. Nevertheless, it is worth noting that PNS may also affect the mother behaviour, in which case rodent pups might be more affected as their development occurs postnatally.

Compared to the rodent literature, studies in farm animals are scarce. A variety of studies have been conducted using either synthetic stress inducers, such as ACTH, or more ethologically relevant stressors. One general characteristic of these studies is that the stress treatments often do not realistically mimic a farm environment in terms of stress intensity which questions the practical consequences of such findings in a farm environment. Nevertheless, the effects of PNS on the HPA axis have been reported for species such as cattle, pig, goat, sheep, and poultry. For example, calves that have been prenatally stressed by subjecting the mothers to transport

stress during early/mid gestation, showed a slower decrease in cortisol than controls in response to restraint test (Lay et al. 1997). Pigs born to mothers restrained and injected with ACTH weekly during mid gestation showed increased and prolonged plasma cortisol responses under stressful conditions (social mixing) (Haussmann et al. 2000). In goats exposed to transport in isolation or ACTH injections during the last third of pregnancy, offspring did not differ in their basal cortisol concentrations but showed increased activity of the sympatho-adrenomedullary system (Roussel et al. 2005). Offspring of pregnant ewes exposed to isolation stress twice a week during the last 5 weeks of gestation showed elevated basal cortisol at 25 days of age, but did not differ from controls in the cortisol response to stress (Roussel et al. 2004).

The observed disparity in these results may be due to the use of different stressors and the time during pregnancy when the stressor was applied, but also to the different species being studied. In fact, as previously mentioned in the rodent literature, in farm animals there is also evidence for time dependency for the effects of PNS on the offspring (Couret et al., 2009; Jarvis et al., 2006; Kranendonk et al., 2006). In pigs, oral administration of hydrocortisone-acetate during early, mid or late gestation decreased the response to an ACTH challenge in piglets only when the stressor was applied to the mothers in early and late gestation (Kranendonk et al. 2006). In another study, social mixing in pregnant sows increased CRH mRNA expression in the PVN and amygdala in female offspring prenatally stressed during the second trimester, but females prenatally stressed during the third trimester only showed greater CRH mRNA expression in the amygdala. Females prenatally stressed during the second trimester also took longer to return to stable social behaviour after weaning than control. Both prenatally stressed groups showed increased and prolonged cortisol response to stress (social mixing) in comparison to control (Jarvis et al. 2006). Different type of stressors such as isolation, restraint and social mixing (among others) have been studied and different results have also been found. When social mixing was applied during late pregnancy in sows, Otten et al. (2010) reported no effect on total plasma cortisol concentrations in the piglets as opposed to the

lowered basal plasma cortisol found in piglets whose mothers had been exposed to restraint stress (Otten et al. 2000). In two other studies where ewes were also stressed during late pregnancy, basal levels of cortisol in lambs were found to be elevated in relation to controls when the PNS consisted of social isolation in the presence of a dog (Roussel et al. 2004) but a later study found no differences when the PNS consisted of the same isolation protocol or transport in isolation (Roussel-Huchette et al. 2008). Similarly, in goats, stress during late gestation imposed by transportation in isolation also had no influence on basal cortisol concentration in PNS kids compared to controls (Duvaux-Ponter et al. 2003).

Parallel to the findings of an altered responsiveness of the HPA axis, farm animal studies have also looked at the impact that PNS has on behaviour and adaptation of the animals to their environment. Specifically, in sheep, some of the experiments conducted to date have focused on isolation (Roussel et al., 2004; Roussel et al., 2008), aversive handling (Coulon et al. 2011), shearing (Kenyon et al. 2002) and repeated transportation (Roussel et al., 2008) as sources of prenatal stress. The results of these studies showed evidence for altered stress reactivity, cognitive function and behaviour in lambs subjected to PNS, namely: increased locomotion in isolation and increased cortisol concentrations (Roussel et al., 2004), increased fearfulness (Coulon et al. 2011, 2015), and decreased birth weight (Corner et al. 2010). However, contradictory outcomes have also been reported: reduced reactivity to a fear-eliciting stimulus and increased exploratory behaviour (Roussel et al., 2008), increased birth weight (Roussel et al. 2004), and weight at 3months (Roussel et al., 2008).

In sheep, stress during pregnancy has also been shown to affect maternal outcomes that can indirectly influence lamb survival. The period that follows lambing is of the utmost importance for lamb survival. Most lamb deaths occur during the first 24hr of life due to birth trauma, inadequate nutrition, hypothermia (Nowak et al. 2000). Maternal factors contributing to these death causes include adequate display of maternal behaviour such as licking and grooming the lamb which will help it dry and is important for the formation of the olfactory memory that will allow the mother to selectively care only for her

own lambs. Failure to develop the ewe-lamb bond will result in the mother not accepting the lamb to access the udder and suck (Dwyer et al. 2016), which will lead the lamb to starvation, hypothermia and death. Stress has been shown to alter maternal behaviour but the direction in which this happens seems to vary depending on the stressor. In two different studies using aversive handling or a combination of different stressors, stressed sheep displayed either higher grooming towards their offspring (Hild et al. 2011) or no differences in this behaviour (Coulon et al. 2014). However, in the second study ewes in the stress group refused to accept the lamb at the udder more frequently depending on ewe temperament (Coulon et al., 2014). Other maternal factors such as the quality and quantity of colostrum are also important during the first 24hr of the lamb's life since the ingestion of colostrum is important to maintain adequate levels of nutrition and thermoregulation, to acquire passive immunity and also to the establishment of lamb-ewe attachment (Nowak & Poindron 2006). Colostrum quality has been shown to be affected by maternal stress. For example, in pigs heat stress during gestation tended to decrease IgG content in colostrum (Machado-Neto et al. 1987) and similar results were observed in cows (Nardone et al. 1997). However, psychological stress in pigs during the last third of pregnancy did not affect colostrum IgG levels (Tuchscherer et al. 2002, Couret et al. 2009).

Not all factors related to lamb survival are dependent on maternal behaviour. Lamb behaviour is also critical in determining survival. The speed at which the lamb is able to stand and suck is related to its probability of survival (Dwyer 2003). One factor know to affect these behaviours is birth weight, with lambs that are born lighter being slower to stand and suck (Dwyer & Morgan 2006). However, birth weight can be affected by prenatal stress, but the direction in which this happens is not clear as both positive (prenatally stressed lambs were heavier - Roussel et al., 2004) and negative (prenatally stressed lambs were lighter - Corner et al., 2010) outcomes have been observed.

In addition to the direct effects of stress during pregnancy on the offspring, factors such as maternal parity (Coulon et al. 2011), sex of the lamb (Roussel

et al. 2005, Chojnacki et al. 2014, Averós et al. 2015) and age of the offspring (Sloboda et al. 2002, Roussel et al. 2005) all seem to contribute to the impact of PNS. For example, in an experiment conducted in goats, the locomotor activity response to novelty in one month old prenatally stressed (transport) female kids was increased in comparison to control females, whereas male kids tended to be less active than control male kids (Roussel et al., 2005). The effects of offspring age were observed in sheep where a single maternal injection of betamethasone had little effect on HPA function in offspring at 6 months of age but led to significantly elevated basal and stimulated (intravenous injection of CRH plus AVP) plasma cortisol concentrations at 1 year of age (Sloboda et al. 2002).

These findings highlight the importance of looking at the effects of PNS considering that characteristics related to the offspring (sex, age) or to the dam (parity) may influence the direction of the results. Understanding how these variables interact with each other may elucidate which groups of animals are at higher risk of suffering the consequences of a stressful prenatal environment.

1.4 MECHANISMS FOR PNS

The Placenta

The hypothalamic–pituitary–adrenal (HPA) axis has been of particular focus in uncovering the mechanisms relating to the impact of maternal stress on the offspring (Weinstock 2005, Harris & Seckl 2011, Khulan et al. 2012). One proposed mechanism through which prenatal stress may alter fetal growth and development is increased exposure of the fetus to maternal cortisol. The maternal glucocorticoids reach the fetus through the placenta and are regulated by the balance of 11ß-hydroxysteroid dehydrogenase (11ß-HSD) type 1 and 2. 11ß-HSD-2 converts cortisol into cortisone (Klemcke 1995, Seckl 1998) and is considered of primary importance in excluding maternal cortisol from the fetus (Benediktsson et al. 1997). The other isoform of this enzyme, 11ß-HSD type 1, has lower affinity for cortisol and converts cortisone to cortisol (Seckl & Walker 2001). There is some evidence in rat models that prenatal stress during the last week of gestation can reduce the

expression of placental 11β-HSD2 (Mairesse et al. 2007, Peña et al. 2012). Additionally, in previous studies in rats where 11β-HSD-2 was blocked during pregnancy, allowing an increased transfer of glucocorticoids from the mother to the fetus, the offspring displayed elevated basal HPA axis activity and increased anxiety-like behaviour in aversive situations (Welberg et al. 2000).

Molecular mechanisms: Epigenetics

One suggested mechanism by which the exposure to prenatal stress is thought to programme offspring behaviours and physiology is the modification of the phenotype by epigenetic mechanisms. These are modifications to the genome that do not involve a change in the nucleotide sequence but that can alter gene expression. Two of the most widely studied of these mechanisms are DNA methylation and histone acetylation (Mathers & McKay 2009). The former consists of methylation of cytosine bases adjacent to guanine bases (CpG islands) and is a major epigenetic mark (Hunter 2012). CpG islands can be found within the promoter or enhancer regions of genes and methylation at these sites is typically a silencing mark, resulting in decreased gene expression (Illingworth & Bird 2009). Histones are proteins around which DNA is packed into chromatin, either tightly and transcriptionally silent, or more open and allowing for active transcription. Histone acetylation is typically associated with a transcriptionally active state (Hunter 2012).

These types of epigenetic alterations are susceptible to environmental cues, which can alter the epigenetic marks and, consequently, gene expression and cell/tissue function. This may be particularly important for early life (in utero) exposures, since during this period the genome undergoes major changes in epigenetic marks (Reik et al. 2001). The mechanistic connection between environmental exposures and altered health through epigenetic processes includes several key stages which Mathers and McKay (2009) have termed the four “Rs” of epigenomics. According to the authors this chain of events involves (a) Receiving the environmental cues, (b) Recording it through altered epigenetic patterns, (c) Recalling the resulting epigenomic

pattern through subsequent cell divisions and (d) Revealing the consequences of the altered epigenomic pattern in the cell's transcriptome.

The GR, AVP, CRH and proopiomelanocortin (the precursor for ACTH) genes are located in brain areas involved in regulation of the HPA axis and appear to be susceptible to epigenetic changes associated with the early life environment. These genes are targets for the effects of PNS since they are stress-responsive genes (Hunter, 2012). Previous studies on PNS or glucocorticoid exposure in late gestation focusing on the expression of these genes reported that the hippocampus shows decreased expression of GR (Darnaudéry & Maccari 2008, Seckl 2014) and MR (Darnaudéry & Maccari 2008, Brunton 2010) and the amygdala has increased (McCormick et al. 1995, Seckl 2014) or decreased (Lee et al. 2011) expression of GR and increased CRH (Meaney et al. 2007, Brunton & Russell 2010). This suggests impaired glucocorticoid negative feedback regulation of the HPA axis. Fewer binding sites for cortisol in the hippocampus decreases HPA axis sensitivity to negative feedback, which will produce higher activation of the HPA axis. The enhanced CRH expression in the amygdala may account for the behavioural effects (e.g. altered emotional reactivity) of PNS on the offspring. Intra-amygdala administration of CRH has proven to be highly anxiogenic (Meaney et al. 2007). However, the epigenetic processes that could account for these alterations in gene expression have mainly been studied in relation to early postnatal life models. Weaver et al. (2004) studied the influence of maternal behaviour on DNA methylation and demonstrated that increased pup licking and grooming and arched-back nursing by rat mothers produces offspring with lower levels of DNA methylation and higher levels of histone acetylation of the GR 1-7 promoter in the hippocampus. These alterations induced increased transcription of the glucocorticoid receptor (GR) gene, which is important for the HPA axis feedback loop (Meaney & Szyf 2005).

PNS studies focusing on epigenetic outcomes have not been conducted in sheep. However, undernutrition during pregnancy was shown to cause epigenetic alterations on the lamb brain (Stevens et al. 2010, Begum et al. 2012). Nevertheless, the vast majority of the literature has focused on rodents (e.g. chronic variable stressors - Mueller & Bale 2008). In male mice

offspring, Mueller and Bale (2008) reported increased expression of CRH mRNA in the central nucleus of the amygdala and reduced methylation in the CRH gene promoter CpG island. In sheep, the outcomes of periconceptional or gestational undernutrition were associated with increased histone acetylation and decreased DNA methylation on the promoter region of the GR gene in the hippocampus of the fetal brain (Stevens et al. 2010, Begum et al. 2012). These epigenetic effects, together with altered gene expression, may predispose to altered behavioural phenotypes and HPA axis dysfunction. Whether they can also be induced by other early life insults in sheep, such as psychological stress in the prenatal or neonatal periods, has thus far received little attention.

1.5 PNS AND THE IMMUNE SYSTEM

Glucocorticoids are known to have suppressive effect on immune system (Selye 1955) and have been used clinically for immunosuppression (Franchimont 2004)**.** PNS alters HPA axis function and consequently the circulating concentration of glucocorticoids. Thus, it is not surprising that PNS has been associated with alterations in immune function as well. In general, PNS has been found to have an inhibitory effect on the immunity of the offspring (Merlot et al. 2008), affecting both passive and innate immunity. Additionally, the type of stressor, the timing of application in relation to pregnancy and sex of the offspring seem to affect the immune outcomes (Howerton & Bale 2012). Most of the studies have been conducted in rodents but there is some literature on pigs, sheep and non-human primates.

Passive immunity

Passive immunity, the transfer of antibodies from the mother to the fetus through the placenta or to the neonate via suckling (Borghesi et al. 2014, Coe & Lubach 2014), seems to also be affected by the type/intensity of prenatal stressor. For example, physical stressors (foot shock) in rodents decreased offspring immunoglobulin (IgG) levels at birth and later ages as opposed to psychological stressors (bystander stress: witnessing foot-shocks

applied to another animal without receiving them itself) where IgG levels were decreased only at birth (Sobrian et al. 1992). Interestingly, a later study using noise as a psychological stressor was not able to reproduce the same results, as IgG levels were found to be decreased only at 21 days of age (Sobrian et al. 1997). Nevertheless, differences in results have also been observed depending on the sex and time when the stressor was applied in relation to pregnancy. In squirrel monkeys, chronic social maternal stress decreased IgG levels in males but increased them in females (Coe & Crispen 2000). Furthermore, maternal IgG acquisition seemed to be affected only if the stress period covered the end of gestation, which corresponds to the period of active transplacental transfer of maternal IgG, while stress occurring during mid-gestation had no effect on IgG acquisition (Coe and Crispen 2000).

The effects of maternal stress on the circulating concentrations of IgG have also been described in farm species. In pigs, maternal stress induced by restraint during late gestation significantly decreased IgG concentrations in suckling piglets at 1 and 3 days of age (Tuchscherer et al. 2002). Similar results were observed in calves where offspring born to heat-stressed mothers during late pregnancy presented lower levels of plasma IgG than controls (Tao et al. 2012). The decreased IgG levels in the offspring did not seem to be related to the IgG content in the colostrum, as both studies were unable to find differences at this level. Nevertheless, psychological stress (individually vs. group housing) applied throughout pregnancy in sows did not produce any differences in piglet IgG concentration in plasma (Sorrells et al. 2006). The same results were found when a different psychological stressor (social mixing) was applied either during early or late pregancy (Couret et al. 2009).

Innate immunity

Innate immunity is a non-specific inflammatory response. It involves the release of cytokines (IL1, TNFα and IL6) that are necessary to activate the acute phase response (APR) in order to destroy the invader pathogen. The

APR includes the synthesis of acute phase proteins (APP – e.g. Haptoglobin (Hp), serum amyloid A (SAA), and C-reactive protein (CRP)) which can be quantified individually and used to assess the immune response to the triggering event (Petersen et al. 2004). The literature on the effects of PNS on the innate immune response suggests that the results also vary according to the type/severity of stressor, timing of application and sex of the offspring. Maternal restraint stress during late pregnancy increased interleukin-6 (IL-6) mRNA expression after *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with phytohemagglutinin in adult rats (Vanbesien-Mailliot et al. 2007). In contrast, psychologic maternal stress (sound startle in dark room, daily for six weeks) in rhesus monkeys did not affect plasma IL-6 protein levels in response to recombinant human interlekjin-1 beta (IL-1β) (Reyes & Coe 1997). Maternal restraint stress applied during late gestation increased the magnitude of serum IL-6 and SAA responses to lipopolysaccharide (LPS) in piglets, but tumor necrosis factor alpha (TNF-α) was decreased in males only (Collier et al. 2011). In contrast, maternal stress in the form of social mixing, applied either in early or late gestation in sows, did not affect liver IL-6 mRNA expression in piglet plasma in response to LPS challenge (Couret et al. 2009).

The literature in sheep is very scarce and mostly involves looking at the effects of undernutrition during pregnancy. Eckersall et al. (2008) reported that the innate immune system of the offspring was affected depending on the timing of maternal undernutrition and the APP being considered. Lambs showed no alterations in plasma Hp protein levels but decreased plasma SAA protein concentrations post-vaccination when the undernutrition period was applied during late but not early gestation. Very little is known about if and how other prenatal stressors, such as social stress, can influence the development of the lambs' immune system and consequently alter their susceptibility to diseases and immune responsiveness to stressful stimuli.

1.6 PNS AND BRAIN MORPHOLOGY

Normal brain function depends on the formation and maturation of synaptic circuits (Bourne & Harris 2008). Synaptic circuits consist of hundreds of

billions of neurons which are interconnected by specialized cell junctions called synapses (Colón-Ramos 2009). Dendrites, which are processes arising from neuronal cell bodies (soma), receive synaptic input from the axon of the preceding neuron through a synapse and pass the signal over to the soma and to the correspondent axon, always in a unidirectional way (Colón-Ramos 2009). At the dendrite level the synaptic input is received by small protrusions of various lengths and shapes which are named dendritic spines. Dendritic spines are accountable for the majority of excitatory synapses (Bourne & Harris 2008). Therefore, alterations in their density or morphology can alter neuronal function at a synaptic level (Segal 2005, 2010). Along with alterations in HPA axis function and behaviour described above, it might be expected that extra-hypothalamic structures involved in the regulation of the HPA axis would also be affected by PNS. Indeed, stress experienced during pregnancy has been shown to produce alterations in these corticolimbic structures (prefrontal cortex, amygdala and hippocampus) including alterations in dendritic morphology and spine density in the hippocampus (Mychasiuk et al. 2012) and prefrontal cortex (Murmu et al. 2006, Mychasiuk et al. 2012) and in the number of neurons and glial cells in the amygdala (Kraszpulski et al. 2006). In rodent PNS studies, impaired cognitive performance has been associated with specific alterations in brain morphology. Reduced postnatal hippocampal weight, reduction of neurogenesis in the dentate gyrus, decreased spine density of pyramidal dendrites in the cornu ammonis 3 hippocampal subfield, decreased synaptic density in the hippocampus and suppression of long-term potentiation (LTP- a cellular model for investigating the synaptic basis of learning and memory Bliss & Collingridge 1993) have been demonstrated in the offspring born to prenatally stressed mothers (Ulupınar 2009). However, morphological alterations in the brain and learning performance are dependent on the stressor duration and intensity, similar to other systems/functions affected by PNS. Accordingly, results such as enhanced neonatal neurogenesis, differentiation of hippocampal neuronal processes and LTP have also been reported as a consequence of short-lasting and mild PNS exposure (Fujioka et al. 2001, 2006), with opposing results being linked to severe PNS (Fujioka et al. 2006).

Although the majority of studies have been conducted in male rats, in studies where this was not the case, sex-dependent effects of PNS have been reported. For example chronic variable PNS (a different stressor is applied each day for a certain period of time) increased spine density in hippocampal CA1 pyramidal neurons in males but not in females (Bock et al. 2011). On the other hand, maternal bystander stress produced similar increases in CA1 spine density in both males and females (Mychasiuk et al. 2012). In another study focusing on the prefrontal cortex, where different information is integrated into emotional regulation and cognitive-related behaviours (Heidbreder & Groenewegen 2003), PNS reduced the spine frequencies of pyramidal neurons in male, but not female, rat offspring (Murmu et al. 2006). In parallel with the observed brain morphological effects observed, offspring of prenatally stressed rats have also demonstrated impaired cognitive performance in learning tests (Chapillon et al. 2002, Gué et al. 2004). However, here the duration and intensity of stress exposure and gender vulnerability also influence the outcomes and contradictory results have been found (Ulupinar 2009). Nevertheless, in general, it seems that learning deficits, reductions in hippocampal neurogenesis, LTP and dendritic spine density in the prefrontal cortex are more readily seen in prenatally-stressed males than females in rats (Weinstock 2007).

Sheep research in this field is very scarce. Nevertheless, two recent studies using different types of stressors (aversive handling - Coulon et al. 2013, chronic variable stress - Petit et al. 2015) reported that PNS lambs had a higher spine density on pyramidal neurons in area CA1 as well as a higher ratio of stubby spines to other more mature and synaptically stable spines in area CA1. Furthermore, in one of the studies the medial prefrontal cortex also presented increased spine density on pyramidal neurons (Coulon et al. 2013). Interestingly, these morphological findings were correlated with decreased expression of genes associated with development and regulation of spine morphology in these brain areas, suggesting that alterations in the expression of these genes could in part mediate the observed changes in spine density and morphology. To my knowledge there are no other studies in sheep related to this topic. Thus, questions about possible gender

differences and the effects of other types of prenatal stressors are still to be addressed.

1.7 PNS AND FEAR LEARNING AND MEMORY

Despite the extensive studies on the consequences of PNS on innate fear and anxiogenic behaviour in the offspring (e.g. Wilson et al. 2013), the evidence regarding alterations in the processing of fear-associated learning and memory due to PNS is still very scant in farm animals.

Associative learning is an adaptive process through which an individual learns to anticipate an event by associating two stimuli, such that the occurrence of one comes to predict when the other will occur. Fear conditioning is a form of associative learning very popular in research that has been used in many species (Kim & Jung 2006). In this behavioural paradigm, an unconditional stimulus (US- something that naturally elicits an unconditioned response, e.g. electric shock elicits a post shock freezing response in rodents) is paired with a conditioned stimulus (CS – something neutral that does not elicit an unconditioned response on its own, e.g. a sound) so that an association is made between the CS and US (Curzon et al. 2009). Eventually, presenting the CS alone will elicit a conditioned response. During fear conditioning there are two different types of learning: contextual and cued. The former involves placing an individual in a novel environment and applying the US (Curzon et al. 2009). If the animals have made an association between the environment and the US they will show a conditioned response when later placed in the same environment. Cued fear conditioning is similar, but a CS is added to the context. When the animal is presented with the CS in subsequent sessions a CS-induced fear response is observed, and this will happened regardless of the context (Curzon et al. 2009). If, after the acquisition of fear conditioning, the animal is repeatedly exposed to the context/CS in absence of US a decline in conditioned fear responses is observed. This is a form of inhibitory learning known as extinction and it does not involve forgetting or memory erasure (Myers et al. 2006).

Important brain regions involved in the acquisition of cued and contextual fear and fear extinction include the amygdala, the hippocampus, the prefrontal cortex (Sotres-Bayon et al. 2006, Curzon et al. 2009). Apart from being involved in the extra-hypothalamic regulation of the HPA axis (McEwen 2007), the amygdala processes fear-associated learning and memory (Lee et al. 2011) as it is a locus for the formation, storage, and retrieval of CS-US associations during Pavlovian fear conditioning. Both the basolateral nucleus of the amygdala (BLA) (Campeau & Davis 1995, Nader et al. 2000) and CeA (Wilensky et al. 2006, Zimmerman et al. 2007) have been identified as being involved in the acquisition and consolidation of fear memory. Further, lesions of the BLA (pharmacological or electrolytic) are reported to prevent fear expression during testing when the lesion occurs either before conditioning or between training and testing (Maren et al. 1996). In addition, using drugs that temporarily inactivate or prevent protein synthesis in the CeA reveal the importance of this nucleus in fear acquisition and consolidation (Wilensky et al. 2006, Zimmerman et al. 2007). The hippocampus is involved in contextual conditioning. It assembles contextual representations (such as the setting in which associations are made) and conveys them to the BLA where emotional meaning is added to context (Ledoux 1995). The prefrontal cortex also plays a role in fear learning and memory processing by integrating different information into emotional and cognitive-related behaviours (Heidbreder & Groenewegen 2003).

As outlined above, stressful events cause the release of glucocorticoids which bind to GRs throughout the central nervous system to alter behaviour. Interestingly, the literature regarding the underlying mechanisms of cued-fear conditioning has shown that experimental manipulation of glucocorticoid receptor signalling can influence the acquisition and encoding of this type of fear memory (Pugh et al. 1997, Hui et al. 2004). For example, GR deletion in the CeA of mice prevented conditioned fear behaviour and was associated with decreases in expression of CRH and the proto-oncogene cFos (Kolber et al. 2008). Additionally, intracerebroventricular delivery of CRH reversed the conditioned fear impairment in these mice (Kolber et al. 2008).

The combination of the findings cited above, together with the fact that there is higher expression of GR (McCormick et al. 1995, Seckl 2014) and CRH (Meaney et al. 2007, Brunton & Russell 2010) in the amygdala of PNS offspring, would lead to the hypothesis that PNS, which produces more fearful offspring, might also affect fear to a conditioned stimulus by enhancing it. However, there is evidence indicating cognitive dysfunction caused by PNS and different results have been reported. Studies in the rodent literature suggest that PNS impairs fear extinction learning, with PNS offspring showing enhanced freezing behaviour during the extinction phase of fear conditioning (Wilson et al. 2013, Li et al. 2014). In another study focusing on the training and recall phases of fear conditioning, PNS animals showed greater fear behaviour (Sadler et al. 2011). These findings support the view that maternal stress during pregnancy may be a risk factor for the development of fear or anxiety-related disorders. Nevertheless, the findings of Lee et al. (2011) suggest that fear acquisition is not affected while fear expression is attenuated in PNS offspring, indicating that the acquired fear memory may not be effectively maintained in maternally stressed mice. The impaired fear retrieval in PNS animals fits with findings by Cai et al. (2006) who reported diminished recall in rats when glucocorticoids were administered immediately after reactivation of a contextual fear memory. Contextual fear was also analysed in the same study (Lee et al. 2011) and was reported to be impaired. Furthermore, although glucocorticoid plasma levels were higher in PNS offspring than in controls, GR expression was found to be reduced in the amygdala and hippocampus of these PNS mice, and systemic injection of glucocorticoids immediately after fear conditioning restored memory consolidation. Although these findings fit with the known role of BLA GRs in fear memory consolidation, decreased GR expression in the amygdala goes against results from previous studies. Nevertheless, it is interesting that in another study PNS enhanced conditioned fear (Griffin et al. 2003). Notably, the difference between these two studies was the type of PNS used: more severe in the former (daily restraint for 6h) and milder in the latter (exposure to a novel environment and subcutaneous injection of saline). These opposing effects are in agreement with general findings in the literature where the type of stressor used during the prenatal period can

influence the outcomes in the offspring. Therefore, the fact that the findings of Lee et al. (2011) conflict with previous studies may be related to the type of stressor used and different results may be found using different PNS. The literature on PNS effects on cued-fear acquisition is very scarce and additional research is needed to further understand how these interactions work. Additionally, to the best of my knowledge there is no work done on PNS effects on fear learning and memory in farm animals.

1.8 SUMMARY

From the research that has been conducted on PNS in sheep, most of the studies have focused on either a combination of stressors or on the use of one single stressor. However, the frequency and severity with which the stressors are applied does not seem to mirror the farm environment reality, at least in the UK (e.g. 1hr transportation twice a week for six weeks; transportation plus being penned for 1hr, and then returned to the original farm repeated five times; combination of various unpredictable aversive events daily for seven weeks). Moreover, sheep studies have also shown that behavioural consequences appear to emerge later in life, suggesting that prenatal social stress may have a long lasting impact on the offspring. Nevertheless, studies focusing on the neonatal period and how prenatal stress may affect key factors related to lamb survival are lacking. Similarly, despite the efforts made to investigate how prenatal stress may affect lamb behaviour there is only a couple of studies that have focused on improving the physiological understanding of how those behavioural changes may relate to molecular and morphological changes at the brain level (Coulon et al. 2013, Petit et al. 2015). The effects of prenatal stress on key brain structures involved in regulating the HPA axis stress response have rarely been investigated in sheep.

1.9 DEVELOPMENT OF A WORKING HYPOTHESES

1.9.1. Experimental Rationale

Throughout their production cycle, domestic livestock may experience various stressors and varying magnitudes of stress that can inhibit health and

productivity. A previous study on the conditions under which sheep are kept in the UK (DEFRA project AW0509) have identified various common husbandry elements that can be considered as stressors, considering the characteristics of this species. Based on the estimated likelihood of a prenatal effect, and on the extent to which ewes under UK management conditions are exposed to them, some of the most important stressors include: under-nutrition, handling and restraint, competition for feed, social mixing, high stocking density, competition for lying area, and poor pain management. Several of these relate to the gestational social environment, a subject that has not received much attention from a PNS perspective. Sheep are a gregarious species that in natural conditions live in social stable groups (Stolba et al. 1990). Introducing sheep into social groups different from those that are naturally formed will introduce uncertainty and unpredictability into their normal husbandry (Rault 2012). Furthermore, modern stress research shows that situations of uncertainty or unpredictability are a major source of stress (Maier & Watkins 2005). It is noteworthy that social instability is typical under high density situations, which makes this type of stressor relevant for sheep from a practical point of view. Therefore, as a consequence of both the possible importance and the novelty of this aspect of ewe gestation management, this project will focus on the role of the social environment during pregnancy and on unpredictable and uncontrollable events as a source of PNS and a possible determinant of lamb postnatal performance.

1.9.2. Objectives

The present project was divided into two main experiments. The main objective was to test how PNS affects neonatal lamb behaviour and factors related to lamb survival, lamb behaviour at later stages in life, and epigenetic regulation of stress axis function in lambs. The two experiments tested different sources of PNS but they were designed in such a way that the conditions reflected normal husbandry practices on sheep farms in the UK.

In the first experiment the aim was to investigate the importance of an unpredictable and uncontrollable social environment during gestation as a source of prenatal stress. It was investigated if repeated exposure to social mixing and high stocking density during the last third of pregnancy results in

alterations to lamb vitality, emotional reactivity and cognition (fearfulness and fear learning), immunity and growth. Epigenetic alterations at the HPA axis level and brain morphological alterations were also investigated.

In the second experiment the aim was to investigate the effects of unpredictable and uncontrollable events, known to provoke stress in sheep, on the development of the offspring. It was investigated how repeated exposure to a dog and an unpredictable feeding schedule during the last third of pregnancy would affect lamb viability, emotional reactivity and cognition (fearfulness and fear learning), immunity and growth.

Knowing how the prenatal environment affects neonatal development, and how this is then expressed in terms of offspring phenotype, will provide knowledge on how to improve production management conditions by minimizing sources of prenatal stress in order to improve lamb survival and productivity.

1.9.3. Hypothesis

As stated above, this project was divided into two major experiments, from which different hypotheses were proposed:

Experiment 1

1) Social mixing and high stocking density are sources of prenatal stress in sheep and will have a negative impact on factors contributing to lamb survival, lamb behavioural reactivity and health during the first 48hr of life (**Chapter 2**).
2) Social mixing and high stocking density will also have a negative impact on physiological responses to immunological challenges, brain morphology, epigenetic regulation of the HPA, fearfulness, fear learning and lamb growth later in life (**Chapter 3**).

Experiment 2

1) Three different levels of maternal gestational stress (HIGH: dog exposure, unpredictable feeding, high stocking density; MEDIUM: no dog exposure, predictable feeding, high stocking density; LOW: no dog exposure, *ad libitum* feed,, low stocking density) will differentially affect factors contributing to lamb survival, lamb behavioural reactivity and health during the first 48hr of life. High gestational stress will have greater negative impacts on the lamb than environments where stress is decreased (**Chapter 4**).

2) Three different levels of maternal gestational stress (HIGH: dog exposure, unpredictable feeding, high stocking density; MEDIUM: no dog exposure, predictable feeding, high stocking density; LOW: no dog exposure, *ad libitum* feed,, low stocking density) will differentially affect the physiological response to immunological challenges, fearfulness, fear learning and lamb growth later in life. High gestational stress will have greater negative impacts on the lamb than environments where stress is decreased (**Chapter 5**).

CHAPTER 2

Low space allowance and social instability during the last third of pregnancy in sheep: impacts on the new-born lamb

2.1 INTRODUCTION

Prenatal stress (PNS) may be defined as factors with a negative biological impact on pregnant mothers that consequently influence the development of the unborn offspring (Braastad 1998). Numerous studies have focused on the effects that this type of stress can have on the offspring indicating that PNS alters stress-coping ability and behaviour in aversive situations. Further, alterations in exploratory, social, sexual and maternal behaviour, emotionality, and impaired learning ability and motor development have been reported (Braastad, 1998; Fride & Weinstock, 1984; Fujioka et al., 2001; Lordi et al., 1997; Marchlewska-Koj et al., 2003; Thompson, 1957; Velazquez-Moctezuma et al., 1993; Weinstock et al., 1992).

In farm animals such as sheep (Roussel et al., 2004), goats (Duvaux-Ponter et al., 2003; Roussel et al., 2005), cattle (Lay et al. 1997) and pigs (Jarvis et al. 2006), the effects of PNS have been shown to affect the behaviour and adaptation of the offspring to the environment. Recent studies in sheep have investigated the effects of maternal stress on offspring behaviour (Hernandez et al., 2010; Coulon et al., 2011), cognition (Erhard et al., 2004; Dwyer et al., 2012), physiology of the stress response (Roussel et al., 2004 and 2008) and growth (Roussel et al. 2004 and 2008), and both positive and negative outcomes have been observed. For instance, PNS has been shown to both increase (Roussel et al., 2004) and decrease (Roussel et al., 2008) fearful behaviour in lambs. Some of these studies have looked at the impact of PNS on lamb birth weight where PNS was also shown to both decrease (Corner et al., 2010) or increase birth weight (Roussel et al., 2004). Lamb birth weight is an important factor for lamb survival: low birth weight is associated with increased lamb mortality in the neonatal period, whilst high birth weight is associated with dystocia and maternal death (Alexander 1974). Other factors that are important for neonatal lamb survival include the ability to thermoregulate, latency to stand and suck and the ingestion of colostrum in order to acquire passive immunity. Despite their importance for the success of the neonatal lamb, to the best of my knowledge, no PNS studies in sheep have yet addressed if and how maternal stress impacts on these factors.

Social instability has been shown to be a cause of maternal stress and to have consequences for the offspring of some species (pigs, Jarvis et al., 2006; Otten et al., 2010; guinea pig, Sachser & Kaiser, 1996; rodents, Brunton & Russell, 2010) but to the best of my knowledge there are no studies that have investigated the effects of social instability as a prenatal stressor in sheep. Additionally, unpredictability and instability of the surrounding environment are known to be a major source of stress (Maier and Watkins, 2005) and also a characteristic of factors related to husbandry practices. Therefore, as a consequence of both the possible importance and the novelty of this aspect of ewe gestation management, this study focused on the role of the social environment during pregnancy as a source of PNS and a possible determinant of lamb postnatal performance. Additionally, possible effects of sex of the lamb and parity of the ewe were investigated. Parity has been shown in previous studies to influence maternal behaviour with primiparous ewes expressing lower levels of maternal care (Dwyer, 2003; Ekiz et al., 2007; O'Conner et al., 1992; Otal et al., 2009). Hence, dams of different parities may also be affected differently by stress. Sex is a factor known to influence many systems and behaviours. For example, in one study female lambs vocalized more than males when subjected to fear tests (Averós et al., 2015), suggesting higher behavioural reactivity in females in stressful situations. Additionally, the effects of prenatal stress have also been shown to differ according to the sex of the lamb (Roussel et al. 2005, Chojnacki et al. 2014).

The aim of this study was to use a gestational stress model in sheep to test the effects of social maternal stress on lamb behaviour, survival and growth for the first 48hr of life. Additionally, other lamb behaviour and health outcomes were investigated at later stages of life (see Chapter 3). The objective in this study was to investigate if repeated exposure to social mixing and high stocking density during pregnancy result in alterations to lamb viability, emotional reactivity or cognition, passive immunity acquisition and growth. Knowing how the prenatal environment affects neonatal development, and how this is then expressed in terms of offspring phenotype, will help to gain knowledge on how to improve production

management conditions by minimizing sources of prenatal stress in order to improve lamb survival and productivity. Additionally, knowing if certain potential social stressors are in fact not stressful in terms of lamb outcomes (i.e. no effects) is also useful from a production management perspective.

2.2 METHODOLOGY

All experimental procedures were performed in a research farm setting and completed under the authority of the Home Office Animals (Scientific Procedures) Act (1986) with approval from the local ethics committee. Animal care was carried out according to UK legislation and DEFRA guidelines.

2.2.1 Ewe Experimental Procedures

Seventy-seven Scottish mule ewes (Scottish Blackface X Blue-faced Leicester, n=36 primiparous, n=41 multiparous) were naturally mated to 1 of 2 Suffolk rams in November 2013 and predicted to give birth between the 7th and 27th of April 2014. Given the novelty aspects of this study there was no previous data to conduct a proper power calculation. Ewe sample size was estimated based on sample size from previous prenatal stress studies conducted in sheep. Ewes were examined via ultrasound in January 2014 (approximately 10 weeks of gestation) and the ones bearing twins were selected to take part in the study. They were moved to the experimental shed for the last third of gestation (9 weeks before the earliest expected parturition). Given that ewes were naturally mated there was variation in the stage of gestation at any given time point. In the present study there was a maximum of 18 days between the most and least advanced stage of gestation. This means that on the day ewes were moved into the experimental shed, gestation stage varied between 9 and 12 weeks of gestation. From here onwards periods of gestation will be addressed referring to the most advanced stage of gestation.

Ewes were divided into 11 groups balancing for parity (primiparous and multiparous): six control groups (n= 42, 22 primiparous and 20 multiparous) and five stress groups (n=35, 19 primiparous and 16 multiparous). Each

group comprised seven ewes housed in straw-bedded pens, with acoustic and visual contact between pens. The shed had a natural ventilation system and ewes were kept under a natural light regime until one week before the expected lambing date. From then onwards, during the natural dark periods they were kept under artificial light in order to allow for rapid identification of imminent births. All animals had *ad libitum* access to water and hay throughout the period they were kept inside the experimental shed. On the 17th of February (~14 weeks of pregnancy), concentrated feed in the form of pellets (Premium 18 Nuts, Harbro Ltd., Scotland) was added to the ewes' diet to meet their daily nutrition requirements (approximately 300g/day/ewe). Concentrate feeding was provided once a day in the morning. The amount of concentrated feed was doubled on the 16th week of pregnancy (approximately 600g/ewe/day) to meet the requirements for maintenance and fetal growth. Feed was provided twice a day, in the morning and afternoon, on a predictable schedule. On week 18, concentrate was adjusted again (approximately 800g/ewe/day) keeping the same feeding schedule.

Control group ewes were kept in stable social groups until the end of gestation. The stocking density used was 2.70m^2/ewe and ewes had access to 71cm feedface each on average. The stress group ewes were housed with a stocking density of 1.19m^2/ewe and 36cm/ewe feedface (Figure 2.1). Additionally, these individuals went through two social mixing events, one at 14 and another at 16 weeks of pregnancy. Once the seven individuals were grouped with new pen-mates they were allocated back to a stress pen different from the one they were in before. Although there were two social mixing events, due to number restrictions, each new formed group always had two pairs of ewes that had been pen-mates previously prior to the first social mixing event. All ewes were identified with a sprayed number (from 1 to 77) on both laterals of their body using a marker spray for animals (Super Sprayline Stock Marker, Ritchey, New Zealand). Vaccination with Heptavac P Plus (Intervet®) for the active immunization of sheep as an aid in the control of lamb dysentery, pulpy kidney, struck, tetanus, braxy, blackleg, black disease and clostridial metritis was done to all ewes four weeks prior to the expected start of lambing.

At the beginning of weeks 13, 14, 16 and 18 of pregnancy saliva, faecal and blood samples were collected from each ewe for later analysis (Table 2.1). Sampling was carried out inside the experimental pens by two trained handlers. All ewes were weighed and had their body condition score assessed by a trained technician on the day they entered the experimental shed and on each sampling day. Saliva samples were collected by introducing a cotton bud in the ewe's oral cavity and allowing it to chew for 30 seconds. Cotton bud tips were placed in plastic tubes and centrifuged at 2,190 × g for 15 min at 4°C and the resulting saliva was used to assess levels of cortisol. Faecal samples were collected directly from the rectum, placed into individual plastic bags and homogenised by manual compression for later analysis of glucocorticoid metabolite (GCM) concentrations. Blood samples were collected by venepuncture of the jugular vein into vacutainer tubes containing heparin (BD Vacutainer® Blood Collection Tunes, Plymouth, UK) and centrifuged at 2,190 × g for 15 min at 4°C. Plasma was separated and aliquoted in 1ml tubes to assess ß-hydroxybutyrate (BOHB). All samples were frozen at -20°C until future analysis.

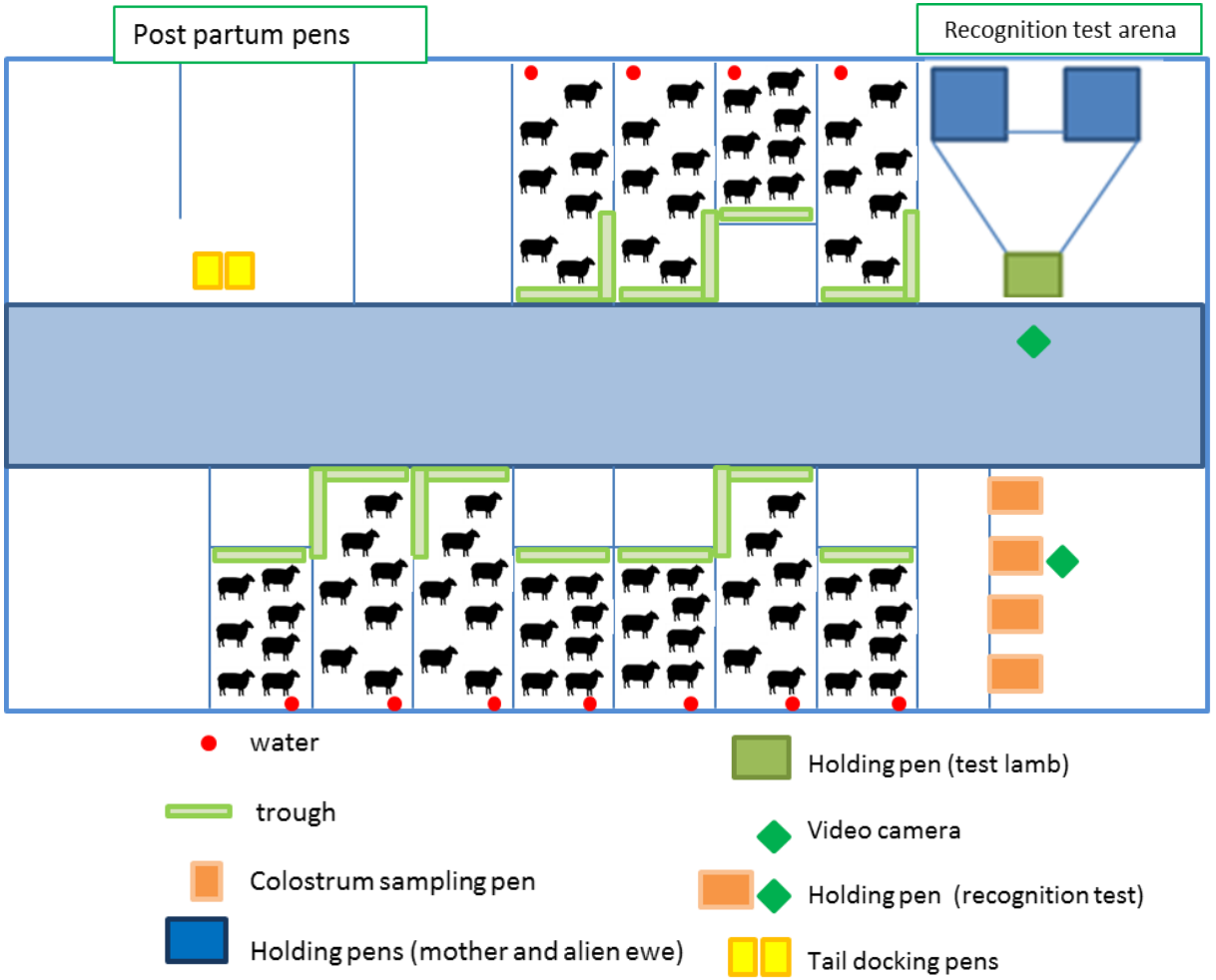


Figure 2.1 Shed layout for gestational treatments, lambing and neonatal period.

Seven days prior to the predicted start of the parturition period, the stress group was released into the same conditions as the controls and ewes remained under observation at all times. At the first signs of imminent birth (e.g., not coming to the feed trough at feed times, pawing the ground, restlessness, getting up and down, possible interest in other lambs/lambing ewes, contractions) a high definition video camera (Canon legria hfs21) was set up in front of the pen to record ewe and lamb behaviour (see section 3.3.2 Offspring experimental procedures). Ewes and lambs were assisted during parturition only if it was considered to be vital to ensure the survival of either, i.e. if no further progress had been made either one hr after fluids were first expelled or if two hr had passed after parts of the lamb were presented; immediately if the lamb was head first and both legs back, if the lamb was backwards and stuck, or if no progression for the second lamb´s birth was observed one hr after the birth of the first lamb.

Table 2.1 Time points of social mixing, sampling, weighing and body condition assessment.

Week 13	**Week 14**	**Week 16**	**Week 18**
03/02/2014	17/02/2014	03/03/2014	17/03/2014
-Body marking	- 2nd Body condition score, weighing	- 3rd Body condition score, weighing	- 4th Body condition score, weighing
-Faecal collection	-Saliva & Faecal collection	-Saliva & Faecal collection	-Saliva & Faecal collection
-1st Body condition score	-Blood sampling	-Blood sampling	-Blood sampling
	-Social Mix	-Social Mix	-Concentrate feed offered
	-Concentrate feed offered	-Concentrate feed offered	

2.2.2 Offspring Experimental Procedures

From the 149 lambs that were born, 136 (n=72 control, 45 females and 27 males; n=64 stress, 32 females and 32 males) were used in the experimental procedures of this study. Lambs excluded from the statistical analysis are detailed under the statistical methodology section 2.2.3 below. All lambs were reared with their mothers and treated as a single flock as soon as they left the lambing pen. Male lambs remained intact throughout the study and all lambs were tailed docked between 36 and 37hr of age. Males were weaned at 15 weeks of age and females at 19 weeks of age. Age at weaning was between 13 and 15 weeks due to lambs being born within a maximum of a 18 day interval. As discussed for gestation period from here onwards periods related to lamb age will be mentioned in relation to the age of the eldest lambs.

2.2.2.1 *Measurements from birth to 6 hr*

All births were video recorded from the moment the first twin was born until 2 hr after the birth of the second lamb for later behaviour analysis (birth was defined as the moment the lamb's hips passed through the birth canal). Lamb behavioural progression (stand and suck; see Table 2.2) and lamb bleating were also recorded live from birth (0min) to 2hr of life in an interval manner using Pocket Observer software (Noldus Information technology, Netherlands) on Workabout Pro (Psion, UK) devices: 0-30mins, 50-60mins, 80-90mins, 110-120 mins after birth. When the second lamb was born within a first twin observation period, the ongoing observation would carry on until the end of the 30 min following the birth of the second twin. If the second lamb was born within an interval between observations a new observation was begun. Observation intervals would be reset according to the second lamb's birth. In all cases, observations for each lamb would stop once it had reached two hr of life.

Lamb rectal temperature was measured using a digital thermometer at the end of the 0-30 min observation and again at two hr. If the second lamb was

born within the first 30 min of life of the first lamb, 30 min temperature of both lambs was only measured when the second born reached 30 min of life.

Approximately two hr after parturition, ewes and lambs were moved to the colostrum sampling pens (1x1m with straw floor) located inside the experimental shed (see Figure 2.1). At this point lambs were assessed by a technician by stomach palpation on the degree of fullness of the stomach. Lambs that were empty and/or not seen performing sucking behaviour were assisted to suck. A colostrum sample, mix from both teats, was immediately collected (10mL of colostrum from each teat) into two 10mL plastic tubes and frozen at -20°C for future analysis on IgG content (this procedure was related to another project and lab methodology will not be reported in this PhD thesis). Once lambs were dry, they were spray marked (Super Sprayline Stock Marker, Ritchey, New Zealand) with one of two colours: red or blue. At 24 hr old red lambs were blood sampled to assess plasma IgG content and cortisol concentrations and then subjected to a test for the ability of the lamb to recognise the ewe, while blue lambs were blood sampled at 48 hr to assess cortisol concentrations. Colour allocation was balanced for sex of the lamb, sex combination in the litter and order of birth (Table 2.3). This procedure was adopted to minimize the number of procedures conducted on any one lamb. Ewes and lambs were kept in the colostrum pens up to 6 hr to allow for the dam-offspring bond to establish fully. During this period water and hay were provided *ad libitum*.

After this period, animals were released into the post-partum pens together with other lambed ewes and lambs. Postpartum pens (see Figure 2.1) were located at one end of the experimental pen and consisted of large pens where ewes and lambs could move freely as a flock with *ad libitum* access to water and hay. Lambs and ewes remained in these pens for approximately 48hr after lambing and were then released to the outside as a single flock.

Table 2.2 Ethogram for lamb behavioural progression after birth.

Behaviour	Definition
stand	lamb supporting weight on all four feet for the first time for at least five seconds
suck	Lamb with head under ewe, has teat in its mouth, making sucking movements of head or noises, may be wagging tail, usually standing still unlike with unsuccessful suck, can sometimes see swallowing movements.
bleating	Any vocalisations made by the lamb (may be low or high pitched).

Table 2.3 Measures taken from lambs at different ages during neonatal period.

	Lamb Age					
Measures	0min	30min	2hr	24hr	36hr	48hr
Behaviour landmarks		All lambs		---	---	---
Temperature		All lambs	All lambs	All lambs	---	All lambs
Weigh, Crown-rump length				All lambs	---	---
Blood sample		---		Red lambs	---	Blue lambs
Recognition test				Red lambs	---	---

2.2.2.2 *Measurements at 24 hr*

At 24hr of age (+/- 2h) rectal temperature was measured for all lambs. Lambs were weighed using a digital hanging scale (PCE-HS 50 series, PCE Instruments ®) and crown-rump lengths measured (by placing a flexible tape measure on the occipital bone and extending it to the base of the tail) for later calculation of ponderal index (PI - measure of leanness of a lamb calculated as a relationship between mass and length (weight/(crown-rump^3)). Red lambs were blood sampled. Blood sampling was carried out in the post-partum pen and blood was collected by venepuncture of the jugular vein into Vacutainer® 4ml plastic tubes coated with ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer® Blood Collection Tunes, Plymouth, UK). Samples were then centrifuged at 2,190 × g for 15 min at 4°C. Plasma was separated from blood, aliquoted in 1.5ml plastic tubes and kept at -20C for later analysis of IgG and cortisol content (see section next section for detailed laboratory methodology).

Lambs and ewes were then moved into the recognition test arena to test the lamb's ability to discriminate its mother from another parturient ewe (alien ewe) at a distance and at close quarters. The maternal recognition test (Nowak et al., 1987) was carried out in a Y shaped arena built at one end of the experimental shed. It consisted of a concrete floor (4.5m x 3m) marked in a checked pattern and fenced by 1m high opaque metal barriers. The base of the Y consisted of one holding pen (1x1m) for the lamb with opaque walls and at the opposite end (4.5m away) were 3 pens side by side, two of which contained the dam ewes (stimulus pens). The two ewes were separated by an empty pen (1x1m) to prevent them from being too close to each other and facilitating discrimination of choice of a ewe by the lamb. The arena was virtually divided into 3 zones: A and B, contact with the ewe zones; and C, neutral zone (see Figure 2.2). Lambs could make contact with the ewes by placing the muzzle through the bars, but they were unable to reach the udder.

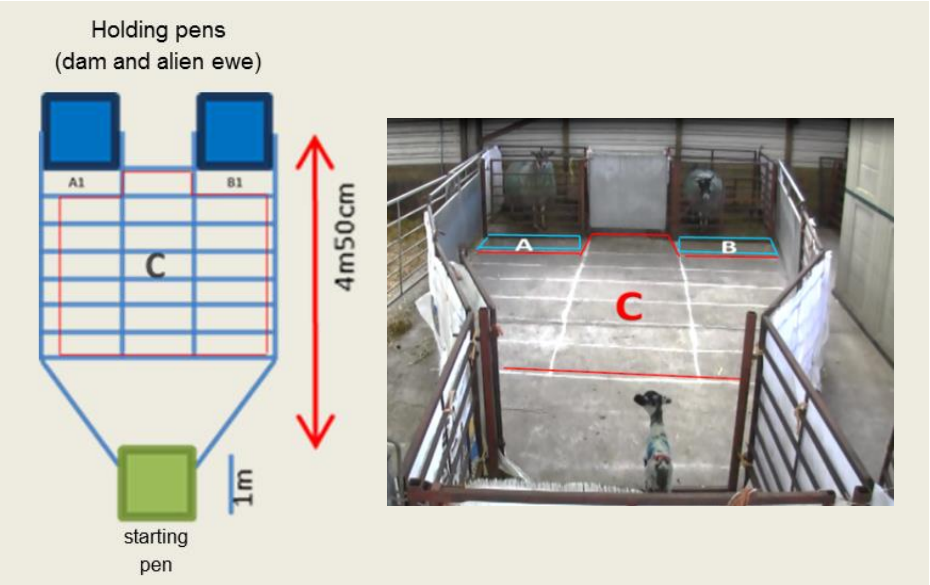


Figure 2.2 Recognition test arena layout

Only lambs marked in red were submitted to the recognition test (n=29 control, 17 females and 12 males; n=27 stress, 14 females and 13 males). Lambs were moved from the post-partum pen to the testing arena with their dams. The dam of a lamb to be tested was placed in one of the holding pens, and another ewe that had lambed at approximately the same time (lambs aged within 24 hr of test lambs) was placed in the other, without her own lambs. The side of the holding pen where the dam of the test lamb was placed was chosen randomly. The tested lamb was placed in the waiting pen immediately after being separated from its own dam, facing the ewes in a standing position. The test started as soon as the holding pen door was opened, for a 3 min duration, and was recorded on video using a high definition camera (Canon legria hfs21) for later analysis with The Observer XT 11.5 software (Noldus, Information Technology, Netherlands). Behaviours recorded were: lamb latency to go to its own dam, first choice (mother or alien ewe), time spent close to each ewe (A and B zones), time spent in the neutral zone (zone C) and activity (number of squares crossed during a test - a lamb was considered to have crossed a square when both forelegs had stepped over the white stripes on the floor).

During the test, the sibling of the tested lamb and the lambs from the alien ewe were kept in a pen away from the testing arena. Once the test was

finished all the lambs were immediately reunited with their dams and returned to the post-partum pens.

Recognition testing was considered to reach an end point whenever being in the tested arena put the welfare of the lamb or ewe at risk, i.e. lamb or ewes getting trapped in any part of the gates or ewes making more than three attempts to escape or successfully jumping out of the holding pen.

Physiological analysis

The IgG concentration in the lamb plasma was measured using a quantitative enzyme-linked immunosorbent assay (ELISA) test kit (Biopanda reagents, ELISA-OG-001, UK) following the manufacturer's guidelines and quantified using a spectrophotometer, filter 450 nm (Thermo Scientific™ Multiskan™ FC Microplate Photometer). Samples were first run using the manufacturer recommended dilution (1/400000). When results registered an IgG concentration below the level of detection of the kits, lower dilutions were used (1/100000, 1/50000, or 1/25000).

Cortisol concentration was assessed using an in-house developed ELISA assay (following the method of Al-Dujaili et al. 2009, 2012). Unless otherwise stated, all chemicals were purchased from Sigma. Lamb plasma samples were first processed for extraction of cortisol by mixing 5ml diethylether to 0.5 mL of plasma, vortexing for 10 seconds, centrifuging at 2500g for 15 min and freezing at -80ºC overnight. Thawed supernatant was transferred to a 5ml glass tube and dried down using vaporized liquid nitrogen. Samples were then reconstituted in assay buffer (0.025 M phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA)) using a 2:1 ratio. For the cortisol ELISA a 96 well plate was coated with 200µl/well of F-BSA conjugate (0.5mg/mL, diluted, 1:200) in PBS and incubated overnight at 4ºC. F-BSA conjugate was discarded and the plate was washed three times with washing buffer (0.015 M PBS, pH 7.4, containing 0.05% Tween 20) and then blocked with 200µl/well of blocking buffer (0.025 MPBS, pH 7.4, containing 0.5% BSA) and left to incubate at 37 ºC for one hr. The buffer was discarded and the plate was loaded in duplicates with either 50µl of sample/well or 50µl

of cortisol standard/well (cortisol standard: 10 µg/mL, at six different concentrations - 1.0 – 500ng/mL - diluted in assay buffer) obeying the defined plate design, followed by 100µl/well of antibody solution (anti-sheep cortisol, 1:20000). Blanks and quality controls were constituted by assay buffer only or a mixture of assay buffer and standard (500ng/mL), respectively. After a two hr incubation period in the dark at room temperature (18-22 °C) the plate was washed again three times with washing buffer and loaded with 100µg/well of antibody + enzyme (donkey anti-sheep antibody + horseradish peroxidase enzyme 1/10000). Another one hr incubation period at room temperature in the dark followed, after which the plate was washed three times and loaded with 100µg/well of substrate solution (0.2 M acetate/citrate buffer pH 4.2 - 500µl 3,3',5,5'-tetramethylbenzenide, 0.4% + 30ml sodium acetate, 10mmol/L + 100µl hydrogen peroxidase, 0.6% [35%, Merck 108600]) followed by 10-15 min of incubation at RT in the dark. At the end of this period 50µl/well of stop solution (sulfuric acid 1:10, 95-97% Merck 100731) was added to stop the reaction. The absorbance was measured at 450 nm on a Multiskan FC spectrophotometer (Thermo Scientific, UK) using SkanIT Software 2.5.1.

2.2.2.3 *Measurements at 36 and 48hr*

All lambs were tail docked between 36 and 37hr of age using the rubber ring method. Lambs and ewe were moved from the post-partum pen to the tail docking pen ($1m^2$) inside the experimental shed (Figure 3.1) where tail docking took place.

At 48 hr rectal temperatures were measured from all lambs, and blue lambs were also blood sampled by venepuncture from the jugular vein (using the same procedure as described above). Samples were treated as previously described for later analysis of cortisol content. Both lambs and the ewe were returned to the post-partum pen after sampling.

At this stage lambs were also ear tagged and were ready to leave the testing shed. Ewes and respective litters were released onto pasture as a single flock after this procedure.

2.2.3 Statistical Methods

Most of the statistical analyses were conducted using the restricted maximum likelihood procedure (REML) in Genstat (Genstat 64-bit, 16th edition). The REML test procedure is similar to an analysis of variance but it allows for data to be unbalanced, which was often the case for the present data. On some occasions, non-parametric tests like the Wilcoxon and Chi-square tests or a survival model, such as the Proportional hazards model, were used (Table 2.4).

Where REML was used, the effects of treatment, ewe parity, lamb sex, and degree of assistance given to the ewe at parturition were fitted in the model as fixed factors. Lambing pen and ewe and lamb identification were fitted as random effects as ewes produced more than one lamb in the dataset and more than one lamb was born in the same pen. On some occasions, fixed and random models were slightly altered and the alterations made will be addressed below for each data set.

Data either followed a normal distribution or were transformed (as described in Table 2.4) to meet the assumptions of a REML procedure. The transformation used was made on theoretical grounds - what would be expected to work given the nature of the response variable. However, on some occasions, the first choice transformation did not work and other transformation options were used that more closely satisfied the statistical assumptions underlying the analysis. Hence, there will be some occasions in which original data followed the same distribution but different transformations were used. Where significant differences were detected by the REML procedure, post hoc Student's T tests were used for pairwise comparisons to determine where significant differences lay. The level of probability determined as statistically significant was $p<0.05$. When a transformation was used, confidence intervals were calculated using the original mean ± 2*s.e.. The same transformation applied to the mean was applied to this result in order to obtain a back-transformed confidence interval.

From the 77 ewes under this study, one aborted (control primiparous), one died of prolapse before lambing (stress multiparous), and one was not pregnant (control primiparous). From the 74 ewes that lambed, one was identified as being infected with fluke (control primiparous), two gave birth to mummified lambs (stress), one lambed a deformed lamb (control), one was euthanized after lambing due to prolapse (control) and another one had triplets (control). All these ewes and respective lambs were removed from the study and excluded from analysis. In total, there were 68 ewes left that were considered under trial.

Data referring to ewes was analysed as part of another PhD project (Yusof 2018). Some of the results relevant to the interpretation of lamb data are presented in appendix 2.

Limitations

Adjusting P-values to take account of multiple testing and so ensure the overall error rate is maintained at (say) 5% can make it extremely difficult to find statistical evidence of differences. How difficult this becomes depends on the number of comparisons being made. Hence a balance often needs to be struck between avoiding undue strictness and so still collecting useful findings, and also controlling as far as possible the risks of false conclusions.

The P-values are much more informative than simply whether formal statistical significance was achieved or not at some arbitrary level (e.g. 5%) set many years ago out of historical necessity. Instead, they reflect the strength of evidence of effects rather than a binary decision outcome. Rather than formally adjusting the P values themselves assuming the variables are independent of each other, caution was exercised in interpreting these P values. The P-values themselves reflect the relative strength of evidence of treatment effects and can act as a yardstick for further investigation. Where borderline statistical significance has been achieved, this has been interpreted cautiously, even more so where there was no apparent biological explanation for the finding. The same approach was taken to interpret data in Chapter 3.

Lamb bleating behaviour

From the 149 lambs born, six litters (13 lambs) were not included in the analysis for lamb bleating behaviour over the first 30 min of life. Lambs were excluded due to one lamb in the litter being a mummy (stress: female=2 + mummy=2), being born with a deformity incompatible with life (control male + deformed lamb) or due to being born in a triplets litter (control: female=3), from a dam that contracted fluke during pregnancy (control: female=2) or that had a uterine prolapse after parturition (control: female=2). In total, 136 lambs (n=72 control, 45 females and 27 males; n=64 stress, 32 females and 32 males) were considered to be under trial.

For the statistical analysis of lamb bleating behaviour, six additional lambs had missing observations (data were lost) (stress: females=2, males=4) and were not included in the analysis. In total, bleating behaviour from 130 lambs was analysed (control=72, 45 females and 27 males; stress=58, 30 females and 28 males).

Lamb behaviour landmarks

From the 136 lambs under trial, on three occasions observations had to be interrupted before lambs performed standing behaviour and lambs had to be taken to the hot box due to low body temperatures. These three lambs were excluded from the analysis (control: female=1; stress: female=1, male=1).

Analysis of sucking behaviour excluded nine lambs from the 136 originally under trial. Lambs were excluded for missed behaviour during live observations which was not possible to retrieve from video files (control: female=2, male=4; stress: female=3). These lambs were checked at two hr after birth and assessed as being full (stomach full of milk) and therefore were not assisted to suck. Lambs that were not seen performing sucking behaviour for the first two hr of life and that were assessed as being empty at this time point were assisted to suck by a technician and assigned a value of 2hr for the latency to suck. This happened to 9 lambs (control: female=4,

male=1; stress: female=1, male=3). A total of 127 lambs were considered for this analysis.

Lamb body temperature

Lamb body temperatures were measured at four different time points. Analysis of the temperature taken at 30 min and 2hr after birth included all the 136 lambs under trial. For temperature at 24hr and 48hr, seven lambs and respective siblings were excluded from the analysis for being rejected by the dam (control: female=1; stress; female=1), dying (stress female=1), having to be euthanized due to injuries inflicted by the dam (control: female=1, male=1), or the dam having to be kept in stocks as it was showing rejection behaviour towards the lambs (control litter: female=1, male=1). Additionally, two other lambs (stress males) and their siblings had to be excluded from the analysis of 48hr temperature for dying before reaching this age. A total of 124 and 120 lambs were included in the 24hr and 48hr temperature statistical analysis, respectively.

24hr Body weight

For the body weight and PI statistical analysis, birth assistance was excluded from the fixed model. Assistance to birth is an event occurring after birth and, therefore, could not affect weight.

Body weight and PI taken at 24hr did not include four lambs and their respective siblings, from the original 136. Two of these lambs died (control; female=1; stress: female=1) and one had to be euthanized due to injuries inflicted by the dam (stress female) before 24hr of life. Another lamb (control male) was missing data (the sibling of this lamb was kept as a valid data point).

IgG concentration in plasma

Plasma samples for the analysis on the concentration of IgG were taken from 52 lambs. However, only 48 samples (control: female=12, male=12; stress: female=14, male=11) were considered for the statistical analysis (one sample

was missing, another was unsuitable for processing and a third one had IgG concentrations below the detection level of the assay). One lamb (stress male) that had to be fed artificial colostrum was excluded from the analysis, as feeding artificial colostrum has been shown to lower circulating IgG (personal communication Dr Ann Mclaren). Indeed, this lamb presented the lowest concentration of IgG from all the lambs analysed.

Cortisol

Due to issues related to lab methodology/quality of plasma samples it was not possible to produce a reliable data set for the concentration of cortisol in lamb plasma. Therefore, there are no data for cortisol. However, a discussion on the possible reasons for the referred issues can be found in appendix 1 *Cortisol in lamb plasma*.

All cortisol samples throughout this PhD thesis were analysed at the same time and the same issues were encountered. Therefore, the appendix 1 will be referred to in forthcoming chapters.

Recognition test

During the recognition test lamb behaviour was assessed as to answer the following questions: a) is the proportion of time the lamb spends with the dam, alien ewe, both ewes, or in the neutral zone different between groups; b) is there an overall difference in the proportion of time spent with the dam compared to the alien ewe; c) is the proportion of time spent close to the alien ewe and the dam greater than chance; d) is the latency to reach the mother different between groups, and e) do lambs choose the dam first over the alien ewe. For each of these questions different statistical analyses were used: a) REML fixed and random models were used; b) Wilcoxon Matched-Pairs test; c) Student's t test; d) Survival analysis (Proportional hazards model); e) Chi-square test (see Table 2.4).

As latency to approach mother times were both censored and skewed, survival analysis methodology was adopted for the statistical analysis. Cox proportional hazards regression models were fitted. Central to proportional hazards models is the concept of the hazard rate or function, *h(t)*. This is

defined to be the probability that a lamb approaches its mother at time *t*, conditional on it not having approached its mother prior to this time. Naturally, this hazard will change as time elapses. The lower the "hazard" rate at any given time, the less likely a lamb is to approach its mother at that instant, conditional on it now having already approached its mother. The "hazard" of a lamb approaching its mother at a given time relative to the hazard of a lamb with baseline levels of covariate(s) is modelled. This enables assessment of whether or to what extent changes to any covariates (e.g. sex or treatment) alter the relative "risk" of approaching the mother at any time point. The fitting of a frailty term (the equivalent in survival analysis to fitting a random effects term in a mixed model) is an extension of the proportional hazards model.

From the 58 lambs (control: female=19, male=12; stress: female=14, male=13) that were tested for the ability to recognise the dam, one was excluded as the video recording for this animal was missing (control: female=1) and another had the test aborted at the beginning because the ewe jumped out of the holding pen at the start of the test (control: female=1). On four other occasions (control: female=1, male=1; stress: female=1, male=1) the test had to be terminated earlier due to ewe jumping out of the holding pen and lambs were excluded from the analysis. These four lambs were only kept as data for the analysis on first choice and latency to go to the dam as they were able to visit dam/alien ewe before the test reached an ending point.

This project was conducted alongside another PhD study, which focused on the ewe outcomes in these experiments. Therefore, the data collected referring to the ewes have been analysed by another student but will be reported here where relevant for the understanding of the impacts of PNS on the lamb.

Table 2.4 Data distribution and transformations.

	Data	Distribution	Transformation	Tests
Early behaviours	Latency to stand and suck	Positively skewed	Log 10	REML
	30min bleats (frequency)	Positively skewed	Log 10	REML
Temperature	30min temp	Negatively skewed	Reverse log10	REML
	2hr temp	Positively skewed	Log10	REML
	24hr and 48hr	Normal	---	REML
Weights	Birth	Normal	---	REML
	Ponderal index	Positively skewed	Square root	REML
Recognition test	Proportion time spent with dam, alien ewe, ewes, neutral zone (comparison between groups)	Normal	Arcsine	REML
	Overall proportion of time spent with dam vs. alien	Not normal	---	Wilcoxon Matched-Pairs test
	Proportion of time spent with dam vs. alien (comparison within group)	Not normal	---	Wilcoxon Matched-Pairs test
	Proportion time (greater than chance) spent with dam, alien ewe, ewes, neutral zone	Normal	Angular	Student's t test
	Latency to reach dam	Normal	---	Proportional hazards model
	First choice (dam or alien) higher than chance	Not normal	---	Chi-square test
	Activity	Positively skewed	Square root	REML
Blood analysis	IgG	Normal	---	REML
	Cortisol	No data available	---	---

2.3 RESULTS

2.3.1 Bleating Behaviour during the first 30 min after birth

The number of vocalisations made by lambs in the first 30 min after birth was affected by an interaction between treatment and sex (F=6.88, d.f.=104.1, p=0.010, Figure 2.3a). Female lambs from the control group vocalized significantly more than control males and stress females, while no differences were found in the stress group (Figure 2.3a).

Treatment also affected bleating behaviour depending on whether assistance was given to the lamb at parturition or not (F=15.61, d.f.=115.3, p<0.001, Figure 2.3b). In the control group lambs vocalized more if they had been assisted at birth (lambed manually or repositioned) than if they had not and also more than assisted lambs in the stress group, while in the stress group assisted lambs vocalized less than unassisted lambs (Figure 2.3b). There were no effects of treatment (F=0.00, d.f.=8.4, p=0.961), sex (F=2.26, d.f.=109.7, p=0.136), parity (F=0.10, d.f.=61.0, p=0.755), or birth assistance alone (F=0.78, d.f.=122.9, p=0.378).

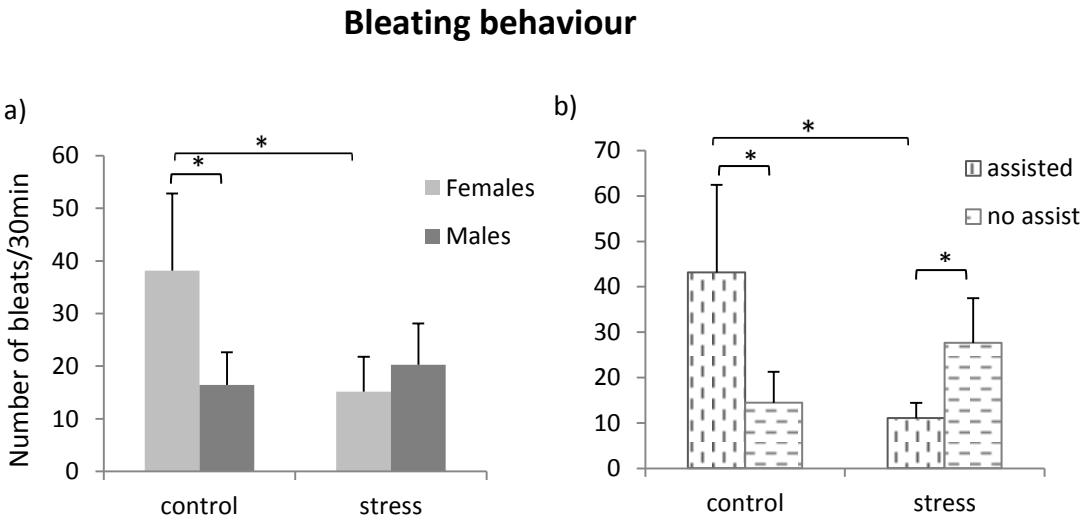


Figure 2.3 Number of lamb bleats during the first 30 min after birth dependent upon a) sex of the lamb and b) assistance given at birth. Female bleated more than males in control the group and than females in the stress group. Assisted lambs bleated more than unassisted lambs in the control group but the opposite was true for the stress group. Graph bars represent back transformed means and errors bars represent CI. Stars (*) represent where significant difference lies.

2.3.2 Lamb Behaviour Landmarks

Treatment had no effect on the time taken by lambs to stand for the first time after birth. Females were faster to stand than males. Parity had no effects on lamb latency to stand. Lambs that were assisted at birth were faster to stand than those that were not (Table 2.5).

There were no effects of treatment, parity, sex or assistance at birth on the latency to suck (Table 2.5).

Table 2.5 Effects of parity, sex, assistance to birth and treatment on latency to stand and suck. Latencies are presented as back transformed means (confidence interval).

		Stand latency (min)		Suck latency (min)	
treatment	control	17.69 (14.99;20.89)	p=0.972	50.68 (41.96;61.21)	p=0.646
	Stress	17.77 (14.85;21.27)		56.21 (49.64;63.66)	
sex	Female	15.02 (12.77;17.69)	p<0.001	53.68 (46.11;62.50)	p=0.812
	Male	20.93 (17.89;24.50)		53.07 (45.59;61.78)	
parity	Primiparous	17.94 (15.27;21.07)	p=0.830	51.86 (44.35;60.65)	p=0.763
	Multiparous	17.53 (14.72;20.89)		54.93 (46.97;64.24)	
assistance	assisted	15.02 (12.24;18.44)	p=0.004	55.06 (47.08;64.39)	p=0.311
	Unassisted	20.93 (18.38;23.84)		51.72 (43.83;61.07)	

2.3.3 Lamb Body Temperature

PNS affected 30min temperature in a parity-dependent manner (F=6.11, d.f.=61.0, p=0.016, Figure 2.4). Lambs born to multiparous ewes had significantly higher temperatures at 30 min after birth than those born to

primiparous ewes in the control group but no differences were found in the stress group. There were no effects of sex (F=0.69, d.f.=115.8, p=0.407), or birth assistance (F=0.04, d.f.=130.5, p=0.849) on 30min temperature.

Temperatures measured at 2hr were not affected by treatment (F=1.17, d.f.=8.9, p=0.309), sex (F=0.04, d.f.=122.3, p=0.842), parity (F=0.40, d.f.=63.9, p= 0.532), or birth assistance (F=0.47, d.f.=130.4p=0.492) (data not shown).

PNS also affected temperature at 24hr but in a sex-dependent manner (F=4.98, d.f.=97.6, p=0.028, Figure 2.5). Females had slightly lower temperatures than males in the control group at 24 hr old, whereas this relationship seemed to be altered by stress in the opposite direction; however, post-hoc analysis revealed no significant differences. There were no effects of treatment (F=0.70, d.f.=8.7, p=0.425), sex (F=0.04, d.f.=109.1, p=0.839), parity (F=2.63, d.f.=57.8, p=0.110) or birth assistance (F=0.00, d.f.=117.7, p=0.956) on 24hr temperature.

There were no effects of treatment (F=0.04, d.f.=8.2, p=0.850), sex (F=0.05, d.f.=96.2, p=0.816), parity (F=1.56, d.f.= 54.9, p=0.217), or birth assistance (F=1.94, d.f.=112.1, p=0.166) on temperatures measured at 48hr (data not shown).

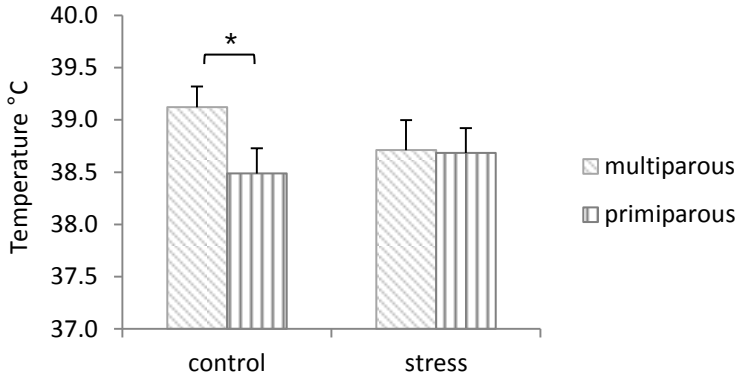


Figure 2.4 Lamb body temperatures at 30min after birth dependent upon an interaction between treatment and parity. Lambs from multiparous ewes had higher body temperature than lambs from primiparous ewes in the control group. Graph bars represent back transformed means and error bars represent CI. Stars (*) represent where significant difference lies.

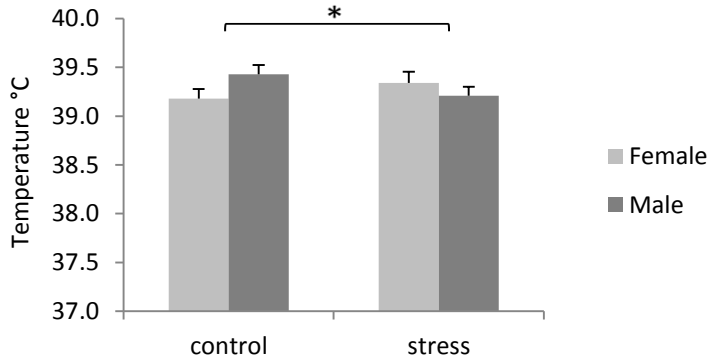


Figure 2.5 Lamb body temperatures at 24hr after birth dependent upon an interaction between treatment and assistance at birth. Females had lower body temperature than males in the control group, while the opposite was true for the stress group. Although post hoc analysis revealed that this difference was not significant. Graph bars represent back transformed means and error bars represent CI. Stars (*) represent where significant difference lies.

2.3.4 24hr Body Weight and Ponderal Index

PNS did not affect 24hr body weight (F= 1.93, d.f.=7.1, p=0.207) or PI (F=3.13, d.f.=6.6 p=0.122). Males were heavier than females at birth (mean weight (Kg) (±s.e.): females=4.76 (±0.085), males=5.18 (±0.097), p=0.002) and lambs born to multiparous were heavier than lambs born to primiparous (mean weight (Kg) (±s.e.): lambs multiparous=5.32 (±0.107), lambs primiparous=4.63 (±0.100), p<0.001). PI was higher in lambs born to multiparous than lambs born to primiparous (PI (±s.e.): lambs multiparous =0.68 (±0.116), lambs primiparous=0.66 (±0.105), p=0.031) but was not affected by treatment (F=3.13, d.f.=6.6, p=0.122) or sex (F=0.85, d.f.=100.5, p=0.358) (data not shown).

2.3.5 IgG Concentration in Plasma

There was no significant effect of treatment (F=0.01, d.f.=8.3, p=0.921), sex (F=2.11, d.f.=37.0, p=0.155), or parity (F=0.02, d.f.=43.0, p=0.879) on plasma IgG concentration. However, lambs that had an assisted birth presented lower concentrations of IgG in plasma than unassisted lambs (F=5.92, d.f.=42.9, p=0.019; Figure 2.6).

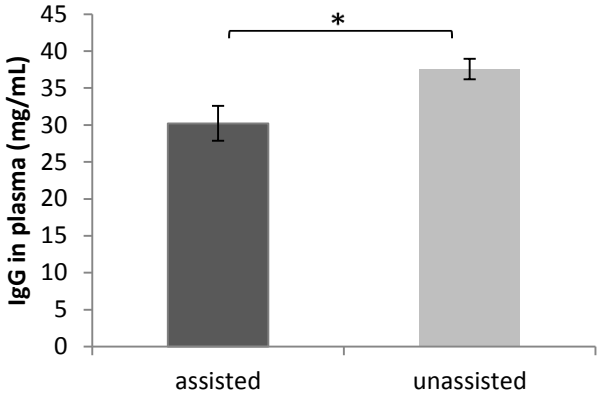


Figure 2.6 IgG concentration levels in lamb plasma depending on assistance at birth. Lambs that were assisted at birth had lower plasma levels of IgG than lambs that were unassisted. Graph bars represent means and error bars s.e.. Stars (*) represent where statistical significance lies.

2.3.6 Recognition Test

All the tested lambs approached a ewe within the 180 sec test duration. Overall the proportion of lambs which approached their dam first did not differ significantly from chance (dam=32, alien=24, χ^2 =1.14, df=1, p= 0.285). Similarly neither control (χ^2 =1.69, df=1, p=0.194) nor stress group lambs were found to approach their mother first significantly above chance (χ^2 =0.04, df=1, p=0.847) However, lambs born to primiparous ewes chose their dam first more than chance (Dam=22, Alien=7, χ^2 =7.76, df=1, p= 0.005) but lambs born to multiparous ewes did not (Dam=17, Alien=10, χ^2 =1.81, df=1, p=0.178, Figure 2.7).

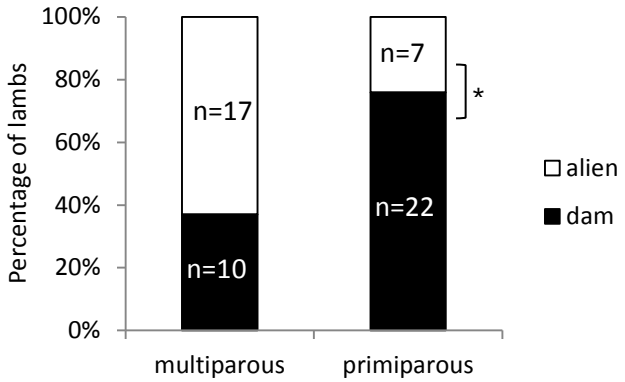


Figure 2.7 Percentage of lambs that choose to go to dam or alien ewe first during the maternal recognition test, dependent upon ewe parity. Lambs born to primiparous ewes chose own dam more often than chance, but lambs from multiparous did not. Stars (*) represent where significant difference lies.

There was no difference between treatment groups in the proportion of time they spent close to ewes (back transformed time proportion (CI): control=0.58 (0.50;0.67), stress=0.68 (0.60;0.76), F=2.88, d.f.=7.0, p=0.134), close to the dam (back transformed time proportion (CI): control=0.41 (0.30;0.52), stress=0.50 (0.38;0.61), F=1.37, d.f.=7.9, p=0.276) or close to the alien ewe (back transformed time proportion (CI): control=0.10 (0.06;0.15), stress=0.13 (0.08;0.18), F=0.78,d.f.=4.5 p=0.422). However, on average the percentage of time spent close to ewes was higher than predicted by chance (angular scale mean=53.16, se=2.032, p<0.001). Lambs also spent more time close to the dam than to the alien (83.48 vs 29.48 secs; Z=201, p<0.001) and more than would be expected by chance (angular scale mean=60.87, se=2.946, p<0.001).

There was no significant effect of treatment (Control= 28.90 (17.78, 41.47), Stress= 30.44 (14.03, 60.81); $\chi2(1)=0.06$,p=0.810), sex (F= 27.53 (16.80, 37.15), M=41.47 (22.65;60.81); $\chi2(1)=0.01$,p=0.900), parity (Multiparous=50.23 (31.09, 61.08), Primiparous=21.49 (14.03, 28.84), $\chi2(1)=1.68$,p=0.190), or birth assistance (assisted=41.47 (14.03, 59.18), unassisted=30.44 (22.65, 53.90) ;$\chi2(1)=0.05$,p=0.830) on the latency to reach the mother.

No differences were found in total activity between treatment groups (F=0.01, d.f.=8.0, p=0.968), sexes (F=1.06, d.f.=46.5, p=0.308), parity (F=0.17, d.f.=45.7, p=0.684), or assistance given to birth (F=0.17, d.f.=47.0, p=0.683) (data not shown).

2.4 DISCUSSION

Two different husbandry systems addressing the normal conditions in which pregnant ewes are kept were investigated during the last third of pregnancy. The control group represented the better management in the UK with ewes being kept in stable social groups and at lower stocking densities. The stress group was designed to test the effects of social instability and high stocking density experienced by the ewes. It was hypothesised that stress treatment would negatively impact the development of the lambs in comparison to the control group.

The obtained results showed that the effects of treatment were only observed as a result of an interaction with other factors such as parity, sex or assistance at birth, suggesting that the effects of prenatal treatment on the lamb, during the neonatal period, were very subtle in nature.

Female lambs bleated more than males during the first 30min of life but prenatal treatment eliminated this difference. A similar result involving parity and treatment was observed for the body temperature at 30min after birth where a difference was seen between lambs from multiparous and primiparous ewes in the control but not in the stress group. Body temperature at 24hr also varied depending on treatment and lamb sex.

Other lamb outcomes important for lamb survival such as latency to stand and suck, IgG concentration in plasma, body weight and the ability to recognise the mother were not affected by prenatal treatment. However, the effects of other factors such as parity, sex and assistance at birth on the above mentioned outcomes were observed.

2.4.1 Lamb Bleating Behaviour

Vocalizations are used by the ewe and the lamb to identify one another (Alexander, 1977; Shillito, 1975; Walser et al., 1981) and lamb vocalizations are thought to be related to their degree of need (Brunelli et al., 1994; Weary and Fraser, 1995). In the present study, females vocalized more than males in the control group but no sex differences were found in the stress group. The findings in the control group are in agreement with previous studies where female lambs of approximately 24hr of life vocalized more than males when in a stressful situation (novel arena and novel object tests) (Averos et al., 2015). Further, in a PNS experiment on goats, three month old females tended to vocalize more than males in the control group when exposed to a novel arena test (Roussel et al., 2005). These studies suggest that females naturally vocalize more than males when exposed to a stressful situation and that there are sex differences in the stress coping strategy, with females adopting a more emotional and males a more passive strategy. Indeed, it is well established that females usually respond to stressful situations expressing higher levels of emotionality than males (Boissy et al., 2005; Hernandez et al., 2010).

Interestingly, in the present study sex differences in lamb vocalizations were absent in the stress group mainly because PNS decreased vocal activity in females to a similar rate to that of males. Studies conducted in sheep and goats have shown PNS may have different effects on the offspring, either reporting no impact of PNS on the bleating behaviour of 24hr old lambs (Averos et al., 2015) or a tendency to increase the number of vocalizations in three months old male kids (Roussel et al., 2005). Of course the different age at testing, the different test, the different species, and the different type and timing of maternal stressors used may have contributed to these different outcomes. This further suggests that the direction of the effects of PNS seems to be not only sex-specific but also dependent on other factors, all contributing to the final offspring phenotype. Additionally, earlier studies conducted on guinea pigs regarding social instability as a PNS factor showed that the development of offspring from stressed dams was altered in a sex-

specific fashion at a behavioural, endocrine and brain level (Sachser and Kaiser, 1996). Similar results have been described in other rodents and, to a lesser extent, in the pig literature, where prenatally stressed males were shown to suffer a feminization process with loss or attenuation of male-typical traits. It was observed that males show lower testes weight and shorter anogenital distance (AGD) (rat: Dahlöf et al., 1978; pig: Lay et al., 2008), altered mating (guinea pig - Kemme et al., 2007), lower testosterone levels, and decreased fertility (pig - Ashworth et al., 2011; mice - Crump & Chevins, 1989; guinea pig - Kemme et al., 2007; rat - Ward, 1972) whereas prenatally stressed females go through a masculinization process showing longer AGD, higher testosterone, and more masculine courtship, play, and social behaviour (guinea pig - Kaiser & Sachser, 1998; rat - Kinsley & Bridges, 1988; mice - Vom Saal et al., 1990).

The mechanism that underlies this type of effect is not yet well defined but one hypothesis is that increased maternal stress allows maternal cortisol to cross the placenta more readily, thereby affecting fetal adrenal, ovarian, and/or testicular function (Barrett et al., 2014). Previous studies show that under stressful conditions maternal cortisol levels rise and that the placental enzyme (11b-hydroxysteroid dehydrogenase, type 2, or 11b-HSD2) which inactivates it, preventing cortisol from reaching the fetus, may be dysregulated (Mairesse et al. 2007, O'Donnell et al. 2012). Consequently, the increased fetal exposure to cortisol may affect fetal or placental androgen activity (Barrett et al. 2013). Indeed, in animal models prenatally stressed females fetuses show increased levels of testosterone compared to controls (Ward & Weisz, 1980, 1984) and in males there appears to be an impediment for the typical prenatal testosterone surge during the "male programing window" in early pregnancy (Vom Saal et al., 1990; Ward & Weisz, 1980, 1984). The reason why in the present study bleating behaviour was altered by PNS only in females remains unclear. One possible explanation may be that the period during which different behaviours differentiate may be altered between males and females. For instance, the critical periods for hormonal influences on male-typical and female-typical sexual behaviour are different in the rat (Christensen & Gorski 1978). Additionally, there is also some evidence suggesting the production of 11b-

HSD2 is more responsive to environmental cues in the female placenta than the male placenta (Clifton 2010).

Bleating rate was also affected by treatment depending whether assistance was given during parturition. Lambs from the control group that were assisted at birth bleated more frequently than the ones that were born unassisted. Darwish & Ashmawy (2011), who reported similar results, also reported that dams assisted through parturition and with longer births showed decreased maternal behaviour. This is in line with previous studies that suggested an existing link between lamb bleat and the quality of maternal care received (Dwyer & Lawrence, 1999; Garcia Gonzalez & Goddard, 1998). Alternatively, assistance given at birth may have affected lamb behaviour. Lambs that are assisted at birth have previously been reported to be slower at achieving lamb behavioural landmarks (Dwyer 2003). This delay in behaviour is thought to be caused by possible hypoxia periods that may happen during a complicated birth (Haughey 1991), which can inflict injuries to the fetal central nervous system and impair locomotor activity in lambs (Haughey 1980). This could prevent the lamb from getting physically closer to the dam and leave it with vocalization as the only mechanism to get the ewe's attention, increasing the bleating rate as was observed in the control group. Nevertheless, PNS seems to alter this relationship between birth assistance-bleating behaviour as assisted lambs in the stress group vocalized less than the unassisted ones and also less than the assisted lambs in the control group. Given that maternal behaviour arises from and is regulated by an interplay between the dam and the cues received from the lamb (Nowak et al., 2011), it is possible that the maternal stressors used in the present study had a positive effect on later maternal behaviour, enabling ewes to buffer the negative effects that assistance at parturition usually has on maternal care. Studies in sheep where maternal behaviour was either challenged shortly after parturition (Roussel et al., 2006), or observed in its spontaneous state (Coulon et al., 2014; Hild et al., 2011), have shown that maternal stress can have both negative and positive effects on maternal behaviour. Accordingly, the rat literature suggests that the intensity of maternal stress may have different outcomes for maternal behaviour: mild maternal stress may improve maternal behaviour by increasing time spent in the nest and nursing (Muir et

al., 1985), while severe stressors reduce those behaviours (Smith et al., 2004). The nature and schedule of the stressors used in this study seems to have had a mild impact on the ewes (Yusof 2018). Thus, it is not possible to rule out a positive effect of PNS on maternal behaviour of dams that were assisted at parturition which, as a result, influenced lamb bleating behaviour.

2.4.2 Lamb Behaviour Landmarks

Lamb readiness to stand and suck is a major factor affecting lamb survival (Cloete, 1993; Dwyer et al., 2001; Owens et al., 1985). Lambs that are quicker to stand have a greater ability to maintain body temperature and are also faster to suck successfully (Dwyer & Morgan, 2006). Early colostrum ingestion ensures an adequate level of nutrition and passive immunity, as well as lamb bonding to the ewe (Nowak et al., 1997). Previous studies in sheep have shown that progress through the sequence of neonatal behaviours is usually delayed in male lambs, lambs born to first parity ewes, and lambs that required assistance to be delivered (Dwyer, 2003; Dwyer et al., 2005). Overall, in the present study ewe parity had no impact on lamb latency to stand but males were slower than females. Interestingly, PNS had no effects on latency for lambs to either stand or suck. These results are in line with the negative findings from past studies conducted in goats (Baxter et al. 2016) and in sheep (Hild et al., 2011), where aversive handling was used as a source of PNS in mid and late pregnancy, respectively. It is important to note that both studies applied the same stressor, although at different stages of pregnancy and in different species; nevertheless, no differences in offspring behaviour were found. Coulon et al. (2014) also reported an absence of effects of PNS on the latency to stand and suck in lambs, despite using a more intensive schedule and various different prenatal stressors. Once more, it seems that the type and timing of PNS applied influences the degree to which behaviour is affected and that some behaviours are subject to change while others are not. It is interesting to note that regardless of the stressor used prenatally, these behaviours seem to remain unaffected. Perhaps, behaviours with such biological importance for lamb survival are less susceptible to prenatal programing.

2.4.3 Lamb Body Temperature

Body temperature and birth weight are two of the main factors that influence lamb survival. Previous studies have associated low birth weight (Dalton et al., 1980; Fogarty et al., 2000) and low body temperature (Obst & Day 1968) with impaired survival in lambs. Dwyer & Morgan (2006) have shown that lightweight lambs have lower body temperatures over the first days of life and are slower in their behavioural progression to stand and suck, although they found no effect of ewe parity on lamb temperature. The present study showed that PNS affects temperature at 30 min after birth depending on the parity of the dam. At this time point body temperature was higher in lambs born to multiparous ewes than lambs born to primiparous ewes in the control group but no difference was found in the stress group. Thermoregulation is dependent on heat loss and heat production. Heat production is related to metabolism of the lambs' brown fat energy reserves and the increase of muscular activity by shivering (Nowak & Poindron, 2006). Heat loss is influenced by lamb surface area (smaller lambs have a higher surface area to volume ratio and lose heat more easily than bigger lambs) (Alexander 1974), the weather conditions (Egan et al., 1972), lamb behaviour after birth (standing and sucking quickly), and the dryness of the lamb (maternal grooming behaviour) (Dwyer & Morgan 2006). It seems unlikely that lamb weight or latency to stand and to suck had any influence on heat loss in the present study, as no differences between groups were found for these factors. This may indicate that the observed differences in body temperature were either related to a difference in maternal grooming or to a difference in the heat production mechanism.

Grooming is a behaviour that helps in drying the lamb and stimulates thermoregulation and respiration (Alexander, 1988; Lent, 1974; Nowak et al., 2000). Prenatal stress has been shown to alter maternal behaviour but the direction in which this happens seems to vary. In two different studies using aversive handling as a prenatal stressor, stressed sheep displayed higher grooming towards their offspring (Hild et al., 2011), while this behaviour was decreased in stressed goats (Baxter et al., 2016). Yet, in another study using different types of stressors, treatment had no impact on maternal behaviour

of sheep (Coulon et al., 2014). It is not improbable that grooming behaviour was affected by prenatal stress in the present study.

Heat production generated by non-shivering thermogenesis is a process that involves oxidation of brown fat adipose tissue controlled by triiodothyronine (T_3), which is produced from thyroxine (T_4) in brown fat adipose tissue by a process involving the enzyme 5-monodeiodinase (Dauncey 1990, Brent 1994) and mitochondrial uncoupling protein (UCP1). Previous studies, comparing difficulty of birth (Darwish & Ashmawy 2011) and different breeds of sheep (Dwyer & Morgan 2006), have found that low concentrations of T_3 and T_4 are related to a reduced ability to maintain temperature after birth in the lamb. Rooke et al. (2010) studied nutrition restriction as a form of PNS and showed that T_3 concentrations in nutrition-restricted lambs were greater than in control lambs, although this did not translate into a difference in rectal temperatures. However, it is important to point out that not only is nutrient restriction different in nature from the PNS used in the present study, but also that it was applied during a different stage of pregnancy (early-mid pregnancy - day 1 to 90). Therefore, it is possible that the difference in lamb rectal temperatures at 30min after birth observed in the present study may have been caused by an altered thermogenic response in relation to altered T_3 and/or T_4 levels.

It is interesting that only multiparous offspring were affected by PNS. There were no differences in the temperatures of lambs from primiparous ewes between stress and control groups. In fact, the analysis of ewe faecal glucocorticoid metabolites (Yusof, 2018) showed that primiparous ewes had higher concentrations in all of the stages of pregnancy (week 12, 14 and 16) analysed when compared to multiparous ewes. This may indicate that primiparous ewes had a different perception of the environment conditions than multiparous ewes. The primiparous ewes used in this study had been in pasture most of their life, hence, the simple novelty of being confined in a limited amount of space may have been in itself perceived as a stressor and a ceiling effect may have been achieved by these conditions in both treatment groups. That is, the conditions for primiparous ewes in the stress group may not have created a higher stressful environment than the conditions for primiparous ewes in the control group. Hence, if stress affected

lamb thermogenesis, either by affecting ewe behaviour (licking), lamb heat production, or a combination of both, it might be expected for this difference to be more accentuated in lambs of multiparous than in primiparous ewes, as was the case here.

At 24 hr after birth, there was a significant interaction between PNS and sex, with female lambs having a lower temperature than males in the control group but the opposite being the case in the stress group. However, these differences were not significant. The significant effect of the PNS by sex interaction on temperature comes from the fact that the direction in which female and male temperature varies is opposite in the control in relation to the stress group. Indeed, temperatures differed by only 0.1 °C, which seems unlikely to affect lamb survival. Further, this variation in temperature is not observable at 48hr, suggesting that this effect is indeed of little biological impact.

2.4.4 24hr Body Weight

Lamb weight at birth is an important factor for lamb survival. Lower birth weights have been associated with lower body temperatures and slower lambs when it comes to standing and sucking (see above). Previous studies in sheep have found an association between PNS later in gestation and lower birth weight (Roussel et al., 2004), while others did not find any association (Roussel et al., 2008). In pigs, social stress applied during early, mid or late gestation does not seem to affect piglet birth weight (Jarvis et al. 2006, Couret et al. 2009, Rault et al. 2013, Rutherford et al. 2014). In contrast, in glucocorticoid models where adrenocorticotropic hormone (ACTH) or hydrocortisone-acetate (HCA) were used to increase sow's cortisol levels during gestation, piglet birth weight was shown to be increased (late gestation - Kanitz et al., 2006; Otten et al., 2007), decreased (pooled weights all 3 stages of gestation - Kranendonk et al., 2006), or not affected (mid gestation - Lay et al., 2008; Otten et al., 2007). In sheep, cortisol injection were shown to decrease birth weight (mid gestation Corner et al., 2010). These results suggest that birth weight can be affected by increased cortisol during pregnancy but that the effects depend on the time when

treatment is applied in relation to pregancy. Moreover, the effects of cortisol of sythetic origin seem to be more pronounced than the effects of endogenous cortisol. This could be related to the fact that synthetic glucocorticoids have higher affinity to the GR and thus have more potent actions on the HPA axis than the endogenous ones (Karssen & de Kloet 2007). However, maternal stressors may also vary in their efficiency in increasing maternal cortisol levels. Indeed, in some of these studies no cortisol differences in the pregnant sows were found between treatments. Yet, where stressed sows showed higher concentrations of cortisol, piglet birth weight reamined unaffected (Jarvis et al 2006). It is not clear at this stage whether sheep and pigs are less likely to show effects of PNS on birth weight or if stronger stressors are needed to produce a difference in birth weight. Nevertheless, in the present study no differences in the concentration of maternal faecal glucocorticoid metabolites were found between groups (Yusof, 2018 data not shown) and the absence of differences in lamb weight at 24hr may have been a consequence of the former.

Not unexpected, is the fact that in the present study the lambs of primiparous ewes were lighter than those of multiparous ewes, and females were lighter than males at birth. These results reflect the effects of parity and sex on birth weight which have been described in previous studies (Gardner et al., 2007). There is a 'physiological imprint' in the uterus after a first pregnancy that increases vascularisation (Khong et al., 2003) and enables greater blood volume expansion during the second pregnancy (Campbell & MacGillivray 1984), which facilitates greater fetal growth in subsequent pregnancies. As for the sex difference, previous studies suggest that males appear to grow faster than respective females *in utero* (de Zegher et al., 1999; Loos et al., 2001). This seems to be related to the presence of a Y-chromosome and the products of sry gene (transcriptional regulator that controls a genetic switch in male development) activation, like androgens (Haqq et al. 1994), that have sex-specific effects on fetal growth.

2.4.5 IgG Concentration in Lamb Plasma

In the present study no differences in IgG plasma concentration were found between treatment groups. However, lambs that were assisted at birth showed lower levels of IgG in plasma. In ruminant species (cow, sheep and goat) colostrum consumption has a fundamental role in passive immune transfer and in the survival rate of new-borns (Hernández-Castellano et al., 2014; Lascelles, 1979; Stelwagen et al., 2008), as they are born hypo-gammaglobulinemic.

Prenatal stress has been linked to alterations in the passive immunity of the offspring. Studies conducted in pigs (which have the same passive immunity system as ruminants; Borghesi et al., 2014), using restraint as PNS during the last third of gestation, demonstrated significantly decreased serum IgG in piglets during the first days of life (Tuchscherer et al., 2002). This difference was attributed to an effect of PNS on decreased permeability of the neonatal gut to antibodies, since no differences were found on maternal colostrum content of IgG, although less effective sucking behaviour may also have been involved. However, in later pig experiments using group vs. single housing throughout the pregnancy (Sorrells et al. 2006) or social mixing during the last third of gestation (Couret et al., 2009), no differences were found in plasma IgG of piglets from different treatment groups. Similarly, the present study also revealed no differences between groups in plasma IgG content at 24hr of age, although the IgG levels to ensure passive immune transfer were likely achieved (while no IgG value is universally accepted to characterize failure to acquire passive immunity in lambs, some authors use the reference IgG concentration <15 mg/mL; Alves et al., 2015). The reason for getting different outcomes in Tuchscherer et al. (2002) study might be due to the use of different prenatal stressors. In fact, similar studies conducted in rodents suggest that different stressors may affect passive immunity differently. For example, in one study bystander stress (witnessing foot-shocks applied to another animal without receiving them itself) was less effective in decreasing offspring antibody levels than electric shock (Sobrian et al. 1992).

In the present study, the concentration of IgG in plasma was affected by the assistance given at birth, with lambs being assisted showing lower levels of this immunoglobulin. Assistance given at birth has been shown to delay the behavioural progression of the neonatal lamb (Dwyer, 2003; Dwyer et al., 2005), which can delay the ingestion of colostrum and consequently decrease the levels of plasma IgG. Yet this did not seem to be the case here, as assistance given during birth did not affect lamb latency to suck in the present study. However, it is possible that subsequent sucking behaviour may have been affected, either by being less able to suck or by having less sucking bouts (see section 2.4.1 for a brief explanation on the effects of complicated births). Alternatively, mother behaviour may have also contributed to the present results, as ewes that are assisted during parturition usually show delayed maternal behaviour (Arnold & Morgan 1975, Poindron & Le Neindre 1980).

2.4.6 Recognition Test

Lambs did not prefer the dam from a distance, and times spent close to the dam did not differ between groups. However, lambs were attracted by ewes, as expressed in the greater amount of time spent close to them than random. Lambs from both the control and stress groups also showed a preference for the dam once at close quarters, as expressed by more time than random spent close to the dam then the alien ewe.

In this study, lambs spent more time close to the dam than to the alien ewe but did not choose to approach the dam first from a distance more than would be expected by chance. These results are in agreement with previous reports stating that lambs' ability to discriminate between their own dam and an alien ewe at close quarters is observable at ages varying from 12 to 24hr. Distal recognition is only achieved at three days of age (for a review see Nowak et al., 2011).

In contrast to our results, another study (Coulon et al., 2014) found that PNS affected lamb choice by increasing the preference for the dam over the alien ewe, expressed in greater amounts of time spent close to the dam. This possibly reflects higher emotional reactivity in PNS lambs that was also seen

in later stages of life (Coulon et al., 2011). However, in an experiment where space restriction was used as a prenatal stressor, a test for lamb sociability (testing the attraction for another lamb at 2-3 days of age) revealed limited effects of PNS treatment (Averós et al. 2015). Although the two studies mentioned represent different challenges, attraction for dam vs. attraction for a conspecific, it is possible that the main reason for different outcomes is the different types of stressors used.

Another interesting finding of the present study was that lambs born to primiparous ewes chose their dam first more than at chance levels but lambs born to multiparous ewes did not, suggesting that lambs from primiparous ewes were able to discriminate their dam from a distance. First of all it is important to note that it is not expected for lambs at the age of 24hr to be able to discriminate their dam at a distance, as this is an ability that is usually seen from three days of age onwards (see Nowak et al., 2011 for a review). Yet present results suggest that maternal behaviour may have had an important role in the development of this ability. Indeed, maternal care during the early periods of life is important for the establishment of the ewe-lamb bond, which determines how attached they are to each other and the efforts they will put in to reuniting once separated. It is well established that a stronger ewe-lamb bond is formed in the case of multiparous ewes (Dwyer et al. 2003, Muñoz et al. 2009, Rooke et al. 2010). Hence, there seems to be no certain explanation for the present finding but given that the establishment of the ewe-lamb bond is dependent on the interplay between ewe-lamb behaviour (Nowak et al., 2011) it is possible that, for some reason yet to be explained, lamb behaviour may have contributed to the present findings.

The fact that PNS did not affect lamb preference for the dam in this study does not necessarily mean that maternal social instability and reduced space are innocuous for the lamb. Instead, the effects of this type of stressor may only become evident when added to other stressful postnatal experiences. Indeed, this cumulative effect has been observed in lambs where the combination of space limitation during pregnancy with early maternal separation resulted in lambs showing higher reactivity in a sociability test (Averós et al., 2015).

2.5 CONCLUSION

The social factors and space allowance conditions used as prenatal stressors in this study were aimed at reproducing the normal husbandry environments experienced by some housed pregnant sheep in farms across the UK. The results from this experiment suggest that these conditions appear to be perceived by the ewes as mild stressors (Yusof 2018). As a consequence, only subtle differences in the development of the lamb during the neonatal period (from birth until 48hr) were seen in the offspring.

Birth weight and latency to stand and suck, which are known to be important factors associated with lamb survival, were not affected by PNS. Body temperature, which is also of key importance for lamb survival, was only mildly affected by PNS as a result of an interaction with ewe parity. However, temperatures did not reach hypothermic values and subsequent checks showed that lambs recovered well from this initial difference. Nevertheless, these lambs were born in sheltered conditions and it is plausible that temperature differences to such a degree (0.5ºC) may have a different impact on lambs born outside in the field, since the effects of wind and evaporation (of rain or amniotic fluid) seem to be cumulative and both cause lambs to die at temperatures that they could otherwise tolerate if dry or in still air (Alexander 1958, 1986). Other factors that enhance lamb survival and wellbeing in the longer term, such as the ability to recognise the dam and the acquisition of passive immunity, were also not affected by PNS.

Nevertheless, the absence of prenatal stress impacts on the neonatal lamb does not necessary mean that there are no consequences as these may only become triggered as a result of an interaction of pre- and postnatal experiences. The reduced bleating rate during the first 30min of life in PNS lambs that were assisted to be born may be seen as an illustration of such an interaction. Importantly, it is possible that behaviours with such biological importance for lamb survival, such as the ones considered here, have a low degree of vulnerability to prenatal programing and only highly stressful environments are capable of inflicting a change.

CHAPTER 3

Low space allowance and social instability during last third of pregnancy in sheep: effects on brain morphology and epigenetic regulation of the HPA axis.

3.1 INTRODUCTION

It is well established from the literature that prenatal stress (PNS) affects the development of the offspring. Previous studies in which the offspring was evaluated at different stages in life have shown PNS can have effects in early or later life. For example, in a study where a single injection of betamethasone (a synthetic corticosteroid used as a stress inducer) was administered to sheep on gestational day 104, little effect was observed on HPA axis function in six month old offspring but elevated basal and stimulated plasma cortisol concentrations were observed at one year of age (Sloboda et al., 2002). This type of result highlights the importance of following the offspring at several different times during their life.

The effects of PNS have been studied across different systems and functions of the body (see Chapter 1). Behaviour and cognition, epigenetic alterations and expression of genes in brain areas involved in regulation of the HPA axis, brain morphology and immune system are fields that have been the subject of study in previous work done in sheep and other species. Alterations to these functions and systems are related to an altered HPA axis function as a result of PNS (for a review see (Kapoor et al. 2006). For example, increased activity of the HPA axis (increased concentrations of basal cortisol) was present in prenatally stressed lambs of 25 days of age and increased exploratory and activity behaviour at 8 months of age (Roussel et al.,2004). Another two companion studies focusing on the effects of PNS on brain morphology in sheep reported increased fearfulness (Coulon et al., 2011) paired with alterations in brain morphology (Coulon et al., 2013) in PNS lambs such as higher spine density on pyramidal neurons in cornu ammonis 1 (CA1) of the hippocampus of PNS lambs, as well as a lower ratio of more mature to more immature spines. Spine morphology is critical for regulating excitatory synaptic physiology (reviewed in Nimchinsky et al., 2002). Expression of genes involved in brain development and spine morphogenesis was also reported to be decreased in hippocampus and prefrontal cortex in PNS compared to control lambs, suggesting a cognitive impairment (Coulon et al., 2013).

The activity of the HPA axis is governed by corticotropin-releasing hormone (CRH) and arginine vasopressin, which in turn are regulated by GR-mediated feedback at multiple levels of the axis and subject to inhibition from extra-hypothalamic sites. Activation of the GR in the hippocampus is associated with the inhibition of hypothalamic CRH synthesis and decreased HPA activity (Sapolsky et al., 2000). Being rich in GR and MR, the hippocampus is particularly vulnerable to glucocorticoid excess, especially in early life (Charil et al., 2010). Moreover, the rodent literature suggests that epigenetic programming of HPA function occurs at multiple levels of the HPA axis in addition to effects on hippocampal GR expression (for a review see Turecki & Meaney, 2016). PNS has been reported to decrease the expression of the GR gene in the hippocampus, suggesting impaired glucocorticoid negative feedback regulation of the HPA axis (Darnaudéry & Maccari 2008). In one study in mice, PNS altered the methylation levels of CRH and GR genes (decreased and increased, respectively) which correlated with altered gene expression in the hypothalamus and hippocampus (Mueller & Bale 2008).

The literature also points to the effects of PNS on the immune system, generally indicating an inhibitory effect. However, stimulatory effects of PNS on the immune system have also been found (for a review see Merlot et al., 2008). Another field that has been subject of study regarding PNS effects is cognitive function. The results from studies where a fear learning paradigm was used to assess the capacity of associative learning suggest that PNS contributes to the development of fear or anxiety-related disorders. These tests usually involve two types of fear: learned (or conditioned) fear and innate (or unconditioned) fear. Innate fear causes an inherent response, usually causing avoidance reactions to a biologically relevant stimulus. Learned fear is caused by an association between a predictable stimulus and its effects, acquired by repeated exposure (Nestler et al., 2009). Interestingly, PNS offspring in these studies showed impaired fear extinction learning (Li et al., 2014; Wilson et al., 2013) and greater fear behaviour during the training and recall phases of fear conditioning (Sadler et al. 2011). However, other studies reported no effects of PNS (Green et al. 2011). These differences in results may have been related to the different PNS protocol used.

All of the above represent points of interest from an animal welfare perspective but also from an economic point of view. For example, an adequate immune response in the offspring is an important landmark in lamb productions systems. It ensures that lambs' first line of defence mounts an adequate response to pathogens, decreasing the chances of contracting disease and leading to better welfare and less economic loss. Similarly, animals that are behaviourally more reactive are at risk of more injuries and are more difficult to manage in a farm environment, representing potential additional economic costs and decreased welfare.

In the previous chapter, the effects of the prenatal treatment were found to be very subtle as they were only expressed as a function of other factors such as parity, sex and assistance to birth. Moreover, outcomes important for lamb survival such as latency to stand and suck, IgG concentration in plasma, body weight and the ability to recognise the mother were not affected by prenatal treatment. The present study continues from the previous chapter, with the aim of understanding the effects of PNS on behaviour, immune response, cognition and neuronal alterations in lambs at later stages of life using the same gestational stress in sheep. Additionally, following the lambs during the post-neonatal period will help to better understand how altered behaviours and functions observed at early stages of life are maintained, potentiated or attenuated as the lamb progresses in age. For this purpose, the potential effects of PNS were assessed on the lambs' capacity to mount an innate immune response to vaccination and on brain morphology, and genetic and epigenetic alterations at the HPA axis level, together with the behavioural response to a fear learning paradigm. In sheep, to my best knowledge, the establishment of a fear conditioning paradigm has never been attempted. Hence, the objective regarding this part of the experiment was to develop a fear conditioning protocol for sheep and to investigate the possible effects of PNS on fear learning and memory.

3.2 METHODOLOGY

3.2.1 Ewe Experimental Procedures

Ewes used in the present chapter were the same ewes used in the previous chapter. Maternal treatments applied during late pregnancy are described in Chapter 2.

After the first 48hr of life of the lamb, all ewes and lambs were released onto pasture and managed as a single flock. These animals were brought into the experimental shed only during periods of data collection, after which they were again released into the field.

3.2.2 Offspring Experimental Procedures

Lambs used for data collection periods described in this chapter were the same lambs tested during the neonatal period. The only treatments applied to these animals occurred during the last third of their prenatal life and are described in Chapter 2.

During the post-neonatal period different data collection points were used at different lamb ages (Table 3.1). At 7 weeks all lambs were vaccinated and weighed, and a subset was blood sampled to measure the immune response, and another subset was euthanized for brain sample collection. At 13 weeks of age a subset of lambs was used in a fear conditioning paradigm to assess fear learning ability and all male lambs were weighed. At 17 weeks all lambs were weighed.

Table 3.1 Data collected during the post-neonatal period and number of lambs sampled at each age.

Lamb age	Data collection	Sample	Females		Males		Total
			Control	Stress	Control	Stress	
7 weeks	Immune response	Blood	12	11	8	9	40
	Weight	-	39	29	25	26	119
	Brain histology	Hippocampus	10	10	10	10	40
	Brain epigenetic alterations	Hippocampus Hypothalamus					
13 weeks	Fear conditioning	Behaviour	4	4	3	3	14
	Weight	-	-	-	15	16	31
17 weeks	Weight	-	29	19	15	16	79

3.2.2.1 *Immune response to vaccination*

At approximately 7 weeks of age, lambs and ewes were brought from the field into the experimental shed in order to be vaccinated. A subset of 40 lambs were assessed for their immune response to vaccination (n=20 control, mean age in days (±s.d.)=47.9 (±0.79); n=20 stress, mean age in days (±s.d.)=47.8 (±0.72)). These lambs were chosen balancing, as far as possible, for age, sex, parity of the ewe and prenatal treatment. Vaccination was carried out over a period of 17 days to respect the lamb age at vaccination day (all lambs were 7 weeks +/- 1 day of age on the day that they were vaccinated). The lambs not chosen to be blood sampled were all vaccinated on the same day, one day prior to the beginning of the vaccination period.

Lambs scheduled to be euthanized for brain collection were not vaccinated or blood sampled. All lambs were weighed on the same day they were vaccinated using a weighing crate inside the experimental shed. To assess the immune response blood sampling was carried out by venepuncture of the

jugular vein immediately before (for baseline levels) and 24 hr after (for immune response levels) vaccination with Ovivac P Plus® (MSD Animal Health) for the active immunization of lambs as an aid in the control of pulpy kidney, tetanus, braxy and blackleg caused by Cl.perfringens type D, Cl.septicum, Cl.tetani and Cl.chauvoei. Blood was collected into two vacutainer 4ml plastic tubes coated with EDTA (BD Vacutainer® Blood Collection Tubes, Plymouth, UK). Samples were then centrifuged at 2,190 × g for 15 min at 4°C. Plasma was separated from blood, aliquoted in 1.5ml plastic tubes and kept at -20°C for later quantification of acute phase proteins (Haptoglobin (Hp) and serum amyloid A (SAA)).

Hp concentrations were determined in collaboration with Professor David Eckersall (at ReactivLab, University of Glasgow) using a colorimetric assay based on the method described by (Eckersall et al., 1999) and modified as described in International Patent Application WO 2012/085497 A1 and validated by Crawford et al., (2013). The Hp assay was performed on an ABX Pentra 400 analyser (HORIBA UK Limited) using a calibration curve with a lower limit of detection of 0.02 mg/mL and a top Hp standard of 1.48 mg/mL. Hp values greater than this were automatically diluted by the machine. In summary, 3 µl of plasma sample were directly pipetted into cuvettes (HORIBA UK Limited) and placed inside the analyser. Reagents (R1, R2 and R3) were added automatically by the machine as follows: 0secs -150µl of R1 (haemoglobin); 48secs - 135µl of R2 (chromogen); 72secs – 37.5µl of R3 substrate (Hydrogen peroxide (H2O2)). All samples were tested in duplicate and read at 660nm.

SAA was measured using a commercial ELISA kit (Tridelta Development, PLC, Dublin, Ireland) according to the manufacturer's instructions. Samples were diluted 1:500 (as per manufacturer’s recommendation) in sample diluent buffer (Tridelta Development Ltd) and tested in duplicate. All plasma samples showing SAA values greater than 300 ng/mL (upper level of the range of the bovine standards used) were further diluted as required and re-analysed.

3.2.2.2 *Brain morphology, gene expression and methylation*

For brain tissue collection 40 lambs (n=20 control, mean age in days (±s.d.)=48.9 (±0.79); n=20 stress, mean age in days (±s.d.)=48.8 (±0.72)), twins to those vaccinated and blood sampled for immune response to vaccination, were euthanized. These lambs were chosen balancing, as far as possible, for age, sex and prenatal treatment. Euthanasia was performed after the 24hr blood sample post-vaccination of the twin. This procedure took place in the experimental shed in a visually isolated corner with an intravenous injection in the jugular vein of pentobarbital sodium (Euthatal, solution for injection 200mg in 1ml, Merial, UK), followed by transportation of the lamb to the site of brain extraction and processing. The period of time between euthanasia and complete brain extraction was on average 648.6 secs (10min49secs) (s.e.m.=13.7). For the extraction of the brain, a bone flap above the brain location was removed and extraction was completed by cutting the remaining meninges and the cranial nerve attachments. Brains were then cut longitudinally and the right hemisphere, used later for histological analysis, was preserved in Golgi Cox solution (Gibb & Kolb 1998, Cook & Wellman 2004) for 38 days (replacing with fresh solution every two days) followed by immersion in 30% sucrose in saline (8.5g NaCl + 300g Sucrose + 1L Water) for 15-17 days, always at room temperature. The left hemisphere hippocampus was dissected and snap frozen in liquid nitrogen and kept wrapped in paper foil at -80°C. The left hemisphere hypothalamus was divided into four parts: frontal/medial, frontal/lateral, caudal/medial and caudal/lateral; and kept in RNAlater at -20°C. Samples were then analysed for GR expression and methylation (see Real time PCR- gene expression within this section).

Histological analysis

After the Golgi Cox staining period of the right hemisphere, the hippocampus was dissected and sagittal sections (200mm) were cut with a vibratome.

Sections were mounted on gelatine-covered slides and went through a sequence of histology staining solutions (Table 3.2) in order to be alkalized, developed, fixed, dehydrated and cleared before being cover-slipped using Permount™ as mounting medium (Fisher Chemical™, cat. SP 15-100).

Histological analysis was conducted for the assessment of dendritic spine type and density (number of spines/µm) on terminal segments of apical dendrites of pyramidal neurons (10 neurons per brain) in the hippocampus CA1 area (ventral/temporal) of 20 brains (n=10 control, 5 males and 5 females; n=10 stress, 5 males and 5 females). Using light microscopy, pyramidal neurons were identified by their characteristic triangular cell body, with one prominent apical dendrite and basal dendrites emerging from the base of the cell body (Figure 3.1). Each terminal segment was traced at magnification ×1000. Images generated with Image J software (ImageJ 1.47v, http://imagej.nih.gov/ij) were used to count dendritic spines over a length of 30-50 µm on each dendrite. Four different types of spines were considered for this study: mushroom (globular prominent head with a thin and short neck; 0.5-2.5 µm size range), thin (smaller globular and circular head with a long and narrow neck; 0.5-4 µm size range), stubby (short protrusions with no clear neck), or filopodia (only long necks without globular heads) according to previously-established classifications (Peters & Kaiserman-Abramof 1969, Crick 1982, Rochefort & Konnerth 2012).

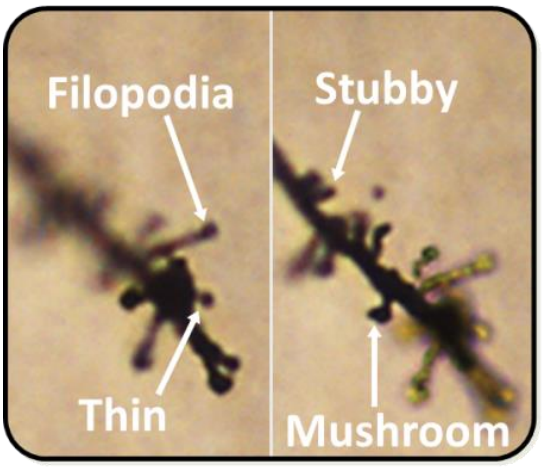


Figure 3.1 The four different types of spines present in the apical dendrites of pyramidal cells in the CA1 area of the hippocampus.

Table 3.2 Sequence of histology staining solutions used

Solution	Specification of reagents	Staining step	sequence timings
dH2O	Distilled water	washing	2 X 5 min
NH4OH 2:1	**Ammonium hydroxide** standard stock solution, ~28% (Sigma-Aldrich)	alkalinisation	30 min
dH2O	Distilled water	washing	2 X 2 min
D19	High contrast developer (Kodak, cat. 146-4593)	development	2 min
dH2O	Distilled water	washing	2 X 2 min
Rapid Fixer	Non-hardening black and white fixer (Ilford, cat. 1984262)	Fixing	10 min
dH2O	Distilled water	washing	2 X 5 min
50% ETOH	Ethanol 50% (diluted from Ethanol 95% (v/v) (Fisher, CAS 64-17-5)	dehydration	5 min
70% ETOH	Ethanol 70% (diluted from Ethanol 95% (v/v) (Fisher, CAS 64-17-5)	dehydration	5 min
95% ETOH	Ethanol 95% (v/v) (Fisher, CAS 64-17-5)	dehydration	5 min (blot afterwards)
100% ETOH	Ethanol absolute (Fisher, CAS 64-17-5)	dehydration	5 min (blot afterwards)
Xylene	(Fisher; c8h10/cas 1330-20-7)	clearing	2 X 5 min

Real time PCR- gene expression

GR gene (Nr3C1) expression was analysed in hippocampus and hypothalamus tissue samples using quantitative real-time polymerase chain reaction (RT-qPCR). A portion of approximately 30mg of tissue was cut directly from the frozen hippocampus always targeting the same region of this structure across samples in order to standardize the section of tissue being analysed. As for the hypothalamus, all analysed samples corresponded to the anterior/medial section of this structure in order to target the paraventricular nucleus (PVN) which is located in this subregion.

Samples were treated as per manufacturer's recommendations (Qiagen, AllPrep DNA/RNA Mini Kit, UK) for RNA extraction. This kit also allowed for parallel DNA extraction, which was also obtained for later analysis of GR gene methylation. After extraction, RNA concentrations in hippocampus and hypothalamus samples were determined using an ND-1000 spectrophotometer (NanoDrop, Labtech). RNA samples (900 ng) were further treated with DNase 1 (RQ1 RNase-Free DNase, Promega, UK) according to manufacturer's recommendations to ensure there was no DNA contamination.

DNase-1 treated RNA samples (450 ng) were used for cDNA synthesis using reverse transcription by Omniscript RT kit (Qiagen, UK), in a 20µl reaction according to the manufacturer's protocol. cDNA obtained was diluted (4 fold) and used as a template in each RT-qPCR reaction. Each sample was run in duplicate on a LightCycler 480 (Roche, UK) using LightCycler®480 SYBR Green I Master (Roche, UK). Briefly, 10µl of SYBR green Mastermix, 2µl template, and 0.6µl (10pmol/µl) of forward (Fwd) and reverse (Rev) primers (see table 3.1) and water were added to achieve a total volume of 20µl. The reaction was subjected to an initial incubation at 95°C for 5min, then 45 cycles of 95°C for 10sec, 58°C for 15 sec and 72°C for 15 sec. Expression of GR gene was calculated using the advance relative quantification method (E-Method) to obtain the ratio of the target gene against the reference gene/housekeeping gene (see below). Efficiency value was calculated by including relative standards to generate a relative standard curve, calculated by making serial dilutions (1:4, 1:16, 1:64, 1:256) of the target and reference

genes. To ensure there was no genomic contamination, PCR was also run on -RT samples.

To find a suitable housekeeping gene a validation of four reference genes (table.3.1) - beta2-microglobulin (B2M), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), succinate dehydrogenase complex subunit A flavoprotein (SDHA), and Cyclophilin (PPIA) - was performed. YWHAZ (for hypothalamus) and PPIA (for hippocampus) were the most suitable reference genes for the tissues tested, and were thus used for normalization of the qPCR data.

Table 3.3 Selected candidate genes and reference genes used in Ovis aries and their primers: sequence, annealing temperature, amplicon size in base pair, PCR efficiency and accession number.

Gene name		Sequence (5' to 3')	AT/Amp[a]	Eff[b]	Accession no.
Glucocorticoid receptor (NR3C1)	Fwd	TTATATGCAGGATATGACAGCTCTGTT	58/126	1.93	NM_001114186.1
	Rev	CCTGAATCCTGGTATTGCCTTT			
B2M	Fwd	GCCAAATTACCTGAACTGCTATGTG	58/73	1.84	NM_001009284.2
	Rev	CCCGTTCTTCAGCAAATCGA			
YWHAZ	Fwd	AGACGGAAGGTGCTGAG	58/123	1.9	NM_001267887.1
	Rev	CGTTTGGGATCAAGAACTTT			
SDHA	Fwd	CATCCACTACATGACGGAGCA	58/90	1.83	XM_012125144.1
	Rev	ATCTTGCCATCTTCAGTTCTGCTA			
Cyclophilin (PPIA)	Fwd	CATACAGGTCCTGGCATCTTGTC	58/100	1.8	NM_001308578.1
	Rev	TGCCATCCAACCACTCAGTCT			

[a] Annealing temperature/amplicon size in base pair

[b] Efficiency.

Pyrosequencing – DNA methylation

DNA methylation was analysed using the pyrosequencing method. Genomic DNA was extracted from hippocampus and hypothalamus samples as previously described (see RT-qPCR above). Bisulphite conversion of genomic DNA was performed by EpiTect Bisulphite kit (Qiagen). Briefly, 600 ng of genomic DNA were treated with sodium bisulphite, followed by de-

sulphonation and was then eluted in 25µl elution buffer. 5µl were then used in a 50µl PCR reaction containing 25µl HotStar Taq master mix (Qiagen) and 10pmol of each primer (forward: biotinylated-AGTTTAGGAGTGGATTTGGAGT; reverse: CTCCTCCACCCCCACCTCTC).

Primers for GR were selected to amplify a 188bp region containing the promoter region exon 1F. This region is homologous to exon 1_7 region in the rat, which contains a binding site for the nerve growth factor-inducible protein A (NGFI-A) (McCormick et al. 2000). NGFI-A has previously been shown to be sensitive to environmental conditions, varying its expression in the same direction as GR expression (Weaver et al. 2004). Additionally, NGFI-A binding site methylation was reported to also be altered by environmental conditions (Weaver et al., 2004). Comparison of GR exon 1F genomic sequences of rat (AJ271870), sheep (HM204706.1) and cow (Chromosome 7: 56,235,966-56,350,171 ENSEMBL) showed that NGFI-A binding site is highly conserved across these species.

PCR was performed as follow: 95°C for 15 min, followed by 45 cycles of 94°C for 30 sec, annealing temperature of 52°C for 30 sec followed by extension at 72°C for 30 sec, with a final extension of 10 min at 72°C. PCR products (8 µl) were run on 1.6% agarose gel to check product specificity. 20 µl of PCR product was used for methylation analysis by pyrosequencing. Single-stranded template for pyrosequencing was prepared using PyroMark Q24 Vacuum Prep Workstation (Qiagen). Briefly, biotin-labelled PCR products were captured by streptavidin sepharose high performance beads (GE Healthcare) in the presence of binding buffer (Qiagen). Captured PCR product was then washed with 70% ethanol, followed by alkali denaturation. Denatured PCR product was washed to remove salt before annealing to 0.3 µM sequencing primer (sequencing primer: ATTTTCTCTATAATTTCTCTTCTT) at 80°C. Pyrosequencing assay covered 48 bases containing 5 CpG sites and was performed in duplicate for each sample using Pyromark Q24 instrument (Qiagen). Percentage of cytosine methylation of individual CpG sites was quantified by PyroMark Q24 2.0.6 software.

3.2.2.3 *Fear learning paradigm*

At approximately 13 ± 1 weeks of age a subset of the remaining intact litters and respective dams was brought into the experimental shed. On the same day lambs were spray marked on body sides with numbers for identification and with a spray dot between the shoulder blades to facilitate the marking of activity during later video analysis. Fourteen lambs (n=7 control, 3 males and 4 females, mean age in days (±s.d.)=90.4 (±4.5); n=7 stress, 3 males and 4 females, mean age in days (±s.d.)=91 (±6.3)) were submitted to a fear learning paradigm to assess if prenatal stress had an effect on acquisition, expression and extinction of fear conditioning to a combined auditory and visual cue. Lambs were chosen balancing, as far as possible, for age, sex (between groups and within the litter) and prenatal treatment.

The acquisition, expression and extinction of fear conditioning and the recall of extinction were assessed in a 4-day aversive learning paradigm (modified from Stevenson et al, 2009). Four ewes and their respective offspring were tested simultaneously. Each testing unit consisted of two adjacent $1m^2$ pens with straw bedding, one for the ewe and one for the lambs (see Figure 3.2). Each unit had water in both pens, a small lamp (Jansjo LED Clamp Spotlight, IKEA) mounted in the corner of the lambs' pen and a radio set to emit white noise placed on the outside of the lambs' pen. A camera (Canon legria hfs21) was placed over each testing unit in order to record lamb behaviour during testing for later analysis. Dam and offspring had visual/auditory contact but tactile contact was not possible. Each testing unit was visually isolated from the other.

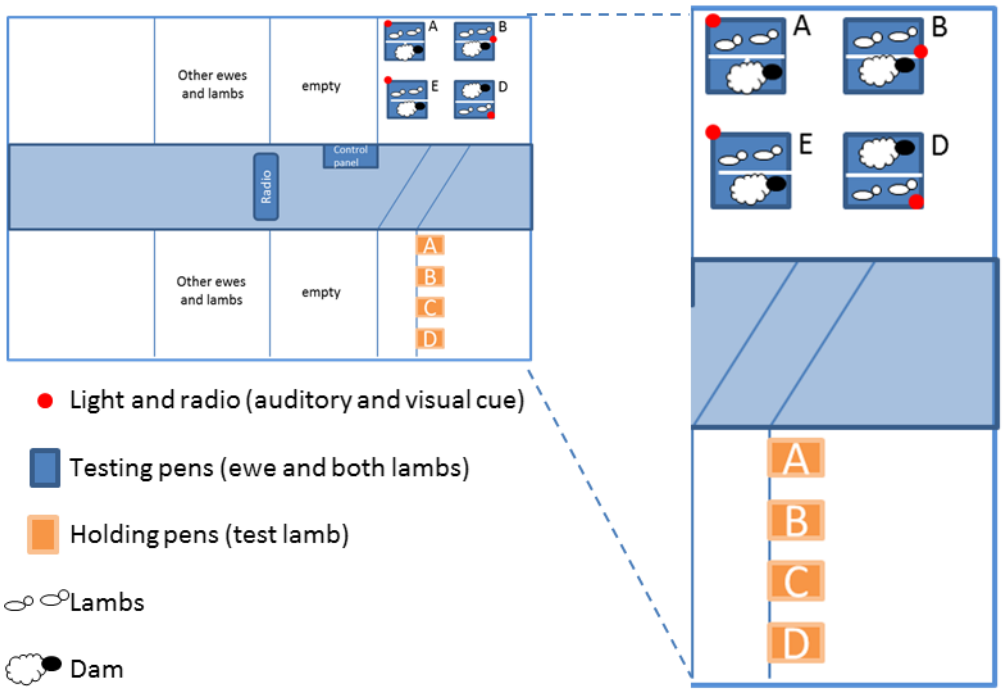


Figure 3.2 Shed layout for fear conditioning testing.

On day 1, animals were subjected to 2 hr of habituation to the experimental setting where the conditioned stimulus (CS) - white noise paired with the turning on of the lamp - was presented 10 times at random intervals of time for 30 secs.

On day 2, animals were subjected to fear conditioning. This session consisted of five repetitions of the same procedures. The conditioning session started with a period of 30min habituation followed by presentation of the CS for 30 seconds every 30 min. Behaviour inside testing units was filmed during the 5min preceding every exposure to CS and the 30secs of CS presentation. At the end of the 30 seconds of cue exposure, the test lambs were moved into individual holding pens (1m^2, straw bedding) across the shed (unconditioned stimulus (US) – resulting in separation and isolation of the tested lamb from the mother and the twin lamb for 5 min (Figure 3.2), while siblings remained in the test unit. The test lamb in any given test unit (A, B, C or D) was always placed in the holding pen of the corresponding letter (Figure 3.2). Holding pens were placed next to each other and lambs

were visually but not acoustically isolated from one another. During this 5 min period behaviour was recorded for future analysis by a CCTV video camera placed in each pen connected to a high memory storage computer using Geovision Digital Surveillance System software (ezCCTV Ltd, Herts, UK). Each lamb was then restrained for 30secs by a handler at the end of the isolation period. At the end of the second isolation period a blood sample was taken (as described in 3.3.2.1) to assess levels of cortisol. All lambs were then returned to the respective testing units after each isolation followed by a 30min resting period before a new repetition of the conditioning session would begin. As other ewes and lambs were all in the same shed where the experimental work was done, a radio was used to mask the sound of the cue for lambs that were not being trained or tested at that moment.

On day 3, animals were assessed for the expression and extinction of fear conditioning, consisting of presenting the CS 15 times in the absence of the US. Animals were placed in the testing units and after 30min were presented with the CS, with 4min30secs intervals between CS presentations. Behaviour was video recorded continuously throughout the session for later analysis. All animals were returned to the original pens at the end of this session.

On day 4, all animals were assessed for the recall of extinction. Animals were placed in the testing units and after 30min were presented with the CS five times alone, with 4min30secs intervals between CS presentations. Behaviour was filmed as previously described. All animals were returned to the original pens at the end of this session.

For conditioning and extinction, video analysis of ear position in relation to head, head angle in relation to cue and body posture (Table 3.4) was carried out on the 30secs of cue presentation videos, by scan sampling the footage every second. Subtle changes in ear postures have been linked to affective state in sheep; aligned ear postures have been associated with a neutral state and ears backwards with unpleasant uncontrollable situations, whereas ears held forward was associated with negative but controllable situations and ears in the asymmetric position with sudden events and negative contrast situations (amount of food expected not meeting expectation) (Boissy et al., 2011; Reefmann et al., 2009a). These changes in ear posture

possibly reflect heightened or reduced states of alertness in the animal, and might be affected by the surrounding environment. Similarly, sheep body posture changes in accordance to the surrounding environment. More stiff postures like standing and staring are adopted in the presence of a potential predator or other disturbance (Geist 1971) as opposed to a lying resting posture in the absence of imminent danger. It was hypothesized that alterations in ear postures from more relaxed to postures more indicative of negative experiences during the course of conditioning would be indicative of successful learning. In contrast, it was hypothesized that ear posture alterations in the opposite direction would indicate successful extinction. The same is expected regarding alterations in body postures. Both the test lamb and its sibling were analysed for differences in behaviour during presentation of the CS, where sibling lamb behaviour was used to compare to the test lamb. During the 5 min isolation (US) period, videos were analysed for the number of lamb attempts to escape, activity and body posture (Table 3.5). Activity was assessed by placing a grid of four equally sized squares over the screen. Since different behaviours were assessed during the cue presentation and isolation, the behaviours in isolation cannot be used to validate the fear conditioned paradigm. Instead these behaviours were used to assess the effects of prenatal treatment on the lamb response to a stressful situation (i.e. isolation). All video behaviour analysis was done using The Observer XT 11.5 software (Noldus Information Technology, Netherlands). Additionally, the number of vocalizations was recorded live by one observer.

Table 3.4 Ethogram for behaviours analysed during 30 secs cue presentation videos during fear conditioning and extinction.

Behaviour	Position	Definition
Ear position (Figure 3.3a)	Aligned	ears in line with the frontal plane, auricles are pointing towards the floor
	Forwards	ears aligned with **or** ahead of the frontal plane and auricles are pointing forwards
	Backwards	both ears are rotated backward and held behind the frontal plane
	Asymmetrical	two ears are in two distinct positions relative to the frontal plane
Head angle (Figure 3.3b)	Looking in cue direction	the intersection between the cue line and the imaginary line that divides the lamb's head longitudinally must be at an angle between 0-90° or 270-360°
	Looking away from cue	the intersection between the cue line and the imaginary line that divides the lamb's head longitudinally must be at an angle between 90-270°
Body posture	Lying head up	lamb rests on sternum with head elevated above the level of the back
	Lying resting	lamb rests on sternum with head below the level of the shoulder blades
	Standing	weight supported on all four legs , with only the feet touching the ground.
	Kneeling	lamb supported on both knees and back feet with back legs extended
	Sitting	Lamb has hindquarters on the ground with front legs extended

a) b)

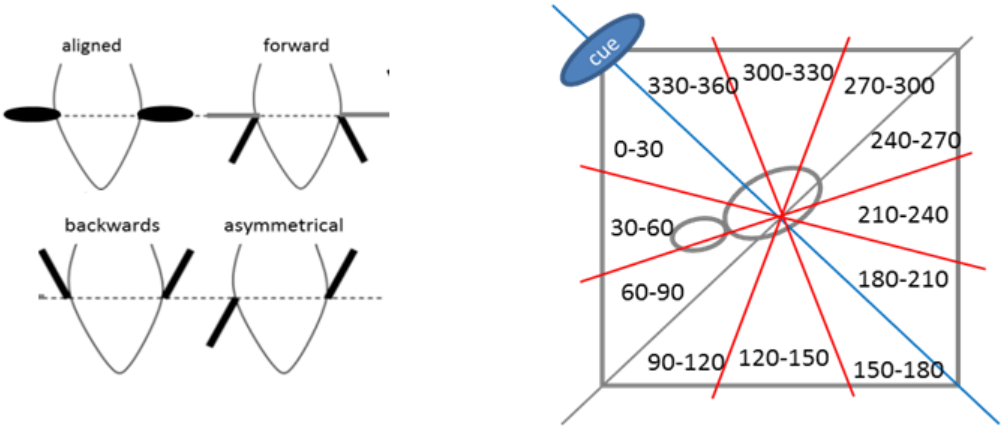


Figure 3.3 Representation of the different ear positions (a) (*image adapted from Boissy et al. (2011)) and representation of the different angles of head position in relation to cue (b).

Table 3.5 Ethogram for behaviours analysed during the 5 min isolation (i.e. US) period videos during fear conditioning

Behaviour	Position	Definition
Activity (state)		lamb is standing and the spray dot between the shoulder blades crosses a line limiting a square when the lamb moves
Escape attempt (event)		lamb jumps against the wall with all four feet from the floor off the ground
Body posture (state)	Lying head up	body weight supported by the length of the body, head elevated in relation to body
	Lying resting	body weight supported by the length of the body, head at the same level as shoulder blades
	Standing	body weight supported only by all four legs
	Kneeling	lamb weight is supported by knees and back legs
	Sitting	lamb is supporting weight on extended forelegs whilst the hindquarters rest on the floor

3.2.2.4 *Weight change*

Weight change was evaluated at three time points: at 7 and 13 weeks of age for males, and at 17 weeks of age for both females and males. At seven weeks 119 lambs (n=119; n=64 control, 39 females and 25 males; n=55 stress, 29 females and 26 males) were weighed as part of the vaccination protocol (see section 3.3.2.1).

Weaning of male lambs was carried out at 13 weeks of age. A decision to wean only males at this time point was made because females were still small and delaying the weaning time allowed them to reach heavier weights faster. Since males had already matured sexually they had to be separated from the flock to avoid possible mating. Thirteen week weight (weaning weight) was recorded for 31 males (n=15 control, n=16 stress). Females were weaned at 17 weeks of age only 4 days before slaughter. On this same day, males (n=31, 15 control 16 stress) and females (n=48, 29 control and 19 stress) were weighed.

Four days after the 17 week weighing, all lambs were transported to a commercial slaughter house and were slaughtered on the same day. After slaughter, hot carcase weights were measured at the slaughter house.

3.2.3 Statistical Methods

Statistical analysis was conducted using the restricted maximum likelihood (REML) procedure in Genstat (Genstat 64-bit, 16th edition). Fixed and random models for this procedure varied by adding or removing factors according to the data being analysed. However, treatment, ewe parity and lamb sex were always fitted into the fixed model and lambing pen and ewe into the random model. This model will be referred to as main model from here onwards and the alteration made will be mentioned below for each data set. On one occasion (isolation repetition during fear conditioning), a repeated measures REML was used to analyse the data. Alterations to the main fixed and random models will also be addressed below.

When fitting mixed models there were cases in which it was not possible to estimate the pen variance component. It was evident that this was arising because the algorithm was attempting to estimate a highly negative variance component for pen. This situation arises when the variability between pens is considerably less than the variation within pens. Biologically, this should not happen but there was no available reason to justify the removal of any data points, which would have remedied the issue. Consequently, the only available option was to drop the variance component for pen from the model.

This means that some of the results have to be interpreted with more caution. Where the pen variance component is dropped, comparisons between pen (e.g. comparisons between treatments) will be less significant than if pen was in the model and the opposite will be true for comparisons within pen (e.g. parity). Comparisons at the stratum lower than this (i.e. between lambs from the same ewe within a pen), such as between sex of lambs, are not affected.

Occasions where pen had to be dropped from the random model will be specified below for each data set.

Immune response to vaccination

To assess the immune response to vaccination pre-vaccination values were subtracted from post-vaccination values. This allowed for looking at differences in the magnitude of immune response. For the analysis of SAA, 70% of the pre-vaccination values were below the detection level of the assay, which meant it was not possible to evaluate the exact concentration of these samples. A decision was made to compare groups only for post-vaccination values, which means that there is no comparison of magnitude of response but only a comparison of values of the post-vaccination results. For this analysis no alterations were made to the main model.

Plasma from post vaccination showed higher levels of Hp than plasma for pre-vaccination on two animals (1 female and one male from control group). This suggested that there was an on-going acute phase reaction on pre-vaccination sampling day and a decision to exclude these two animals from the analysis was made.

Brain Morphology, Gene Expression and Methylation

The main model used for the statistical analysis of brain morphology (type and proportion of spines in the hippocampus) and gene expression was not altered. For methylation, position of CpG island was added as a fixed factor to the main model as five different positions of CpG islands were analysed. However, this factor was removed when looking at overall methylation status.

Although coronal sections from 40 brains were prepared, histological analysis (more precisely the process of finding a good quality dendrite) is a very time consuming process and due to time constraints, results presented in this PhD thesis will only refer to hippocampus from 20 brains.

Fear Learning Paradigm

For the analysis of fear conditioning and extinction two lambs were being observed at any given time and two repetitions within the same session were being compared. Hence, lamb status (tested or sibling) and session repetition (1^{st} and 5^{th} for conditioning, and 1^{st} and 15^{th} for extinction) were added to the main model as fixed effects. The random model was also altered by adding lamb identification, given that there was more than one observation per lamb. For this analysis the final model included lamb status, session repetition, treatment, sex and parity in the fixed model and lambing pen, ewe and lamb in the random model. Additionally, pen was dropped from the random model in the statistical analysis for *"ears in aligned"* and *"ears backwards"* position during conditioning for 2 way interactions between factors (i.e. treatment*sex, sex*parity, parity*session, etc). For the analysis on the extinction period, pen was dropped from random model for *"time lying with head up"* in all models.

During isolation the majority of lambs remained active and alert. Only one lamb (male, stress group) laid down during the isolation period, therefore no data analysis was performed for body posture.

During presentation of CS during conditioning repetitions no lamb was seen lying resting, sitting or kneeling, therefore statistical analysis was only conducted on *"standing"* and *"lying head up"* body postures. Given that only two behaviour postures were observed and because they are not

independent (if standing increases lying with head up has to decrease), statistical analysis will be presented for one posture only, which in this case will be "*lying with head up*". For the extinction analysis, only two animals were seen kneeling and seven animals were seen lying resting, five of them only for one second and the other two for 13 and 5 secs (first repetition - only males - 3 stress and 1 control; 15th repetition – only females - 2 stress and 1 control). Therefore it seemed unreasonable to present these data as results would definitely be skewed. No lambs were seen sitting. Consequently, no statistical analysis was performed on these three body postures. Standing and lying head up were treated as mentioned for conditioning.

Although video recording took place at all stages of this experiment, due to time constraints it was not possible to analyse all the data. A decision was made to focus on the data referring to the acquisition instead of the expression of fear conditioning and extinction. Hence, for behavioural reaction to cue presentation (CS) and isolation period (US) on day 2 only the first and fifth repetitions were analysed. On day 3 (extinction session), behavioural reaction to the first and fifteenth repetitions of CS were analysed. Extinction recall session data from day 4 was not analysed.

Weight change

To analyse the average daily gain (ADG) to 7 weeks, no alterations were made to the main model. The statistical analysis for ADG to 13 weeks was only carried out on males (as females were not weighed at this time point); however, an alteration to the main model had to be introduced since some these lambs became single at 7 weeks (if their sibling was used for brain sampling). For this reason litter status (single litter or intact litter) was added to the main fixed model (sex was removed from the model as only one sex was being analysed).

For ADG at 17 weeks statistical analyses were run separately for females and males as they were weaned at different time points and therefore experienced different growing conditions (i.e. females stayed with the dam for the entire 17 weeks, whereas males only until the 13th week) in the time

between 7 and 17 weeks. Hot carcase weight was also analysed separately for females and males for the same reason. On both 17 week and hot carcase weight models, litter status was added to the main fixed model.

All models for ADG at the different time points had lamb identification added to the random model, as on some occasions 2 lambs of the same ewe were weighed.

Because lambs were weighed as a group at each weigh date, there was variation in age at these different time points. Due to this variation, ADG was considered to be a more accurate measure of weight change than total weight gain. Therefore, calculation of ADG was made by subtracting birth weight to 7 week, 13 week or 17 week weight and dividing it by the number of days of life at weighing day.

From the 149 lambs born only 119 were weighed/analysed at 7 weeks. 30 lambs were excluded from this analysis (17 control, 3 males, 13 females, 1 deformed; 13 stress, 6 males, 5 females, 2 mummies) due to natural death, rejection by the dam, death by injury inflicted by the dam, triplets or one lamb born deformed or as a mummy. For the weights of males at 13 weeks, only 31 from the 51 males alive were analysed as 20 had been euthanized for brain procedures at 7 weeks. These same males were weighted at 17 weeks. Only 48 females were alive and weighed at 17 weeks as 20 had been euthanized for brain procedures at 7 weeks.

For each of the above referred models, data either followed a normal distribution or were transformed (as described in Table 3.6) to meet the assumptions of the REML procedure. The transformation used was made on theoretical grounds - what would be expected to work given the nature of the response variable. However, on some occasions, the first choice transformation did not work and other transformation options were used that more closely satisfied the statistical assumptions underlying the analysis. Hence, there will be some occasions in which original data followed the same distribution but different transformations were used. Where significant differences were detected by the REML procedure, post hoc Student's t tests were used for pairwise comparisons to determine where significant

differences lay. The level of probability determined as statistically significant was $P<0.05$. When a transformation was used, confidence intervals were calculated using the original mean ± 2*s.e.. The same transformation applied to the mean was applied to this result in order to obtain a back-transformed confidence interval.

Table 3.6 Data distribution and transformations.

	Data	distribution	transformation	tests
Immune response to vaccination	Hp	Positively skewed	Log 10	REML
	Saa	positively skewed	Square root	
Brain	Spine density	Normal		REML
	Spine morphology	Proportion	Arcsine	
	GR methylation and expression	Normal	---	
Fear Learning Paradigm	Ear position	Percentage	Angular	REML
	Head angle	Percentage	Angular	
	Body postures	Percentage	Angular	
	Escape attempts	Positively skewed	Log 10	REML
	Number of vocalizations	Positively skewed	Square root	
	Activity	Positively skewed	Square root	
Weights (ADG)	7 weeks	Normal	---	REML
	13 weeks	Normal	---	
	17 weeks males	Normal	---	
	17 weeks females	Normal	---	
	Hot carcase males	Normal	---	
	Hot carcase females		Log10	

3.3 RESULTS

3.3.1 Immune Response to Vaccination

Hp concentration in plasma was higher at post-vaccination than at pre-vaccination ($F=431.82$, $d.f.=37.0$, $p<0.001$; Figure 3.4), indicating that, overall, the animals mounted an immune response to vaccination. Treatment had no effect on the magnitude of the Hp response to vaccination (back transformed means ($F=0.52$, $d.f.=6.3$, $p=0.498$). However, parity affected the Hp response in a sex-dependent manner ($F=4.77$, $d.f.=30.9$, $p=0.037$). Males born to multiparous ewes showed a lower magnitude of Hp response than males born to primiparous ewes, whereas the female response was not affected by dam parity (Figure 3.5).

Statistical analysis on SAA concentration in plasma for the post-vaccination results showed no effects of the main factors treatment ($F=0.27$, $d.f.=5.0$, $p=0.625$), sex ($F=0.38$, $d.f.=34.8$, $p=0.540$) and parity ($F=2.41$, $d.f.=16.8$, $p=0.139$). No other interactions between main factors were found (data not shown).

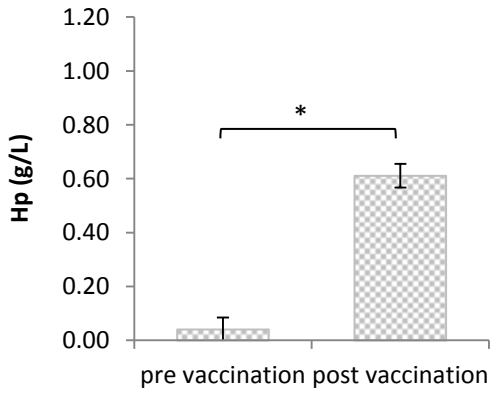


Figure 3.4 Concentration of Hp in pre- and post-vaccination plasma. Post-vaccination levels of Hp in plasma were higher than pre-vaccination values. Graph illustrates back transformed means and error bars represent CI. Stars (*) represent where statistical significance lies.

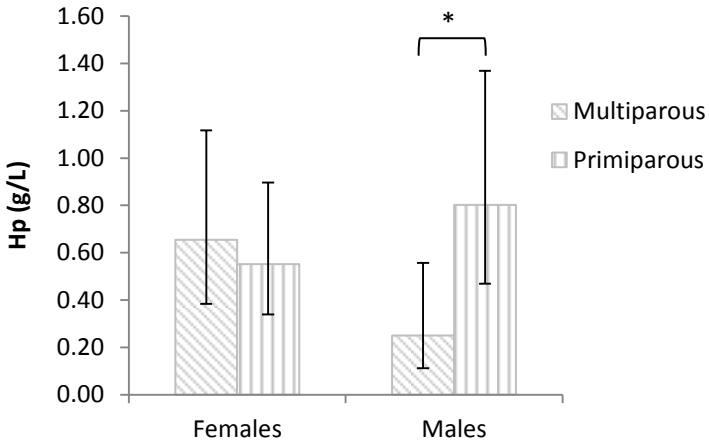


Figure 3.5 Magnitude of immune response measured by the difference between post- and pre-vaccination of plasma Hp concentration. Males from multiparous ewes had smaller magnitudes of Hp response to vaccination than males from primiparous. Graph illustrates back transformed means and error bars represent CI. Stars (*) represent where statistical significance lies.

3.3.2 Brain Morphology

Spine density (number of spines/µm) in apical dendrites of neurons in the CA1 area of the hippocampus was not affected by treatment, sex or parity alone (Table 3.7). Instead, there were differences in spine morphology between treatments depending on sex of the lamb. There was a treatment by sex interaction for filopodia spines ($F=8.47$, d.f.=13.0, $p=0.012$; Figure 3.6a), with spine proportion being lower in stress males in comparison to control males and stress females but no differences being observed between males and females in the control group. Stubby spine proportion was also affected by an interaction between treatment and sex ($F=11.03$, d.f.=12.4, $p=0.006$; Figure 3.6b). Control males presented higher proportion of this type of spines than control females and also than stress males and stress females, but no differences were observed between males and females in the stress group. The interaction between treatment and sex was also observed for the proportion of thin spines ($F=19.90$, d.f.=12.8, $p<0.001$; Figure 3.6c), where females presented higher proportion of this type of spine than males in the control group and also than stress males and stress females, but no sex differences were found in the stress group. Mushroom spine proportion was

only affected by parity (F=8.83, d.f=15.5, p=0.009). Primiparous offspring had higher proportions of this type of spine than multiparous offspring (back transformed mean of mushroom proportion (upper and lower CI): Lm=0.13 (0.107;0.148), Lp=0.17 (0.151;0.194)) (data not shown).

Table 3.7 Effects of treatment, sex and parity on spine density in apical dendrites of neurons in the CA1 area of the hippocampus.

		Mean ± s.e.	$F_{statistic}$	df	p_{value}
Treatment	Control	1.009 ± 0.028	2.08	6.4	p=0.196
	Stress	1.070 ± 0.031			
Sex	Female	1.022 ± 0.031	0.59	14.4	p=0.453
	Male	1.058 ± 0.029			
Parity	Primiparous	1.017 ± 0.031	1.02	13.7	p=0.330
	Multiparous	1.062 ± 0.029			

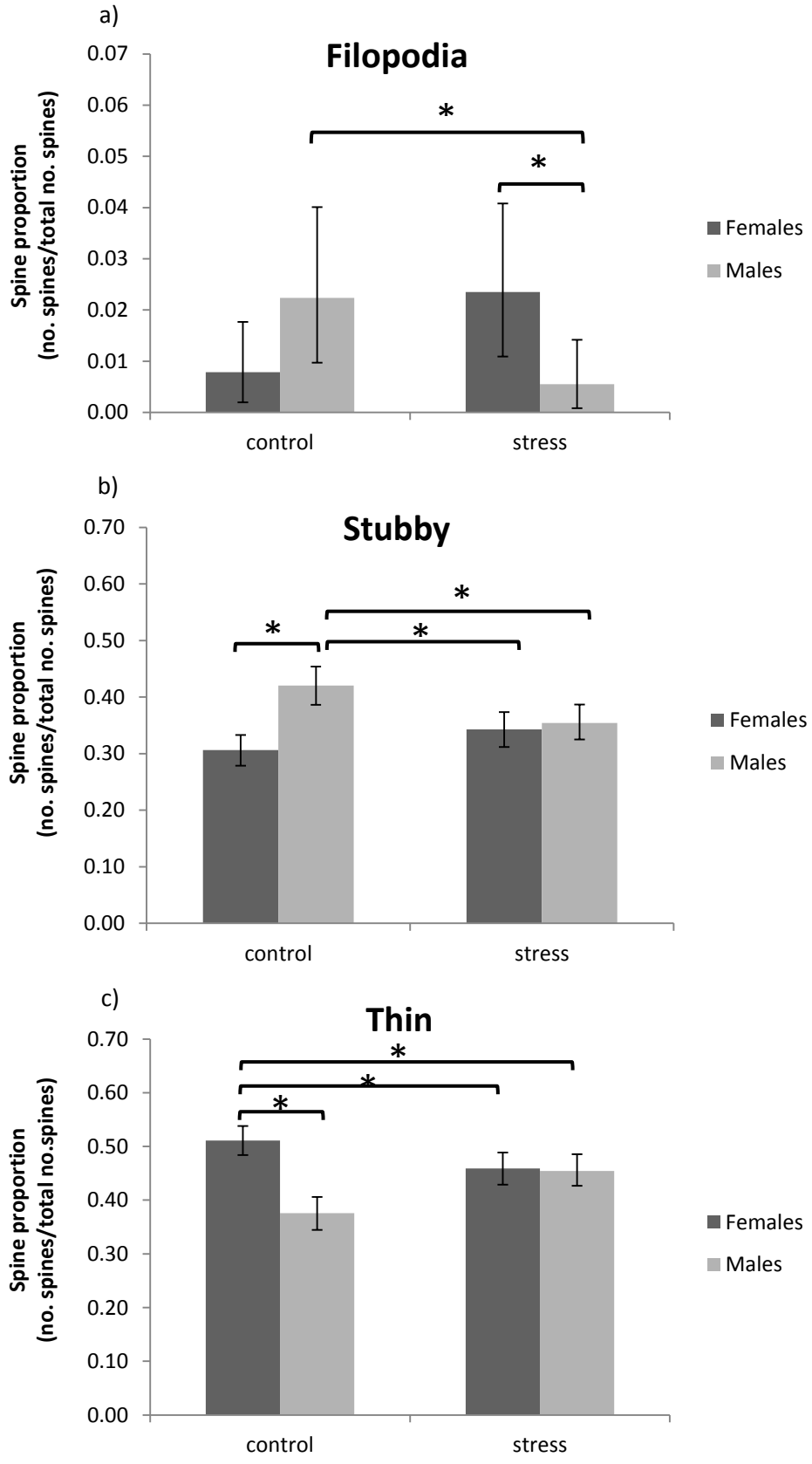


Figure 3.6 Dendritic spine porportions a) filopodia, b) stubby, and c) thin in apical dendrites of neurons in the CA1 area of the hippocampus dependent upon an interaction between sex and treatment. Graph bars illustrate back transformed means and error bars represent CI. Stars (*) represent where statistical significance lies.

3.3.3 Glucocorticoid Receptor Methylation and Expression

GR gene expression in the hippocampus was not affected by treatment (F=1.00, d.f.=7.3, p=0.349), sex (F=0.68, d.f.=35.4, p=0.416), or parity (F=1.46, d.f.=34.3, p=0.236) (data not shown). However, in the hypothalamus GR gene expression was higher in control than in prenatally stressed lambs (mean gene expression ± s.e.: control=0.10 ±0.002, stress=0.09 ±0.002, p=0.013; Figure 3.7a) and also in females than in males (mean gene expression ± s.e.: F=0.10 ±0.002, M=0.09 ±0.002, p=0.005; Figure 3.7b), but it was not affected by parity (F=1.03, d.f.=35.0 p=0.317).

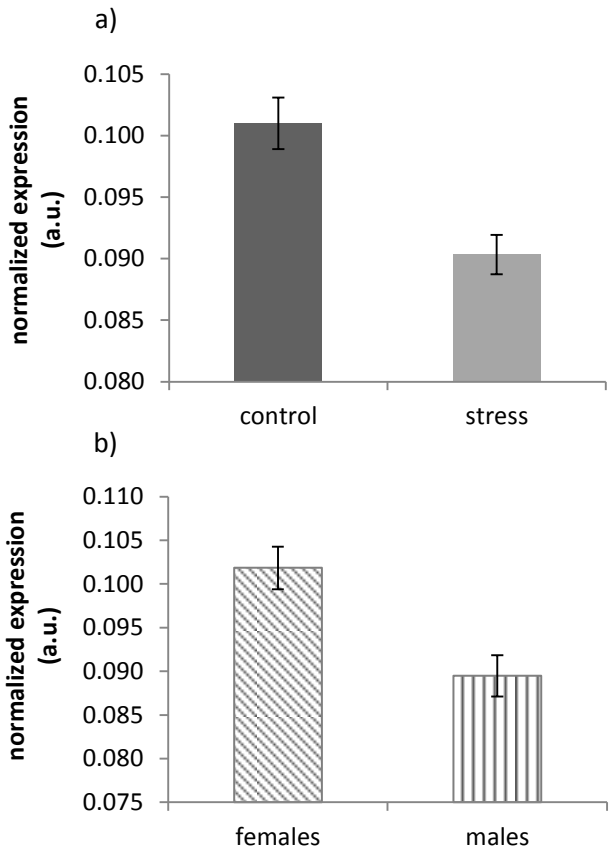


Figure 3.7 GR gene expression in the hypothalamus dependent upon a) prenatal treatment and b) parity. Gere expression was higher in control than in the stress group and higher in females than in males. Graph bars illustrate the means and error bars s.e..

Methylation of the GR gene in the hippocampus was affected by treatment and parity, with control lambs and lambs born to multiparous ewes having higher levels of methylation than their counterparts (Table 3.8a). Females also had a tendency to express higher levels of methylation than males (Table 3.8a). However, at the hypothalamus level there was only an effect of sex, where females had higher levels of GR gene methylation than males (Table 3.8b). No effects of treatment or parity were found in the hypothalamus (Table 3.8b).

Table 3.8 Percentage of methylation of GR gene in a) hippocampus and b) hypothalamus

a)

		Hippocampus			
		Mean ± s.e.	**F_{stat}**	**df**	**p_{value}**
Treatment	Control	1.24 ± 0.03	7.15	6.2	0.036
	Stress	1.13 ± 0.03			
Sex	Female	1.23 ± 0.03	3.9	35.2	0.056
	Male	1.14 ± 0.03			
Parity	primiparous	1.11 ± 0.03	11.97	33.8	0.001
	multiparous	1.26 ± 0.03			

b)

		Hypothalamus			
		Mean ± s.e.	**F_{stat}**	**Df**	**p_{value}**
Treatment	Control	1.44 ± 0.04	0.48	5.3	0.517
	Stress	1.45 ± 0.03			
Sex	Female	1.52 ± 0.04	4.67	32.9	0.038
	Male	1.38 ± 0.04			
Parity	primiparous	1.45 ± 0.04	0	35.7	0.997
	multiparous	1.45 ± 0.05			

3.3.4 Fear Learning Paradigm

During fear conditioning (CS presentation period) and extinction, four different types of ear postures were analysed during cue presentation:

forward, backwards, aligned and asymmetric. For the isolation period (US) lamb behaviour was analysed for activity, attempts to escape and number of vocalizations.

During the conditioning period (CS presentation) no treatment effects were observed on any of the ear positions considered (Table 3.9). However, repetition within session had an effect on ear position forwards and asymmetric. The percentage of time with ears forward was higher on the first repetition of conditioning than on the fifth repetition ($F=7.65$, $d.f.=26.7$, $p=0.010$; Figure 3.8a). Additionally, there was a tendency for test lambs to show a higher percentage of time with ears in this position than their siblings (back transformed mean percentage of ears forwards (upper and lower CI): sibling=47.9 (18.66;78.00), test =71.9 (41.04;94.31), $p=0.080$). Asymmetric ear posture percentage was higher in the fifth than in the first repetition of conditioning ($F=4.68$, $d.f.=26.6$, $p=0.040$; Figure 3.8b) and higher in the siblings than the test lambs (back transformed mean percentage of ears asymmetric (upper and lower CI): sibling=20.1% (7.92;36.12), test=6.8% (0.44;16.93), $p=0.023$). The percentage of time spent with ears backwards was affected by parity and sex. Lambs from primiparous ewes spent more time with ears in the backwards position than lambs from multiparous ewes (back transformed mean percentage of ears backwards (CI): Lprimi=27.4 (8.96;51.29), Lmulti =5.5 (0.01;21.62), $p<0.001$) and males more than females (back transformed mean percentage of ears backwards (CI): M=19.1 (4.11;41.61), F=10.8 (0.76;30.30), $p=0.041$). Aligned percentage of time was not affected by any of the analysed factors.

Lambs looked more in the direction of the cue if they were born to multiparous ewes compared with primiparous ewes (back transformed mean of time looking in cue direction (%) (upper and lower CI): Lmulti=34.8 (13.18;60.41), Lprimi=3.1 (0.70;17.81), $p=0.024$). Treatment ($F=0.40$, $d.f.=5.5$, $p=0.550$), sex ($F=1.56$, $d.f.=14.0$, $p=0.232$), session ($F=0.64$, $d.f.=27.0$, $p=0.432$) and lamb status ($F=0.58$, $d.f.=11.9$, $p=0.459$) did not affect this behaviour (data not shown).

Body posture during CS presentation during conditioning varied depending on a treatment by sex interaction ($F=8.34$, $d.f=7.7$, $p=0.021$) and a treatment

by lamb status interaction (F=8.56, d.f.=7.5, p=0.020). Male lambs from the stress group spent a higher percentage of time lying with their head up than females but this sex difference was not seen in the control group (Figure 3.9a). Siblings from the control group spent more time lying with the head up than siblings in the stress group, but test lambs remained unaffected (Figure 3.9b).

During the isolation (US) sessions during conditioning there were no observable effects of treatment, sex or parity on lamb activity, attempts to escape, or number of vocalizations. However all three behaviours were affected by session, showing a decrease in frequency with increasing number of sessions (Figure 3.10). Escape attempts decreased dramatically from the first isolation session to the fifth (F=19.04, d.f.=12.1, p<0.001; Figure 3.10a), as well as vocalizations (F=41.17, d.f.=12.6, p<0.001; Figure 3.10b) and activity (F=92.42, d.f.=12.6, p<0.001, Figure 3.10c).

During extinction, no effects of treatment were found on any of the ear positions (Table 3.10). An effect of lamb status was found on the percentage of time spent with ears backwards (F=5.41, d.f.=42.5, p=0.025). Tested lambs exhibited a higher percentage of time with ears backwards than their siblings (Figure 3.11a). Percentage of time with aligned ears was affected by a sex by repetition of extinction interaction (F=5.52, d.f.=22.5, p=0.028), with males showing an increase in the percentage of aligned ears on the 15th repetition compared with the first session but with females remaining unaffected (Figure 3.11b). Ears forward and asymmetric percentages were not affected by any of the analysed factors.

Looking in the direction of the cue during extinction was affected by treatment depending on parity (F=4.74, d.f.=12.4, p=0.050). Whereas parity did not affect the percentage of time lambs spent looking in the direction of the cue in the control group (back transformed mean of time looking in cue direction (%) (upper and lower CI): Lmulti=20.3 (1.46;52.84), Lprimi=19.9 (1.29;52.65), p=0.976), there was a tendency for lambs from multiparous ewes to look more in the direction of the cue than lambs from primiparous in the stress group (back transformed mean of time looking in cue direction (%) (upper and lower CI): Lmulti=55.9 (13.78;93.33), Lprimi=4.4 (2.29;29.57), p=0.059).

For body posture during extinction male lambs spent a higher percentage of time lying with head up than females (back transformed mean of time spent lying with head up (%) (upper and lower CI): M=91.4 (67.62;99.99), F=55.7 (29.71;80.05), p=0.047).

Table 3.9 Effect of treatment on ear position rates during conditioning session. Means are reported as back transformed (upper and lower CI).

	Ear position rate	Control (CI)	Stress (CI)	$F_{statistic}$	df	p_{value}
Conditioning	Forwards	62.9 (25.02;93.20)	57.6 (17.38;92.51)	0.03	7	p=0.860
	Backwards	16.62 (0.82;46.41)	12.92 (0.00;45.07)	0.04	7	p=0.839
	Aligned	0.2 (0.0;0.91)	0.3 (0.0;0.99)	0.04	2	p=0.858
	Asymmetric	9.7 (1.00;25.71)	14.8 (2.52;34.63)	0.24	7	p=0.642

Table 3.10 Effects of treatment on ear position time percentages during extinction session. Means are reported as back transformed (upper and lower CI).

	Ear position rate	Control (CI)	Stress (CI)	$F_{statistic}$	df	p_{value}
Extinction	Forwards	42.63 (17.98;69.46)	36.4 (11.64;65.83)	2.83	7	p=0.762
	Backwards	20.9 (8.09;35.93)	28.5 (14.53;44.93)	0.3	7	p=0.602
	Aligned	0.6 (0.0;2.71)	1.4 (0.25;3.33)	0.47	7	p=0.491
	Asymmetric	22.4 (8.19;36.34)	16.7 (5.35;32.78)	0.13	7	p=0.730

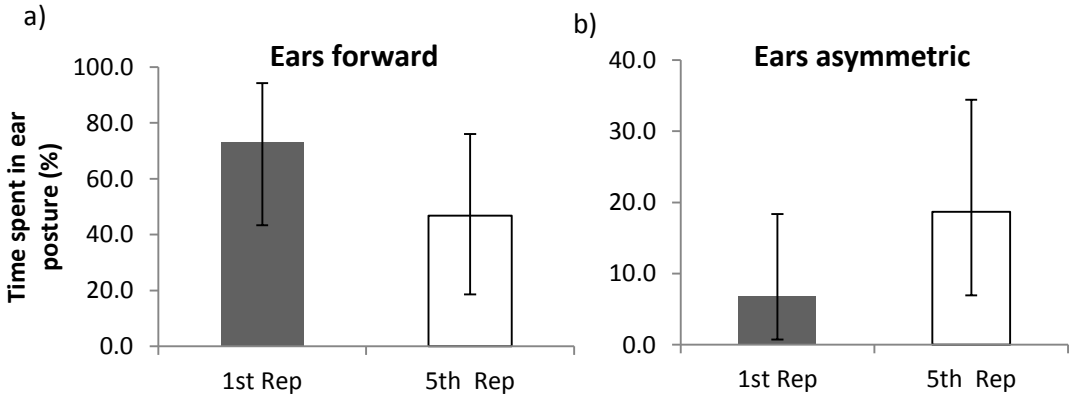


Figure 3.8 Percentage of time spent with ears a) forward and b) asymetric during fear conditioning (CS) depending on the session repetition. Percentage of ears forward was higher during first than during fifth repletion of conditioning, while percentage of ears in asymmetric position varied in the opposite way. Graph bars illustrate back transformed means and error bars represent CI.

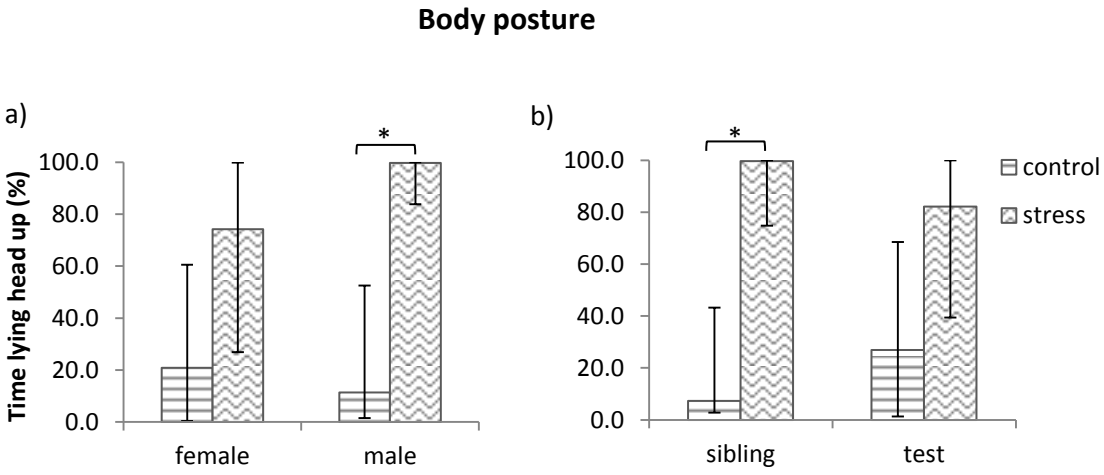


Figure 3.9 Percentage of time spent lying with head up during conditioning depending upon an interaction between a) treatment and sex and b) treatment and lamb status. Male and sibling lambs from the stress group spent more time lying with their head up than males and siblings in the control group Graph bars represent back transformed means and error bars represent CI. Stars (*) represent where statistical significance lies.

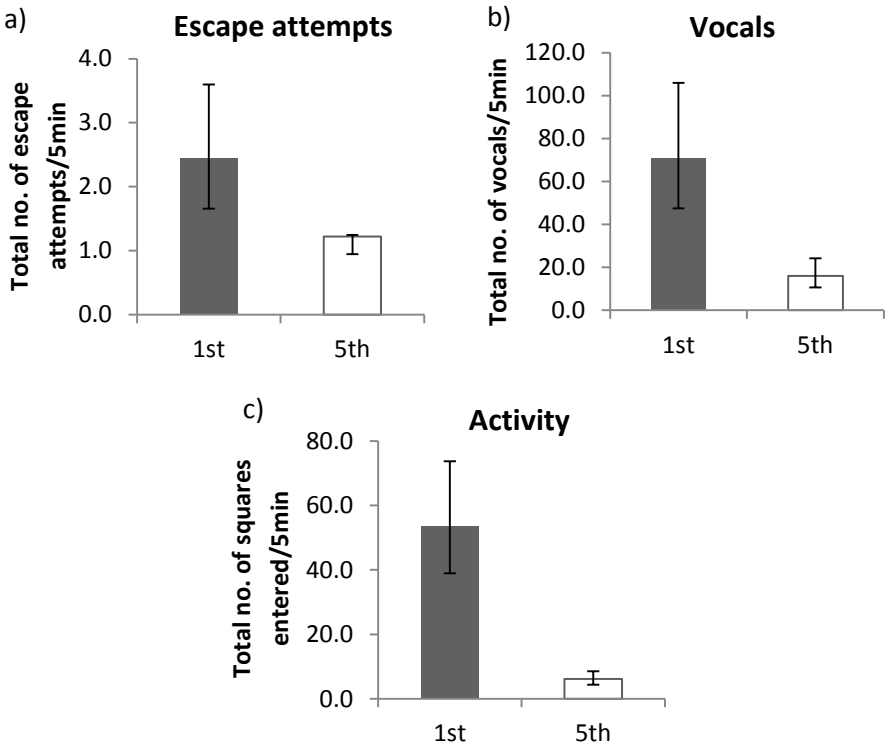


Figure 3.10 Total number of a) escape attempts, b) vocalizations and c) activity during the five min of first and fifth isolation periods that occurred during conditioning session. Graph bars represent back transformed means and error bars represent CI.

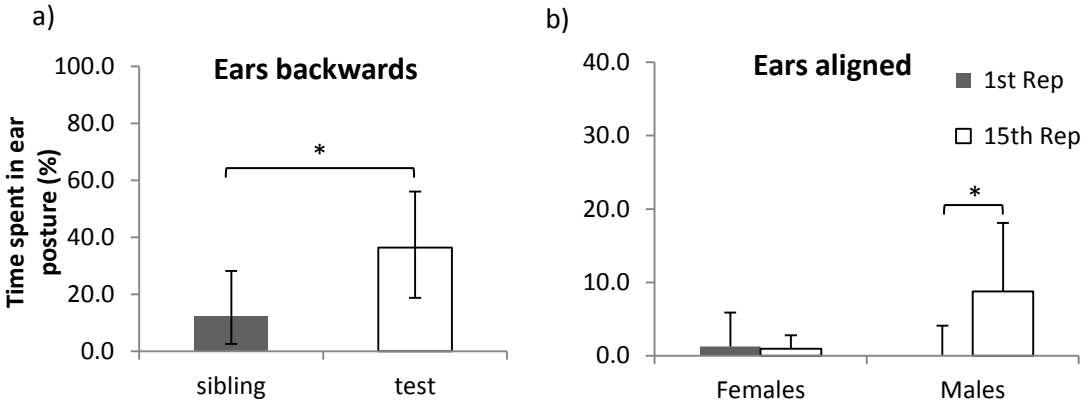


Figure 3.11 Percentage of time spent with ears a) backwards and b) aligned during fear extinction depending on lambs status and on the interaction between session repetition and lamb sex, respectively. Percentage of ears in backward position was higher in test than in sibling lambs and percentage of time with ears aligned was higner during 15th repetition than during first in males. Graph bars illustrate back transformed means and error bars represent CI. Stars (*) represent where statistical significance lies.

3.3.5 Weight Change

Treatment had no effect on ADG to 7 weeks. However, an interaction between parity of the ewe and sex of the lamb was found (F=4.83, d.f.=79.2, p=0.031; Figure 3.12). Males from multiparous ewes were heavier than females but no differences were found between sexes in primiparous offspring (Figure 3.12).

No effects of treatment were found on male ADG to 13 weeks (F=0.21, d.f.= 7.7, p=0.659) (data not shown). However, primiparous male offspring that had become single at 7 weeks of life showed greater growth rates than males from intact litters but no differences were found in multiparous offspring (F=6.54, d.f.=21.9, p=0.018; Figure 3.13). This same pattern was observed for male 17 weeks weights (F=7.85, d.f.=22.8, p=0.010; Figure 3.14a). Interestingly, at this time point an effect of treatment depending on litter status was also observed (F=6.66, d.f.=21.3, p=0.017; Figure 3.14b). Males from intact litters showed the same ADG independently of the treatment group but males from single litters showed increased ADG in the stress group compared to the control (Figure 3.14b).

Male hot carcase weight was affected by parity depending on the offspring litter status in the same manner as for ADG to 13 and 17 weeks, with lambs from multiparous ewes and single litters presenting heavier carcases than lambs from primiparous ewes and intact litters (mean weight (Kg) ± s.e.: Primiparous intact litter=18.47 ± 0.90; Primiparous single litter=22.93 ± 0.74; p<0.001) but no differences between multiparous offspring (mean weight (Kg) ± s.e.: Multiparous intact litter=22.94 ± 0.88; Multiparous single litter=21.89 ± 1.07; p=0.416). Surprisingly, the difference between treatment groups for single litters observed for ADG to 17 weeks changed for hot carcase weight and a difference between intact and single litters became evident only in the stress group (mean weight (Kg) ± s.e.: Stress intact litter=20.33 ± 0.948; Stress single litter=23.74 ± 0.87; p=0.016; Figure 3.15).

Females ADG to 17 weeks was not affected by treatment (F=0.43, d.f.= 8.5, p=0.528), parity (F=2.94, d.f.= 8.7, p=0.122) or litter status (F=1.96, d.f.= 31.4, p=0.157) (data not shown). Hot carcase weight was not affected by

treatment or litter status in females but carcases from female lambs from multiparous ewes were heavier than from females from primiparous ewes (Table 3.11).

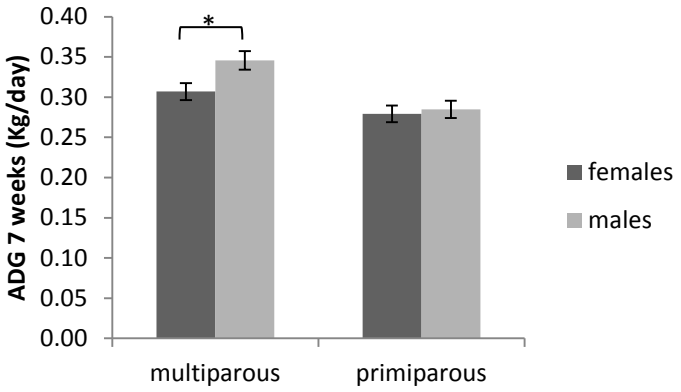


Figure 3.12 ADG to 7 weeks dependent upon an interaction between parity of the dam and sex of the lamb. Females from multiparous ewes gained less weight than males but this difference was not see in lambs from primiparous ewes. Graph bars represent the means and error bars represent standard errors. Stars (*) represent where statistical significance lies.

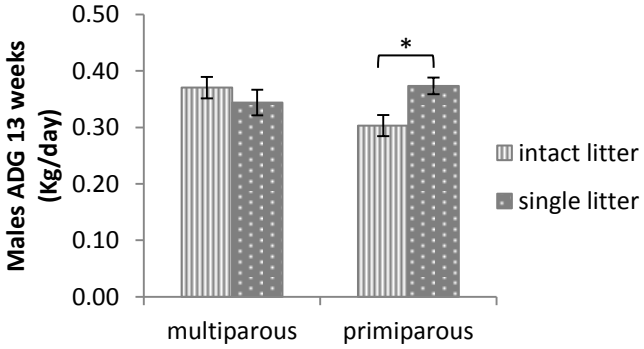


Figure 3.13 ADG to weaning (13 weeks) dependent upon an interaction between parity of the dam and litter status. Lambs from primiparous ewes whose litter remained intact until weaning gained less weight than lambs that became single at seven weeks. Graph bars represent the means and error bars represent standard errors. Stars (*) represent where statistical significance lies.

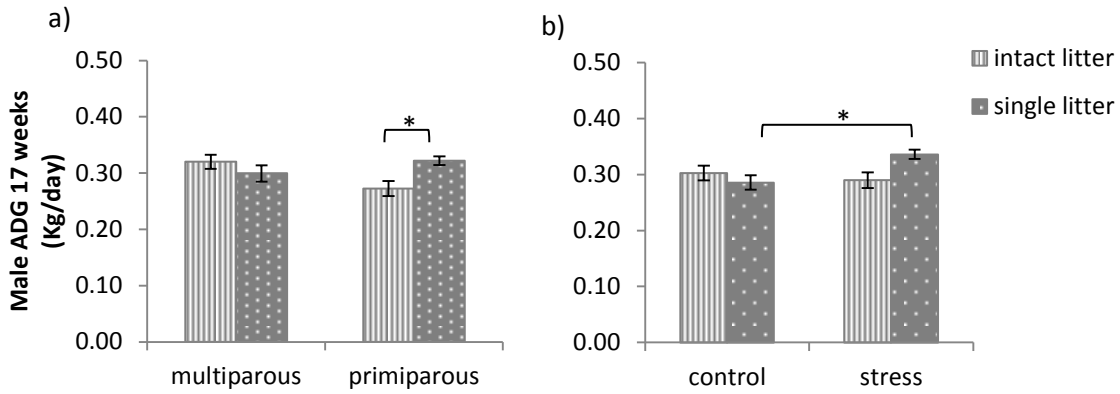


Figure 3.14 ADG to slaughter dependent upon an interaction between parity of the dam (a) or treatment (b) and litter status. Lambs from primiparous ewes whose litter remained intact until weaning gained less weight than lambs that became single at seven weeks. Lambs that became single in the stress group gained more weight than in the control group. Graph bars represent the means and error bars represent standard errors. Stars (*) represent where statistical significance lies.

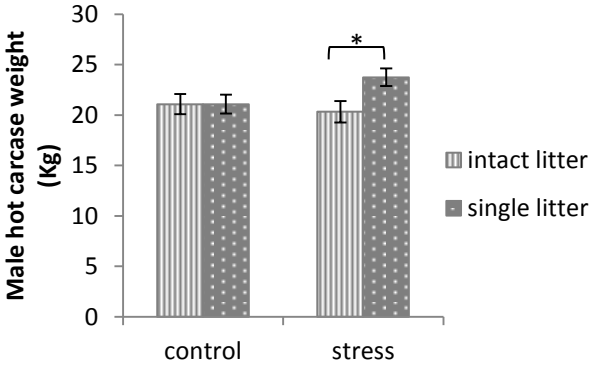


Figure 3.15 Male hot carcase weight dependent upon an interaction between treatment and litter status. Graph bars represent the means and error bars represent standard errors. Star (*) represents where statistical significance lies.

Table 3.11 Effects of treatment, parity and litter status on female hot carcase weight.

		Mean (CI)	$F_{statistic}$	df	p_{value}
Treatment	Control	20.27 (23.71;17.34)	0.07	3.5	p=0.800
	Stress	19.78 (22.80;17.14)			
Parity	Primiparous	18.49 (20.84;16.41)	95.57	2.1	p=0.008
	Multiparous	21.63 (24.38;19.19)			
Litter status	Intact	20.00 (22.34;16.87)	0.64	10.3	p=0.549
	Single from 7wk	21.23 (29.44;15.31)			
	Single from 13wk	18.84 (21.04;16.87)			

3.4 DISCUSSION

Overall, PNS effects at later stages of the lambs' life were subtle in nature as they were always expressed as a function of interactions with other factors. Hence, these results followed the same trend seen for the neonatal period (Chapter 2).

Prenatal treatment did not alter the capacity to mount an adequate acute phase immune response to vaccination. However, parity of the dam influenced how well males responded to vaccination but not females.

Spine density in the pyramidal cells of the hippocampus CA1 was not affected by PNS. However, differences between treatment groups in the proportion of filopodia, stubby and thin types of spines were observed in a sex dependent manner. The mushroom spine type was not affected by PNS.

GR gene expression was not affected in the hippocampus but was higher in control than in stress lambs in the hypothalamus. Levels of GR methylation in the hippocampus were higher in the control than in the stress group but did not differ in the hypothalamus.

Results seem to show some evidence for fear learning in sheep but fear extinction seemed to be impaired or more repetitions of CS were needed for extinction to take place. Nevertheless, males seem to respond better to extinction than females. No effects of PNS were observed in the analysed sessions of the fear learning paradigm.

Weight measured at different time points was only affected by treatment at 17 weeks of age as the result of an interaction with a post-natal event, which was the loss of a sibling at 7 weeks of age. This was only true in the case of male lambs, as no effects of treatment were found for female weights at any given time point.

3.4.1 Response to Vaccination

The results in this study showed that elevated levels of Hp or SAA were present in post-vaccination samples indicating that vaccination stimulated an acute phase response in lambs. However, prenatal treatment did not alter the response of these proteins to vaccination. The immune response to vaccination results are in agreement with the results from Chapter 2, where 24hr IgG plasma levels also remained unaffected by prenatal treatment.

It is clear from the literature that PNS has consequences for the immune system. In general, PNS has been found to have an inhibitory effect on the immunity of the offspring (for a review see Merlot et al., 2008), though the nature of the stressor and the time in relation to when it is applied during pregnancy seem to play a major role in the immune outcomes. For example, T cell response to specific antigens is decreased in three day old monkeys whose mothers were stressed during mid-late pregnancy but increased when the same stressor was applied during early pregnancy (Coe et al. 1999). In piglets, heat stress during late pregnancy resulted in decreased IgG concentrations (Machado-Neto et al., 1987) but maternal restraint stress

applied during the same period of gestation increased the magnitude of IL-6 and SAA responses to LPS (Lipopolysaccharide - the major molecular component of the outer membrane of Gram-negative bacteria, recognized by the immune system as pathogen invasion, Alexander & Rietschel 2001) in 25 day old piglets (Collier et al. 2011). In sheep, undernutrition from days 65 to 125 of pregnancy decreased the lamb SAA response to vaccination but SAA levels did not differ when undernutrition was applied during the first 65 days of pregnancy (Eckersall et al. 2008).

The present findings indicate that the acute phase response was not affected by the PNS applied in this study, which may be directly related to the type/intensity of stressor used which was possibly not strong enough to produce an effect on innate immunity. In fact, ewes were only introduced into new social groups twice which may have worked more like an acute rather than a chronic stressor. Further, the decrease in space allowance, which was intended to act as a chronic stressor may not have been low enough to cause the expected elevated levels of stress on ewes. In other studies where the effects of PNS on the immune response of the offspring was evaluated, stressors were more frequently applied to the mothers (restraint 5 min daily for 28 days - Collier et al. 2011; heat stress permanently for 14 days - Machado-Neto et al. 1987), which may explain the absence of a treatment effect in the present study. Still, the immune response to vaccination results are in agreement with the results from Chapter 2, where 24hr IgG plasma levels also remained unaffected by prenatal treatment. Although these refer to two different types of immunity, passive (IgG) and innate (Hp, SAA), the lack of a treatment effect in both seems to further indicate that the prenatal stressors applied were not severe enough to affect this feature of the lamb's health. Alternatively, ewes could be buffering the effects of stress on their offspring via the placenta as suggested by Boersma & Tamashiro (2015). The same authors further suggest that this buffering function may also serve to distinguish between short term/moderate stressors from long term/more severe stressors.

Another finding of this study was that the response of Hp to vaccination was lower in males born to primiparous ewes than those born to multiparous

ewes, but females remained unaffected. It is well established that innate immune response differs between males and females (for a review see Klein & Flanagan 2016). For example, the levels of IL-6, a pro-inflammatory cytokine that stimulates the production of Hp (Moshage 1997), have been reported to be higher in male mice compared with females following administration of LPS (Naor et al. 2009). Interestingly, another study in pigs looking at TNF-α and IL-1 β also detected higher levels of these pro-inflammatory cytokines in males compared with females but no differences were found in SAA or Hp in response to LPS challenge (Llamas et al., 2006). Similarly, there were no direct sex differences in Hp or SAA in the present study. However, the immune response of males and females differed depending on dam parity. To my knowledge, the only study found focusing on the effects of maternal parity on innate immune response was done in pigs (Miller et al. 2013). This study looked at the number of circulating lymphocytes or phagocytes (neutrophils and monocytes) in piglets as a way to monitor the innate immune response and no effects of dam parity were found, though sex of the piglet was not taken into account. At present, there is no definite explanation for the effect of the interaction between parity and sex shown in this experiment. However, it is possible that latent sex differences are emphasized by parity either prenatally, by the stress buffering capacity of the ewe, or postnatally, by ewe-lamb interactions. Still, the exact mechanism for this finding is yet to be understood.

3.4.2 Brain Morphology

In this study, stress applied during pregnancy did not affect spine density in the hippocampus of 7 week old lambs. However, spine morphology was affected by prenatal stress in a sex-dependent manner. Filopodia spines presented a higher proportion in females than males from the stress group but no sex differences were found in the control group. Stubby spines showed a higher proportion in males than females in the control group and the opposite was true for thin spines, however sex differences disappeared in stress groups for both these spine types. Mushroom spines were not affected by PNS.

The effects of PNS on spine density have been previously investigated and the rodent literature indicates that PNS increases spine density in brain areas such as the hippocampus CA1 (Bock et al., 2011; Martínez-Téllez et al., 2009; Murmu et al., 2006; Mychasiuk et al., 2012). More recently, two experiments conducted in sheep have shown similar results (Coulon et al., 2013; Petit et al., 2015). In contrast, in the present study, spine density did not vary between treatment groups. As mentioned earlier (see Chapter 1, General introduction section), the effects of prenatal stress depend on factors such as: type of stressor used, time of when stress was applied in relation to pregnancy, sex of the offspring and studied species. For example, in rats exposure to chronic PNS increased spine density in hippocampal CA1 pyramidal neurons only in males (Bock et al., 2011), whereas prenatal bystander stress increased CA1 spine density in both males and females (Mychasiuk et al., 2012). Fujioka et al. (2006) have also suggested the importance of intensity-dependent effects of prenatal stress, as short-lasting, mild PNS enhanced neurogenesis and differentiation of processes of hippocampal neurons, while long-lasting, severe PNS had opposing effects. The stressors used in the present study only affected some of the studied lamb outcomes and always as a result of an interaction with other variables. It is possible that the different outcomes for spine density obtained in this study are a result of the type and/or intensity of PNS used.

Another important feature of brain histology is dendritic spine morphology, which is critical for regulating excitatory synaptic physiology (reviewed in Nimchinsky et al., 2002). Alterations in shape and volume of spines correlate with the extent to which transduced signals are spread within the dendrite, emphasising a critical relationship between spine morphology and neuronal function (Harris & Kater, 1994; Yuste et al., 2000). These alterations are also associated with neurodevelopmental disorders characterized by cognitive impairment (Fiala et al., 2002). Although PNS did not affect spine density in this experiment, spine morphology was affected by PNS in a sex dependent manner. One interesting aspect was the fact that the proportion of stubby and thin spines varied between males and females in the control group, but no differences were found in the stress group. Interestingly, the opposite was

true for filopodia spines. Previous studies in rats have described a reduction in sex differences in hippocampal spine density (Bock et al 2011), neurogenesis (Mandyam et al., 2008) and gene expression as a result of PNS (Biala et al., 2011). It is possible that spine morphogenesis is affected in the same way. As mentioned in Chapter 2, one possible mechanism underlying these sex-specific responses may be the PNS-induced process of feminization of males and/or masculinization of females. Previous PNS experiments conducted in rats have reported reduced levels of plasma testosterone in males (Gerardin et al., 2005; Richardson et al., 2006; Rodríguez et al., 2007) and estradiol in females (Del Cerro et al. 2010). It has been suggested that sex hormones can modulate the activity of brain-derived neurotrophic factor an important modulator of the formation and maturation of dendritic spines, on which estrogen has a positive regulatory effect on expression and signalling (for a review see (Chan & Ye 2017). It is possible that in the present study, PNS altered the levels of sex hormones, offsetting sex differences in spine morphology. The reason why this affected stubby and thin spines but not filopodia is not clear. Interestingly, mushroom spines were not affected by treatment. As opposed to stubby spines, which are considered an immature type of spine, and thins, that have a high turnover rate and form weak synapses (Holtmaat et al. 2005), mushroom spines are the most mature and stable type of spines and their spine head volume is positively correlated with synapse strength and age (Harris & Stevens, 1989; Kasai et al., 2003; Petrak et al., 2005; Schikorski & Stevens, 1997). Perhaps, these more mature spines are less sensitive to stress and less prone to suffer alterations. Indeed, previous studies looking at the effects of chronic stress in rats (Castañeda et al. 2015) and mice chronically treated with corticosterone (Wang et al. 2013) have found decreased density of thin and stubby spines but not of mushroom spines in the hippocampus CA1 area. Coulon et al. (2013), who looked at the effects of PNS on brain morphology in lambs, observed a decrease in mushroom spine ratio in PNS offspring, as opposed to Petit et al. (2015), who found no differences in the ratio of this type of spine. However, the nature and schedule of the stressor applied and age of the offspring were different from the ones used here (Coulon et al., 2013 - lamb age: one month, PNS: aversive human

interactions 10 min, twice daily, during the last 5 weeks before parturition; Petit et al., 2015 **-** lamb age: new-born, PNS: daily exposure to various unpredictable and negative routine management-based challenges during the last third of gestation). Once again the different types of PNS used makes it difficult to make direct comparisons across studies and may be the reason for obtaining different outcomes.

Further, the hippocampus is an important regulator of the neurocircuitry of the HPA axis response to stress (Ulrich-Lai & Herman 2009) and disruption of its neural morphology plays a role in the dysregulation of glucocorticoid release in response to stress exposure (Conrad, 2008; Snyder et al., 2011). Therefore, the alterations in spine morphology observed here may reflect alterations in synaptic transmission in PNS lambs that can ultimately contribute to increased fear and cognitive impairment often seen in these offspring (Jarvis et al., 2006; Lay et al., 1997; Roussel et al., 2005; Roussel et al., 2006). Importantly, males and females have shown different ratios of stubby and thin spines in the hippocampus under control conditions, and their dendritic morphology responded in opposite directions to the same PNS. This indicates that females and males may have different susceptibility to PNS.

3.4.3 Glucocorticoid Receptor Methylation and Expression

GR gene expression in the hippocampus was not affected by treatment but levels of methylation were higher in the control than in the stress group. The opposite was true for gene expression in the hypothalamus, which was higher in control than in stress lambs, and for methylation levels, which did not differ between groups.

The hippocampus and the hypothalamus are two important structures in the regulation of the HPA axis. Both are rich in GR and act as centres for the negative feedback of cortisol, important in the mediation and termination of the stress response (de Kloet et al., 1998; Frim et al., 1990; Keller-Wood & Dallman, 1984).

In rodents, a postnatal stressful environment (low maternal care or maternal separation) is associated with decreased GR expression in the hippocampus

and PVN hypothalamic nucleus and a higher hormonal response to stress (low maternal care, Francis et al., 1999; Liu et al., 1997; maternal separation, Ladd et al., 2000, 2004). Other studies also looking at the quality of maternal care, reported levels of 80–100% methylation of the conserved site in promoter 1_7, which is thought to correspond to a binding site for the transcription factor NGFI-A, assumed to be involved in the expression of 1_7 transcript (Weaver et al. 2004, 2005, 2007). Together, these results suggest the DNA methylation-dependent expression of glucocorticoid receptors as an integral part of the regulatory mechanisms controlling HPA axis activity, where less expression of this receptor corresponds to decreased negative feedback and therefore a more reactive HPA axis response.

In the present study, the levels of methylation of the promoter region of the GR in the hippocampus were higher in control than in prenatally stressed lambs. However, it is important to note that the levels of methylation were very low (<2%), suggesting two things: that binding of NGFI-A to promoter 1F was not likely to be inhibited and that differences in methylation found between treatment groups are most likely of little biological value. Indeed, no differences between groups were found in GR gene expression in the hippocampus. Nevertheless, it is possible that other epigenetic mechanisms regulating gene expression, such as histone acetylation, opposed the group differences in methylation resulting in no net effect on the GR expression. Additionally, the analysed sequence for gene expression may not have targeted the right transcript. Previous studies of the GR in pigs showed multiple alternative first exons each of which is expressed under the control of a unique promoter (Reyer et al., 2013). Hence, usage of alternative promoters drives the expression of different transcript variants (Landry et al., 2003).

Similarly to the present results, a previous study in rodents using perinatal stress (maternal separation) also failed in finding the high levels of methylation in GR reported by Weaver et al. (2004, 2005, 2006), although abnormal behaviours (anxious-like behaviour and hyperactivity) and HPA axis dysregulation (increased corticosterone levels) were observed (Daniels et al. 2009). The disparity of findings in different rodent studies may be

related to the differences in perinatal environment, i.e. altered maternal behaviour versus maternal separation. Intensity of the stressor may also influence the outcomes. For instance, exposure to a series of stressors increased hippocampal NGF levels (which occur in conditions where the HPA axis is dysregulated) but subsequent studies where only maternal separation was used as a stressor failed to reproduce the same results (Faure et al., 2007; Marais et al., 2008). Again, this reinforces the relevance and nature of early stressors. However, in two other studies conducted in sheep (Coulon et al., 2013, Petit et al., 2015), where the intensity and nature of prenatal stressors was different from the ones used here, no alterations in GR gene expression in the hippocampus were also observed (methylation levels were not evaluated).

As for the hypothalamus, no differences were found in methylation but gene expression of the promoter region of the GR was higher in the control than in the stress group lambs. Again, discrepancies between gene expression and methylation status may have different causes (see above). This decrease in GR gene expression in prenatally stressed lambs suggests an impairment of the HPA axis at the hypothalamus level and is in concordance with the previous decreased levels of this receptor found in the hypothalamus in studies on perinatal stress in rodents.

It is not very clear why the GR expression was affected only in the hypothalamus but it is possible that the effects of PNS on GR expression vary between tissues. For example, GR expression was reported to be increased in the hypothalamus, decreased in the hippocampus and not affected in the pituitary in adult offspring of sheep that were undernourished around conception period (Begum et al. 2013). Additionally, GR expression also varies within tissues (e.g. hippocampus - Herman et al., 1989). For example, in the hippocampus GR is enriched in CA1 and dentate gyrus (Joels 2008). Hence, the selected zone for sampling in the hippocampus may have had an influence on the present results. It is possible that the zone sampled for gene expression analysis did not include the CA1 area which is where the GR is more expressed. Research on prenatal stress and the hippocampus shows that the CA1 area of the hippocampus is one of the

regions particularly susceptible to changes following prenatal stress, apart from the dentate gyrus (Bock et al., 2011; Levitt et al., 1996). Therefore, missing this particular subfield may have contributed for the present results.

Although the present results point to the possibility of impairment of the HPA axis at the hypothalamic level, this did not translate into behavioural alterations at least when siblings to these lambs were tested in a stressful situation (isolation test – see section 3.4.4 Fear learning paradigm). It would have been interesting to see if cortisol levels were elevated in these animals, in which case it would suggest they were particularly good at buffering the behavioural alterations usually seen in prenatally stressed offspring. Unfortunately, cortisol results were not possible to obtain. Alternatively, it is possible that the alterations seen at the hypothalamus level are not enough to produce a phenotypical alteration. Indeed, previous studies have highlighted the importance of the GR:MR (MR: mineralocorticoid) ratio in the hippocampus. An increase in this ratio results in a more efficient feedback and facilitates recovery from stress (de Kloet et al. 1998, Joëls 2008, Liebl & Martin 2013). Both MR and GR contribute to negative feedback inhibition of the HPA axis. MR is another steroid receptor, mainly expressed in the limbic brain regions (Joëls 2008), which shows high affinity for glucocorticoids and is almost completely occupied during normal physiological concentrations of endogenous glucocorticoids. However, previous studies on the MR expression in response to altered glucocorticoid levels suggest that MR signalling is a dynamic process with possibly significant changes in its occupancy (Kalman & Spencer 2002) and therefore may add to the biological effects of altered glucocorticoid hormones. In a study conducted in Japanese quails using corticosterone injections to the egg as a prenatal stressor, prenatally stressed individuals presented a higher GR:MR ratio in the hippocampus, although GR expression was not affected (Zimmer & Spencer 2014). In the present study the GR:MR ratio was not evaluated but if indeed this was increased it could have been one factor contributing to the absence of behavioural differences. The present results highlight the importance of analysing the different levels of the HPA axis when looking at the effects of prenatal stress on the alterations of the HPA axis activity.

3.4.4 Fear Learning Paradigm

Studies in rodent literature have reported the effects of PNS on fear conditioning and memory. This experiment had two main objectives: to develop a fear conditioning protocol for sheep and to investigate the possible effects of PNS on fear learning and memory.

The present study seemed to indicate there was some degree of fear learning in the lambs as shown by an increase in the percentage of time spent with ears in asymmetric posture over the course of the conditioning phase. However, results suggested that fear extinction was impaired or that the number of times the CS was presented needed to be higher in order for extinction to take place. During the conditioning phase (CS presentation) the percentage of time lambs exhibited ears in the asymmetric position was higher in the last compared with the first repetition of conditioning. Higher percentages of asymmetric ear position have been previously observed when sheep are exposed to sudden events (Boissy et al., 2011) but also in situations of negative contrast (amount/quality of food expected not meeting expectations, (Boissy et al., 2011; Reefmann et al., 2009) and isolation from conspecifics, suggesting that asymmetric ear posture is associated with negative situations. The increase in percentage of time spent with ears in this position during the fifth repetition of conditioning may be related with the anticipation of a sudden negative event which was the introduction of the separation panel and separation from dam/sibling. More importantly, this result seems to be indicative that a learning process was in progress and, if at first presentation of the cue it meant nothing more than an unfamiliar sound, then by the fifth repetition lambs learnt it was indicative that something sudden and unpleasant was about to happen. The percentage of time with ears in this position was also different between test lambs and their siblings with test lambs presenting lower percentages than siblings. Again this seems to indicate that test and sibling lambs were learning different things or that the cue had different salience for them both. In rats, a neutral stimulus can also acquire aversive value by observation of another rat undergoing fear conditioning (Bruchey et al., 2010; Kim et al., 2010). It is possible that a similar process happened in this experiment. Alternatively,

events that followed the CS, i.e. the US, may also have been perceived as aversive by the sibling lambs given that after cue presentation the sibling went through a combination of sudden movements, removal of test lamb and a brief period of visual isolation from the dam (the visual barrier between lambs and dam was only removed after test lambs were placed into the isolation pens). Why siblings were more reactive than test lambs to the suddenness of the cue does not have an obvious explanation, although a feature of the design of the fear conditioning paradigm may be one of the underlying causes for this result. When the CS finished, a panel was introduced between the dam and the lambs. For the sibling, the time between the end of the CS and the following events (removal of test lamb and brief visual isolation) was shorter than for the test lambs (being moved into the isolation pen). In fear conditioning, a delay between the CS and US is known as trace conditioning and results in a less effective association between the CS and US being formed (Hall & Stewart 2010). Hence, it is possible that sibling lambs formed a stronger association between the CS and US than test lambs due to there being less trace conditioning in sibling lambs than in test lambs, which translated into a higher percentage of ears in the asymmetric position in the sibling lambs.

During the conditioning phase (CS presentation) lambs spent more time with the ears in forward position and less time with the ears in asymmetric position in the first repetition compared with the last. High proportion of ears forward and asymmetric in sheep have been previously associated with negative situations such as separation from group members (Reefmann et al., 2009a,b). However, other authors (Guesgen et al. 2016) suggest that the association of ears forward with negative situations is not linear and that ears forward may be a specific behavioural strategy adopted in a situation of isolation from conspecifics. Additionally, in a study conducted in sheep by Boissy et al. (2011), time spent with ears forwards was increased in negative but controllable situations but ears were presented backwards if the animal had no control over the same negative situation. Ears are used to gather information from the surrounding environment which can then be integrated and judged (Manteuffel, 2006). If ears in forward position is related to the

intention of gathering information (i.e. representing a state of attention/alertness) in addition to being related to negative situations, the drop in percentage of time spent with ears forward over the course of conditioning may indicate that the lambs habituated to the presentation of the CS. However, the decrease in ears forward was accompanied by a parallel increase in the amount of time spent with ears in the asymmetric position, suggesting that lambs were learning to associate cue presentation with the following events. Interestingly, there was an overall tendency for test lambs to present ears forward for a higher percentage of time than their siblings. One possible explanation for the lack of statistical significance between test and sibling lambs may be that the events following cue presentation were perceived by both lambs as having some degree of negativity. Nevertheless, this result suggests that sibling and test lambs were learning different things. Given that the siblings were suggested to be better conditioned than the test lambs (see above), the decrease in forwards position is possibly reflecting an increased association with the US in relation to the test lambs, represented by the increase in asymmetrical position.

The body posture results during fear conditioning (CS) suggested that prenatally stressed males showed less fear overall than control males and than control and stress females, as they spent more time lying with head up. However this difference was not observed between control and stressed females. However, there was no effect of repetition; hence it is not possible to link this behaviour with acquisition of fear conditioning. Moreover, behaviour was not analysed before the cue presentations; therefore, it is not possible to infer if this difference in behaviour was observed in response to the CS or if this was a trait also observed under basal conditions.

An effect of prenatal treatment on the percentage of time spent lying head up during the CS period of conditioning was also found between sibling and test lambs. Sibling lambs from the stress group seemed to show less fear than siblings from the control group but this difference was not observed in the test lambs. This finding may be a result of siblings actually being better conditioned than test lambs and, because of this, effects of prenatal treatment were observed in this cohort but not in the test lambs.

Nevertheless, there was no effect of repetition which makes it impossible to know in which direction this behaviour was varying and to link it with the acquisition of fear conditioning.

During the isolation (US) period that followed cue presentation, test lambs showed a consistent decrease from first to subsequent isolations in all analysed behaviours: attempts to escape, vocalizations and activity. A high number of vocalizations has been interpreted as an indication of distress or fear in ewes and cattle in isolation (Torres-Hernandez & Hohenboken 1979; Boissy & Le Neindre 1997) and the number of jumps as an attempt to escape isolation and novelty situations in ungulates (Boissy & Bouissou, 1988; Vandenheede et al., 1998). Notably, no differences were found between PNS and control lambs in the frequencies of those behaviours. Decreased expression of such behaviours, either as a consequence of habituation or learned helplessness, is one of the expected outcomes of repeated exposure to a stressor (Freeman & Manning 1979). Hence, it seems plausible that lambs could have developed reduced aversion to isolation and become habituated to the procedure. Habituation to a procedure may depend on the predictability of the stressor (Hargreaves & Hutson 1990). In this experiment the isolation period was short and predictable and nothing aversive, apart from the isolation itself, was inflicted upon the lambs. Additionally, although the walls of the isolation pens were opaque, the lambs could hear other lambs and ewes and could see the neighbour lamb if they jumped or reared on the wall. Previous studies have suggested that the sight of conspecifics decreases the aversion to isolation (Parrott 1990; Baldock & Sibly 1990). The finding that no differences between treatment groups in activity, bleating and escaping behaviour were found in the present study may indicate that the PNS used in this study was not strong enough to induce behavioural alterations in response to repeated stress in lambs. Similarly to the present findings, in two other studies conducted in lambs (Roussel et al., 2004) and kids (Roussel et al., 2005), no differences were found between PNS and control offspring in behavioural responses (vocalizations and jumping) either to a single isolation episode (lambs) or to five isolation episodes in consecutive days (kids), although PNS altered behavioural reactivity in other

tests performed in the kids. In rodents, PNS has been reported to increase immobility in tests designed to induce stress (Secoli & Teixeira 1998, Drago et al., 1999, Morley-Fletcher et al., 2003, 2004, Abe et al., 2007). Moreover, results have shown that this is true independently of the type of stressor applied during the last week of gestation (for a review see Weinstock 2017). Why behavioural reactivity to isolation seems to be more resistant to PNS effects in sheep is unknown.

In the extinction phase lambs were assessed for the expression of fear conditioning. During this period, male lambs spent more time with ears in an aligned position during the last repetition of extinction than during the first but females did not show any differences. No differences were found in the percentage of time spent with ears in forward or asymmetric posture but instead the percentage of time spent with ears backwards was higher in test than in sibling lambs. However, no effects of PNS treatment were found.

The increase in the percentage of time male lambs spent with ears in aligned position was higher in the last compared with the first repetition of extinction. This ear posture has been associated in previous studies with neutral, relaxed and positive situations (Reefmann et al. 2009a, 2009b, Boissy et al. 2011) and the present result suggests that male lambs were increasingly less fearful towards the last cue repetition during extinction. This finding may be indicative that fear extinction was acquired to some degree in males but was impaired in females. This finding is in agreement with rodent research, which also indicates impaired fear extinction processing in females (Baker-Andresen et al., 2013; Matsuda et al., 2015).

Test lambs also presented an increased percentage of ears backwards posture compared to sibling lambs. In sheep, the ears backwards posture has previously been associated with unpleasant/uncontrollable situations (negative contrast shift in size of a reward, Boissy et al., 2011; castration pain, Guesgen et al., 2016, Briefer et al., 2015; isolation, Briefer et al., 2015). It is reasonable to think that test lambs present more of this posture than siblings during extinction, given that to them the cue was paired with subsequent isolation. However, because there were no differences between the first and last repetition of extinction it is not possible to associate this

result with fear extinction rate. Moreover, other ear postures thought to be indicative of fear and unpleasant situations (forwards, asymmetric or backwards) did not show a decrease as might be expected over the course of extinction. This may suggest some impairment in the rate of extinction or that an increase in the percentage of time spent with ears in an aligned posture is more important as an indicator of fear extinction than significant decreases in the other ear postures. Perhaps, a higher number of CS presentations during extinction would have been necessary for clearer extinction to be observed. Additionally, as previously mentioned (see above), isolation may not have been as aversive to the test lambs as expected, hence alterations in ear postures within conditioning and extinction sessions were more subtle than anticipated.

PNS did not produce any effects in any of the two stages of the experiment (conditioning and extinction). The species where fear conditioning has been the most studied has been rodents and PNS effects have been variable. Extinction learning has been both reported to be impaired (Markham et al., 2010; Wilson et al., 2013) by PNS or not affected (Green et al. 2011), while contextual fear learning (see Chapter 1 section 1.7) remained unaffected (Markham et al., 2010; Wilson et al., 2013). Similarly, acquisition of fear learning during conditioning has also been reported to be affected by PNS in opposite ways, either being impaired (Markham et al. 2010) or remaining unchanged (Green 2011). Differences in the PNS protocol may be the cause for the different effects observed since these studies used either a variable PNS (Markham et al., 2010; Wilson et al., 2013) or a repeated immobilization PNS protocol (Green 2011). Similarly, and in light of the general findings of the present study, it is plausible that the stressors used to induce maternal stress were not strong enough to produce clear behavioural differences in the offspring.

3.4.5 Weight Change

In the previous chapter, an effect of sex and parity on birth weight was found (males heavier than females and lambs from multiparous ewes were heavier

than lambs from primiparous ewes. Interestingly, at seven weeks of age, an interaction between parity and sex became evident. The present results showed that males from multiparous ewes were heavier than females but sex seemed to have no effect on the weight of offspring from primiparous ewes. One possible explanation for these results could be differences in milk production between primiparous and multiparous ewes. Previous studies have shown that milk production in sheep may vary with parity. Sheep in first lactation produce lower quantities of milk than multiparous sheep (Agnihotri & Rajkumar, 2007; Casoli et al., 1989) and milk yield in the ewe is a major determinant of the strength of the ewe-lamb bond, with a threshold level below which the weaning process starts (<1000cc/day) (Arnold et al., 1979). Parallel to this, a decrease in frequency and duration of sucking is also observable after four or five weeks of the lamb's life (Gordon & Siegmann 1991) but lambs are able to digest pasture by three weeks of age. If, in fact, primiparous ewes in this study had lower production of milk than multiparous ewes then this may have affected their offspring feeding behaviour by imposing an earlier/greater shift from milk to grass intake, either by dam behaviour or by lamb energy needs, or both (Pryce 1992). One drawback of this earlier shift is that the amount of solid food intake is not enough for the lamb to maintain its energy balance and as a consequence its ADG decreases (Bechet et al., 1989). The fact that multiparous ewes are better prepared to respond to the lamb's nutritional needs may have allowed male lambs to express their increased growth potential compared to females in multiparous offspring, an effect that would not have occurred in the primiparous offspring. Additionally, as previous studies have shown, when a feeding shift occurs, as a result of the weaning process, heavier lambs suffer a higher drop in growth rates than do lighter lambs (Fraser & Saville 2000). Given that males were heavier than females at the starting point, they would have suffered a steeper decrease in ADG than females to a point where sex differences became unnoticed.

At 13 weeks of life, when weaning of males occurred, approximately half of these lambs had been single offspring for the previous six weeks (when siblings were euthanized for brain procedures). Present results showed that

primiparous male offspring that had become single at seven weeks of life had greater growth rates than primiparous male offspring from intact litters at 13 weeks of life. Between six and 13 weeks lambs already acquire part of their energy from grazing but milk will still be a part of their diet. Lambs have been reported to suck up until between 150 (Arnold et al. 1979) and 180 days of life (Geist 1969), depending on the breed. The sudden loss of a twin means that, at least for a period of time, the ewe will still be producing sufficient milk for two lambs (twins stimulate up to 35% more total milk production). It is possible that the remaining lamb had taken advantage of the surplus milk production, which would then contribute to a boost in its growth. Interestingly, becoming a single did not seem to affect growth rates in male lambs from multiparous ewes. The reason for this finding is not clear but it is possible that the higher quantity and quality of milk produced by multiparous ewes is enough to secure the optimal growth of twins to a point where becoming a single lamb has little to no effect on growth.

For the weights measured at 17 weeks, female lamb ADG was only affected by parity of the ewe, where multiparous female offspring were heavier than their primiparous peers. Like males, some females became a single litter at seven weeks (due to sibling being used in brain procedures) but others only became single at 13 weeks (when males were weaned) and a third group continued as an intact litter until weaning. Interestingly, the effects of becoming a single lamb did not seem to affect female lamb growth. One possible explanation for this finding is the lower growth potential of females compared to males, which makes it easier for a ewe to feed two lambs adequately. Alternatively, it is possible that females are better than males at buffering the disadvantages of being in a twin litter. Females are known to stay in closer proximity of their mother (Dwyer & Lawrence, 1999 - Suffolk sheep; Guilhem et al., 2005 – wild mouflon sheep; Shillito-Walser & Williams, 1986 – domestic sheep) and to suck for longer periods than males, although males suck more frequently (Atroshi & Österberg 2010). It is possible that female lambs are better than males at maximising the benefits of the ewe-lamb bond. If they are already taking maximal advantage of this relationship

when in an intact litter, becoming a single lamb will not have any advantages on lamb growth.

For the pre-slaughter weight of males at 17 weeks, the same interaction between parity and litter status effect found at 13 weeks was observed. Prior to slaughter males had been weaned for one month. The fact that growth maintained the same pattern as seen at 13 weeks (see above) may indicate that weaning at that age had no influence over growth. Interestingly, the effects of treatment which had not been noticed before seemed to show some expression over the weaning period depending on litter status, with males from litters that had become single at seven weeks showing greater growth in the stress than in the control group but with no difference being found between intact litters. The effects of a prenatal treatment seem to be associated with the process of weaning and only appear later in life. These results partly agree with those from a previous study, where PNS originated heavier lambs one month after weaning, although no treatment effects had been observed at earlier stages of the lambs' life (Roussel et al., 2008). However, the present results have shown that this was only true when comparing single lambs from stress and control groups, which seems to indicate that treatment effects were only expressed as a function of post-natal experiences (i.e. becoming a single lamb). Several studies have shown that post-natal experiences can alter the effects of prenatal stress (Maccari et al. 1995, Sternberg & Ridgway 2003, Averós et al. 2015). It is possible that spending six weeks as a single lamb influenced lamb eating behaviour patterns in a way that better prepared it for the weaning period, which was accentuated by the prenatal treatment.

The pattern of an interaction between parity and litter status was maintained for the hot carcase weights in males. However, a shift occurred: single lamb carcases were heavier than intact litter ones only in the stress group. Similar findings related to ewe parity were seen in female hot carcase weight, where the observed difference in hot carcase weight between primiparous and multiparous female offspring was not found in pre-slaughter weight. This goes against the expected results which would be that carcase weight would vary in the same pattern as pre-slaughter weight, given that lambs were all

treated the same in the time between weighing and slaughter (1hr transport and all slaughtered on the same day of transportation). Reasons for this shift in results are not clear but it is possible that weight differences in internal organs could have been behind such findings. Additionally, a difference in weighing method between live lambs and carcase could also explain the present results.

3.5 CONCLUSION

The experiments conducted in the present chapter represent a continuation of Chapter 2. Here, lambs were studied at later stages of life in order to better understand the effects of potential prenatal stressors on the development of the offspring. In agreement with the results from Chapter 2, only subtle effects of PNS treatment were observed at later stages of life. For example, PNS affected some aspects of brain morphology of the lambs; still, this was evident only in a sex-dependent manner and only more immature neuronal structures were affected. Other effects of PNS treatment were observed for lamb weights but also in a sex-dependent way and males seemed to be affected but females did not. However, in this particular case the observed effects of PNS were expressed as a result of a post-natal event, which was the fact that some lambs lost a sibling due to euthanasia or weaning. These results highlight the importance of postnatal events and their contribution to determining the outcomes of PNS but also that sex is an important feature to take into account when studying such outcomes.

The lack of clearer effects of the PNS used in this study is possibly a reflection of the general findings throughout the literature, where the nature of the applied stressors, the timing in relation to pregnancy and the species being studied all contribute to the variable outcomes observed in the offspring. In the present case, it further suggests that the selected stressors were not strong enough to elicit a strong physiological stress response in the dams and/or that ewes are particularly successful at buffering the effects of external environment. Still, although no behavioural differences between

treatment groups were found in the offspring at later stages in life when presented with a stress-eliciting situation (i.e. fear conditioning), results from brain morphology and gene expression analysis suggest that prenatal treatment had some influence over the development of the offspring. This is further confirmed by the behavioural differences observed at the very early stages in life (see Chapter 2). Together, these results mark the importance of adopting a multidisciplinary approach when it comes to study the potential effects of prenatal stress, with the risk of dismissing the importance of certain prenatal environment aspects when not doing so.

Another objective of this phase of the experiment was to validate a fear conditioning paradigm in sheep and to investigate possible effects of PNS on the acquisition and extinction of the fear response. Although no effects of PNS were found, the results from this test revealed that lambs show fear learning capacity but that fear extinction seemed to be impaired in females compared to males when using the 'ears aligned' measure. Possible issues related to the design of the experiment and the behaviours used to assess the fear response may have been the cause for the lack of a clearer difference in behaviour between first and last repetitions within conditioning and extinction phases. Further investigation towards developing a more adequate protocol is needed.

CHAPTER 4

Maternal housing systems as a source of prenatal stress in sheep: impacts on the neonatal lamb.

4.1 INTRODUCTION

In a study conducted to evaluate the husbandry procedures for pregnant sheep in the UK, it was found that 50% of sheep farms included in the study housed all their ewes at some point prior to lambing (DEFRA project AW0509). The housing of animals implies that these individuals, usually left to roam freely the vast majority of time, go through a period of time where they are exposed to new husbandry routines and, consequently, potential unpredictable and uncontrollable events. One example of this type of event is the delivery of concentrate feed. This is an uncontrollable event from the ewes' perspective and may be susceptible to a certain degree of unpredictability in a farm setting. The introduction of uncertainty and unpredictability are a major source of stress (Maier & Watkins 2005). The amount of evidence indicating that predictable events are less stressful than unpredictable ones is also considerable (Weinberg & Levine 1980). An unpredictable feeding schedule during pregnancy in sheep may therefore represent a potential stressor and act as a source of prenatal stress for the fetus, ultimately affecting its development. Moreover, even where feeding schedules are consistent, there is another potential source of stress caused by feed competition and aggression inherent to feeding period (Arnold & Maller 1974, Dwyer 2017). When ewes are on concentrate feed, feeding periods are usually restricted to one or two times a day and a limited amount is provided, which is eaten in a few minutes.

Another source of stress for sheep is exposure to the presence of a dog. In a housed setting, exposure to dogs may increase as sheep are kept in a more human supervised environment and dogs may be accompanying the person in charge of caring for the sheep. Indeed, in the study mentioned earlier (DEFRA project AW0509) 25% of farms were reported to use dogs in handling pens and sheds when dealing with pregnant ewes and 18% of farms reported having problems with public dogs chasing and worrying their pregnant ewes. In addition, there is some uncertainty of the additional number of farms where the dog is just kept loose.

Dogs represent the threat of a predator to sheep and elevated heart rate, plasma cortisol and ACTH, and body temperature have been reported in

sheep when exposed to either the presence or the recording of a barking dog (Harlow et al., 1987, Baldock & Sibly 1990, Cook 1996, Komesaroff et al., 1998). Exposure to dogs during pregnancy in sheep may therefore adversely affect the development of the offspring. Indeed, lambs whose mothers were exposed to isolation in the presence of a dog during pregnancy showed increased basal cortisol concentrations at 25 days of age, although this difference was no longer present at eight months of age (Roussel et al. 2004).

Previous studies on the effects of long-term daily exposure to various unpredictable stressful events in sheep, and where delayed feeding and exposure to a dog were part of the treatment, have reported a state of chronic stress induced by such treatment (Destrez et al., 2013, Coulon et al., 2014). Where stressors were applied during pregnancy (Coulon et al., 2014, Petit et al., 2015), alterations in the behaviour and brain structures of the lambs were also observed, suggesting that this model (i.e. combination of various acute stressors) may be used to study the effects of maternal stress on the offspring.

The present chapter followed the same line of investigation as in Chapters 2 and 3, focusing on the effects of husbandry stressors during ewe pregnancy on the offspring. The aim of this study was to test the effects of unpredictable and uncontrollable events, known to act as maternal stressors (i.e. feeding system and exposure to a dog), on lamb behaviour, survival, passive immunity and growth for the first 48hr of life. Other lamb behaviour and health outcomes were investigated at later stages of life (Chapter 5). In addition, it was hypothesized that if the unpredictability and uncontrollability of the feeding schedule, the presence of the dog and potential feed competition were eliminated, stress exposure would be decreased for sheep and that the negative implications of maternal stress would be absent or have a positive impact on the offspring. To test this hypothesis, an alternative system was designed where silage was used as an alternative feeding system (food was available *ad libitum*) and sheep husbandry was kept to a minimum (absence of dogs). Hence, in this study there were three prenatal treatments putatively representing different levels of maternal stress. The

control group represented a medium level of stress with normal level of feed competition, the stress group a higher level of stress increased by an unpredictable feeding schedule and dog exposure, and the alternative group a lower level of stress by removing/reducing the feed competition.

Possible effects of sex and parity and temperament of the ewe were also investigated as some of the outcomes evaluated in the lambs may be affected by these factors (see Chapter 2 Introduction for the importance of sex and parity). In sheep, calmer temperament has been shown to positively affect maternal behaviour (more time spent grooming their lambs) (Murphy et al., 1994), a factor crucial for the establishment of the ewe-lamb bond, although in other studies this difference was either not found (Bickell et al., 2011) or reported to be inverse (calmer temperament ewe showing decreased maternal behaviour) (Coulon et al., 2014). Moreover, dam temperament has been shown to accentuate the effects of prenatal stress on lamb fearfulness, with lambs born to high emotional reactivity ewes being more affected (Coulon et al., 2015). Because these factors contribute to differences in natural conditions, it was hypothesized that they would be differently affected by PNS.

4.2 METHODOLOGY

4.2.1 Ewe Experimental Procedures

Eighty four Scottish mule ewes (Scottish Blackface X Blue-faced Leicester, n=43 primiparous, n=41 multiparous) were naturally mated to 1 of 2 Suffolk rams in November 2014 and predicted to give birth between the 7th and 27th of April 2015. Given the novelty aspects of this study there was no previous data to conduct a proper power calculation. Ewe sample size was estimated based on sample size from previous prenatal stress studies conducted in sheep. Ewes were examined via ultrasound in January 2015 (approximately 10 weeks of gestation) and the ones bearing twins were selected to take part in the study. They were moved to the experimental shed for the last third of gestation (9 weeks before the earliest expected parturition). Given that ewes were naturally mated there was variation in the stage of gestation at any

given time point. In the present study there was a maximum of 20 days between the most and least advanced stage of gestation. This means that on the day ewes were moved into the experimental shed, gestation stage varied between 9 and 12 weeks. From here onwards periods of gestation will be addressed referring to the most advanced stage of gestation.

On the day ewes were brought into the experimental shed, a temperament test was performed. Each ewe was socially isolated from the flock for 3 min in an isolation pen inside the experimental shed. Ewes were walked through a runway from the pen where the flock was kept (located at one end of the experimental shed) to the isolation pen, which was 2m x 2m, with 1m high solid walls and dirt flooring. Ewes were visually isolated from the flock but could see outside the pen if they jumped against the walls.

The isolation period was live-streamed to a small room inside the experimental shed where an observer was recording ewe activity and the number of attempts to escape. The number of vocalizations was recorded live by a second observer standing next to the isolation pen. To measure activity a grid of 9 equally sized squares was placed over the screen of the computer and movement was scored when the 2 anterior legs of the ewe crossed a line from a square. Attempts to escape were scored as jumps against the wall, raising all four feet from the floor.

After the temperament test ewes were classified as having a calm or reactive temperament according to their activity. Animals were divided into two groups using a cut-off of eight movements/min for activity. Individuals that had an activity rate higher than eight movements/min were considered to be on the reactive side of the scale and the ones with lower activity were categorized as being animals of less reactive temperament.

Ewes were divided into 12 groups: four control groups, four stress groups and four alternative groups, balancing for parity, date of expected lambing and temperament. Each group contained 7 ewes housed in straw-bedded pens, with acoustic, visual and olfactory contact between pens. The shed had a natural ventilation system and ewes were kept under a natural light regime until one week before the expected lambing date. From then onwards, during the natural dark periods they were kept under artificial light in order to allow for rapid identification of imminent births. Control and stress groups were

kept under a stocking density of 1.28m^2/ewe and allowed 33cm per ewe feedface (Figure 4.1) and the alternative groups were kept at a stocking density of 4.57m^2/ewe and allowed 72cm feedface per ewe. For stress and control groups there was an 8.65m^2 feeding/lambing pen adjacent to the experimental pen, where feed troughs were located (Figure 4.1). Access to the feeding/lambing pen was only allowed during feeding times or when a ewe started lambing and was relocated to this pen. The alternative groups also had a smaller pen (6.5m^2) inside the experimental pen but it was only used for when a ewe started lambing. Ewes were identified with coloured collars with a hanging tag marked with a number between 1 and 84. All animals had access to water and hay *ad libitum* throughout the experimental period.

Approximately on week 14 of gestation, concentrated feed in the form of pellets (Davidsons Super Ewe Nuts, Davidsons, Scotland) was added to the ewes' diet in control and stress groups to meet their daily nutrition requirements (approximately 250g/day/ewe). The amount of concentrated feed was adjusted as the ewes progressed through the gestational period until it reached approximately 1000g/ewe/day by the end of pregnancy. Ewes remained on this diet until they left the shed (table 4.1). The alternative group was fed grass silage (Dry Matter (DM) 251 g/kg; Protein 11.4 g/kg DM; Metabolisable Energy 11.4 MJ/kg DM) available *ad libitum* from the beginning of the experiment. Silage was formulated in order to meet the ewe's dietary requirements and was supplemented with approximately 14g/ewe/day of vitamins and minerals (Norvite Farm Minerals for Cattle and Sheep, Norvite, Aberdeenshire) and 75g/ewe/day of soya bean meal (Sopralin Trouw Nutrition, Ireland).

All ewes were vaccinated on week 16 of gestation, 4 weeks prior to lambing, with Heptavac P Plus (Intervet®) as an aid in the control of lamb dysentery, pulpy kidney, struck, tetanus, braxy, blackleg, black disease and clostridial metritis.

Table 4.1 Concentrate feeding program for control and stress ewes.

week 14 of gestation 16/02/15 – concentrate 250gr→500gr/head/day (adding 50gr/head/day each day)
Week 15 of gestation 22/02/15 – concentrate 500gr/head/day
week 16 of gestation 02/03/15 – concentrate 2 x 300gr/head/day
week 17 of gestation 09/03/15 – concentrate 2 x 400gr/head/day
week 18 of gestation until after lambing 16/03/15 – concentrate 2 x 500gr/head/day – until leaving the shed

Control groups were fed on a predictable schedule. Concentrate feeding was always delivered at the same time of day and paired with an auditory cue (radio sound). Once the radio was turned on concentrate was distributed through the troughs located at the front of the feeding/lambing pen, the experimental pen door was opened and ewes were allowed to come to the trough during the feeding period. Stress groups were under an unpredictable feeding schedule (between the 14^{th} and 19^{th} week of gestation). These animals were either fed at the same time as the controls, following the above procedure, or had their feeding process delayed by 15, 30 or 60 min. The order in which the delay was presented was attributed randomly according to a pre-determined schedule. During delayed feeding periods the door of the experimental pen was open and ewes were allowed to come to the front of the feeding/lambing pen. Food was either visible but not reachable (in a bucket with concentrate positioned in front of the pen) or absent (no bucket in front of the pen). After feeding, and once troughs were empty, all ewes were returned to the experimental pen, and access to the feeding pen prevented until the next feeding time. The alternative groups had silage *ad libitum* and feed troughs were refilled with silage twice a day from the outside of the pens. These ewes were left undisturbed for the entire period preceding lambing (Figure 4.1).

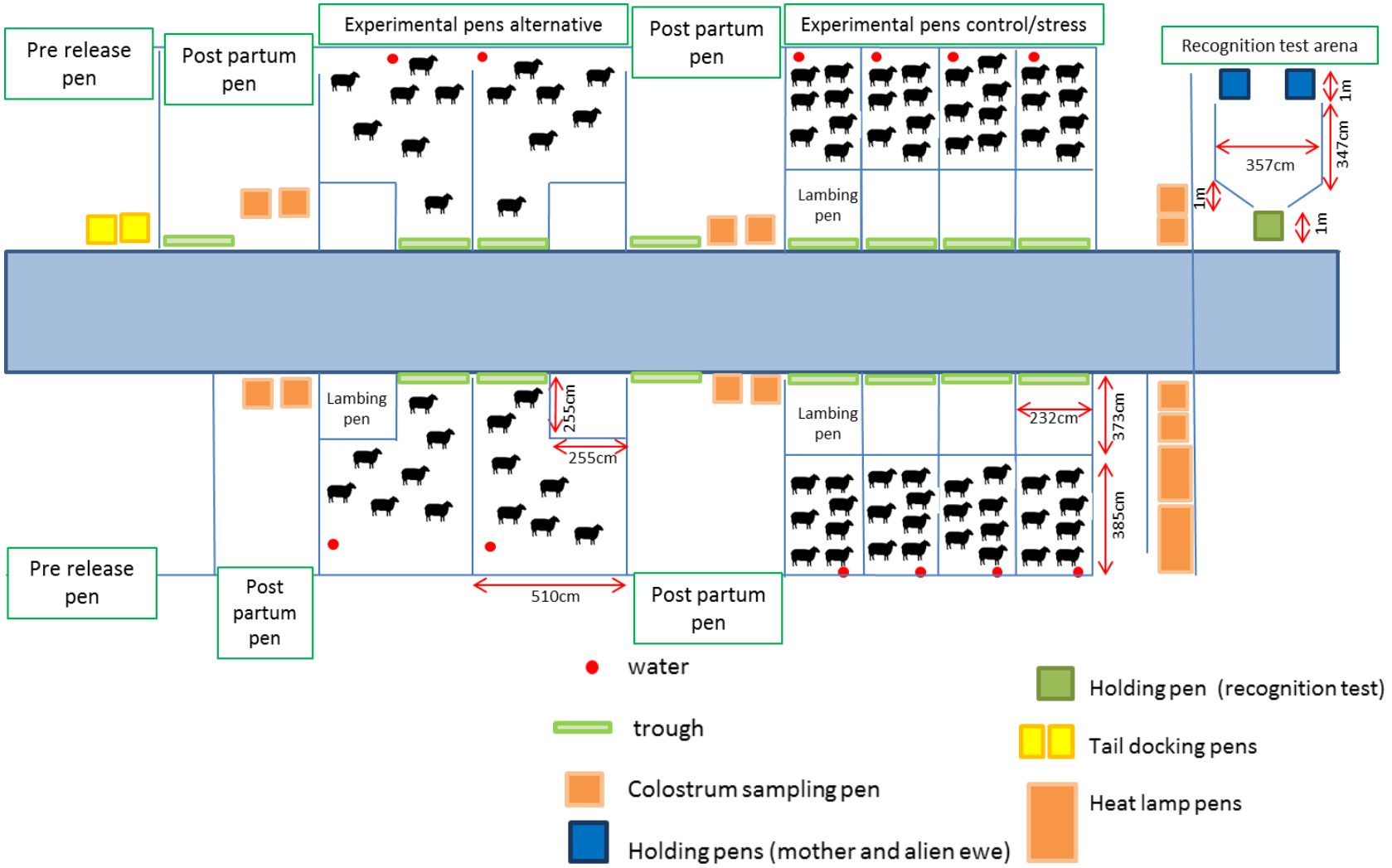


Figure 4.1 Experimental shed: lambing layout.

As an additional stress factor the stress groups were exposed to the presence of a dog once per week during 6 weeks (between the 12th and 17th week of gestation).

The dog exposure pens were located at one end of the experimental shed and all the procedures were conducted with visual isolation from all the other ewes. On the day of exposure two stress groups were brought to the exposure area and each group was penned in one of the two available exposure pens. Exposure to the dog was for 15 min and consisted of a person walking a dog on the lead around the exposure pens occasionally stopping in front of either pen (Figure 4.2). Six different dogs were used and none of the ewe groups was exposed to the same dog more than once.

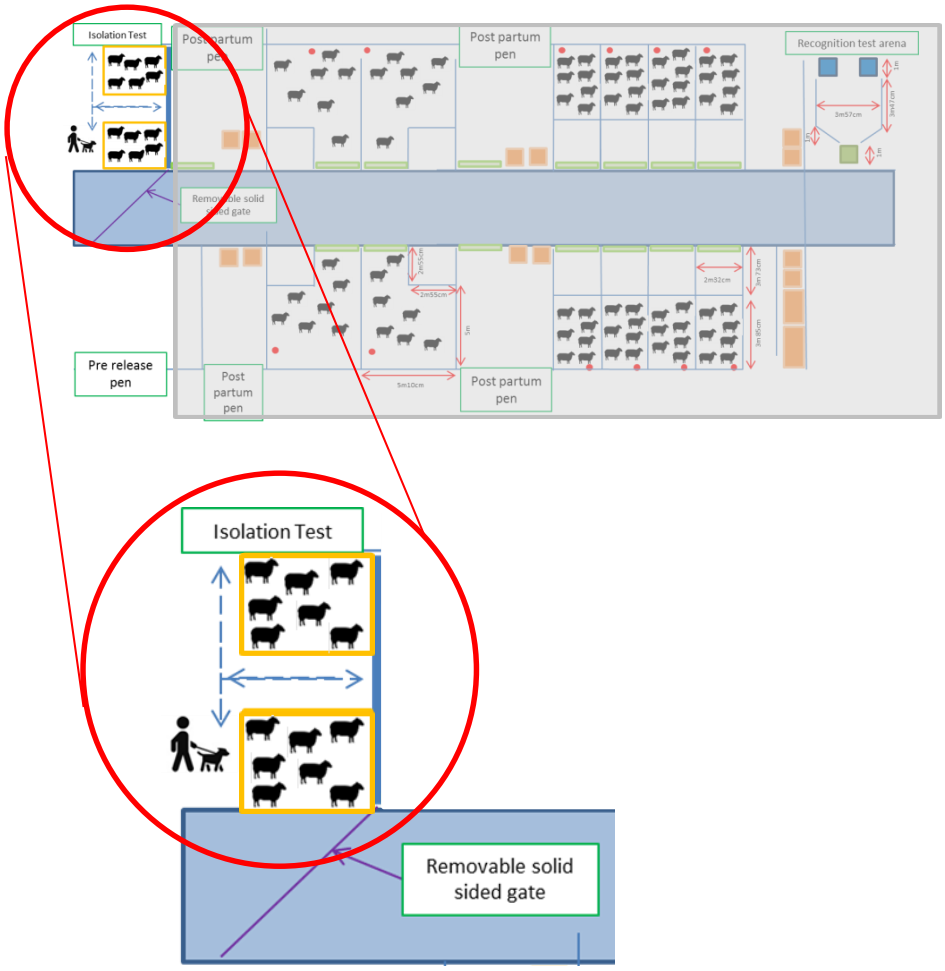


Figure 4.2 Experimental shed: dog exposure layout.

At the beginning of weeks 14, 16 and 18 of pregnancy a blood and a faecal sample were collected from each ewe for later analysis (these procedures and associated data analysis were part of another project conducted by a different PhD student, Yusof 2018). Sampling and sample processing were carried out as described in Chapter 2, section 2.2.1.

One week prior to the predicted start of lambing, stress groups were fed on the same feeding schedule as control groups. Alternative groups remained under the same previous conditions.

At the first signs of imminent birth (e.g., not coming to the feed trough at feed times, pawing the ground, restlessness, getting up and down, interest in other lambs/lambing ewes, contractions) ewes were brought to the feeding/lambing pen (Figure 1). If a birth was missed and occurred inside the experimental pen, the ewe and born lamb were brought to the feeding/lambing pen only after the first 30min of life of that lamb. If a birth was missed and the second lamb was born within 30min of the first born, then the ewe was only moved after the first 30min of life of the second born lamb. If parts were already visible and other signs of lambing were missed then the ewe was only moved 30 min after the first born or 30 min after the second born if born less than 30 min apart from the first born. If there was already one ewe in the lambing pen and a second one started lambing the second ewe was only moved into the lambing pen if the first one was not showing parts or if both lambs had already been born.

The decision to assist ewes and lambs during parturition followed the same criteria as described in Chapter 2. After parturition, ewes and lambs were moved to the colostrum sampling pens and a colostrum sample was collected (see next section). Ewes and lambs were kept in these pens for six hr to allow for the dam-offspring bond to establish properly. After this period, animals were released into the post-partum pens together with other lambed ewes and lambs.

4.2.2 Offspring Experimental Procedures

At the end of the lambing period 169 lambs were born and 155 (n=51 control, 31 females and 20 males; n=52 stress, 29 females and 23 males; n=52 alternative, 28 females and 24 males) were used in the experimental procedures of this study. The reasons why 14 lambs were excluded are detailed under the statistical methodology section. Male lambs remained intact throughout the study and all lambs were tailed docked at 36hr of age. Age at weaning was between 11 and 14 weeks due to lambs being born within a maximum of a 20 day interval. As discussed for the ewe gestation period, from here onwards periods related to lamb age will be mentioned in relation to the age of the eldest lambs.

4.2.2.1 *Measurements from birth to 6 hr*

Experimental procedures for the first 6 hr of life of the lambs were similar to what was described in Chapter 2. Briefly, lamb behaviour was recorded for two hr after birth to assess lamb behavioural progression (latency to stand and suck) and vocalizations for the first 30 min. Rectal temperatures were taken at 30 min and 2hr after birth. However, in contrast to Chapter 3, lambs were weighted and had their crown rump length measured at 6hr after birth instead of at 24hr. At this time point a test for the ability of the ewe to recognise her lambs was performed (data from this test was part of a different PhD and will not be reported in this thesis). Once lambs were dried they were allocated to one of two treatment groups and spray marked: lambs allocated to the red colour were subjected to blood sampling and lamb recognition of the ewe testing at 24 hr old, while blue lambs went through an isolation test at 24 hr, were tail docked at 36 hr and blood sampled at 37 hr. Red lambs were tail docked one hr later than blue lambs.

4.2.2.2 *Measurements at 24 hr*

At 24 hr all lambs had their rectal temperatures measured. Red lambs were blood sampled for analysis of IgG concentration and tested for recognition of the dam ewe. The methodology for these procedures is described in Chapter

2. However, in contrast to Chapter 2, during the recognition test the blue lamb (sibling of the tested lamb) was taken into an isolation pen ($1m^2$) with straw covered floor (Figure 4.3) located in a different shed, visually and acoustically isolated from the experimental shed. The isolation test lasted for 5 min and was video recorded for later analysis of activity (number of squares entered), attempts to escape (lamb tries to or fits its head through the bars of the pen walls) and number of vocalizations using The Observer XT 11.5 software (Noldus Information technology, Netherlands). Once the test was finished all the lambs were immediately reunited with their dams and returned to the post-partum pens. 64 lambs were tested for recognition of the dam (control: female=13, male=10; stress: female=12, male=10; alternative: female=10, male=9) and 68 for the five min isolation period (control: female=16, male=7; stress: female=14, male=11; alternative: female=10, male=10).



Figure 4.3 Isolation shed and pen layout

4.2.2.3 *Measurements at 36 and 48 hr*

All lambs allocated to the blue colour (control: female=17, male=9; stress: female=14, male=14; alternative: female=14, male=12) were tail docked at 36 hr using the rubber ring method. Tail docking took place in tail docking

pens ($1m^2$) inside the experimental shed (Figure 5.1) as described in Chapter 3. To later test the existence of a pain memory association in lambs, a subset of twin lambs (total=68; control: female=11, male=11; stress: female=12, male=14; alternative: female=11, male=9), balanced for sex, litter sex combination and ewe parity, were tail docked either in the presence (conditioned) or absence (unconditioned) of a novel scent Anisole (Sigma-Aldrich). The scent was provided via a scent box (plastic box with multiple holes drilled into it) containing a sponge onto which the liquid Anisole had been pipetted. The scent box was fixed to the side of the pen at approximately lamb head height (Figure 4.4). All blue lambs were blood sampled by venepuncture from the jugular vein one hr after tail docking (using the same procedure as described in Chapter 2, section 2.2.2.2). Samples were treated as previously mentioned for later analysis of cortisol content (cortisol levels were not possible to measure due to issues related with the lab methodology; see Appendix 1). All lambs and ewes were returned to the post-partum pen after sampling.

Forty eight hr after parturition, lamb rectal temperatures were measured again and they were ear tagged. After these procedures lambs were released onto pasture together with their dams as a single flock.



Figure 4.4 Tail docking pen

4.2.3 Statistical Methods

Data in this chapter were analysed by the restricted maximum likelihood procedure (REML) in Genstat (Genstat 64-bit, 16th edition). On some occasions, non-parametric tests (Wilcoxon and Chi-square tests) or a survival model (the Proportional hazards model) were used. The analysis used for each variable is given in Table 4.2. In the cases where a REML procedure was used, fixed and random models were slightly altered to best fit the different data sets being analysed. However, treatment, ewe parity, lamb sex, and ewe temperament were always fitted as factors into the fixed model and lambing pen and ewe as random factors into the random model. This model will be referred to as main model from here onwards and the alterations made to it will be mentioned below for each data set.

For each model, where a REML procedure was used, data either followed a normal distribution or were transformed (as described in Table 4.2) to meet the assumptions of this procedure. The transformation used was made on theoretical grounds (see statistical methods in Chapter 2). Where significant differences were detected by the REML procedure, post hoc Student's t tests were used for pairwise comparisons to determine where significant differences lay. The level of probability determined as statistically significant was $P<0.05$. When a transformation was used, confidence intervals were calculated using the original mean ± 2*s.e.. The same transformation applied to the mean was applied to this result in order to obtain a back-transformed confidence interval.

Within individual experiments various response variables were recorded. In many cases some of these variables were strongly associated with each other, and this would be expected on biological grounds. In such circumstances broadly similar findings of either strong statistical evidence across variables or a lack of statistical evidence for treatment effects across variables is anticipated. Hence, if (for example) three strongly associated response variables (e.g. latency to stand, latency to suck, IgG concentration in plasma) were tested and all found to be statistically significant, it would be overly rigorous to apply a P-value adjustment such as Bonferroni (a conservative adjustment which also assumes independence of the response

variables) and so require each individual P-value to be below the 1% level to declare statistical significance at 5%. On the other hand, it would clearly be wrong to regard the presence of statistical significance in all three variables as giving five times the amount of evidence of differences that would have been existed if only one of the five variables had been recorded and then assessed statistically.

Limitations

Adjusting P-values to take account of multiple testing and so ensure the overall error rate is maintained at (say) 5% can make it extremely difficult to find statistical evidence of differences. How difficult this becomes depends on the number of comparisons being made. Hence a balance often needs to be struck between avoiding undue strictness and so still collecting useful findings, and also controlling as far as possible the risks of false conclusions.

The P-values are much more informative than simply whether formal statistical significance was achieved or not at some arbitrary level (e.g. 5%) set many years ago out of historical necessity. Instead, they reflect the strength of evidence of effects rather than a binary decision outcome. Rather than formally adjusting the P values themselves assuming the variables are independent of each other, caution was exercised in interpreting these P values. The P-values themselves reflect the relative strength of evidence of treatment effects and can act as a yardstick for further investigation. Where borderline statistical significance has been achieved, this has been interpreted cautiously, even more so where there was no apparent biological explanation for the finding. The same approach was taken to interpret data in Chapter 5.

Lamb bleating behaviour

For the statistical analysis of total number of bleats during the first 30 min of life, birth assistance was added to the main model as a fixed factor. Since each ewe produced more than one lamb in the dataset and more than one

lamb was born in the same pen, the random model was also adjusted by adding lamb identification (ear tag number).

From the 169 lambs that were born only 155 were considered for the statistical analysis of the bleating behaviour. Fourteen lambs were excluded from the analysis due to missed birth (control female=1, stress males=2), being delivered by the veterinarian as a result of an exceptionally difficult birth and having missing data (control: female=1, male=1), being a sibling to a stillborn (control: male=1, still born=1; alternative: male=1, still born=1), having an anatomical anomaly and dying at 9 days of age (stress: male=1, male anatomical defect=1) or being in a triplets litter (alternative: female=2, male=1).

Lamb behaviour landmarks

The main model for the analysis of lamb latency to stand and to suck was adjusted by adding birth assistance and lamb identification as fixed and random factors, respectively. One hundred and fifty three lambs were analysed for these behaviours. The same lambs as mentioned for bleating behaviour were excluded from this statistical analysis plus two more that had to be moved into the hot box before showing standing behaviour due to a drop in body temperature (alternative: female=1, male=1).

For the latency to suck the factors used in the fixed and random model were the same as for latency to stand. Lambs that were not seen performing sucking behaviour for the first two hr of life were assisted to suck by a technician and assigned a value of 2hr for the latency to suck. This happened to 13 lambs (control: male=1; stress: male=2; alternative: female=6, male=4). One hundred and fifry four lambs were included in this analysis. The same lambs as for standing latency were excluded except for one (alternative female) due to being taken to the hot box before having the opportunity to stand but was recorded as being assisted to suck later at 2hr of life.

Lamb body temperatures

The main model for the analysis of body temperatures at the four different time points was adjusted by adding birth assistance to the main model as a fixed factor and lamb identification as a random factor. Nine lambs from the 169 born were excluded from the analysis (sibling to a still born control: male=1, still born=1; alternative: male=1, still born=1; anatomical anomaly stress: male=2; triplets litter alternative: female=2, male=1) for all the four temperatures taken at different time points. In addition to these, at 2hr a litter had missing data (alternative: female=1, male=1), at 24hr three additional litters were excluded due to death of one sibling (control: female=2; stress: female=1, male=1; alternative: male=2) and at 48hr one litter (stress: female=1, male=1) and one lamb (alternative: male=1) had missing data (not recorded when measured).

Lamb body weight and ponderal index

For birth weight and ponderal index, no adjustments were made to the fixed model. However, lamb identification was added to the random model. Weights and ponderal index of 158 lambs were analysed. 11 lambs were left out of the analysis due to sibling being a still born (control: female=1, male=1; alternative: male=1, no noted sex=1), sudden illness of the sibling and subsequent euthanasia (stress: female=1, male=1), death of the sibling after 4hr of life (control: females=2), being born in a litter of triplets (alternative: female=2, male=1).

IgG in plasma

The statistical model for the analysis of IgG levels in the lamb plasma was only adjusted by adding birth assistance as a factor to the fixed model. Fifty five plasma samples were analysed from the original 66 samples taken. Ten lambs that had to be fed artificial colostrum were excluded from the analysis, as feeding artificial colostrum has been shown to lower the levels of circulating IgG (personal communication Dr Ann Mclaren) (control: female=1, males=3; stress female=1; alternative: female=2, male=1). Another lamb

(stress male) was excluded because it was born with an anatomical defect (no anus) and died two days later.

Two samples were read as zeros in the lab assay. These samples were not real zeros (i.e. they had some concentration of IgG) as indicated by their absorbance above zero. Therefore, a decision was made to attribute the lowest detectable value by the assay to these samples (i.e. 0.75mg/mL).

Recognition test

Only birth assistance was added to the fixed model for the analysis of the recognition test data. From the 64 lambs tested for the ability to recognise the mother (control: females=10, males=3; stress: females=9, males=8; alternative: females=10, males=9) two lambs (alternative: female=1, male=1) were excluded from the analysis because the test reached a predetermined end point before reaching the three min duration due to one of the ewes jumping out from holding pen. Additionally, three lambs were excluded from the analysis because they were ill at the time of testing (alternative: female=1, male=1) or were lambed by the vet and were untouched by the ewe during the early hr of life (control male=1).

Five different questions were being asked during the recognition test (see statistical methods in Chapter 2) and different statistical analyses were used (see Table 2.3).

As latency to reach the mother were both censored and skewed, a survival analysis methodology was adopted for the statistical analysis (see statistical methods in Chapter 2).

24 hr Isolation test

The isolation test statistical model had birth assistance added to the fixed model but no alterations were made to the random model. From the 71 lambs that were tested for behaviour during isolation, 3 were excluded due to missing data (video footage not found).

Table 4.2 Data distribution and transformations.

	Data	Distribution	Transformation	Tests
Early behaviours	Latency to stand and suck	Positively skewed	Log 10	REML
	30min bleats frequency	Positively skewed	Log 10	REML
Temperature	30min and 2hr	Negatively skewed	Reverse log10	REML
	24hr and 48hr	Normal	---	REML
Weights	Birth	Normal	---	REML
	Ponderal index	Normal	---	REML
Recognition test	Proportion of time spent with dam , alien ewe, ewes, neutral zone (comparison between groups)	Normal	Arcsine	REML
	Overall proportion of time spent with dam vs. alien	Not normal	---	Wilcoxon Matched-Pairs test
	Proportion of time spent with dam vs. alien (comparison within group)	Not normal	---	Wilcoxon Matched-Pairs test
	Proportion time (greater than chance) spent with dam, alien ewe, ewes, neutral zone	Normal	Angular	Student's t test
	Latency to reach dam	Normal	---	Proportional hazards model
	First choice (dam or alien) higher than chance	Counting data	---	Chi-square
	Activity and vocalizations	Positively skewed	Square root	REML
Blood analysis	IgG	Normal	---	REML
	Cortisol	No data available	---	---

4.3 RESULTS

4.3.1 Bleating Behaviour During The First 30 Min After Birth

Treatment did not affect lamb bleating behaviour (F=1.35, d.f.=8.2, p=0.311). Lamb sex affected bleating behaviour depending on parity of the dam (F=7.91, d.f.=121.5, p=0.006) and assistance given to birth (F=5.22, d.f.=117.0, p=0.024). Females bleated more than males when born to primiparous ewes but this difference was not found in multiparous offspring (Figure 4.5 a). When birth was assisted, males were also observed to bleat less than females but this sex difference was not observed in the case of unassisted births (Figure 4.5 b). Dam temperament also affected lamb bleating behaviour ((F=4.71, d.f.=66.8, p=0.033), with offspring from reactive dams bleating more frequently than offspring from dams of calmer temperament (back transformed mean number of bleats/30 min (upper and lower CI): Reactive dam lambs=33.7 (23.86;47.36), calm dam lambs=20.5 (14.60;28.58), Figure 4.5 c).

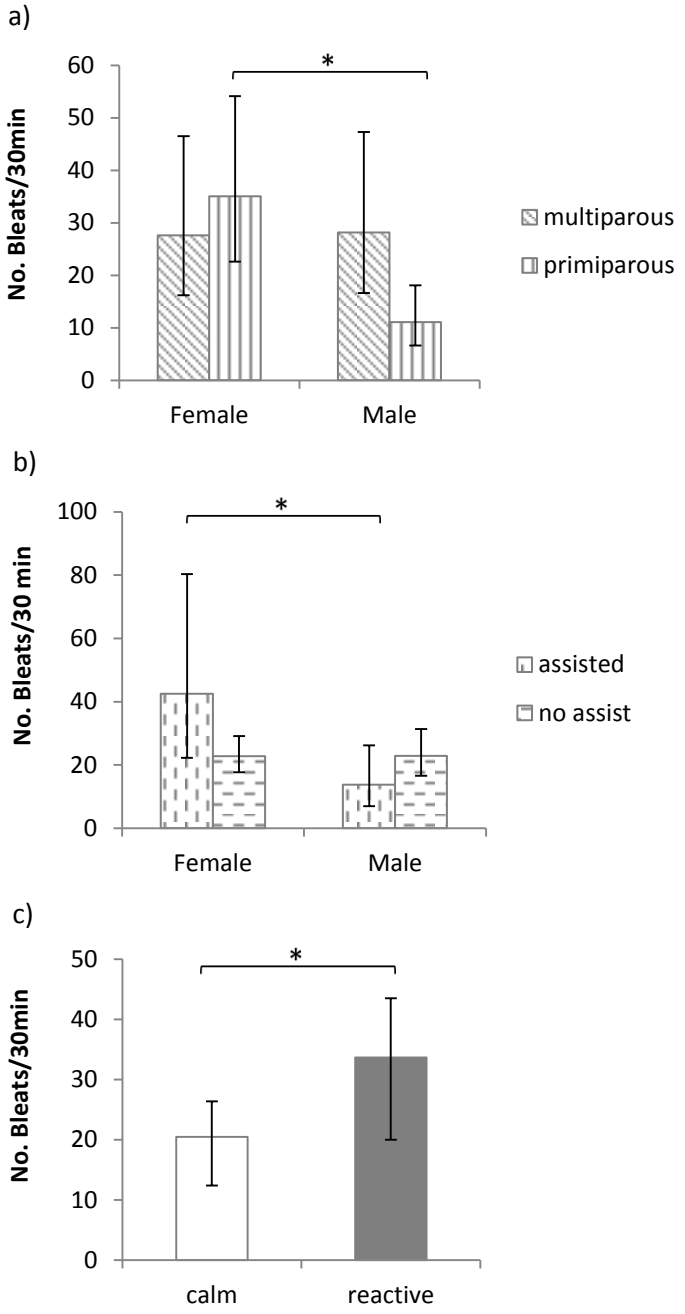


Figure 4.5 Number of lamb bleats during the first 30 min after birth depended upon a) an interaction; between lamb sex and parity of the dam; b) an interaction between lamb sex and assistance given to birth and c) ewe temperament. Females bleated more than males when born to primiparous ewes and when assisted at birth. Lambs born to reactive ewes bleated more than lambs born to calm ewes. Graphs bars illustrate back transformed means and error bars represent CI. Stars (*) indicate where statistical significance lies.

4.3.2 Lamb Behaviour Landmarks

Latency to first stand up was affected by interactions between birth assistance and treatment (F=3.97, d.f.=125.9, p= 0.021) and between birth assistance and temperament (F=6.30, d.f.=132.0, p=0.013). Lambs took longer to stand if the birth was assisted than if it was not in the control group, but no differences were found in either the stress or alternative groups (Figure 4.6 a). Lambs from ewes with a reactive temperament that were assisted at birth were slower to stand up compared to lambs that were not assisted but birth assistance did not affect latency to stand when lambs were born to calm ewes (Figure 4.6 b). Additionally, females had a tendency to be faster to stand than males (back transformed mean latency to stand (min) (upper and lower CI): F=16.0 (14.7;20.3), M=18.7 (13.9;18.4), p=0.069). Parity had no effect on lamb latency to stand (F=1.90, d.f.=70.4, p=0.172).

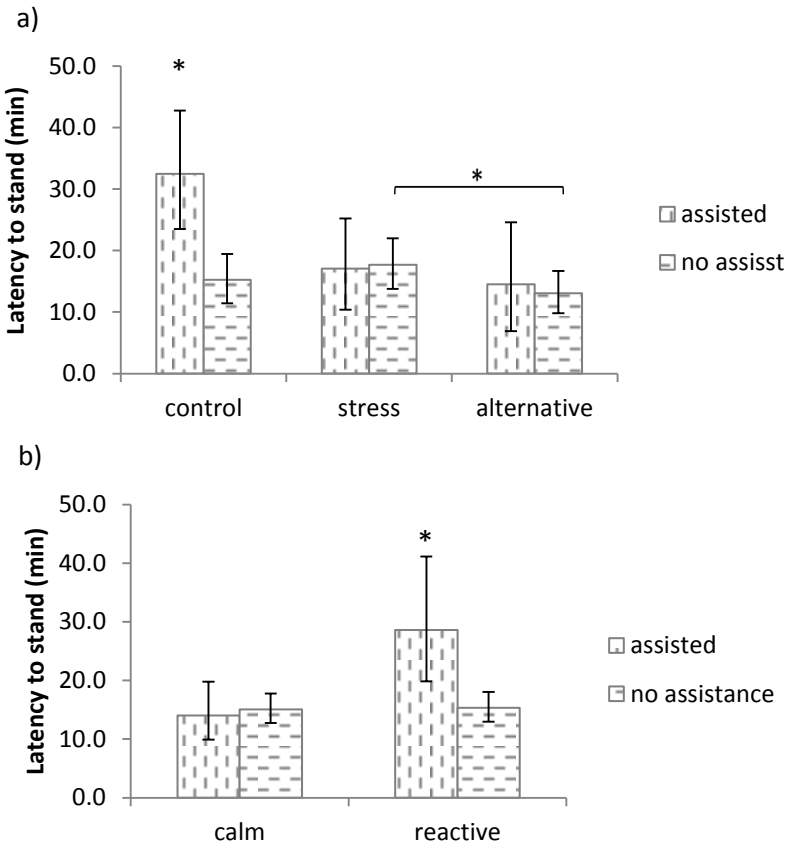


Figure 4.6 Lamb latency to stand after birth (min) dependent upon a) an interaction between birth assistance and a) treatment and and b) temperament. Prenatal treatment decreased the latency to stand in lambs that were assisted at birth. Graphs bars illustrate back transformed means and error bars represent CI. Stars (*) indicate where statistical difference lies.

Lamb latency to suck was not affected by any of the factors analysed, although there was a tendency for females to be faster to first suck than males and for lambs that were assisted to birth to be slower than unassisted (table 4.3).

Table 4.3. Effects of treatment, sex, parity, assistance to birth and temperament on latency to suck.

Main Factors	Groups	Mean (CI)	$F_{statistic}$	df	p_{value}
Treatment	Control	54.95 (47.42;68.87)	0.57	8	p=0.586
	Stress	56.89 (48.75;70.46)			
	Alternative	51.52 (42.17;63.24)			
Sex	Female	51.40 (44.57;59.29)	3.69	112.4	p=0.057
	Male	60.50 (51.76;70.79)			
Parity	Primiparous	55.72 (46.99;66.07)	0	69.4	p=0.999
	Multiparous	55.72 (46.56;66.68)			
Assistance	Assisted	62.09 (50.00;77.09)	3.22	143.7	p=0.075
	Unassisted	50.12 (44.87;55.98)			
Temperament	Calm	56.49 (47.64;66.99)	0.04	67.8	p=0.835
	Reactive	55.08 (46.03;65.92)			

4.3.3 Lamb Body Temperature

There were no observed effects of the main factors included in the statistical analysis for 30min temperature (Table 4.4).

At 2hr, temperature was affected by an interaction between treatment and birth assistance (F=3.82, d.f.=109.4, p=0.025). Lambs assisted at birth in the stress group had lower temperatures than if not assisted, but this difference was not observed in either control or alternative groups (Figure 4.7). Sex (F=0.36, d.f.=143.1, p=0.552), parity (F=0.55, d.f.=68.2, p= 0.460) and temperament (F=0.01, d.f.=68.2, p= 0.933) did not affect 2hr temperature.

Table 4.4 Effects of treatment, sex, parity, assistance to birth and temperament on 30min temperature.

Main factors	Groups	Mean (CI)	$F_{statistic}$	Df	P_{value}
Treatment	Control	38.33 (38.00;38.62)	0.98	5.4	p=0.220
	Stress	38.13 (37.77;38.45)			
	Alternative	37.89 (37.47;38.26)			
Sex	Female	38.14 (37.88;38.37)	0.05	117	p=0.815
	Male	38.11 (37.83;38.36)			
Parity	Primiparous	37.95 (37.63;38.24)	2.9	68.9	p=0.093
	Multiparous	38.29 (37.99;38.56)			
Assistance	Assisted	38.04 (37.83;38.39)	0.74	150.6	p=0.392
	Unassisted	38.20 (38.00;38.40)			
Temperament	Calm	38.21 (37.91;38.48)	0.75	68.7	p=0.389
	Reactive	38.03 (37.72;38.33)			

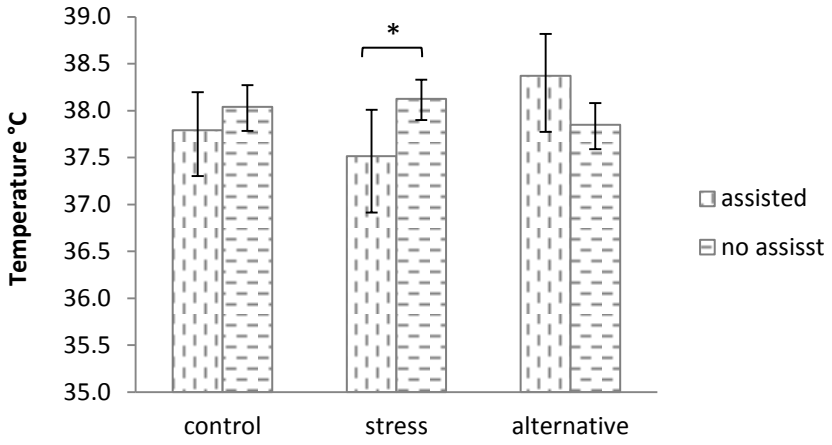


Figure 4.7 Lamb body temperature at 2hr after birth dependent upon an interaction between treatment and assistance at birth. PNS increased the difference in body temperature between assisted and unassisted lambs. Graph bars represent back transformed means and error bars represent CI. Stars (*) indicate where statistical difference lies.

Treatment also affected lamb temperature at 24hr depending on ewe parity ($F=5.00$, d.f.=57.1, $p=0.010$, Figure a)) or lamb sex ($F=3.12$, d.f.=118.6, $p=0.048$, Figure b)). In the alternative group, lambs from multiparous ewes had higher temperatures than lambs from primiparous ewes, whilst in control and stress groups parity did not affect lamb temperature at 24hr (Figure 4.8 a). As for sex differences, only in the control group did females have higher temperatures than males (Figure 4.8 b). In the stress and alternative groups, no sex differences were found. 24hr temperature was not influenced by temperament ($F=0.19$, d.f.=64.6, $p=0.663$) or assistance given to birth ($F=0.82$, d.f.=130.2, $p=0.366$).

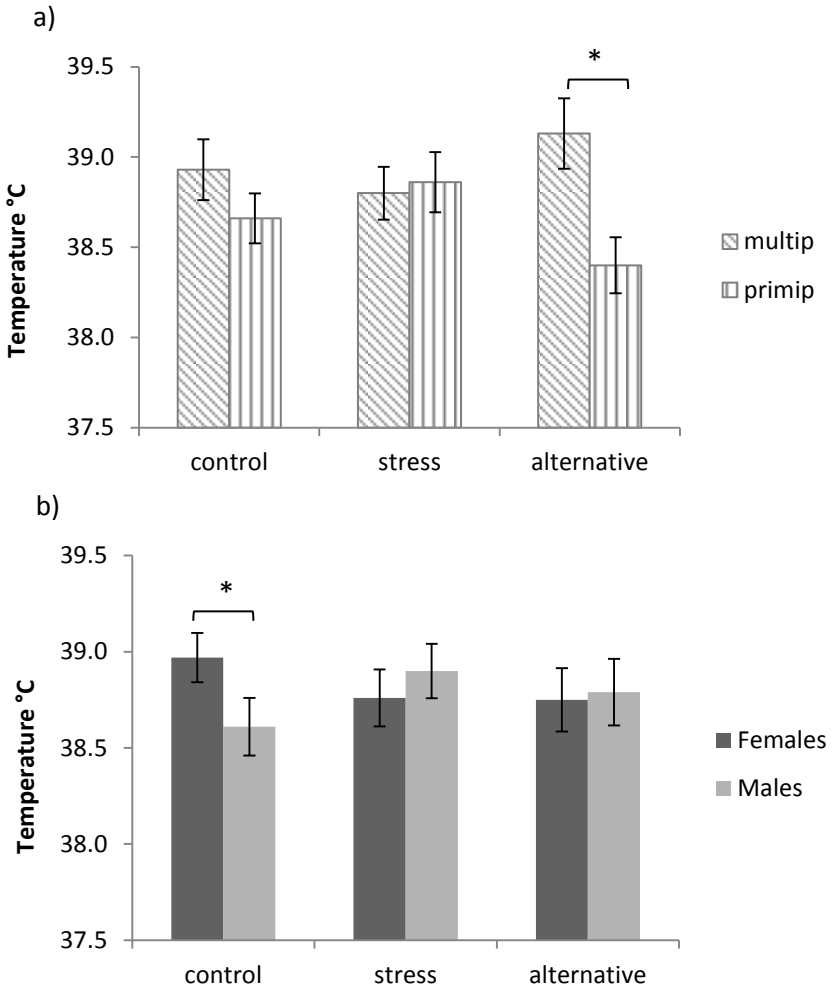


Figure 4.8 Lamb body temperature at 24hr after birth dependent upon a) an interaction between treatment and parity or b) treatment and sex. The alternative treatment increased the difference in body temperature between lambs born to multiparous and to primiparous ewes. Prenatal treatments decreased the difference in body temperature between male and female lambs. Graph bars represent means and error bars represent s.e. Stars (*) indicate where statistical difference lies.

There were no effects of treatment (F=4.40, d.f.=7.6, p=0.054), sex (F=1.14, d.f.=137.6, p=0.288), assistance at birth (F=0.92, d.f.=88.5, p=0.340) or temperament (F=1.08, d.f.=69.1, p=0.302) on 48hr temperatures. Lambs from multiparous ewes had higher temperatures at 48hr than lambs from primiparous (mean temperature at 48hr (°C) (±s.e.): lambs from multiparous ewes=38.82 (±0.058), lambs from primiparous ewes=38.63 (±0.058), p=0.039; Figure 4.9).

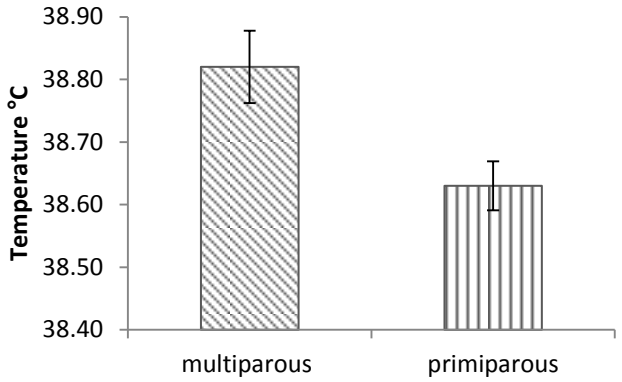


Figure 4.9 Lamb body temperature at 48hr after birth dependent upon parity. Lambs from primiparous ewes had lower temperature than lambs form multiparous ewes. Graph bars represent means and error bars represent s.e..

4.3.4 Lamb Body Weight and Ponderal Index

There was an effect of treatment on lamb weights at 6hr after birth (F=18.27, d.f.=7.1, p=0.002, Figure 4.10), with lambs in the alternative group being lighter than lambs in the control and stress groups. Lambs born to multiparous ewes were heavier than those born to primiparous ewes (mean weight at 6hr (Kg) (±s.e.): lambs from multiparous ewes=5.10 (±0.079), lambs form primiparous ewes=4.37 (±0.076), p<0.001), but sex did not influence lamb weight (F=1.53, d.f.=144.9, p=0.219). Temperament of the ewe had no effect on lamb weight at 6hr (F=0.23, d.f.=73.6, p=0.632).

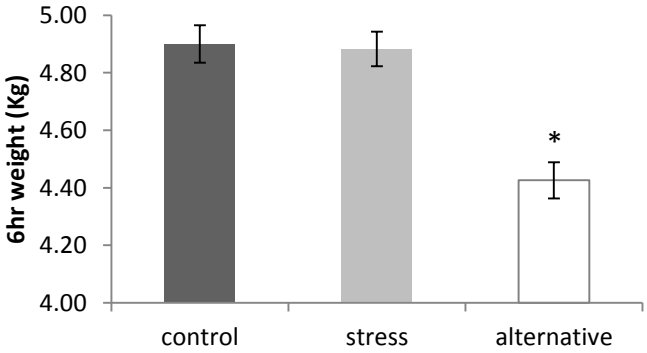


Figure 4.10 Average lamb weight at 6hr depending on treatment group. Lambs in the alternative treatment were lighter than in control and stress groups. Graph bars represent means and error bars s.e. Star (*) indicates where statistical significance lies.

Treatment had no influence on PI (F=1.55, d.f.=9.1, p=0.263). Sex influenced PI, with females having a lower PI than males (mean PI (±s.e.): females=4.12 (±0.087), males=4.56 (0.097), p<0.001). An interaction was found between parity and temperament (F=6.37, d.f.=67.0, p=0.014, Figure 4.11), with lambs from multiparous ewes of calm temperament having a lower PI than lambs from primiparous ewes of calm temperament and multiparous ewes of reactive temperament.

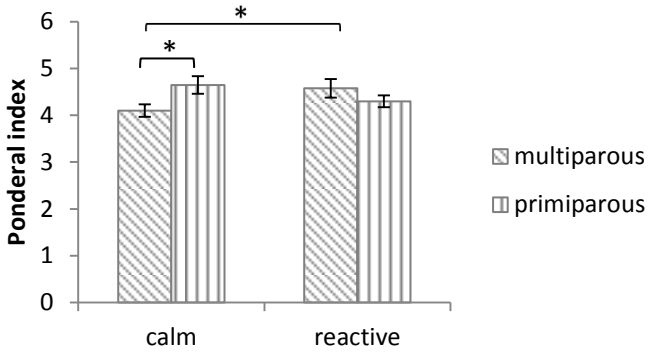


Figure 4.11 Lamb PI at 6hr depending on the interaction between ewe parity and ewe temperament. Lambs from calm multiparous ewes had lower PI than lambs from calm primiparous and reactive multiparous. Graph bars represent means and error bars s.e. Stars (*)indicate where statistical significance lies.

4.3.5 IgG Concentration in Plasma

There was an effect of treatment in the concentration of IgG in lamb plasma (F=14.37, d.f.=8.0, p=0.002, Figure 4.12 a). Lambs from the alternative treatment group had the lowest circulating levels of this immunoglobulin, whilst there were no differences between the stress and control groups. An effect of parity was also found (F=4.87, d.f.=40.5, p= 0.033, Figure 4.12 b), with lambs from multiparous ewes showing higher levels of IgG than lambs from primiparous ewes. Temperament (F=3.19, d.f.=41.2, p= 0.082) and sex (F=0.04, d.f.=43.3, p= 0.841) did not affect plasma IgG levels.

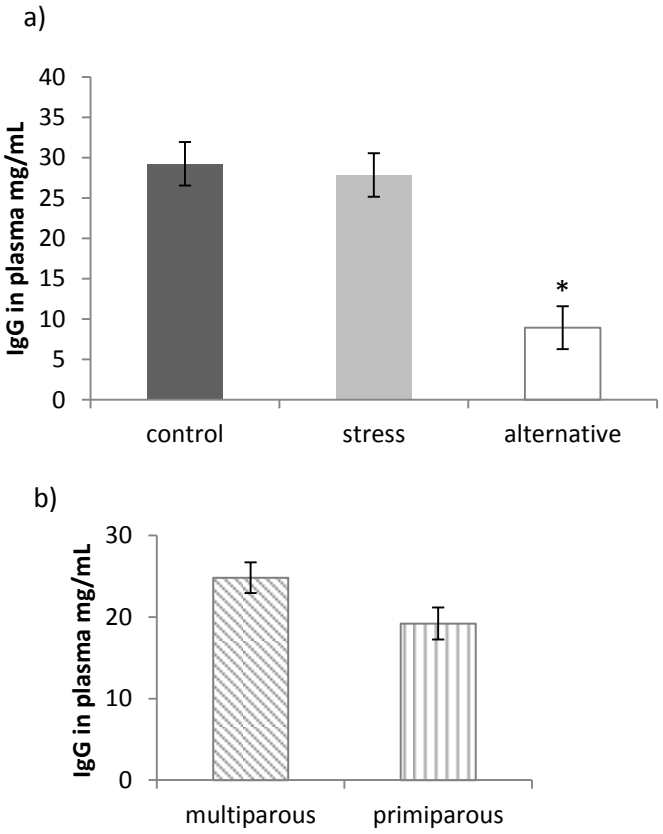


Figure 4.12 IgG concentration levels in lamb plasma depending on a) treatment and b) parity. Lambs in the alternative treatment had lower levels of IgG than in control and stress groups and lambs from primiparous ewes had lower levels than lambs from multiparous ewes had lower levels than lambs from multiparous ewes. Graph bars represent means and error bars s.e. Star represents statistical significance ($p<0.05$).

4.3.6 Recognition Test

Not all lambs made a decision to visit ewes (Figure 4.13 a). Overall, during the recognition test lambs did not show a preference to approach the dam first more often than the alien ewe (dam=34, alien=21, χ^2=3.07, df=1, p=0.080). Similarly, in none of the treatment groups did lambs choose to approach the dam first significantly above chance level (Figure 4.13 a). However, lambs from multiparous ewes chose their dam first significantly more than chance (χ^2=7.00, df=1, p=0.008) but lambs from primiparous ewes did not (χ^2=0.03, df=1, p=0.853) (Figure 4.13 b). There was no significant effect of treatment (X^2(2)= 2.491, p=0.288), sex (X^2(1)=0.54, p=0.46), parity

($X^2(1)=2.32$, p=0.13), temperament ($X^2(1)=0.72$, p=0.40) or birth assistance ($X^2(1)=0.17$, p=0.68) on the latency to reach the mother.

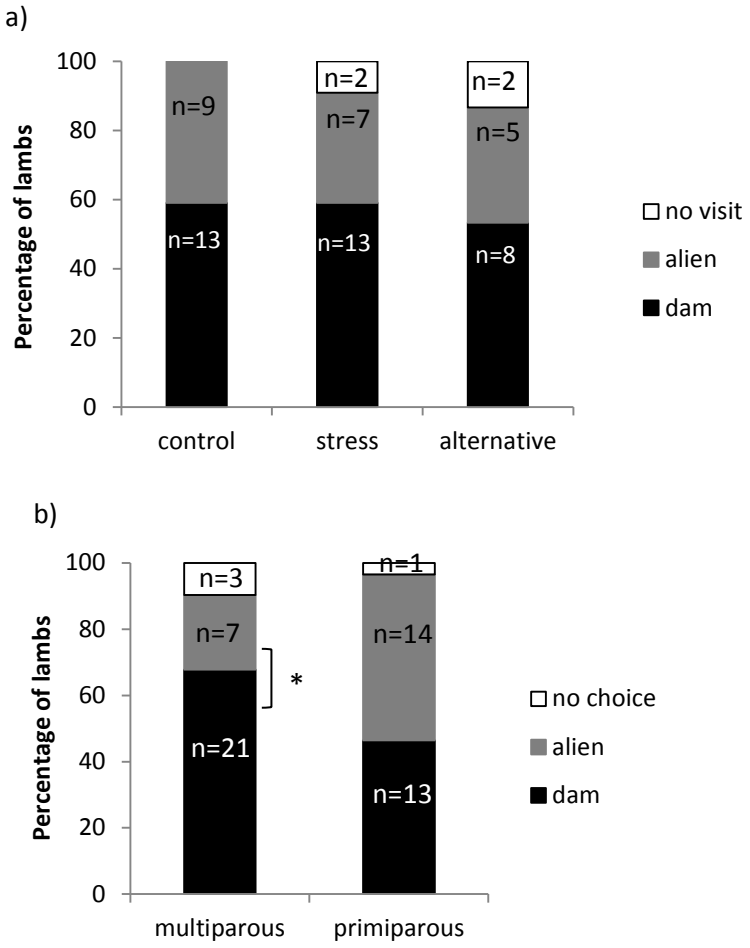


Figure 4.13 Percentage of lambs that choose to go to dam or alien ewe first, or that did not approach the ewes during the maternal recognition test, according to a) treatment or b) parity of the ewe. Graph bars represent percentage of lambs choosing ewe or alien. Star (*) represents statistical significance.

Treatment did not affect the proportion of time the lambs spent close to the dam (F=0.30, d.f.=8.1, p=0.746) or close to ewes (F=0.09, d.f.=8.6, p=0.918) over the duration of the test. There were also no significant effects of parity (F=0.00, d.f.=45.1, p=0.990) or temperament (F=1.81, d.f.=46.5, p=0.185). Proportion of time spent close to the alien ewe was not affected by any of the main factors: treatment (F=0.59, d.f.=8.8, p=0.577), sex (F=0.00, d.f.=50.9,

p=0.968), parity (F=2.58, d.f.=47.9, p=0.115), assistance given to birth (F=0.34, d.f.=48.9, p=0.565) and temperament (F=1.26, d.f.=49.8, p=0.267).

However, an interaction between sex of the lamb and assistance given to birth was found (F=7.30, d.f.=33.7, p=0.011, Figure 4.14). Assisted female lambs spent less time close to the dam than unassisted lambs (back transformed proportion of time spent close to dam (CI): assisted females=0.03 (0.0;0.20), unassisted females=0.34 (0.19;0.50)) but this difference was not seen in males (back transformed proportion of time spent close to dam (CI): assisted males=0.54 (0.20;0.86), unassisted males =0.38 (0.19;0.59)).

This same pattern was observed for total time spent close to ewes (back transformed proportion of time spent close to ewes (CI): F-assisted=0.0 (0.0;0.29), F-unassist=0.45 (0.30;0.61); M-assisted=0.71 (0.40;0.94), M-unassist =0.56 (0.37;0.75)).

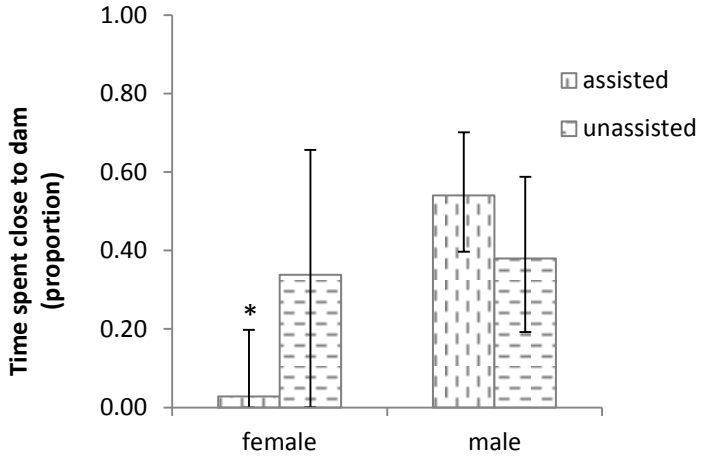


Figure 4.14 Proportion of time lambs spent close to their dam depending on the interaction between sex and assistance at birth. Assisted females spent less time close to dam than unassisted females but this difference was not see in males. Graph bars represent back transformed means and error bars upper and lower CI. Star (*) indicates where statistical difference lies.

Lambs did not spend more time close to ewes higher than what would be predicted by chance (angular scale mean proportion=43.67, se=2.650, p=0.647). However, from the time lambs spent close to ewes, overall proportionally more time was spent close to the dam than the alien (proportion of time spent close to dam or alien ewe (secs): dam=0.72, alien=0.26; Z=233.5, p<0.001). At the treatment level, this was only true for the control and stress lambs (proportion of time spent close to dam or alien ewe (secs): control - dam=0.76, alien=0.24; Z=34.50, p=0.002; stress – dam=0.77, alien=0.23; Z=11.0, p<0.001; Figure 4.15) but not for the alternative lambs (alternative - dam=0.63, alien=0.37; Z=31.0, p=0.341; Figure 4.15).

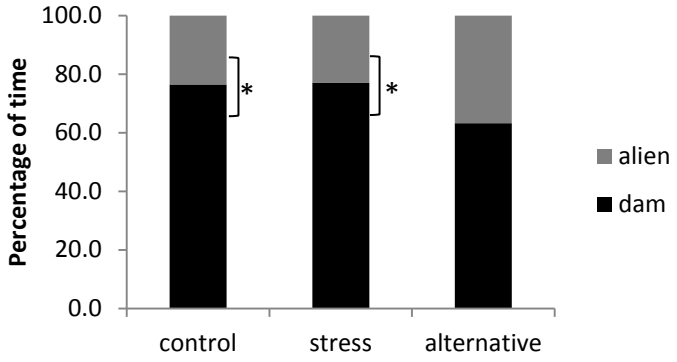


Figure 4.15 Percentage of time (in the original scale) spent close to the dam and alien ewe when considering total time spent close to ewes. Lambs in control and stress groups spent more time close to dam than to alien ewe but lambs in the alternative group did not. Stars (*) indicate where statistical significance lies.

Lamb activity during the recognition test was dependent on an interaction between parity and treatment (F=4.92, d.f.=32.5, p=0.014). Lambs from the control and stress treatments had similar activity levels independently of parity, whereas in the alternative group lambs from multiparous ewes were less active than lambs from primiparous ewes (Figure 4.16). Sex of the lamb (F=0.68, d.f.=50.8, p=0.413), birth assistance (F=0.67, d.f.=49.8, p=0.418) and temperament of the ewe (F=1.24, d.f.=49.1, p=0.270) did not influence lamb activity during the recognition test. Likewise, lamb vocalization during

the recognition test did not differ between treatment (F=0.30, d.f.=9.1, p=0.7.44), sex (F=0.378, d.f.=48.1, p=0.544), parity (F=0.65, d.f.=51.0, p=0.424), assistance to birth (F=0.14, d.f.=50.7, p=0.711) or temperament (F=0.43, d.f.=49.5, p=0.513) groups.

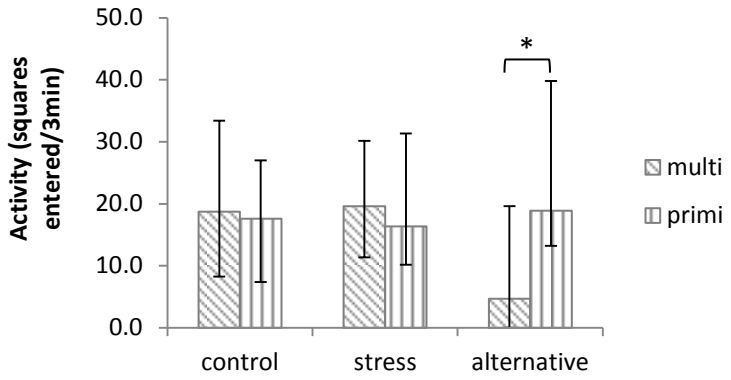


Figure 4.16 Lamb activity levels during the recognition test depending on the interaction between parity and treatment. Lambs from multiparous ewes had lower activity than lambs from primiparous in the alternative group but this was not seen in control and stress groups. Graph bars represent back transformed means and error bars upper and lower CI. Stars (*) indicate where statistical significance lies.

4.3.7 24 Hr Isolation Test

There were no significant effects of the main factors on lamb activity or bleating rate (Table 4.5).

However, the rate of escaping attempts was significantly affected by treatment (F=6.71, d.f.=6.9, p=0.024) and parity (F=6.07,d.f.=60.9, p=0.017). Lambs from the alternative group performed fewer attempts to escape than lambs from the stress group and showed a tendency to attempt to escape less than controls during the five min test (Figure 4.17 a). Lambs born to primiparous ewes also had a lower rate of attempting to escape than lambs from multiparous ewes (Figure 4.17 b). Sex of the lamb (F=0.19, d.f.=60.3, p= 0.666), assistance to birth (F=2.59, d.f.=47.8, p= 0.114) and temperament of the dam (F=0.32, d.f.=61.0, p=0.573) did not affect the rate of lamb attempts to escape.

Table 4.5 Activity and bleating rates of lambs during a five min isolation test at 24hr of age depending upon treatment, sex, parity, assistance to birth and ewe temperament.

		Activity rate				Bleating rate			
Main factors	**Groups**	**Mean (CI)**	**$F_{statistic}$**	**Df**	**P_{value}**	**Mean (CI)**	**$F_{statistic}$**	**Df**	**P_{value}**
Treatment	Control	58.64 (39.51;81.90)	0.73	8.5	p=0.511	60.96 (48.47;75.17)	1.26	7.4	p=0.338
	Stress	64.75 (44.56;88.72)				58.63 (46.39;72.30)			
	Alternative	47.65 (29.32;70.41)				47.80 (35.62;61.78)			
Sex	Female	63.33 (46.92;81.43)	1.83	57.5	p=0.181	57.08 (46.31;68.57)	0.17	59.2	p=0.683
	Male	50.61 (36.07;67.60)				54.23 (43.74;65.84)			
Parity	Primiparous	54.05 (39.39;71.03)	0.33	57.5	p=0.566	53.93 (43.69;65.25)	0.24	58.8	p=0.628
	Multiparous	59.60 (44.14;78.07)				57.38 (46.80;69.37)			
Assistance	Assisted	57.78 (45.55;81.52)	0.03	61	p=0.867	52.53 (45.05;68.03)	0.57	59.7	p=0.454
	Unassisted	55.83 (43.82;69.29)				58.94 (50.91;67.35)			
Temperament	Calm	55.56 (40.09;72.56)	0.07	58.3	p=0.797	55.52 (44.74;66.80)	0.00	59.4	p=0.972
	Reactive	58.03 (42.20;76.39)				55.77 (44.97;67.73)			

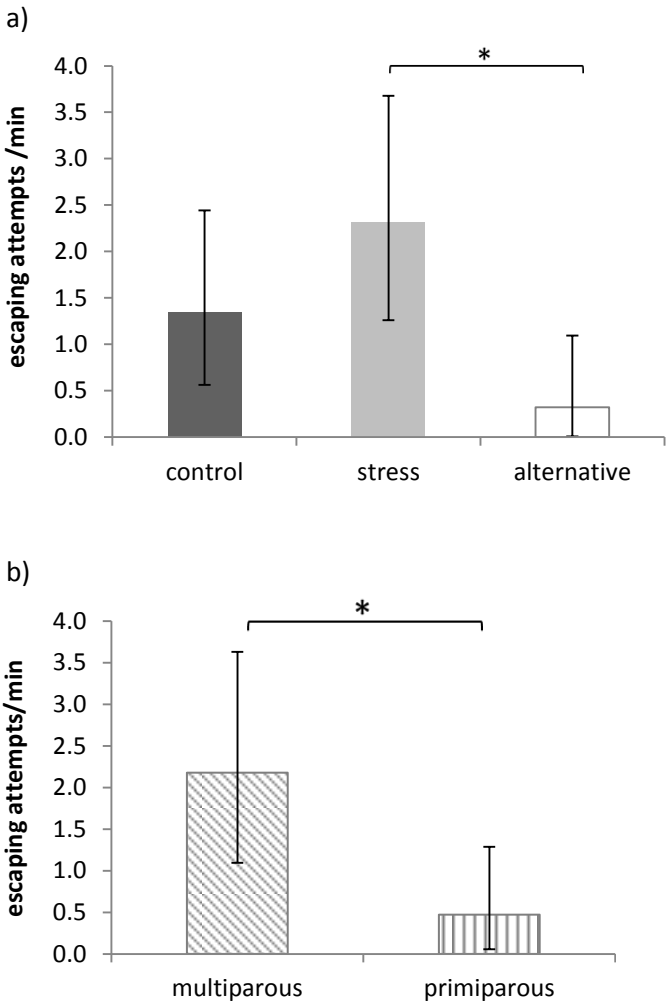


Figure 4.17 Rate of escaping attempts per min during the 24hr isolation test depending on a) treatment and b) parity. Lambs in the alternative group attempted to escape less than lambs from the stress but not than lambs in the control group. Graph bars represent back transformed means and error bars upper and lower CI. Stars (*) indicate where statistical significance lies.

4.4 DISCUSSION

Three different prenatal environments addressing the normal husbandry conditions in which pregnant ewes are kept in the UK were investigated. Each system aimed at representing a different level of stress exposure with the control group simulating standard management in the UK, and the stress and alternative groups simulating a higher and a lower stress environment in comparison to controls. It was hypothesised that stress treatment would negatively impact the development of the lambs and that these impacts would be absent in the alternative and control groups. The alternative lambs were hypothesised to have better outcomes than in the stress and controls.

The majority of effects caused by the prenatal treatment were seen in the lambs from the alternative group and suggest that the proposed alternative husbandry system did not work in favour of the lambs. These lambs weighed less at birth and had a lower concentration of circulating IgG than lambs from the control and stress groups. They also seemed to have some impairment of their capacity to distinguish their own dam, as there were no differences between the amount of time they spent with dam and alien ewe. Lambs in the alternative group also displayed lower rates of escaping attempts during the isolation test than lambs in the stress group, suggesting either decreased fearfulness or a general decreased level of activity.

The husbandry conditions designed to impose higher stress on the ewes did not negatively affect the lambs as hypothesised. No direct consequences of prenatal treatment were found in lambs from the stress group in comparison to the control group. Interactions between treatment and other factors (assistance at birth, sex, parity) were also observed for latency to stand, body temperature and activity during the recognition test, suggesting that treatment effects observed were subtle.

4.4.1 Lamb Bleating Behaviour

Bleating behaviour during the first 30min of life was affected by lamb sex depending on dam parity and birth assistance, with females bleating more frequently than males in the case of primiparous offspring and when birth had

been assisted. These sex differences were not observed in the case of multiparous offspring or when births were unassisted. An explanation for these results may be related to the degree of maternal care being offered by the ewe. Primiparous ewes are known to express lower levels of maternal care (O'Conner et al., 1992; Dwyer et al., 2003; Ekiz et al., 2007; Otal et al., 2009) and ewes that have been assisted at lambing show decreased interest in the lamb (Darwish & Ashmawy 2011). Vocalization in lambs is known to indicate their degree of need for maternal care (Brunelli et al., 1994; Weary & Fraser 1995). Thus, a decrease in ewe maternal behaviour would justify an increase in lamb bleating rates.

It is interesting that female lambs that were assisted at birth or born to primiparous ewes vocalized more than males in the same conditions. If the decrease in maternal care, as a consequence of having been assisted to parturition or being a primiparous ewe, is in fact a source of neonatal stress for the lamb it is possible that the sex differences found in bleating behaviour reflect sex differences in stress coping strategies, with females opting for a more emotional and males a more passive strategy. Indeed, in a previous study involving fear tests (novel arena and novel object tests), female lambs of approximately 24hr of age were found to vocalize more than males, which also stood passively more frequently than females (Averós et al., 2015). Additionally, in the same study female lambs also showed higher social motivation than males. This could have further contributed to the more reactive behaviour seen in females in the present study, as an expression of higher maternal need in females. Previous studies have suggested that female lambs tend to stay in closer proximity with their mothers than males do (Shillito-Walser & Williams 1986). This behaviour is possibly related to the sexual segregation observed in sheep and in many ungulate species that happens when males leave the flock to form male only groups (for a review see Guilhem et al., 2005).

Not unexpected was the fact that offspring from reactive dams bleated more frequently than offspring from dams of calmer temperament. Temperament, measured as vocal and locomotor activity in sheep, is a trait that has a strong genetic basis (Bickell et al., 2009). In sheep, temperament of lambs born to

calm or reactive ewes and later cross-fostered is not affected by the genotype of the rearing mother, suggesting that temperament is mainly determined by genetic transmission rather than behaviours learned from the mother (Bickell et al., 2009). Hence, the observed increase in lamb bleating behaviour in the offspring of reactive ewes is possibly a consequence of the inherited maternal temperament. However, temperament has been described in sheep as being a consistent trait over time (Murphy et al., 1994; Bickell et al., 2009b) and the effects of temperament were not observed at later stages (e.g. isolation test at 24hr) in the lambs in the present experiment. Therefore, it is not possible to rule out the influence of other non-genetic factors on lamb bleating behaviour. Being gestated by a reactive ewe may act as a prenatal stressor itself and result in lambs that react more in stressful situations (i.e. transition from uterus to the outside environment). Alternatively, lamb bleating frequency could be a response to the bleating of the mother as vocalizations are known to be used by the ewe-lamb dyad as a means to identify one another (Shillito & Alexander 1975; Alexander, 1977; Shillito Walser et al., 1981).

In contrast to the previous experiment (Chapter 2), there were no observable interactions of prenatal treatments with lamb sex or birth assistance. The main reason for the lack of a treatment effect on the lamb bleating behaviour in the present experiment may be the different prenatal stressors used. As mentioned earlier in this thesis, the outcomes on the offspring depend not only on the time when stress was applied during pregnancy but also on the species and the type of stressors used. Given that the time when stress was applied (last third of pregnancy) and that the species being studied were the same in both experiments, the type of stressor is most likely the cause for the different findings.

4.4.2 Lamb Behaviour Landmarks

An interaction between prenatal treatment and lambing assistance on latency to stand was found. In the control group, lambs that were assisted to birth were slower to stand than unassisted lambs. The effect of birth assistance on

the delay of behaviour progression of the neonatal lamb has been previously seen (Dwyer 2003; Dwyer et al., 2005). Interestingly, prenatal treatment seemed to have a buffer effect on this since birth assistance did not affect latency to stand in lambs from either the stress or alternative groups. Previous studies in sheep (Hild et al., 2011; Coulon et al., 2014) and goats (Baxter et al. 2016) did not find any effect of prenatal treatment on latency to stand but they also did not find any effects of assistance given to birth overall. However, different stressors were used in these studies, which may be the cause for the different findings. In rats, mild maternal stressors have been shown to have different outcomes than more aversive ones. For instance, mild PNS decreased, whereas more severe PNS increased locomotion (Mychasiuk et al. 2011). This same type of effect is also seen at the maternal level; mild maternal stressors have been reported to improve maternal behaviour by increasing time spent in the nest and nursing (Muir et al., 1985), as opposed to a reduction in those behaviours caused by more aversive stressors (Smith et al., 2004). Additionally, studies in sheep have also found that maternal stress, such as repeated isolation associated to the exposure of a dog during pregnancy, had positive effects on lambs by increasing exploratory behaviour in novel stimulus situations, indicating a reduced fear reaction at later stages in life (Roussel et al. 2004). Likewise, it is possible that the treatments used in the present experiment, both in the stress and alternative groups may have been perceived as mild stressors by the ewes. Consequently, these treatments may have had a positive outcome for the neonatal lamb when in face of a stressful situation (i.e. progressing from intra-uterine to external environment with the additional stress of being assisted at birth). Although it may be easier to understand why treatment applied to the stress group may have been perceived as a mild stressor, the alternative system was designed to represent a low stress environment. However, one particularity of the alternative system was that human contact maintained with the ewes during the experiment was minimal in relation to the control and stress groups (this was a consequence of the design rather than a programmed feature). This decrease in contact may have made these ewes more aware of the human presence in the experimental shed, which may have been perceived as mildly aversive.

Another finding from the present study was that lambs from reactive ewes that were assisted at birth were slower to stand than all other lambs. One possible explanation for this may be that the assistance given at birth (i.e. human contact) may have been more stressful for the reactive ewes than for the calm ewes, which may have lead to longer lambing periods in this group. Longer lambing periods are known to affect lamb early behaviour as a consequence of associated hypoxia periods (Haughey 1991) that impair locomotor activity in lambs (Haughey, 1980). Anecdotally, it was observed that some of the ewes in this study could not cope with being brought into the lambing pen at the front of the experimental pen and had to be released back into the experimental pen. This may suggest that human contact during the lambing period may be an acute stressor, with possible consequences for lambing duration.

As in Chapter 2, parity did not affect lamb latency to stand but females showed a tendency to be faster to stand than males. The observed sex effect on latency to stand has previously been shown in other studies (Dwyer, 2003; Dwyer et al., 2005). Latency to suck was not affected by prenatal treatment or parity but females tended to be faster than males and lambs assisted to birth slower than unassisted ones, following the same trend as latency to stand. This is in agreement with results from previous studies which have shown that lambs that are quicker to stand are also quicker to suck successfully (Dwyer & Morgan 2006).

4.4.3 Lamb Body Temperature

Lamb body temperatures measured at 30 min were not influenced by any of the investigated factors. However, at 2hr after birth a difference in temperatures was seen in the stress group, with lambs that had been assisted at birth having lower temperatures than unassisted lambs. Up until 5hr after birth the major cause of hypothermia is excessive heat loss (Eales et al. 1982), which may be influenced by lamb surface area, the weather conditions, lamb behaviour after birth (standing and sucking quickly - Dwyer & Morgan 2006) and the dryness of the lamb (maternal grooming behaviour;

(Lent 1974; Alexander 1988; Nowak et al., 2000). Birth assistance is usually affected by lamb weight: heavier lambs have a higher probability of needing assistance than lighter ones (in the present study this was also true – data not shown). Being bigger, heavier lambs have a lower surface area to volume ratio which helps them in maintaining heat more easily than smaller lambs. Additionally, maternal behaviour was suggested to be increased in mothers from the stress group that had been assisted in parturition (see section 4.4.2 Lamb behaviour landmarks). Hence, both these factors are unlikely to be contributing to the present finding. Early behaviours between lambs assisted to birth and unassisted lambs in the stress group did not differ either. However, there was an overall tendency for assisted lambs to be slower to first suck. In the present study (the number of sucking bouts or time spent sucking was not assessed). One possible explanation for the lower temperatures in assisted lambs from the stress group could be that lambs had less sucking bouts in comparison to the unassisted lambs in the same group during the first two hr after birth.

At 24hr, female lambs had higher temperatures than males in the control group. As opposed to findings from the previous year (Chapter 2), in the present experiment, females in the control group presented higher temperatures than males. Previously, the sex difference in temperature was given little importance as the difference in temperature was only of 0.1°C and it was considered to be of little biological meaning for the lamb. Here, the average difference in temperatures was ~0.6°C, which has been previously shown to have an impact on behaviours related to lamb survival such as latency to stand and suck (Dwyer & Morgan 2006). The sex difference in lamb body temperatures suggests that males and females have a different ability to maintain body temperature, although previous experiments have not found such sex differences (Dwyer & Morgan 2006; Miller et al., 2010; Baxter et al., 2016). However, because results from this experiment vary in the opposite direction from the experiment in Chapter 2, it is difficult to understand which sex would be at an advantage in relation to the other.

Another finding at 24hr was that offspring of multiparous ewes presented higher temperatures than of primiparous ewes but only in the alternative

group. One factor that may influence lamb body temperature, particularly at this stage, is lamb ability to suck (as by 24hr the lamb is dry and has already stood up – two other factors that contribute greatly for body temperature). It is known that multiparous offspring have better maternal care and display less rejection behaviour directed at sucking attempts, which could therefore aid the lamb in maintaining its temperature. However, if parity was the only factor explaining the observed difference in temperature then this would have been visible in the control and stress groups as well. Thus, in addition to the parity effect, as suggested before, the different diet fed to the mothers of the lambs in the alternative group may have affected lamb thermoregulation capacity by affecting heat production.

Heat production is generated by non-shivering thermogenesis, a process that involves oxidation of brown adipose tissue. This process is initiated by the mitochondrial uncoupling protein (UCP1) (Symonds et al., 1995) and controlled by triiodothyronine (T_3) (Dauncey 1990; Brent 1994; Schermer et al., 1996). In large mammals, the increase in UCP1 around the time of birth is also dependent on other endocrine stimulatory factors including cortisol, prolactin, leptin and catecholamines (Symonds et al. 2003), which can also be found in milk. Hence, factors directly affecting the thermogenesis process (availability of T3 and UCP1) or milk composition may contribute to differences in temperatures observed in the offspring.

Indeed, a study using nutrition restriction during pregnancy reported lower levels of T_3 in lambs born to undernourished mothers (Rooke et al., 2010). Although in that study the decrease in T_3 did not translate into a difference in lamb temperature, it is important to note that nutritional restriction was applied only during early-mid pregnancy. Additionally, inadequate nutrition during late pregnancy and ewe lower parity have been shown to reduce colostrum and milk production (Banchero et al., 2015). Ewe parity has also been shown to affect milk quality (Sevi et al., 2000). For example, in one study primiparous ewes had significantly lower milk protein, casein and fat contents compared to multiparous ewes (Sevi et al., 2000). As a consequence, delayed suckling may lead to exhaustion of energy reserves within 6 hr of birth, resulting in depressed heat production (Dwyer et al.,

2016). Additionally, since endocrine stimulatory factors of thermoregulation including cortisol, prolactin, leptin and catecholamines can be found in milk, milk quality is also an important factor for heat production.

Data from the ewes in the alternative group (Appendix 2) suggest that nutritional requirements may not have been met towards the end of gestation. Hence, the observed differences in temperature may have been caused by differences in the heat production mechanism between offspring from primiparous and multiparous ewes, together with external factors involved in that mechanism (i.e. colostrum), which were then accentuated when nutritional pressure was added.

Alternatively, other factors affecting ewe and lamb behaviour, not related to undernutrition, may have affected lamb body temperature. As mentioned above, in the alternative group, the level of human contact during the experiment was lower than in the control and stress groups. This lower contact may have affected the way these ewes perceived human presence during the first 24hr after lambing. This is a crucial period during which a series of behaviours that contribute for the ability of the lamb to maintain body temperature occurs (i.e. standing and sucking, maternal grooming). During this period there were a series of human interventions (temperature measurements, moving ewe/lambs to colostrum pen and collection of colostrum sample, moving ewe/lambs to post partum pen, ewe recognition of the lamb test at 6hr, and weighing and marking of the lambs) that disrupted ewe/lamb behaviour and may have been interpreted as more aversive by the ewes in the alternative group, which were less used to human proximity than ewes in the control and stress groups. Further, this may have been differently experienced by multiparous and primiparous ewes. Indeed, if this is true, it is logical for the lambs from primiparous ewes in the alternative group to have lower body temperatures, since the level of novelty of all the events for these ewes would have been higher than for multiparous ewes. Hence, human presence/contact would have been more aversive to this group of animals.

Interestingly, by 48hr the parity difference in temperature between lambs from multiparous and primiparous mothers becomes evident as a general effect, with lambs from multiparous ewes showing greater temperatures

independently of the prenatal treatment. Again, this may refer to differences in maternal care where lambs from multiparous ewes would be more frequently accepted to suck but also maintaining closer contact to their dams, aiding in the maintenance of body temperature. Nevertheless, as mentioned above, milk production and quality are still two factors that cannot be excluded as causes for the observed results.

The fact that an interaction between parity and treatment is observed at 24hr, but only an effect of parity is observed at 48hr, suggests that the differences in the thermoregulation mechanisms between offspring from primiparous and multiparous ewes may be accentuated by inadequate nutrition.

4.4.4 Lamb Body Weight

Low lamb birth weight has been associated with lambs that are slower to stand and suck, and worse at maintaining body temperature (Dwyer & Morgan 2006). Hence, these lambs also have a lower likelihood of survival. Maternal stress during pregnancy has been shown to have different outcomes for birth weight of the offspring depending on the type of stressors used. Indeed, Fride & Weinstock (1984) reported increased birth weights after predictable prenatal stress and a decrease in birth weight after unpredictable prenatal stress in rats. Similarly, the time when maternal stress is applied and stress severity have variable outcomes for weight (lamb - Roussel et al., 2004; Roussel et al., 2008; piglet - Jarvis et al., 2006). In the present experiment, analogous to results from the 2014 experiment (Chapter 2), lamb weight at 6hr after birth did not vary between stress and control groups. However, lower birth weights were found in lambs from the alternative group.

Ewes in the alternative group were fed silage ad libitum as opposed to the concentrate and hay fed to the control and stress groups. The results from ewe weights (Appendix 2) suggest that this difference in the feeding system may not have been the most adequate for the ewes, as this group presented lower weight and ADG in the pre-lambing period when compared to control and stress ewes. In previous studies, undernutrition of the ewe during the last

third of pregnancy has been shown to be a major factor contributing to a decrease in lamb weight (for a review see (Rooke et al., 2015), which suggests that the differences in weight observed here may have been a consequence of a certain degree of undernutrition in the ewes in the alternative group caused by the different feeding system in this group.

Alternatively, the lower birth weights in the alternative group may not have been a direct consequence of undernutrition experienced by the ewe but rather something related to the combination of the conditions in the alternative husbandry system that affected placenta/fetal growth. Although ewes in the alternative group had lower weight and ADG, body condition score (BCS) and beta-hydroxybutyrate (BOHB) were not different from the control and stress ewes (Appendix 2). These results could suggest that despite being lighter in weight, these ewes were not experiencing undernutrition. Given the diet in the alternative group was different in composition and also fed differently (ad libitum vs specific times of the day in control and unpredictably in the stress groups), different gut mean retention time (MRT - the average time available for digestion of a food) may have contributed to the observed differences in ewe weights. For example, the particle size of forages is one factor that has been shown to influence MRT in the reticulorumen (the first chamber in the digestive tract of ruminant animals), with lower MRT for pelleted compared to chopped versions (Laredo & Minson 1975). Consequently, particle size also influences voluntary dry matter intake. Food composed by bigger particles limits intake as a result of restricted flow of digesta through the gastrointestinal tract (Dulphy & Demarquilly, 1973; Dulphy et al. 1975; Deswysen et al. 1978). Given the difference in particle size in the feed used in the present study (pelleted in control and stress groups vs chopped in alternative group), ewes in the alternative group may have been eating below their energetic requirements. Additionally, at 48hr post-partum, not only did ewes in the alternative group continue to have lower weights but they also had lower BCS in comparison to both the control and stress groups. This seems to suggest that perhaps undernutrition was occurring to some degree from late gestation.

Females had a lower ponderal index (PI) than males. PI is a measure of body proportionality of a lamb, calculated as the relationship between mass and length (weight/(crown-rump^3)). This difference in PI could be reflecting the general finding that females have lower weights than males. However, in the present study females and males did not differ in their weight at 6hr or in crown-rump length. Further, another study in sheep also failed to find sex differences in PI, although female lambs were lighter than their male counterparts (Dwyer et al., 2005). Interestingly, in a meta-analysis looking at the use of PI as an indicative measure of late-gestation fetal growth in sheep, it was concluded that PI was not a very accurate measure (Gootwine 2013). PI was only useful as an indicative measure of late-gestation fetal growth in a few studies. Thus, it is possible that the present result is reflecting this inadequacy of PI as an index for leanness of a lamb.

In the present experiment lamb PI was also affected by an interaction between parity and ewe temperament. Lambs born to multiparous calm ewes had a lower PI than lambs born to primiparous calm ewes and to multiparous reactive ewes. This is an unexpected finding since lambs from multiparous ewes usually present a higher PI than lambs from primiparous ewes (Dwyer et al., 2005).

4.4.5 IgG Concentration In Plasma

Passive immunity in the lambs in the present study was affected by treatment applied during pregnancy. The alternative group lambs presented lower IgG plasma levels in comparison to the control and stress groups. IgG levels were also lower in offspring from primiparous ewes than in offspring from multiparous ewes. A tendency for lambs born to reactive ewes to present lower levels than lambs born to calm ewes was also noted.

In the experiment from the previous year (Chapter 2) no differences between treatments were found. The justification for this was the type of treatment used to impose stress on the pregnant mothers. Alterations in IgG plasma levels have been noted by others as a result of the prenatal treatment (in piglets Tuchscherer et al., 2002); however, it has been suggested that one of

the most important factors affecting passive immunity is the type of stressor used. Prenatal psychological stressors in rodents were shown to be less effective in decreasing offspring antibody levels than physical stressors (for a review see Veru et al., 2014). Accordingly, there were no differences between lambs from the control and stress groups which, again, may be linked to the type of prenatal stressor used in this experiment. Interestingly, the alternative group showed the lowest levels of plasma IgG, even lower than what is usually accepted as necessary to assure passive immune transfer (<15 mg/mL - Alves et al., 2015). Although the alternative prenatal treatment was not aimed at imposing stress on the pregnant ewes, it is possible that the husbandry system used in this group had some negative outcomes for the lambs.

Given that IgG in the new-born is obtained solely by the ingestion of colostrum, any factors affecting milk ingestion may mediate plasma IgG concentration in lambs: quantity and quality of colostrum, the amount ingested by the neonate and the ability of the neonatal gut to absorb immunoglobulins.

The effects of undernutrition on these variables are inconsistent. There are studies reporting no effects of late pregnancy undernutrition on the concentration of IgG in lamb plasma at 24hr of age, although lamb birth-weight was reduced (Khalaf et al., 1979). Others have reported increased IgG status and birth weight when undernutrition was applied in early pregnancy (Muñoz et al. 2008). Undernutrition from mid to late gestation was reported to increase IgG concentrations at 24hr but decrease birth-weight (Hammer et al. 2011). Micronutrient composition of the diet can also affect serum IgG in the lambs (Rooke et al. 2008). For example, excess intake of minerals (mainly iodine content in the mineral mix) has been shown to impair immunoglobulin absorption in lambs, although colostrum IgG content remained unaltered (Boland et al. 2005), whereas an increase in lamb plasma IgG concentrations has been observed following vitamin E supplementation before lambing (Gentry et al. 1992). Undernutrition during pregnancy has also been shown to decrease ewe colostrum yield (Mellor & Murray 1985; Hall et al. 1992; O' Doherty & Crosby 1996) and to influence

behaviour of the mother (i.e. udder refusals) and the lamb (i.e. latency to suck) (Dwyer et al. 2010) that may directly influence the amount of colostrum ingested by the lamb. The volume of colostrum produced by the ewe and ingested by the lamb is positively correlated with the concentration of IgG in lamb plasma, since larger volumes of colostrum are associated with larger amounts of immunoglobulins (Shubber et al. 1979a, 1979b).

It is not possible from the present findings to know the exact cause for the lower levels of IgG in the lambs from the alternative group. Nevertheless, the results seem to suggest that the observed differences may be related to the different diet fed to the ewes in this group. The lower body weight and ADG in late gestation and lower body weight and BCS in the ewes (see Appendix 2) from the alternative group seem to suggest some degree of undernutrition in this group. Additionally, lambs in this treatment group also had lighter body weight at 6hr of life, a consequence often seen in offspring from undernourished ewes during late gestation (Rooke et al 2015). Alternatively, other factors affecting ewe and lamb behaviour, not related to undernutrition, may have affected the volume of ingested colostrum and consequently decreased the IgG concentration in lamb plasma. As previously mentioned, the decrease in human contact may have made the ewes in the alternative group more aware of the human presence during the first 24hr after lambing, a crucial period for the absorption of IgG (Smeaton & Simpson-Morgan 1985). During this period there was a series of human interventions that may have been interpreted as more aversive for the ewes in the alternative group. Consequently, these interventions may have disrupted ewe/lamb interactions with a direct impact on lamb accessibility to the udder and ingestion of colostrum.

Similar behaviour alterations to those mentioned above may be behind the difference in IgG concentration found between offspring from primiparous and multiparous ewes. Since a poorer establishment of the ewe-lamb bond is found most frequently in primiparous ewes (Dwyer et al. 2003, Muñoz et al. 2009, Rooke et al. 2010), behaviour alterations may have been the reason for primiparous offspring to present lower levels of IgG, although these levels were still above those necessary to assure passive immune transfer.

Additionally, differences in the quantity and quality of the colostrum may have contributed to this result, as lower parity ewes usually have less effective lactations than multiparous ewes (Abd-Allah 2013).

4.4.6 Recognition Test

Overall, lambs were not able to identify their dam from a distance but lambs from multiparous ewes chose to approach their dam first more than chance levels. Latency to reach the dam was not affected by any of the main factors. Proportion of time spent with the dam, alien ewe or close to ewes did not differ between treatment groups, but females that were assisted to birth spent less time with the dam than males in general and than unassisted females. Interestingly, overall lambs did not seem to be attracted to the ewes, as the time spent close to ewes did not differ from random. However, from the time lambs spent close to the ewes, more time was spent close to the dam than the alien ewe in the control and stress groups but not in the alternative group.

In this experiment lambs did not show a clear preference to approach the dam first over the alien ewe from a distance, with only 59.1% going to their dam first in the control and stress groups and 47.1% in the alternative group. These results are analogous to the results obtained in the same test in Chapter 2 and demonstrate the lambs' natural low ability to discriminate their dam from another ewe at a distance before reaching the third day of age (for a review see Nowak et al. 2011). However, parity of the ewe affected lambs' first choice, with lambs from multiparous ewes choosing to approach their dam first more than random. Although, overall, lambs did not seem to show the ability to recognise their dam from a distance, this latter result suggests that parity may influence this behaviour. This may be in part related to a difference in maternal behaviour in multiparous compared with primiparous ewes. It is well established that primiparous ewes show poorer maternal behaviour than multiparous ewes (O'Conner et al., 1992; Dwyer et al., 2003; Ekiz et al., 2007; Otal et al., 2009). Previous research using the same type of test suggests that lambs prefer to spend more time close to ewes that show higher acceptance maternal behaviours (low pitched vocalizations, lowered

head, sniffing, less activity) (Terrazas et al. 2002) and that ewes change their behaviour when lambs approach them (Nowak & Lindsay 1992), displaying more acceptance behaviours in the case of the dam and the opposite in the case of the alien ewe (Nowak and Lindsay, 1991). Although this presumes that lambs are in close contact with the ewes, still, it is possible that multiparous ewes were faster at identifying their lamb before it reached the contact zone and hence started showing acceptance behaviour, while primiparous ewes would have kept emitting high pitched bleats. Therefore, a difference in behaviour between multiparous dams and alien ewes would have been clearer than in the case of primiparous dams and alien ewes. Ultimately, this may have affected lambs' first choice and may explain the present results. Alternatively, lambs from multiparous ewes may have been better at recognising their mother and a combination of both ewe and lamb behaviour may have contributed to the better performance observed in these lambs.

Unexpectedly, lambs also did not seem to be attracted to the ewes, spending no more time in close proximity to the ewes than what would be predicted by chance. As previous studies show, by the age of 24hr lambs are attracted by ewes, spending more time close to ewes than away from them in this type of test (Nowak 1991; Terrazas et al. 2002; Bickell et al. 2009b). One factor that may have contributed to this was the design of the experimental setting. Because ewes were exhibiting escaping behaviour (trying to jump out) when inside the holding pens an extra fence (made of wooden pallets) was attached to the front gate of the holding pen to make it higher and not possible to jump over. This extra fence may have impaired lamb visual ability to identify the location of the ewes and therefore decreased its attractiveness to the ewes. Indeed, the orientation response in lambs at this age relies on both visual and auditory cues as lambs show difficulties in finding their mother in a two-choice test, where access to visual or hearing cues is impaired (Nowak 1991). Nevertheless, from the total time lambs spent close to the ewes they preferred to be closer to their dam than to the alien, suggesting that once in close quarters lambs are able to identify their mother. However, this was true only for the control and stress groups, as lambs from

the alternative group did not stay close to the dam more than to the ewe. One reason for this difference may be the different diet fed to the ewes from the alternative group. Past studies show that a diet restriction imposed during pregnancy impairs the ability of the lambs to discriminate their dam (personal communication Dr Tamsin M. Coombs), possibly due to an altered emotional response to stressful events (Strupp & Levitsky 1995), an impaired ability to form memories (Erhard et al. 2004) or to integrate different stimuli (Cravioto & Arrieta 1986). Although the different diet fed to ewes in the alternative group was aimed at fulfilling the dietary requirements of the ewes at each stage of pregnancy, it is possible that the diet was not adequate or that ewes were not consuming the necessary amount to fulfil their metabolic needs. Lamb weight measured at 6hr after birth further suggests this to be the case, as lambs from the alternative group were lighter than the control and stress lambs. Undernutrition throughout gestation has also been shown to reduce the expression of maternal care in sheep (Dwyer et al. 2003) and lamb orientation response during the first day of life relies mainly on the acceptance behaviours displayed by the dam (Terrazas et al. 2002). Hence, it is possible that a combination of altered ewe and lamb behaviour contributed to this result in the lambs from the alternative group.

Another explanation for the observed results may not have a direct prenatal nutritional cause. Lambs in the alternative group had lower levels of IgG in their plasma. The previous section highlighted the lower human contact during pregnancy in the alternative group as a possible cause for altered ewe/lamb behaviour and consequent decrease in IgG levels caused by a lower ingestion of colostrum. It is known that the ingestion of colostrum/milk is important for the establishment of the ewe/lamb bond (Goursaud & Nowak 1999). A weaker ewe/lamb bond may have altered ewe behaviour during the recognition test in comparison to the control and stress groups, showing lower acceptance behaviours which would consequently be less attractive for the lamb. This would also be true from the lamb perspective, as suckling and ingestion of colostrum plays a role in the attachment of the lamb for its mother (Goursaud & Nowak 1999). Hence, in addition to a poorer maternal

acceptance behaviour display it is possible that the lambs in the alternative group were less attached and, therefore less attracted to the ewes.

Another interesting finding from this test was the fact that females that were assisted to birth spent less time close to their dam and close to ewes in general than unassisted females but males were not affected, suggesting that assistance given to birth influences the ability to discriminate the dam and the attractiveness to the ewes depending on lamb sex. However it is possible that these differences are a consequence of the small sample size, as only five males and five females of the ones tested for recognition of the dam were assisted to birth in the present study.

Previous research has shown that lambs that go through difficult deliveries are slower to perform all behaviour landmarks (e.g. sucking, which is important for the establishment of the lamb's preferential bond to its dam (Nowak et al., 1997) and are also less active during the first 3 days of life (Dwyer 2003). Extended deliveries have been associated with injuries to the fetal central nervous system due to haemorrhage or asphyxia (Haughey 1991), which can impair locomotor activity and sucking in lambs (Haughey 1980). Although birth assistance did not influence lamb activity during the recognition test, there was indeed a tendency for lambs that were birth assisted to be slower to suck. Ewe maternal behaviour is also known to be affected (an increased latency to groom the lamb (Dwyer et al. 2001) by the duration of parturition, which may influence lamb behaviour and interfere with the establishment of the ewe-lamb bond, further contributing to this result. Why this has affected females and not males in the present study is not clear, particularly since the literature says that females are more resistant to perinatal insults (e.g. asphyxia and consequent hypoxia) than males (Simon & Volicer 1976; Boksa & El-Khodor 2003; Ingemarsson 2003). It is also unclear why the present results differ from the ones in the same test in the previous year, where birth assistance did not affect lamb behaviour. However it is possible that these differences are a consequence of the small sample size, as only five males and five females of the ones tested for recognition of the dam were assisted to birth in the present study, compared to nine of each in the previous year. Alternatively, lambs that were assisted during this

experiment may have gone through more prolonged parturitions than in the previous year as the system used to define the degree of assistance given did not account for duration of parturition. Hence, although two ewes are marked as having been partially assisted, the duration of parturition may have been different between them.

Parity and treatment had an interacting effect on lamb activity during the recognition test as lambs born to multiparous and primiparous ewes showed differing activity levels in the alternative group, but not in the other two treatments. Lambs born to multiparous ewes had lower levels of activity compared to those born to primiparous ewes. Notably, in the multiparous group two lambs did not leave the starting pen, showing zero activity. Factors related with the different diet fed to the alternative group may explain this result. As mentioned earlier, inadequate nutrition during pregnancy may have detrimental effects on the offspring as well as on maternal care (Dwyer et al. 2003). One of these repercussions is a decrease in activity in stressful situations (personal communication Dr Tamsin M. Coombs; Abecia et al. 2014), possibly used as a passive coping mechanism for fear. Why this affected multiparous and not primiparous offspring remains unclear. In normal conditions nutrition during pregnancy is one of the factors that may affect lamb behaviour in post natal life since it can restrict intra uterine growth, which impairs both vigour and the ability to carry out complex behaviours (Cock et al. 2001). Nutrition of the fetus is, thus, related to the sufficiency of the placenta (Robinson et al. 1999, Greenwood & Bell 2003). Multiparous ewes are known to have more efficient placentas (Ocak et al. 2013) so their offspring should be at lower risk of being affected in their development in the case of inadequate nutrition. Nevertheless, multiparous offspring have also been reported to be more fearful compared to primiparous offspring in another PNS study in sheep (Coulon et al. 2011).

Overall, treatment affected time spent close to the dam and, depending on parity, the activity of the lambs. However these differences were only observed in the alternative group as the control and stress groups remained the same. The different outcomes for the alternative group have been addressed earlier. In the past, studies have shown different outcomes for

control and PNS lambs in this type of test, indicative of increased emotionality in the case of prenatally stressed lambs which show a more pronounced preference for the mother (Coulon et al. 2014). The results from the present study do not show this and are in agreement with the ones from the previous year (Chapter 2), where no detectable differences were found between PNS and control lambs during the recognition test. It is possible that the prenatal treatments used were not stressful enough to have an effect on the fetal development of the lambs.

4.4.7 24 Hr Isolation Test

During the isolation test lambs were assessed for their activity, bleating and escape attempts. Bleating and activity did not show any differences between treatment groups, sex, assistance given at birth or ewe temperament. However, lambs from the alternative group had a decreased escaping rate than lambs in the stress group and a tendency for a decrease in relation to the control group. Parity also affected escaping rate, with offspring of primiparous ewes attempting to escape less than multiparous ewes.

The absence of behavioural response differences between the stress and control groups in the present study is possibly related to the maternal stressors used. Previous studies where a period of isolation was used to test lamb behavioural response in a stressful situation have found different results depending on the type of maternal stressor used. Roussel et al. (2008) reported a tendency for one month old lambs to jump less in an isolation test when born to mothers that had been repeatedly transported during pregnancy in relation to control lambs, but this difference was not present when the maternal stressor was repeated isolation. In this same study, no differences in vocalizations were found.

In addition to above, in the present study the alternative treatment seems to have affected lamb behaviour the most by decreasing lamb attempts to escape. Again, one possible explanation for this result may be related to the diet fed to the alternative ewes, which was suggested above not to be the most adequate. Previous studies where undernutrition was used as a

maternal stressor have found different effects on lamb behavioural response to isolation depending on the time when nutritional restriction (NR) was applied. Results have shown no effects of treatment when NR was applied during the first two thirds of gestation (Erhard et al., 2004 – lambs tested at 18months of age) or during the first or second third of gestation (Simitzis et al. 2009 - ambs tested at 2, 3, 4 and 5 months of age) but NR during the periconceptional period decreased the escaping attempts (Hernandez et al. 2010 – lambs tested at 4months of age). Social isolation in sheep strongly stimulates cortisol release by immediate and maintained activation of the HPA axis (Niezgoda et al. 1987, Minton & Blecha 1990). Moreover, the effects of maternal undernutrition on the offspring also seem to be age-dependent. For instance, in the offspring of NR sheep for the first 70 days of gestation, HPA axis activity is reduced during fetal life (128 days of gestation) but increased in 3 month old lambs (Hawkins et al. 2000). Hence, both age of the lamb when tested and time when treatment was applied may explain the different results in the present experiment.

Alternatively, decreased attempts to escape may have an underlying nutritional cause but may not be directly related to ewe undernutrition during gestation. These lambs had lower levels of IgG in their plasma at 24hr and one possible explanation for this was related to postnatal events. Mothers in this group may have found human presence/interaction during the period around and after lambing more aversive than in the other groups and this may have affected their behaviour towards the lamb, making it difficult for the lamb to access the udder. The consequences of this may have been a lower quantity of ingested colostrum by the lamb than the necessary to meet their energetic requirements. If energy requirements were not being met then lambs may have shown decreased levels of vitality, expressed as decreased attempts to escape. Nevertheless, it is intriguing why this difference is evident for the escaping attempts but not for the activity levels.

A parity effect was also observed in lamb behavioural response to isolation. Lambs from primiparous ewes displayed decreased attempts to escape in comparison to multiparous offspring. One factor that may have contributed for this was lamb early life experience related to maternal care. It is well

established that primiparous ewes exhibit poorer maternal care and are more often separated from their offspring compared to multiparous ewes. (Romero et al. 2009) proposed a theory where early life stress, in the form of a mild challenge to the infant, can be beneficial for his/her development by decreasing stress reactivity, anxiety and fear (amongst other benefits). In fact, results from the experiment done by (Meaney et al. 1991) suggest that mild stress imposed by brief maternal separation is beneficial for rat pups as it decreased stress reactivity in adulthood (reduced HPA axis activation and fear/anxiety levels). Hence, differences in maternal care related to ewe parity may have influenced the way lambs cope with a stressful situation involving separation from the mother. Alternatively, or in conjunction with above, due to some other prenatal mechanisms related to parity (yet to be explained) multiparous offspring become more reactive to stressful situations than primiparous offspring. Indeed, in an earlier experiment in sheep using aversive handling as a prenatal stressor, lambs from multiparous ewes also showed more attempts to escape when compared to lambs from primiparous ewes, although no treatment effects were found (Coulon et al. 2011).

4.5 CONCLUSION

The experimental work developed in this chapter was aimed at designing three different husbandry systems addressing the normal conditions in which pregnant ewes are kept. Each system was designed to pose different levels of stress for ewes: the control group aimed at mimicking the standard management in the UK, the stress group a higher stressful environment and the alternative group a lower stress environment. It was hypothesised that stress treatment would negatively impact the development of the lambs and that these impacts would be absent in the alternative and control groups. The alternative lambs were also hypothesised to have better outcomes than in the stress and controls.

As a general finding, the husbandry system applied to the alternative group did not seem to work in favour of the lambs. It is not possible to know if the

ewes in the alternative group were less stressed than the ewes in the other groups as the effects of the different diet fed in the alternative group may have masked possible positive outcomes of the design of the husbandry conditions in this group. Additionally, the post-lambing period may have been more stressful for the ewes in the alternative group due to human presence, as a consequence of decreased human contact during pregnancy as a result of the experiment design of this group. Results seem to suggest that the main reason for the negative outcomes observed in the lambs from the alternative group were related to the nutritional system adopted, as lambs from these ewes showed low body weight and decreased circulating IgG, outcomes that are usually related to inadequate nutrition of the ewe. Additionally, inadequate nutrition during pregnancy has also been previously shown to affect lamb behaviour. Lambs from the alternative group also showed altered behaviour (decreased escaping behaviour) when submitted to a social isolation test and were the only group in which levels of activity during the recognition test were affected by parity. If on one side, decreased escaping behaviour and activity are interpreted as lower fear, on the other, such decreases in behavioural response could also mean overall decreased energy, given the fact that alternative lambs had lower weights and IgG. Although silage quality and quantity was calculated to meet the ewes' nutritional needs, it is possible that ewes were not eating the amount required to maintain their metabolic needs. As the gestation progresses, fetus growth leaves less space available for other internal organs which may decrease the ewe intake capacity. This is a problem that would possibly not pose an issue in the control and stress groups as the quantity of concentrate needed to meet the ewe nutritional requirements would be less than with silage (1kg concentrate vs 5kg of silage/head/day).

Although the results from both the lambs and ewes (weight, BCS, ADG; for ewe data see Appendix 2) both seem to suggest that the nutritional requirements of the ewe were not being met, other post-gestation causes such as increased ewe aversion to human contact/presence, which were a consequence of the experimental design, can not be ruled out as at least contributing factors for the observed outcomes in the lambs.

As in the previous year (Chapter 2), average body temperatures, which are a major factor contributing to lamb survival, did not reach hypothermic values. Effects of treatment on this outcome were always expressed as a function of some other factors such as assistance given to birth, parity and sex. Differences in temperatures were observed at 2hr and 24hr and always within but not between treatment groups. For example, at 2hr assisted lambs had lower temperatures than unassisted lambs within the stress group but these temperatures were not different from control or alternative lambs. If here, PNS seems to have accentuated the difference in temperature between lambs assisted and unassisted at birth, which was otherwise not seen in the control or alternative groups, at 24hr it eliminated the temperature advantage of females over males seen in the control group. The present results add to other results presented in Chapters 2 and 3 suggesting that prenatal treatment interacts with postnatal factors and lamb intrinsic (sex) or external (ewe parity) factors; however, those interactions are not constant over time, at least for lamb temperature, making it difficult to understand which or what combination of factors influence the ability to maintain body temperature during the first two days of life. Although these differences in temperature were absent at 48hr, the results still suggest that PNS effects need to have other factors taken into account, and that some groups of individuals may be at greater risk of potential hypothermia than others. It is important to point out that these lambs were under human care 24/7 and that differences in temperatures found here may become a risk factor for hypothermia in lambs born in field conditions.

Also important from a lamb survival point of view are factors such as birth weight, latency to suck, the acquisition of passive immunity and the ability to recognise the mother. None of these factors was affected by the maternal stressors used in this experiment. However, latency to stand, which is known to be higher in lambs that are assisted to birth (a fact that the present results confirm), was not affected by assistance given when lambs had been prenatally stressed. Again, as in the previous chapters, these results suggest that the impacts of PNS may seem absent but are subjected to being triggered by post-natal events.

Overall, the present results suggest that maternal treatment had only a minor impact on the development of the offspring as shown by the lack of direct PNS effects on the studied outcomes. However, these effects seem to build upon intrinsic traits of the individual (sex) and/or maternal-related factors (parity), highlighting the importance of taking this into consideration when carrying out such studies.

It is worth noting that only two aspects of normal husbandry conditions were being tested here. In real life circumstances there will certainly be farms in which ewes will have more encounters with dogs, will be handled often for procedures, and possibly be moved and reintroduced into new groups as a consequence of that. It seems very plausible, in light of the present results, to consider that in such circumstances some groups of lambs will be more susceptible to the outcomes of prenatal stress than others, and that being so, the consequences may be more drastic than the ones observed here.

CHAPTER 5

Maternal housing systems as a source of prenatal stress in sheep: impacts on the immune system, growth and the fear response of pre-pubertal lambs.

5.1 INTRODUCTION

In the previous chapter, three different husbandry systems designed to impose different levels of stress during late pregnancy in sheep were investigated for their potential effects on the development of the lamb during its first 48hr of life. In the present chapter, the ability to mount an acute immune response and growth were evaluated in those same lambs at later stages of life. Both the immune system (for a review see Merlot et al. 2008) and growth (Roussel et al., 2008) have been shown in other studies to be affected by PNS. Another outcome that has also been previously shown to be affected by PNS and that is dependent on the age of the individuals is behaviour reactivity (Roussel, et al. 2004; Viérin & Bouissou 2003). Hence, the objective of this study was to investigate the effects of prenatal treatment on immune response, growth and behavioural responses to stressful/fear in lambs at later stages of life.

There has been extensive research assessing behavioural and physiological fear responses, particularly in rodents using different fear paradigms. As a result, many of these fear tests have later been adapted for use in other species, such as livestock (Forkman et al. 2007). One fear paradigm that has previously been used in rats to test the effects of PNS on behavioural fear responses is the fear potentiation test. Fear potentiation is a model widely used in the rodent literature (Korte & De Boer 2003) and it involves exposing the individual to a threatening stimulus (TS) (e.g. predator) and later assessing the fear/anxiety response in a stressful/anxiogenic situation (e.g. plus-maze). Animals exposed to the TS will display enhanced fear behaviour (i.e. fear potentiation) when tested, in comparison to animals that have not been exposed to a TS before testing.

The fear potentiation model has also been tested in sheep (Drake 2006) using a dog as a threatening stimulus and isolation as the stressful test situation and the results seem to suggest that sheep also respond to this type of paradigm. However, the effects of PNS in this type of fear paradigm were not evaluated. Nevertheless, in rats, PNS has been shown to potentiate the startle response when compared to animals that had not been exposed to PNS (Griffin 2001, Meyer & Keele 2014). Using a fear potentiation model

facilitates investigation of the behavioural response to a perceived threat under an increased state of anxiety (Korte & De Boer 2003). Additionally, previous studies have shown that a single exposure to an unavoidable stressor is enough to enhance an animal's fear response (Zangrossi et al. 1992). In view of these findings, PNS may also enhance fear potentiation in lambs.

Similarly to the fear potentiation paradigm, another test that has been widely explored in the rat literature is pain memory association with a specific odour. The olfactory system has direct access to the emotional centres of the brain and seems to be specifically designed for allowing emotional learning and memory. These studies usually involve a fear conditioning paradigm where an odour (CS) is paired with a foot shock (US). The reaction to the later presentation of the odour alone invokes a pain memory and triggers a fear reaction (Sacco & Sacchetti 2010) and avoidance of the odour in a two-odour choice Y-maze (Sullivan et al. 2000, Moriceau & Sullivan 2006). In sheep, odour conditioning has also been studied (Manuelian et al. 2015) and while this study involved aversion to food rather than a painful stimulus, it was demonstrated that sheep were able to associate the presence of a specific odour with an unpleasant event (i.e. post-ingestion discomfort caused by the ingestion of food) (Manuelian et al., 2015). During early life, as part of the husbandry routine, lambs are exposed to procedures that are known to be painful, such as tail docking (Molony & Kent 2007). From the findings above, it was hypothesized that pairing tail docking with a specific scent would create a fear memory, associating the painful tail docking experience to that scent, which might be enhanced by PNS.

The present study continues from the previous chapter, with the aim of understanding the effects of prenatal stress on the immune response, behaviour and growth in lambs at later stages of life. For this purpose, the potential effects of PNS were assessed on the lambs' capacity to mount an innate immune response to vaccination, and also on the behavioural response to a fear potentiation paradigm and pain-scent association memory. These two behavioural tests involved exposing lambs to isolation and follow up on the first isolation test done at 24hr of life. This series of isolations was

designed to test if the lamb behavioural reactivity to a stressful situation was different depending on the prenatal environment but also to understand if that reactivity changes as the lamb progresses in life. Additionally, the effects of the alternative husbandry system (see Chapter 5) on the same offspring outcomes were assessed to investigate the possible advantages of such a system. Possible effects of sex, parity and temperament of the ewe were also investigated as some of the outcomes evaluated in the lambs may be affected by these factors (see Chapter 2 Introduction for the importance of sex and parity, and Chapter 4 for temperament).

5.2 METHODOLOGY

5.2.1 Ewe experimental procedures

Ewes used in the present chapter were the same ewes used in the previous chapter. Maternal treatments applied during late pregnancy are described in Chapter 4. After the first 48hr of life of the lamb, all ewes and lambs were released onto pasture and managed as a single flock. These animals were brought into the experimental shed only during periods of data collection, after which they were again released into the field. Periods of data collection will be described below.

5.2.2 Offspring experimental procedures

The lambs used here were the same ones tested during the neonatal period (see Chapter 4). Lambs were weighed at 9, 14 and 19 weeks of age. In addition, three experimental procedures were carried out as summarised in Table 5.1. At nine weeks all lambs were vaccinated; a subset (n=69) was blood sampled to measure the immune response and another subset (n=126) was tested for pain memory; at approximately 14 weeks a subset of lambs (n=60) was used in a fear potentiation test.

Table 5.1 Data collected during later postnatal period and number of lambs sampled at each age.

Lamb age	Data collection	Sample	Control		Stress		Alternative		
			Females	Males	Females	Males	Females	Males	Total
9 weeks	Immune response	Blood	12	12	12	12	11	10	69
	Weight	-	31	17	29	21	23	21	142
	Pain memory	Behaviour	30	16	29	21	15	15	126
14 weeks	Fear potentiation	Behaviour	21	-	22	-	17	-	60
	Weight	-	30	17	29	21	23	21	141
19 weeks	Weight	-	31	17	29	21	23	21	142

5.2.2.1 *Immune response to vaccination*

At approximately nine weeks of age lambs and ewes were brought back into the experimental shed. All lambs were vaccinated with Ovivac P Plus® (MSD animal health). A subset of 69 lambs (mean age in days (±s.d.): control=61.5 (±1.4); stress=61.9 (±1.3); alternative=61.5 (±1.6)) was chosen to test the immune response to vaccination, balancing as far as possible for sex, age, parity of the ewe and prenatal treatment. One lamb per litter was blood sampled immediately before and 24hr after vaccination for later quantification of acute phase proteins (Haptoglobin, Hp and serum amyloid A, SAA), following the same procedures as described in Chapter 3 section 3.2.2.1.

5.2.2.2 *Pain memory association*

When lambs were brought in for vaccination, a pain memory association test was conducted on a subset of 126 lambs (mean age in days (±s.d.): control=61.7 (±2.9); stress=62.5 (±2.5); n=30 alternative=62.7 (±2.6)). Within each treatment group these lambs were balanced for sex, litter sex combination, ewe parity and scent exposure during tail docking. Pain memory association testing was intended to evaluate the association between a painful event (tail docking) and the exposure to a specific scent (see Chapter 4 section 4.3.2.3). This test was conducted in the experimental shed and consisted of an isolation period of five min for both twins of the same ewe. Each lamb was placed in one of two isolation pens ($1m^2$) located on each side of the shed corridor (Figure 5.1). Each pen had three 2m high walls and a 1m high gate and was visually but not acoustically isolated from the surroundings. To investigate the memory of a possible association between a painful event and a specific scent, a scent box (the same used for tail docking) was placed inside the pen at lamb head height. Litters that had not been exposed to the scent box during tail docking were also tested and used as controls for the litters that were exposed to the scent box. Vocalizations were recorded live by continuous observation and the lambs were filmed for later analysis of activity and attempts to escape the isolation pen using The Observer XT 11.5 software. The ethogram used for the coding of the behaviours has previously been described in Chapter 3 (see table 3.5

under the subsection 3.3.2.3 Fear learning paradigm). The test was stopped if the lamb attempted to escape more than two times in the pen gate direction. At the end of the test lambs were immediately reunited with their own dam.

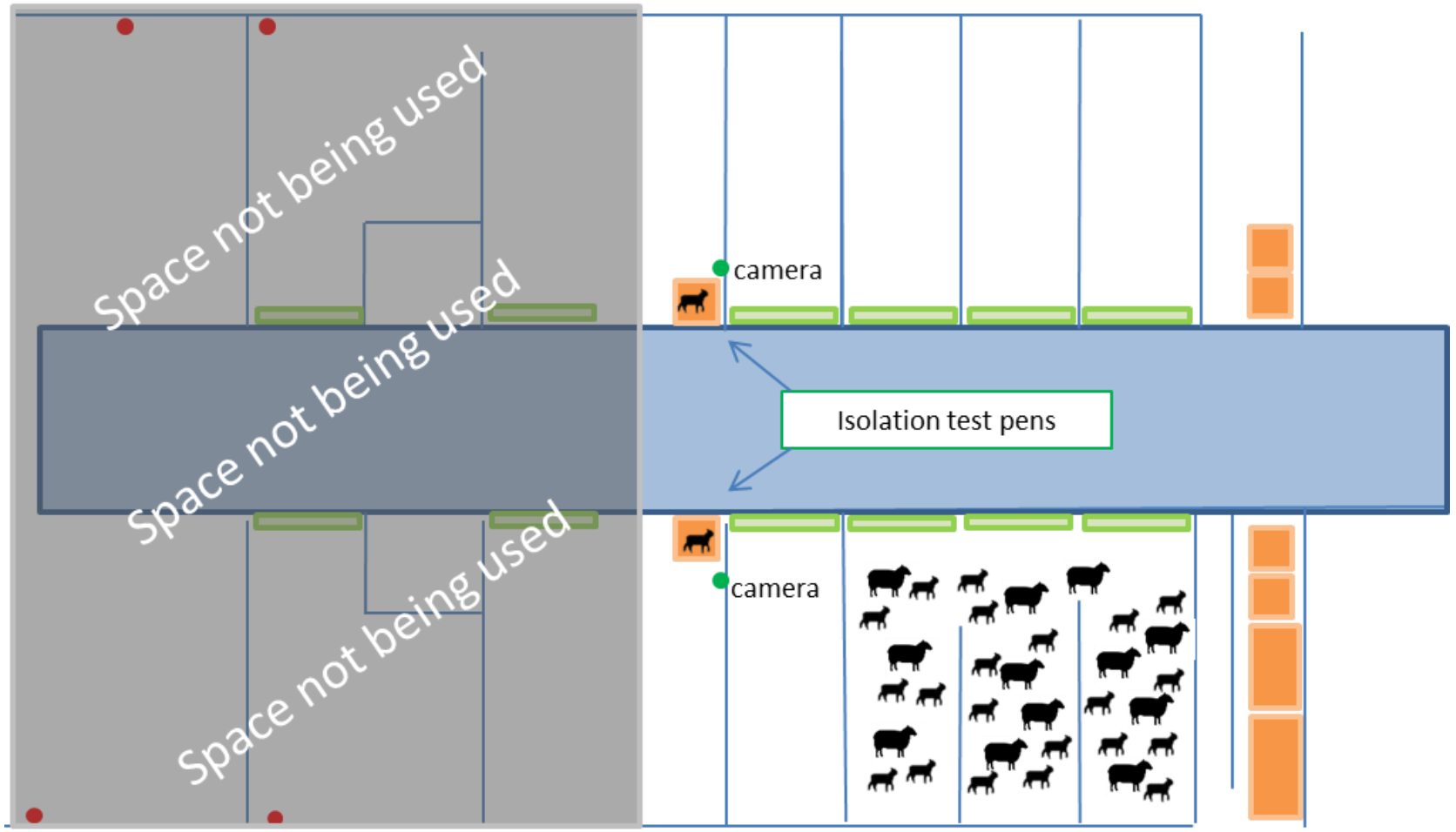


Figure 5.1 Shed layout: Pain memory association test.

5.2.2.3 *Fear potentiation*

Lambs were tested for the effect of prenatal treatment on fear potentiation at 14 weeks. After being brought from the field with their dams, lambs were kept with their mothers and separated into different groups to be tested at different times (either morning or afternoon). Lambs to be tested were spray marked with numbers on the sides of their bodies for identification and with a dot between the shoulder blades to facilitate the marking of activity during later video analysis. All tested lambs went through two five min visual isolation periods separated by a 48hr interval. In the first isolation, half of the lambs were isolated in the presence of a dog (dog/iso) and the other half in the absence of a dog (iso/iso). In the second isolation all lambs were isolated in the absence of a dog.

A total of 60 lambs were tested (mean age in days (±s.d.): control=90.7 (3.8); stress=89.3 (4.2); alternative=89.8 (4.7)). The allocation to one of the 2 groups (dog/iso or iso/iso) was made balancing for age, ewe parity, litter sex combination and treatment.

The test was carried out inside the experimental shed in three isolation pens ($1m^2$) placed side by side with three 2m high opaque walls and a 2m see-through gate. There were three lambs being isolated at a time. Visual but not acoustic isolation from other lambs was ensured (Figure 5.2). For isolation in the presence of a dog, after all three lambs were inside the allocated pen a dog was walked on a lead in front of the pens for the whole duration of isolation, occasionally stopping in front of each pen. For each lamb there was one designated observer recording lamb vocalizations continuously for the five min period. The observers were standing approximately five meters away from the isolation pen and were visible to the lambs. The isolation period was also video recorded for later analysis of lamb activity, body posture and attempts to escape using The Observer XT 11.5 software. The ethogram used for the coding of the behaviours has previously been described in Chapter 3 (see table 3.5, section 3.3.2.3).

Due to available lamb numbers a decision to test only female lambs was made. If males were to be tested as well the best scenario would be only 6 males to compare against females in each treatment group.

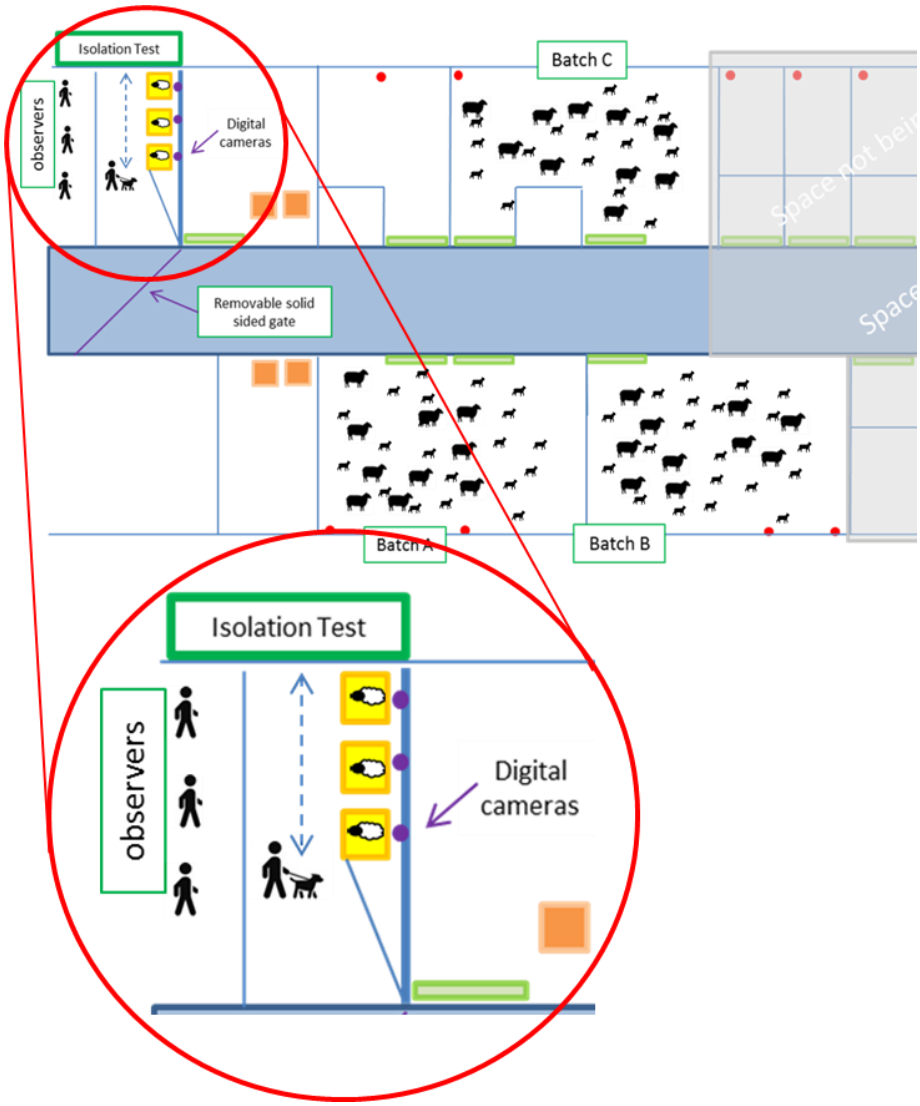


Figure 5.2 Fear potentiation test shed layout

5.2.2.4 *Weight change*

Weight change after the neonatal period was evaluated over a nine week period on three different occasions: at nine weeks as part of the vaccination protocol (see section 5.3.2.1), at 14 weeks when lambs were weaned (all lambs were weaned and weighed on the same day) and at 18 weeks before being sent to slaughter. At each weighing time point 142 lambs were weighed (control: female=31, male=17; stress: female=29, male=21; alternative: female=23, male=21). Four days after the 18 week weighing, 75 lambs (control: female=18, male=6; stress: female=17, male=11; alternative: female=11, males=11) were transported to a commercial slaughter house and were slaughtered on the same day. The remaining 67 lambs (37 females and 30 males) were kept for reproductive studies as part of a different experiment not under the scope of this PhD. After slaughter, hot carcase weights were measured at the slaughter house.

5.2.3 Statistical methods

The statistical analysis of the data presented in this chapter was conducted using a restricted maximum likelihood procedure (REML) in Genstat (Genstat 64-bit, 16th edition). The main factors of PNS treatment, lamb sex and ewe parity and temperament were fitted in the model as fixed factors. Lambing pen and ewe were fitted as factors in the random model. On some occasions, slight alterations to the fixed and the random model were made, which will be addressed below together with other particularities for each data set.

For each model, where a REML procedure was used, data either followed a normal distribution or were transformed (as described in Table 5.2) to meet the assumptions of this procedure. The transformation used was made on theoretical grounds (see Chapter 2 section 2.2.3). Where significant differences were detected by the REML procedure, post hoc Student's t tests were used for pairwise comparisons to determine where significant differences lay. The level of probability determined as statistically significant was $P<0.05$. The level of probability determined as statistically significant was $P<0.05$. When a transformation was used, confidence intervals were

calculated using the original mean ± 2*s.e.. The same transformation applied to the mean was applied to this result in order to obtain a back-transformed confidence interval.

When fitting mixed models there were cases in which it was not possible to estimate the pen variance component. Consequently, the only available option was to drop the variance component for pen from the model (see Chapter 3 section 3.2.3).

Immune response to vaccination

In the statistical analysis of plasma levels of Hp pre-vaccination values were subtracted from post-vaccination values to allow for the evaluation of the response magnitude. For the SAA data, 79% of the pre-vaccination values were below the detectable level of the assay, making it impossible to have a comparison between pre and post-vaccination values. A decision was made to statistically analyse the values for post-vaccination only, therefore sample timing was not added to the model in the case of SAA analysis.

One lamb (stress male) presented lower concentrations of Hp and SAA in plasma from post- than from pre-vaccination, suggesting that there was an on-going acute phase reaction at pre-vaccination and a decision was made to exclude this animal from the analysis as already having a subclinical condition. Two other animals (alternative females) presented lower post-vaccination values of SAA than could be detected by the assay and were eliminated from the analysis. In total 68 lambs were included in the statistical analysis for Hp (control: females=12, males=12; stress: females=11, males=11; alternative: females=11, males=10) and 66 lambs for SAA (the same as for Hp less two females from the alternative group).

Pain memory isolation

The model was altered by adding tail docking and scent to the fixed model and lamb identification to the random model. From the 126 lambs that were tested only 118 were considered for the statistical analysis (Control:

females=27, males=15; stress: females=27, males=20; alternative: females=14, males=15). Lambs that had their test stopped before reaching two min of duration were excluded from the analysis (Control: females=3, males=1; stress: females=2, males=1; alternative: females=1) as it was considered these individuals would have too little data from which to extrapolate. The data were adjusted to rate/min.

Fear Potentiation

Fear potentiation statistical analysis involved alterations to the fixed model due to the nature of the experiment. In this particular case, isolation session (because each lamb was isolated twice) and order of isolation session (ISO/ISO or dog/ISO) were added to the fixed model as fixed factors. The random model remained unaltered. All tested animals were included in the statistical analysis and the data were adjusted to rate/min for each behaviour analysed.

Weight change

The fixed model for the analysis of ADG at 9, 14 and 18 weeks was not altered. For the analysis of hot carcase weight, pen was dropped as a factor from the random model.

Because lambs were weighed as a group at each weigh date, there was variation in age at these different time points. Due to this variation average daily gain (ADG) was considered to be a more accurate measure of weight change than total weight gain. Therefore, calculation of ADG was made by subtracting birth weight from 9, 14 or 18 week weight and dividing it by the number of days of life at weighing day.

From the 169 lambs born, 11 were eliminated from the weight analysis (see section 4.2.3 in Chapter 4 for an explanation of these numbers). By the ninth week of life one lamb had died in an accident involving a gate (control male), three lambs had died (one stress male born without anus, one alternative female was very weak and was euthanized as ewe had little milk and lamb

rejected bottle, and one alternative male could not stand and had difficulties to suck) and five became pet lambs due to rejection by the dam (control litter male/female, stress male, and alternative female) or as a consequence of treatment for a broken leg (alternative male). All these lambs and their siblings were excluded and only 142 lambs were considered for the statistical analysis of the ADG to 9, 14 and 18 weeks. Additionally, one lamb (control female) died before reaching 18 weeks of age. For hot carcase weight, 75 lambs were slaughtered and included in the analysis (control: female=18, male=6; stress: female=17, male=11; alternative: female=11, males=11).

Table 5.2 Data distribution and transformations.

	Data	Distribution	Transformation
Immune response to vaccination	Hp	Normal	---
	Saa	Positively skewed	Log10
Pain memory isolation	Activity	Positively skewed	Log10
	Escaping		
	Vocalizations		
Fear potentiation	Activity	Positively skewed	Square root
	Escaping		
	Vocalizations		
Weights (ADG)	9 weeks	Normal	---
	14 weeks		
	19 weeks		

5.3 RESULTS

5.3.1 Immune Response to Vaccination

Analysis of plasma Hp content showed that overall the lambs were capable of responding to an immune challenge, as higher Hp concentrations were found in plasma samples taken 24hr after vaccination than in those taken before vaccination ($F=118.87$, d.f.=68.0, $p<0.001$; Figure 5.3). Treatment ($F=0.81$, d.f.=8.3, $p=0.479$), sex ($F=1.55$, d.f.=62.8, $p=0.217$), parity ($F=0.65$, d.f=58.0, $p=0.423$) and temperament ($F=0.04$, d.f.=57.2, $p=0.835$) did not affect the magnitude of response of Hp. Similarly, no interactions between the main factors were found (data no shown).

The concentration SAA was not affected by treatment ($F=1$, d.f.=8.0, $p=0.411$), sex ($F=0.0$, d.f.=56.2, $p=0.979$), parity ($F=0.58$, d.f.=57.6, $p=0.448$), or temperament ($F=0.16$, d.f.=56.9, $p=0.693$). However, there was a tendency for SAA concentration to be affected by prenatal treatment depending on the sex of the lamb ($F=3.16$, d.f.=50.3, $p=0.051$), with females from the alternative group having a tendency for higher SAA plasma concentrations than females from the control and stress groups (back transformed mean of SAA (mg/L) (CI): F alternative=174.58 (89.86;185.17);Fcontrol=48.19 (21.59;39.10), Fstress=67.30 (37.84;119.67)). No other interactions between the main factors were found (data not shown).

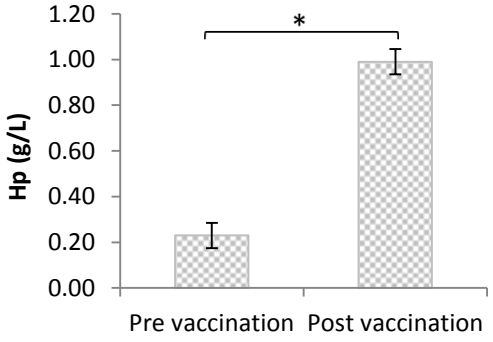


Figure 5.3 Concentration of Hp in pre- and post-vaccination plasma. Post-vaccination concentrations of Hp in plasma were higher then pre-vaccination. Graph bars illustrate means and error bars represent s.e.. Stars (*) represent where statistical significance lies.

5.3.2 Pain memory association

Three different behaviours were analysed during the pain memory association test: vocalizations, activity and attempts to escape. There were no effects of treatment, sex, parity, temperament, tail docking or scent on lamb vocalization rate (Table 5.3) and no interactions between these different factors.

Table 5.3 Effects of main factors on vocalization rate during pain memory association. Means represent back transformed data with 95% confidence intervals.

		Mean (CI)	$F_{statistic}$	Df	P_{value}
Treatment	Control	13.59 (11.85;15.44)	0.65	7	0.550
	Stress	14.78 (13.08;16.59)			
	Alternative	15.03 (12.85;17.38)			
Sex	Female	14.99 (13.56;16.50)	1.01	97	0.317
	Male	13.94 (12.39;15.59)			
Parity	Primiparous	15.40 (13.66;17.24)	2.2	52	0.144
	Multiparous	13.55 (12.02;15.17)			
Temperament	Calm	14.53 (12.85;16.32)	0.01	54.2	0.916
	Reactive	14.39 (12.77;16.11)			
Tail docking	36hr	15.24 (13.76;16.79)	2.68	58.1	0.107
	37hr	13.70 (12.32;15.16)			
Scent exposure	Yes	14.61 (12.99;16.32)	0.07	55.3	0.796
	No	14.31 (12.79;15.91)			

Similarly, activity rate was not affected by any of the main factors. However, there was a tendency for lambs exposed to scent and for lambs that were docked inside the docking pen at 36hr to display a higher activity rate than their counterparts (Table 5.4).

Table 5.4 Effects of main factors on activity rate during pain memory association. Means represent back transformed data.

		Mean (CI)	$F_{statistic}$	Df	P_{value}
Treatment	Control	15.17 (13.27;17.34)	0.67	7.9	0.539
	Stress	16.87 (14.89;19.10)			
	Alternative	16.22 (13.87;18.97)			
Sex	Female	16.79 (15.17;18.58)	1.33	102.6	0.252
	Male	15.38 (13.71;17.26)			
Parity	Primiparous	16.22 (14.39;18.28)	0.05	51.7	0.819
	Multiparous	15.92 (14.26;17.78)			
Temperament	Calm	16.14 (14.32;18.20)	0.01	53.9	0.925
	Reactive	16.00 (14.26;17.95)			
Tail docking	36hr	17.26 (15.52;19.19)	3.98	58.1	0.051
	37hr	14.96 (13.46;16.63)			
Scent exposure	Yes	17.22 (15.35;19.32)	3.2	54.7	0.079
	No	14.96 (13.40;16.71)			

The rate of escaping attempts was affected by an interaction between sex and temperament (F=5.83, d.f.=62.9, p=0.019, Figure 5.4), with females showing higher rates of escaping attempts than males in offspring from calm ewes and than females from reactive ewes. No other effects of treatment (F=0.05, d,f,=7.4, p=0.952), parity (F=0.26, d.f.=47.1, p=0.611), tail docking (F=0.91, d.f.=51.4, p=0.345) or scent exposure (F=0.01, d.f.=49.8, p=0.922) were found.

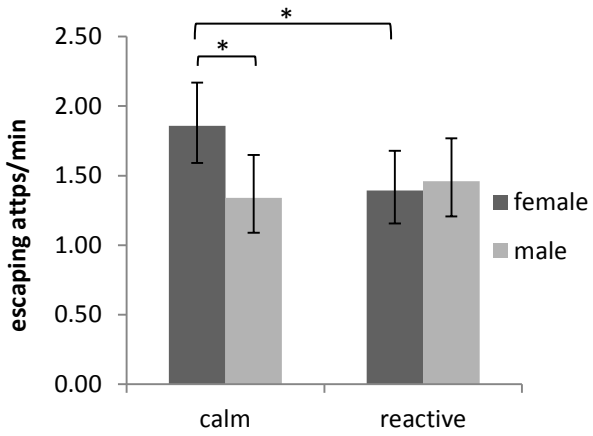


Figure 5.4 Rate of escaping attempts during pain aversion to isolation test dependent upon an interaction between sex and temperament. Females form calm ewes attempted to escape more than males from calm ewes and than females from reactive ewes. Graph bars illustrate back transformed means and error bars represent CI. Stars (*) represent where statistical significance lies.

5.3.3 Fear potentiation

During the fear potentiation test vocalization and activity rate varied depending on the isolation session order (first or second) and type (dog present or absent). Lambs vocalized less when the first isolation was in the presence of a dog compared with the second isolation, but no differences were observed when first and second isolations were in the absence of a dog ($F=55.01$, $d.f.=53.4$, $p<0.001$; Figure 5.5a). The same pattern was observed for activity rate ($F=25.32$, $d.f.=52.7$, $p<0.001$; Figure 5.5b).

There were no effects of treatment ($F=0.39$, $d.f.=8.4$, $p=0.692$), parity ($F=0.50$, $d.f.=49.6$, $p=0.483$) or temperament ($F=0.24$, $d.f.=48.5$, $p=0.626$) on the vocalization rate. Similarly, there were no effects of treatment ($F=0.22$, $d.f.=9.0$, $p=0.810$), parity ($F=0.01$, $d.f.=47.5$, $p=0.942$) or temperament ($F=0.64$, $d.f.=47.6$, $p=0.426$) on the activity rate (data not shown).

Escape rate was affected by an interaction between treatment and parity ($F=3.88$, $d.f.=42.1$, $p=0.029$). Lambs from primiparous ewes displayed more attempts to escape than lambs from multiparous ewes in the alternative group, but no differences were found in the control and stress groups (Figure 5.5c). There was no effect of session order, session number or temperament on the escape rate.

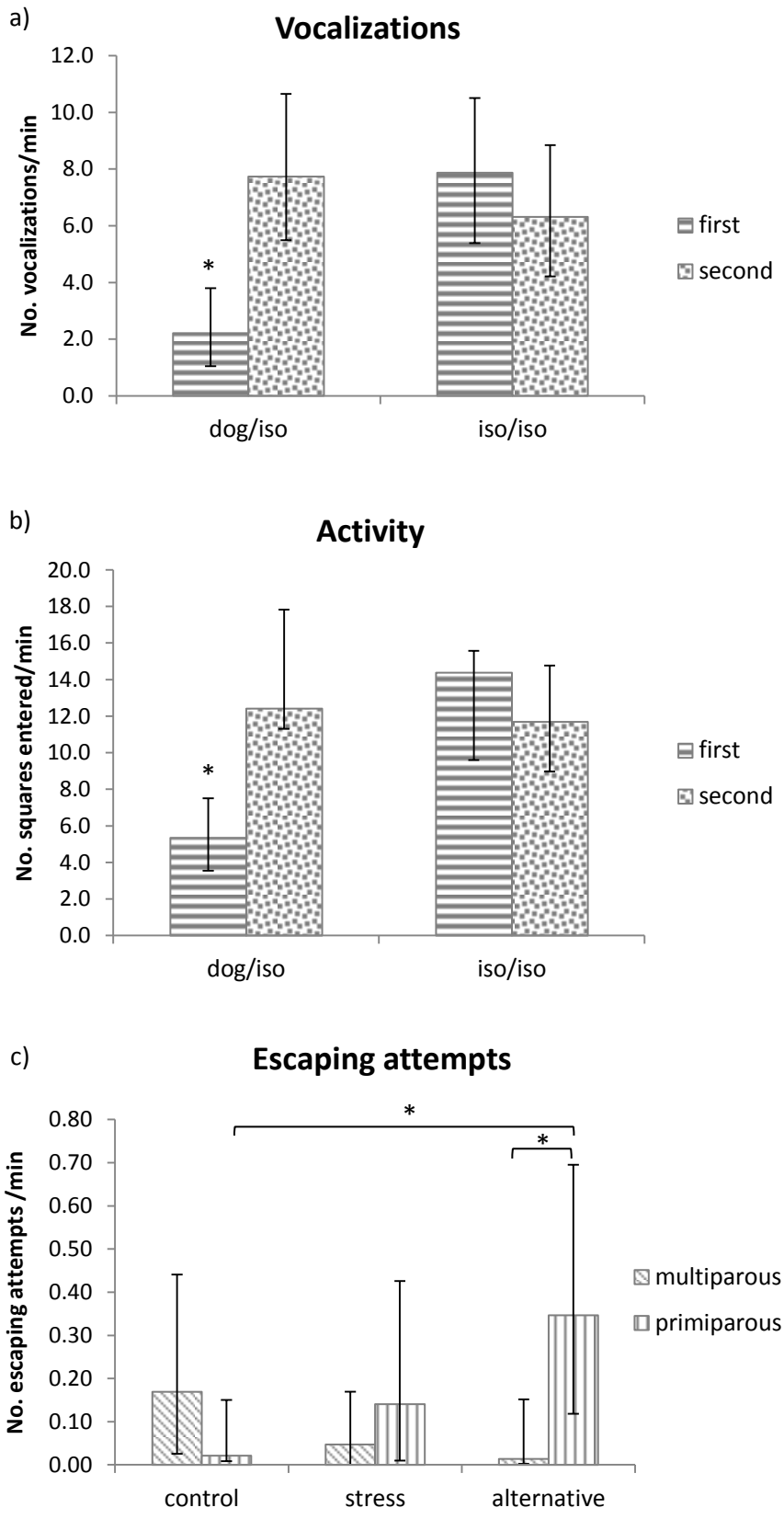


Figure 5.5 Rates of a) vocalization, b) activity and c) escaping attempts during the fear potentiation test. Graph bars represent back transformed means and error bars represent CI. Stars (*) indicate statistical difference.

5.3.4 Weight Change

ADG to 9 weeks was affected by sex of the lamb and parity of the ewe. Female lambs gained less weight per day than males (mean ADG (Kg/day) ±s.e.: F=0.31 (0.006), M=0.35 (0.006), p<0.001) and primiparous offspring gained less weight per day than multiparous offspring (mean ADG (Kg/day) ±s.e.: Lmulti=0.34 (0.007), Lprimi=0.32 (0.007), p=0.031). There was a tendency for treatment to affect ADG to 9 weeks (F=4.04, d.f.=8.2, p=0.060), where lambs from the alternative group had lower ADG than in the stress and control groups (mean ADG (Kg/day) ±s.e: control=0.35 (0.01), stress=0.36 (0.01), alternative=0.33 (0.01); F=4.04, d.f.=8.2, p=0.060) but there were no effects of temperament (F=0.16, d.f.=64.5, p=0.688) (data not shown).

Similar findings were observed at 14 weeks (weaning weights), with slower daily gains for females than males (mean ADG (Kg/day) ±s.e.: F=0.31 (0.006), M=0.35 (0.006), p<0.001) and for primiparous offspring compared with multiparous offspring (mean ADG (Kg/day) ±s.e.: Lmulti=0.34 (0.007), Lprimi=0.32 (0.007), p=0.0017). Treatment (F=0.87, d.f.=8.9, p=0.453) and temperament (F=0.00, d.f.=62.0, p=0.953) did not affect ADG at 14 weeks (data not shown).

When pre-slaughter weights were measured at 19 weeks, still the same effects of sex and parity on ADG were observed. Females had lower ADG than males (mean ADG (Kg/day) ±s.e.: F=0.29 (0.004), M=0.34 (0.005), p<0.001) and primiparous offspring had lower ADG than multiparous offspring (mean ADG (Kg/day) ±s.e.: Lmulti=0.32 (0.005), Lprimi=0.31 (0.005), p=0.038). Treatment (F=0.70, d.f.=8.4, p=0.524) and temperament (F=0.12, d.f.=62.4, p=0.734) did not influence ADG at 19 weeks (data no shown).

No interactions between the main factors were found for ADG taken at the three different time points.

Hot carcase weight was affected by an interaction between parity and treatment (F=4.80, d.f.=32.4, p=0.015). Primiparous offspring had lighter carcases than multiparous offspring in the alternative group, but no differences were found in control and stress groups (Figure 5.6). Additionally,

carcases of the offspring of primiparous ewes from the alternative treatment were lighter than in the control but not than in the stress group (Figure 5.6). Again, females had lighter carcases than males (hot carcase weight (Kg) ±s.e.: F=19.38 (0.36), M=21.34 (0.43), p<0.001) and no effects of temperament were found (F=0.46, d.f.=46.2, p=0.633) (data not shown).

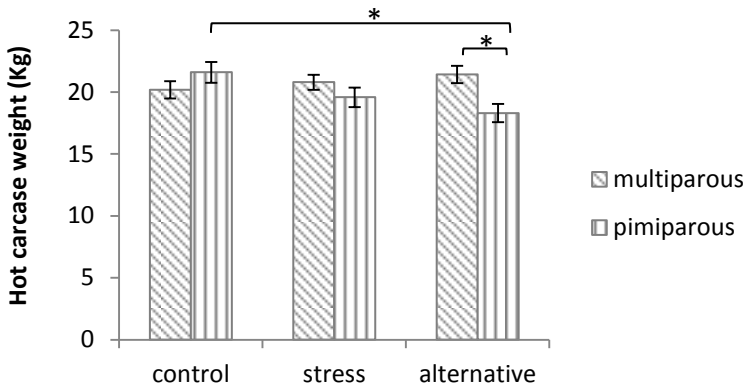


Figure 5.6 Hot carcase weight dependent upon an interaction between treatment and parity. Primiparous offspring had lighter carcases than multiparous offspring in the alternative group. Graph bars represent means and error bars s.e. Stars (*) represent statistical significance.

5.4 DISCUSSION

As a continuation of the work developed in Chapter 4, the aim of the experiments in this chapter was to understand if the prenatal environments designed to represent different levels of PNS would differently affect the immune response, behaviour and growth in lambs at later stages of life.

The acute immune response to vaccination of the lambs was not affected by the prenatal treatment. However, there was a strong tendency for females from the alternative group to show higher levels of SAA in relation to the control and stress groups. Hp levels also differed between males and females.

When testing for memory of a painful event, lambs that had been tail docked in the presence of the scent did not behave differently from lambs tail docked

in absence of the scent or lambs exposed to the scent but tail docked later, showing no evidence of an aversive response to the scent. Additionally, vocalization, activity and escape rates did not differ between treatment groups. However, an interaction between sex and temperament was found for the escape rate.

In the fear potentiation test the results suggest that lambs did not show behaviours compatible with the achievement of fear potentiation, which suggests that the model used here did not work as expected. Further, effects of treatment on the vocalization and activity rate during isolation were not observed. However an interaction between treatment and sex was observed for the escape rate, with lambs from primiparous ewes escaping less than lambs from multiparous ewes in the alternative group but not in the control or stress groups.

Changes in weight over time reflected the expected effects of parity and sex, with males and offspring from multiparous ewes gaining more weight than their female and primiparous counterparts. A tendency for lambs in the alternative group to gain less weight than in the control and stress groups was observed at nine weeks of age. Hot carcase weight was affected by parity in the alternative group but not in the control and stress groups.

Overall the findings suggest that prenatal treatment subtly influenced the lamb outcomes investigated in this chapter, as the observed effects were always a consequence of an interaction with the other factors (sex, parity, temperament). However, when an interaction effect was observed it was always the alternative group that differed from the control and stress groups.

5.4.1 Immune response to vaccination

Prenatal treatment did not affect the Hp response to vaccination. However, for SAA, females in the alternative group tended to have a higher concentration of this APP than females in the control and stress groups but males remained unaffected. Further, all lambs responded adequately to vaccination as shown by the elevated post vaccination levels of both of these proteins in comparison to pre-vaccination levels.

Sex differences in the innate immune response are well established across species and typically, a higher response is observed in females compared to males (Klein & Flanagan 2016). In addition to the effects of sex on the immune response, other external factors like maternal nutrition during pregnancy, microbiome, age and reproductive status may interact with these sex differences and have sex-dependent effects on the immune response (Klein & Flanagan 2016). Indeed, in the present study females from the alternative group showed a strong tendency for higher levels of SAA than females in the control and stress groups but males remained unaffected. Previously (Chapter 4) inadequate ewe nutrition was suggested as a potential cause for some of the observed differences between the alternative and the other two treatments. In fact, previous studies on ewe undernutrition have shown that this type of challenge has adverse effects on lamb immunity, namely, decreased SAA plasma concentrations (Eckersall et al. 2008). This finding is different from the one reported here, where SAA tended to be increased in the females from the alternative group. However, it is important to note that in the mentioned study (Eckersall et al. 2008), the sex of the lambs was not accounted for and the response to vaccination was tested at a much earlier age (8-14 days old) than in the present study (seven weeks old), which may have contributed for the different results. For example, there is evidence in pigs for an increase in the basal concentration of Hp as the individual progresses in age (Piñeiro et al. 2009). Hence, differences in lamb age between this study and Eckersall et al. (2008), may have contributed for the observed increase in SAA concentration in the alternative group.

It is important to note that the tendency of SAA concentrations to be affected by a treatment by sex interaction was not observed in Hp plasma concentrations. Similar results, where one APP is affected and the other is not, have been previously reported (Eckersall et al. 2008) and they suggest that the cytokine driven APP response mechanisms vary with individual APPs.

5.4.2 Pain memory association

Lambs in the present study did not seem to have associated the painful event of tail docking with the presence of a specific scent. Exposure to scent in isolation at a later age did not affect the rate of vocalizations, activity or escape attempts. Additionally, prenatal treatment did not affect behavioural response to isolation. However, females showed higher rates of escaping attempts than males in offspring from calm ewes and than females from reactive ewes, but offspring from reactive mothers did not differ in their behaviour.

Pain memory association with a specific odour has been widely investigated in the rat literature. Nevertheless, to the best of my knowledge it has not been tested in sheep before. These studies usually involve a fear conditioning paradigm. Sheep, as with many other species, can be conditioned to a stimulus (Liddell, 1962) and therefore it would be expected to be possible to condition them to the presence of a specific odour during the painful tail docking procedure. However, it is worth noting that lambs were exposed to the painful stimulus-scent (US-CS) pairing only once, which may not have been sufficient to form associative learning and consequently a CR during later testing. In rodent research pain memory association training involves a training period of approximately 40-45 min with around 10-11 pairings of US-CS (Moriceau et al. 2006, Moriceau & Sullivan 2006, Thompson et al. 2008). Additionally, in the present study the presence of scent and the painful stimulus were presented in a continuous form over the course of an hr, which may have contributed for a decrease in CS salience, i.e. lambs may have habituated to the presence of the scent.

PNS has been reported to produce offspring that are more reactive to fear provoking stimuli and that show increased fear conditioned responding. For example, in one study, PNS rats displayed more freezing following acute footshock and 24 hr later in the same context when the footshock was not presented (Griffin 2001). In the present study, the results suggest that the lambs were not conditioned to the scent, which made it impossible to determine the possible effects of PNS on pain memory association. Nonetheless, it would still be expected to find behaviour differences during

isolation between the PNS and control lambs, based on the fact that isolation itself represents a fear inducing situation for sheep and that behavioural reactivity can be altered by PNS. Nevertheless, no differences between treatment groups were found.

In sheep, it is possible to find contradictory results on the effects of PNS on offspring behavioural reactivity: increased fearfulness (Coulon et al. 2011), reduced reactivity to fear eliciting stimulus (Roussel et al. 2008) and no effects (Roussel et al. 2004). Nevertheless, the severity, schedule and timing when the maternal stressor is applied in relation to pregnancy are all important factors that influence the outcomes observed in the offspring. Therefore, the present results seem to be in line with results in other sections of this experimental work (Chapters 4 and 5), where the effects of maternal stress on the offspring were indeed very subtle. This suggests that the PNS used in this study was not strong enough to produce obvious behavioural differences between groups.

Interestingly, the maternal temperament affected escaping behaviours in females and males. Females attempted to escape more than males in lambs from calm ewes but the reactive temperament of the mother decreased female lambs' reactivity, offsetting the sex differences. Given that temperament, measured as vocal and locomotor activity in sheep, is a trait that has a strong genetic basis (Bickell et al., 2009), it would be expected for offspring from reactive ewes to be more reactive as well. However, other non-genetic factors may have contributed for this result. If we look at temperament as a source of PNS itself (as suggested in Chapter 4 section 4.4.1), a reduction in sex differences is a possible consequence of a more reactive mother. Indeed, an abolishment of sex differences caused by PNS has been described previously in other studies but it has also been observed in other occasions in the present study (e.g. bleating behaviour and proportion of dendritic spines in experiment 1, lamb temperatures in experiment 2).

5.4.3 Fear potentiation

The fear potentiation experiment results suggest that isolation in the presence of a dog did not elicit an anxiety state capable of influencing the fear response to the subsequent isolation. Indeed, there were no differences in behaviour during the second isolation whether the first isolation was in the presence of a dog or not. Therefore, in the present conditions fear potentiation was not observed. Lambs did show a decreased behavioural response to isolation acutely when in the presence of a dog, with decreased activity and vocalizations when compared to isolation only. However, this response was not affected by prenatal treatment.

When in the presence of a potential predator or other disturbance, sheep typically show an 'alarm posture' (Geist 1971). This is a stiff-vigilance posture in which the animal stares at and orientates its ears in the direction of the disturbance. For example, sheep show higher vigilance when a dog was present in an arena test when compared to the presence of a human, a goat or a card box (Beausoleil et al. 2005). Vocalizations also tend to be inhibited in threatening situations such as the presence of a dog (Torres-Hernandez & Hohenboken 1979). In line with these findings, the present results also showed decreased activity and vocalizations when lambs were isolated in the presence of a dog, demonstrating that dogs were indeed regarded as a threatening stimulus and suggesting it was perceived as having the necessary degree of threat. Further, previous studies have also reported higher heart rate, plasma cortisol, ACTH and body temperature when sheep are exposed to a dog or the recording of a barking dog, as opposed to a human or noise (Harlow et al., 1987, Baldock & Sibly 1990, Cook 1996, Komesaroff et al., 1998) adding evidence that the presence of a dog is perceived by sheep as a severe threatening situation.

For fear potentiation to be observed, the threatening stimulus presented prior to exposure to the stressful/fearful situation needs to be a certain severity (for a review see Korte & De Boer 2003). Indeed, previous studies have shown that fear potentiation is more pronounced in rats previously exposed to the presence of a cat (Adamec & Shallow 1993) as opposed to cat odour only (Zangrossi et al., 1992). Hence, one possible explanation for the absence of

a fear potentiation effect here could be that, despite being an acute severe stressor, the dog wasn't threatening enough to the lambs. Hence, it did not elicit and maintain a state of anxiety that would potentiate fear in response to later isolation.

Another factor known to influence fear potentiation is the period of time between exposure to the stressor and subsequent testing. For example, fear potentiation during a plus-maze test was observed directly after acute social defeat (stressor) and 24hr later. However this effect was gone at 48hr after exposure to the stressor (Korte & De Boer 2003). Hence, testing the lambs 48 hr after stressor exposure may have been too long an interval to observe fear potentiation. However, in a similar study in sheep, also using isolation plus dog exposure as a stressor, stressed animals were reported to show reduced activity when re-exposed to isolation (no dog) in comparison to controls (Drake 2006). One difference in that study was the fact that sheep were submitted to isolation in the presence of a dog twice, as opposed to only once in the present study. It is possible that re-exposure to isolation+dog may have helped the sheep to form an association between isolation and the presence of a dog in Drake (2006). Therefore, the subsequent decreased behavioural responses to isolation may have been the result of fear conditioning instead of a fear potentiated response. In fact, it has been shown previously that tailshocks given to rats without fear conditioning did not elicit a fear potentiation response (Grahn et al. 1995).

When (Griffin 2001) studied the effects of PNS on fear conditioning (see section 5.4.2) he also looked at those same effects on fear potentiation. However, this study focused on a variation of fear potentiation paradigm where fear potentiation is achieved through fear conditioning by pairing a neutral stimulus (e.g. light) with a US (e.g. foot shock). In another study using variable PNS, rats from stressed mothers also showed increased fear-potentiated startle compared to controls when exposed to the same fear potentiated startle paradigm (Meyer & Keele 2014). However, the effects of fear potentiation alone were not investigated. To the best of my knowledge, there are no studies investigating the effects of PNS on fear potentiation.

In the present study, a fear potentiation mechanism was not observed, which made it impossible to determine the possible effects of PNS. Nonetheless, as mentioned in the previous section (5.4.2), it would be expected to find differences between treatments in behaviour during isolation. However, no differences in behaviour were found. Again, these results are in agreement with the general findings of this experimental work where the effects of the specific prenatal stressors used are seldom observed and subtle in their behavioural expression. Interestingly, in the alternative group lambs born to primiparous mothers exhibited higher rates of escaping behaviour than lambs born to multiparous ewes. When lambs were exposed to isolation at 24hr after birth (see Chapter 4 section 4.4.7) the results showed that lambs in the alternative group displayed fewer attempts to escape than their counterparts. This suggests that lambs in these groups were less reactive to a stressful situation. Interestingly, at a later stage in life it is still the alternative group that is affected but now in a parity-dependent manner, with primiparous offspring being more reactive than multiparous offspring.

As in the previous chapter, the present results suggest the possibility that the different nutrition in the alternative group may have been one factor contributing to the altered behavioural responses to a stressful situation. Nonetheless, they also suggest that some postnatal factors are contributing to the observed outcomes, as seen by the interaction between treatment and parity which was absent in earlier stages. For example, dam-lamb interactions during early life may have been altered in the alternative group due to simple human presence (see Chapter 4 section 4.4), which may have had repercussions only in later life. Ewes in the alternative group were less used to human proximity than ewes in the control and stress groups and may have perceived it as more aversive and consequently more disruptive. This unintentionally more stressful postnatal environment may have revealed parity differences, which went unseen in the control and stress groups. Furthermore, the different age at which lambs were tested could also be contributing to these findings. For example, maternal peri-conceptional undernutrition was found to decrease the lamb behaviour response to isolation at four but not at 18 months of age (Hernandez et al. 2010). In

another study involving maternal injection of betamethasone, elevated basal and stimulated plasma cortisol concentrations were observed in lambs at one year of age but not before that (Sloboda et al. 2002).

5.4.4 Weight change

In the present study, the females and primiparous offspring always had lower ADG than males and multiparous offspring, respectively, at each weighing point. No treatment effects were observed on ADG to 9, 14 or 19 weeks of age. However, there was a tendency at 9 weeks for lambs in the alternative groups to have lower ADG than in the control and stress groups. The only effect of treatment found depended on parity of the dam and was observed in hot carcase weight. Notably, only in the alternative group was this difference apparent, with carcases from lambs born to primiparous ewes having lower weight than the ones from lambs born to multiparous ewes.

Sex and parity effects on the weight of lambs are well established throughout the literature (Gardner et al. 2007) and are explained by the lower potential of growth naturally observed in females and by the 'physiological imprint' that a first pregnancy leaves in the uterus, allowing greater fetal growth in a subsequent pregnancy (see Chapter 2 section 2.4.4 for an extended explanation).

The results at nine weeks are in line with the findings in Chapter 4 (lambs in the alternative group were lighter than in the other two treatment groups at 6hr of life) and suggest that the alternative husbandry system used in this group affected lamb growth, leaving these lambs disadvantaged in comparison to the other treatment groups. However, lambs in the alternative group do seem to be able to recover from this initial drawback as shown by the absence of treatment effects at later weighing stages (14 and 19 weeks of age). The observed tendency for a lower ADG in the alternative lambs may have been a consequence of an inadequate maternal nutrition in the alternative group (discussed in Chapter 4). However other factors related to the husbandry system may have also influenced lamb growth. For example, as mentioned previously, ewes in the alternative group were particularly

reactive to human presence during peri- and postpartum periods. This may have affected lamb-ewe bond, which consequently affected lamb access to milk. Given that these lambs were already born with the disadvantage of being lighter than lambs in the other treatment groups, a poorer access to milk would have accentuated this difference which is visible until later stages of life. It is not possible to disentangle if a poorer performance of these lambs was due to the initial disadvantage of being born with lower weight and hence a consequence of inadequate maternal nutrition, to postnatal causes inducing a weak maternal bond that negatively affected lamb access to milk or a combination of both. Other authors have found that the effects of prenatal treatment on weight vary according to the time during gestation when undernutrition is applied, with under nutrition during late pregnancy consistently affecting birth weight the most (for a review see (Rooke et al. 2015). These birth weight differences remain at later stages of the lamb's life. The severity of nutritional restriction can also affect birth weight. For example, in a study where three different levels of nutrition (well nourished, moderately undernourished, severely undernourished) were applied during late pregnancy, the greatest differences in birth weight were seen between high and low nutritional planes and they persisted during later stages in the lambs' life (Peart 1967). The fact that, in the present study, this weight difference disappears as the lamb progresses in life may be a consequence of the degree of undernutrition that the pregnant ewes were unintentionally exposed to (note that diet in this group was designed to cover nutritional requirements during this stage of pregnancy) and suggests a compensatory growth strategy adopted by the lambs in this group. In fact, previous studies have shown evidence of lambs adopting this strategy in situations of maternal undernutrition (Cleal et al. 2007).

Treatment interacted with parity for hot carcase weights, although this difference was only seen in the alternative group. It would have been expected to see the same differences in hot carcase and pre-slaughter weights, given that all animals were treated equally in the period of time between pre-slaughter weighing and slaughter. However, hot carcase weight is measured after dressing (the process of removing various parts of the

body of an animal following slaughter) and differences between groups in the weight of internal organs may have been the cause for the present findings. Undernutrition during late pregnancy in sheep has been associated with increased omental, mesenteric, perirenal and pelvic fat (Geraseev et al. 2007), which is attached to the organs that are removed during dressing. The results also show that offspring from primiparous ewes were more affected by the nutritional regime, as primiparous offspring carcases were lighter in the alternative group than in the controls but no differences were found in multiparous offspring. Parity of the dam is known to contribute to the degree of nutritional constraint imposed on the fetus and is likely to have affected carcase composition.

5.5 CONCLUSION

The results presented here continue to show that the majority of effects caused by the prenatal treatment were seen in the lambs from the alternative group and suggest that this system did not work in favour of the lambs. The most probable reason for this outcome is inadequate ewe nutrition during pregnancy. Lambs in this group had lower birth weights and this result was still a tendency at nine weeks of age. However, lambs seemed to recover from this initial disadvantage as they progressed in life. The majority of the results show that the effects of the prenatal treatment used in this group were expressed as a function of other factors, such as sex and parity. Moreover, the prenatal treatment effects seem to increase these parity and sex differences. A good example of the potentiation effect that prenatal conditions may have on pre-existing factors, such as parity, was that lambs born to primiparous ewes in the alternative group had significantly lighter hot carcases than lambs born to primiparous control ewes. The same type of result was observed for the response to vaccination, where females from the alternative group showed increased levels of SAA in comparison to control females.

The findings from the series of isolations again confirmed that the alternative group was the most affected. Few effects of prenatal treatment were found

but always in the alternative group. Lambs in this group showed decreased rate of escape attempts when tested at 24hr of life comparing to stress and control groups, and an increase in this behaviour at 90 days of age if their mother was primiparous in comparison to either multiparous or primiparous controls. However, no differences between groups were found at 60 days of age. These findings suggest that behavioural reactivity was altered according to age of testing. Nevertheless, the location and conditions of each isolation test were not exactly the same, due to some variations inherent to the testing procedure (e.g. presence of scent in pain memory isolation). Hence, it is not possible to exclude the procedure variations as the cause for the observed differences in behaviour at different ages.

Lambs did not seem to have developed aversion to a scent previously paired with a painful procedure. Hence, it was not possible to evaluate the potential effects of PNS on fear triggered by pain memory. A major issue with this experiment was the fact that lambs were exposed to the pair scent/pain only once and over a continuous period of time. Similarly to these results, exposure to the presence of a dog (threatening stimulus) two days prior to a social isolation test did not elicit a potentiation in the fear response of the lambs. Hence, it was not possible to observe the effects of PNS on the fear potentiated response. Possible explanations for the absence of effects include the time between exposure to the threatening stimulus and testing and the salience of the threatening stimulus. The rodent literature shows that the salience of the stressor used influences the length of time during which a potentiated fear response is observed. However, has shown in similar studies in sheep, more than one exposure to the dog may have been necessary in order to observe fear potentiation.

A general finding of this experiment was that the use of dog exposure and a unpredictable feeding system seemed not to have worked as a source of prenatal stress. These stressors have been shown in the past to be stressful to sheep. However, where effects were found on the offspring, the ewes had been more frequently exposed to these stressors and as part of a schedule that included more stressors. Hence, the lack of effects in the offspring observed here suggests that these stressors, applied with a

frequency/intensity that mimics the real farm conditions, are not a major issue for lamb welfare. Additionally, ewes may be particularly successful at buffering the effects of psychological stressors.

CHAPTER 6

General discussion and conclusions

6.1 PROJECT OVERVIEW

A general observation from the PNS literature in sheep is that experiments usually do not reproduce the real life conditions for the pregnant ewes. Therefore, one of the main goals of this project was to replicate farm husbandry practices instead of exposing sheep to stressors known to affect lamb development but that do not mirror the reality of farm life. To tackle this issue, the design of this project was planned partly based on the results from a previous study (DEFRA project AW0509) which investigated the farm conditions in which pregnant ewes are kept in the UK. However, other aspects of ewe management which are known to induce stress (such as competition for feed) were also investigated. Hence, the stressors used as part of the present PNS protocols were chosen based on the extent to which ewes under UK management conditions are exposed to them and on their likelihood of a prenatal effect. Further, the schedule on which stressors were applied was designed to reproduce the intensity/frequency with which ewes encounter such stressors in real conditions. This novel approach ensured that the experimental conditions would better replicate real husbandry conditions in the UK. Potential stressors such as social instability, high stocking density, exposure to a dog and feed competition (unpredictable feeding schedule) were chosen as maternal stressors. Additionally, an alternative system designed to decrease sheep exposure to a stressful environment was also investigated. The effects of prenatal environment on factors known to be important for lamb survival during the critical neonatal period were systematically investigated. Moreover, other lamb outcomes were investigated at later stages of life, up to slaughter age.

6.2 MAIN FINDINGS

The first experiment investigated the effects of maternal social instability and high stocking density during late pregnancy on lamb behaviour, immunity, growth, HPA axis regulation, and brain morphology. The second experiment focused on maternal stress caused by feed competition and repeated exposures to a dog during late pregnancy and its effects on lamb behaviour,

immunity, and growth. An alternative system designed to expose sheep to a low stress environment was also investigated for its possible positive effects on the offspring. The effects of stress on the mothers during both experiments were studied as part of another PhD project (Yusof, 2018).

Experiment 1

The effects of prenatal treatment on the lamb were very subtle in nature as they only manifested as a function of other factors such as parity, sex or birth assistance.

1) PNS decreased the bleating rate in female lambs and in lambs assisted at birth but males and unassisted lambs remained unaffected.
2) PNS decreased body temperature at 30min in offspring from multiparous but not primiparous ewes.
3) PNS increased lamb ADG to 17 weeks in males that had lost a sibling at 7 weeks of age but females were not affected.
4) PNS affected dendritic spine morphology in pyramidal cells of the hippocampus CA1 area by decreasing the proportion of filopodia and stubby spines in males but not in females and increasing the proportion of thin spines in males but decreased it in females. A general finding for spine morphology was that PNS eliminated the sex differences observed in the control group.
5) PNS decreased GR gene expression in the hypothalamus and increased GR promoter region methylation in the hippocampus but did not affect gene expression in the hippocampus or methylation in the hypothalamus.

Experiment 2

The majority of effects caused by the prenatal treatment were seen in the lambs from the alternative group, suggesting that the proposed alternative husbandry system did not work in favour of the lambs.

1) PNS and the alternative treatment decreased lamb latency to stand when lambs were assisted at birth.
2) PNS accentuated temperature differences at 2hr between assisted and unassisted lambs. The alternative treatment accentuated temperature differences at 24hr between lambs from multiparous and lambs from primiparous ewes. Both PNS and alternative treatment eliminated the difference in temperature at 24hr between males and females.
3) Lambs from the alternative treatment had lower acquisition of passive immunity than in control and stressed groups.
4) The alternative treatment decreased lamb birth weight in comparison to control and stress groups and lambs from the alternative treatment also had a tendency to have lower ADG to nine weeks and increase parity differences in hot carcase weight.
5) The alternative treatment decreased lamb ability to recognise its mother at close quarters.
6) Lambs in the alternative treatment displayed lower rates of escape attempts during the isolation test at 24hr of age than stress and control lambs.
7) The alternative treatment increased the escaping rate in isolation in lambs from primiparous compared to multiparous ewes.

The results from these experiments suggest that there may be associations between the different tested maternal conditions during late pregnancy and offspring outcomes but that these associations are dependent on lamb sex and ewe parity. Results also suggest that some effects of PNS may only be seen in the presence of a subsequent insult later in life.

Alterations in the phenotype of an individual can be caused directly or indirectly when environmental pressure acts on one individual (i.e. mother) influencing phenotypic development in another (i.e. offspring). The general lack of clearer effects of PNS in the present study may be interpreted by integrating the results into these two different dimensions: the level of stress

the mothers experienced and their capacity to buffer effects of an eventual stressful environment and the nature of PNS effects on the offspring, short versus long term consequences.

Stress experienced by the mothers

The negative impacts of social mixing (Sevi et al. 2001) and decreased space allowance (Averos et al., 2014; Bøe et al., 2006) on sheep have been previously demonstrated, although these studies did not focus on the effects of these as prenatal stressors. Similarly, dog exposure and unpredictable feeding schedule have been shown to negatively impact on sheep (Komesaroff et al. 1998, Doyle et al. 2011, Destrez et al. 2013). It would be therefore expected to see more obvious differences in the offspring (in comparison to controls) as a consequence of such prenatal environments. One possible cause for the lack of clearer consequences for the lambs may then be related to the level of stress experienced by the mother. Indeed, in the present study there was very little evidence that ewes were stressed by the maternal stressors and there were no differences between treatment groups in maternal GCM (Yusof 2018, Appendix 2) This may have been related to the schedule the stress was applied or on the capacity of sheep in dealing with stress of a social nature.

In the study of Sevi et al. (2001), sheep were submitted to three social mixing events separated only by a one week interval, as opposed to the two social mixing events separated by two week intervals in the present study. This may have contributed to the clearer negative effects of social mixing observed in Sevi et al. (2001), as in the present study ewes may have had more time to adapt to the newly formed groups. Nevertheless, although in Sevi et al. (2001) this difference was observed at the behavioural level, cortisol levels were not affected. Similarly, in the present study maternal GCM were not affected by treatment. Previous studies have identified that the negative effects of social mixing are worsened by increased stocking density (Talebi et al. 2014). The higher stocking density used in this study was on the limit of what is legally the minimum space allowance ($1.2m^2$/ewe) defined by the DEFRA Code of Recommendations for the Welfare of Livestock – Sheep

(DEFRA 2000), which may have attenuated the adverse consequences of social mixing. Indeed, in two studies that investigated the effects of space allowance in sheep, greater behaviour alterations indicative of higher stress were seen when space allowance was decreased from 1 to 0.5m^2 (Bøe et al., 2006) but not so obvious when space decreased from 3 or 2 to 1m^2 (Averos et al., 2014). Similarly to experiment 1, the exposure to a dog and unpredictable feeding schedule in experiment 2 may not have been as stressful as anticipated. One reason for this may be the fact that ewes were exposed to a dog in groups and not in isolation. The presence of conspecifics has been shown to lower the levels of stress and increase the ability to cope with stressful situations in sheep (González et al. 2013). As for the unpredictable feeding schedule, although uncertainty and unpredictability are considered major sources of stress (Maier & Watkins 2005), a study done in dairy calves showed that when fed on an irregular schedule calves did not exhibit altered behaviour (Johannesson & Ladewig 2000). In the present study, the negative effects of unpredictability were probably decreased by the fact that food was always delivered. Moreover, the fact that unpredictability was associated with a pleasant event, which was the delivery of food, may have further contributed for a lower stressful relevance of this event. Therefore, the tested stressful conditions in both experiments of the present study may have been stressful in a low level. In this case, ewes may have been able to cope with the stressful cues without raising the levels of cortisol to a level representative of a chronic stressful situation. However, slight/temporary increases in maternal cortisol may have been enough to cause the behavioural alterations seen in the lambs.

Another aspect to consider is that the stressors used in the present study were social in nature and touched on aspects that are consequences of the human interaction with sheep. Sheep were one of the first animals to be domesticated and simple procedures such as gathering sheep in smaller spaces, using dogs as helpers and occasionally mixing groups have probably been part of sheep husbandry for many years. Hence, it is possible that sheep evolved to have low sensitive to this type of stressors when they are

presented in low levels such as the ones in this study. This would help them buffer the effects of these stressors on the development of the offspring.

Stress effects on the offspring

In experiment 1, the majority of factors contributing to neonatal lamb survival were not affected by prenatal treatment. However, there was some evidence that PNS affected bleating behaviour during the first 30min after birth and body temperature at 30min of life, although these effects were dependent on sex and parity and did not impact on later behaviour or ability to maintain body temperature. Similar results were found for experiment 2. However, different lamb outcomes were affected. Here, the effects of PNS were seen on body temperature and latency to stand depending on sex and assistance at birth. It is true that different PNS treatments were used during experiment 1 and 2 and, as has been discussed on several occasions during this thesis, that alone may be one explanation for the difference in results. However, a general observation on PNS research is that studies are not often repeated. The absence of repetition may indicate that some of the findings may just be statistical coincidences, particularly when various response variables are recorded, as was the case in the present study. In many cases, from a biological point of view, some of these variables are expected to be strongly associated (e.g. for a lamb to suck it has to first stand). In such circumstances broadly similar findings of either strong statistical evidence across variables or a lack of statistical evidence for treatment effects across variables is anticipated. Nevertheless, it was found that only some of these outcomes were affected and that they did not really affect subsequent related behaviours. Moreover, they seem to be affected by interactions between treatment and other factors but not in a consistent way. Hence, the validity of such findings must be interpreted with caution in that they may be the result of statistical chance. Nevertheless, another possible interpretation for the lack of clear effects of PNS in the present study is that perhaps the lamb factors investigated here are of such importance for lamb survival that the degree of vulnerability to prenatal programming is low. However, it is not

possible to rule out the possibility that the stressors used were not stressful enough to elicit clear changes in the PNS lambs compared to controls.

An interesting finding in both experiments was that some of the results observed at later stages of life indicate that the effects of PNS may in fact be present but only evident in the face of a later events. The higher ADG in PNS male lambs at 17 weeks in experiment 1 was observed as a consequence of a postnatal event, which was the earlier loss of a sibling. However this only became evident when facing the challenge of weaning. This type of effect was also observed at an earlier stage when lambs from the stress group showed decreased bleating behaviour during the 30min after birth as a consequence of having been assisted at birth. A similar finding was observed for latency to stand in experiment 2, where PNS decreased lamb latency to stand when lambs were assisted at birth. Indeed, this cumulative effect of pre- and postnatal experiences has been observed before in lambs. For instance, in one study, early maternal separation exacerbated the effects of PNS by increasing fearfulness in those lambs (Averós et al. 2015). In another study assessing pain sensitivity in lambs, neonatal challenges also affected later adult behaviour by increasing the levels of pain-related behaviour during lambing (Clark et al. 2014). Both these examples agree with the double-hit hypothesis which posits that an early life genetic or environmental insult may go by unnoticed but still provoke alterations in the organism, which may emerge in the presence of a subsequent insult, or ‘second hit’ in later life (Walker et al. 2009). Nevertheless, overall there was a relative absence of PNS effects in later life of the lambs. In line with the double hit hypothesis, these results may be explained by the fact that these animals had very few challenges in their life.

Interestingly, in the present case PNS did not seem to be detrimental for the offspring. In fact, the combination of PNS and post-natal challenges increased ADG and decreased the latency to stand.

Previous studies have discussed the outcomes of PNS from an evolutionary perspective. These studies argue that, in evolutionary terms, mothers should maximise the fitness of their offspring by adjusting their phenotype to the environment in which they will live (Monaghan 2008). For example, exposing

the mothers to a dog may have signalled a stressful environment to the offspring which responded by altering its phenotype to one that is faster to stand, which would represent an advantage in a environment where predation is a high possibility. The same goes for the increased ADG in lambs that became a singleton at some point. The feed competition during pregnancy, due to decreased feedface space in experiment 1, may have signalled an environment where the amount of food is unpredictable, to which fetus phenotype adapted but responded with extra growth later when food was more readily available. Hence, extra growth may not be itself an adaptive response but rather a consequence of a phenotype that was selected to respond well in an unpredictable environment. Extra growth and the favouring of neonatal adaptations (i.e. faster standing) may have consequences for later stages in life. The current adaptive theories propose that the effects of adverse conditions may benefit the offspring either in the short (developmental constraints) or long term (predictive adaptive responses) in the expectation of future adaptive advantages (Berghänel et al. 2016). However, the negative effects of PNS can be subtle and difficult to recognize, particularly if they do not appear until later in life (Metcalfe & Monaghan 2001). It is possible that the lack of effect observed in this experimental work is a consequence of the time chosen to evaluate the outcomes. Looking at lamb performance at later stages in life may have revealed different outcomes and a clearer idea of the effects of PNS. In fact the effects of PNS seen at the brain level, both morphology of dendritic spines and gene expression, may be an early indicator of potential future altered behavioural outcomes at later stages of life. The brain alterations did not translate into behaviour alterations, at least at the age that lambs were tested in experiment 1, but it is possible that later life experiences facilitate the emergence of altered behaviour (as explained by the two-hit hypothesis).

An example of how PNS effects may be triggered and changed by later life experiences was illustrated in the alternative group in experiment 2. The prenatal environment had clear disadvantages for the development of these lambs during the neonatal and later periods in relation to control and stress lambs. The available data on weight and BCS of the ewes suggested a possible nutritional cause for the negative impact of the alternative treatment

on the lambs, although it was not possible to tell if this was the only cause. In the period of time between birth and 14 weeks of age, lambs were exposed to a series of isolations designed to test if lamb behavioural reactivity to a stressful situation was different depending on the prenatal environment but also to understand if that reactivity changes as the lamb progresses in life. Lambs in the alternative group showed a decreased rate of escaping attempts when tested at 24hr of life comparing to the stress and control groups, and an increase in this behaviour at 90 days of age if they were born to primiparous ewes in comparison to lambs born to alternative multiparous ewes or primiparous controls. However, no differences between groups were found at 60 days of age. These series of isolations seem to suggest that behavioural reactivity of the lamb changes depending on the age at which they are tested.

6.3 FINAL CONCLUSION

This study indicated that stressors relevant to the pregnant ewe, when applied in a way that better mirrors the husbandry practices on farms in the UK, only show subtle impacts on lamb neonatal behaviour, HPA axis regulation, brain morphology, and growth. Nevertheless, other factors of vital importance for lamb survival during the perinatal period and the behavioural response to stress at later stages were not affected. On the schedule and intensity with which stressors were applied in the present study, there seems to be no major implications for lamb welfare. It is important to note that the stocking densities used in this study represent what is practiced in the majority (82%) of farms in the UK (DEFRA project AW0509) which also corresponds to DEFRA's space recommendation for sheep. Hence, from a farm management perspective this study suggests that, in general, current ewe management practices are not detrimental for lamb welfare. However, given the potential effects that these stressors may have, as shown by the subtle effects found, care should be taken when generalizing these findings. This study was aimed at representing the husbandry conditions found in farms in the UK where ewes are housed, but there are situations which fall on the lower end of the spectrum in what is considered good practice. In such

situations, the effects of higher stocking densities and social instability, increased frequency of dog exposure and competition for feed may pass the threshold at which the animal is able to cope and could produce maladaptive or pathological effects on the offspring. In fact, the results from brain morphology and gene expression analysis suggest that prenatal treatment had some influence over the development of the lamb. Together with the fact that some lamb outcomes were affected as a consequence of the combination of PNS and stressful postnatal events, the observed brain alterations may represent a phenotype that is more vulnerable to environmental stressors but that vulnerability will only express itself later in life. However, the present study followed the lambs from birth to slaughter, which corresponds to the life length of most lambs on farms in the UK. Therefore, later effects of PNS potentially emerging with further insults might not be relevant in welfare terms for most lambs.

Together, the results of the present study mark the importance of adopting a multidisciplinary approach when it comes to studying the potential effects of prenatal stress, with the risk of dismissing the importance of certain prenatal environment aspects when not doing so.

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Appendix 1

Cortisol results

It was not possible to achieve a trusted measurement for lamb plasma cortisol in this experiment. The first approach for cortisol measurement was using an in-house assay that had been validated previously (Al-Dujaili et al., 2009; Al-Dujaili et al., 2012). However, three main issues arose: the sample coefficient of variation (CV%) was too high (>20%), the absorbance range for the standard curve range was too small (0.4 absorbance), and there was insufficient colour development (i.e. low optical density (OD) readings). Causes for obtaining high CVs and insufficient colour development, which could to some degree also explain the short range standard curve, were identified and addressed (Table a). However, this did not correct the problems first encountered for the CV% and standard curve. After these alterations, a subset of samples were run in a different lab (Al-dujaili Lab), where the assay was originally developed. For this ELISA plate the samples were run using that lab's reagents and buffers, and four different standard curves were designed using four different combinations of reagents and standards (our reagents and our standards, our reagents and other lab standards, other lab reagents and standards, and other lab reagents our standards) in order to tackle not only the high CVs but also the standard curve issues. Results showed that high CVs were absent. However, running three different standard curves revealed that previous small range standard curves were related to the preparation of our reagents (see Figure a). Subsequently, a new ELISA was run using a new batch of conjugate and antibody provided by the Al-dujaili Lab and fresh buffers were made. Again, high CVs and very short range standard curves were obtained. These results seemed to indicate that there was a problem with the preparation of the buffers and/or washing solution. Extra care was taken using a trusted water source but still the results remained the same. At this point a decision was made to use a commercial kit (Cortisol (sheep) ELISA kit, Abnova (Taiwan) KA0919), given that results were needed in order to make decisions for the epigenetic part of this project (see Chapter 3). Results obtained with the kit

went as expected: standard curve absorbance values were within the range described by the manufacturer (0.33–2.62nm) and sample and inter-plate CVs were also low. However, a high percentage of samples (80%) had concentrations above the highest detectable possible value for the standard curve (80ng/mL). For these samples a higher dilution was performed (1:4), which put the upper limit of the assay at 320ng/mL (or 883nM/L). Nevertheless, 55% of the samples still showed out of range readings.

The literature shows that the range of cortisol values for newborn lambs at 24hr varies but 90ng/mL was the highest reported result found (Camacho et al., 2012; Dwyer & Morgan, 2006; Paisey & Nathanielsz, 1971). Higher values than this (100-155ng/mL) have been reported in lambs but at an earlier stage of life (1hr after birth) (Dwyer & Morgan, 2006; Wrutniak & Cabello, 1987), and these values usually drop within the next few hr after birth (Dwyer & Morgan, 2006; Paisey & Nathanielsz, 1971). The obtained values from the present ELISA seemed therefore excessively high to be interpreted as having biological meaning and may indicate that there was an issue with the samples. In fact, the manufacturer guidelines for this assay advise not to store samples for more than one month at -20ºC and these samples were stored for several months before being processed. The cortisol ELISA used in the present study was an inverse competitive assay (high sample concentration = low absorbance) so anything that inhibits the enzyme may have caused false high readings. For example, EDTA used as an anticoagulant for plasma collection inhibits enzymatic reactions and these samples were collected using tubes coated with EDTA.

Table a Causes for obtaining high CVs and insufficient colour development and actions taken to address them

		action taken
causes for high CVs	**imprecisions when pipetting the samples**	running two plates on the same day by two different experimenters with different degrees of experience (a lab technician and me –a PhD student)
	excessive time to load the plate	executing only half plate to reduce the loading times
	washer system malfunctioning	substituting automated washing system by hand washing
	poor distribution of antibody in the sample	vortex samples before being loaded into the plate
low colour development	**deterioration of the conjugate**	running two plates on the same day by two different experimenters with different degrees of experience (a lab technician and me –a PhD student)
	low room temperature	Recording room temperature for each plate (18-24^{o}C)
	incorrect preparations of washing solution	Preparation of new washing buffer

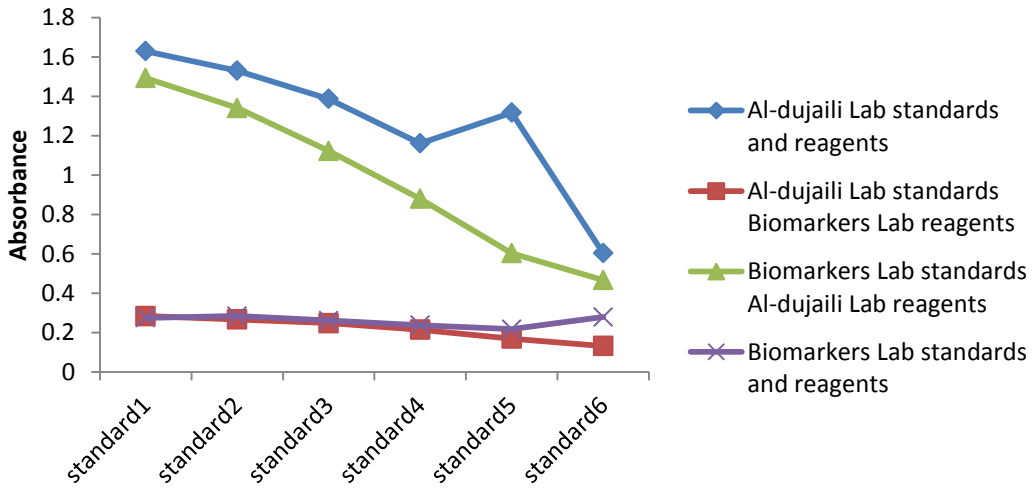


Figure a Four different standard curves using different combinations of reagents prepared in Biomarkers lab and in Al-dujaili Lab.

Appendix 2

Ewe data

Data referent to ewes was analysed as part of another PhD project (Yusof 2018). Some of the results relevant to the interpretation of lamb data are presented below.

Ewe data experiment 1

Overall there was a significant effect of time and parity on the concentration of faecal GCM. Primiparous ewes had significantly higher faecal GCM than multiparous ewes at weeks 11, 13 and 15 ($F=10.93$, $d.f.=3$, $p=0.014$) of gestation. However, there was no effect of treatment on the concentration of faecal GCM throughout gestation.

Ewes from the PNS group showed increased rumination at week 18 of gestation and increased aggression at feedface at week 14, suggesting that treatment may have induced stress in pregnant ewes. Further, aggressive behaviour performed by ewes from the stress group declined over time compared to Control group which was more constant and lower, although not significantly.

Ewe data experiment 2

Ewes in the alternative group had a tendency to have lower weight than ewes in the control and stress groups three weeks prior to the expected start of lambing ($F=3.23$, $d.f.=9.0$, $p=0.088$). This weight difference became significant one week prior to lambing ($F=7.179$, $d.f.=9$, $p=0.014$; mean weight (Kg) ± s.e.: control=82.27 ±1.29, stress= 81.71 ±1.29, alternative=76.02 ±1.29). Ewes in the alternative group also gained less weight compared to control and stress groups in the 15 day period leading up to lambing ($F=11.96$, $d.f.=9$, $p=0.003$; ADG (Kg) ± s.e.: control=0.358 ±0.03, stress= 0.339 ±0.03, alternative=0.142 ±0.03). However, body condition scores

(BCS) and betahydroxybutyrate (BOHB) were not different from the control and stress ewes.

Similar findings were observed at 48hr post-partum, with ewes from the alternative group weighing less than ewes from both the control and stress groups ($F=6.99$, d.f.=9, $p=0.015$; mean weight (Kg) ± s.e.: control=69.08 ±1.22, stress= 70.36 ±1.22, alternative=64.27 ±1.22), and having lower BCS than in the stress group but only tendentiously lower than in the control group ($F=6.43$, d.f.=9.0, $p=0.018$; BCS ± s.e.: control=2.580 ±0.06, stress=2.714 ±0.06, alternative=2.420 ±0.06).