The consequences of epigenetics and fetal programming

for English beef and sheep producers

Kevin Sinclair[1], Kenny Rutherford[2], Jacqueline Wallace[3], John Brameld[1], Reinhard Stöger[1], Ramiro Alberio[1], Dylan Sweetman[1], David Gardner[1], Viv Perry[1], Clare Adam[3], Cheryl Ashworth[4], Jane Robinson[5] and Cathy Dwyer[2]

[1]Schools of Biosciences and Veterinary Medicine and Sciences, University of Nottingham, Sutton Bonington, Leicestershire, LE12 5RD

[2]SRUC, Roslin Institute Building, Easter Bush, Midlothian, EH25 9RG

[3]Rowett Institute of Nutrition & Health, University of Aberdeen, Bucksburn, Aberdeen, AB21 9SB

[4]Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG

[5]College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow

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Executive summary

1. **Aims and objectives:** A comprehensive review of the literature is provided which offers a critical analysis of data pertaining to how fetal programming and epigenetics influence contemporary beef and sheep production traits. In places the review draws heavily on knowledge and experience gained from a wide variety of species but, in particular, rodents and humans where the majority of data resides.

   In this article we provide:
   a. An assessment of possible risk factors and likely exposures affecting beef and sheep production
   b. Evidence of the extent to which commercially important traits are affected
   c. An analysis of effects of specific environmental factors (e.g. parental diet, maternal stress)
   d. A comprehensive overview of epigenetic mechanisms and their role in fetal programming of subsequent development and health
   e. Key industry 'take-home' messages
   f. A list of future research needs

2. **Overview of document:** The article is divided into 7 sections which provide detailed overviews of (i) the general field of developmental programing, (ii) epigenetics and its role in long-term development, (iii) programming of health and wellbeing, (iv) programming of body composition, (v) programming of fertility, (vi) impact of advanced reproductive technologies and (vii) industry relevance and recommendations.

3. **Context:** Although it has been known for some 70 or so years that fetal development can have a lifelong impact on offspring growth, it is only since the 1980s that the extent and impact of prenatal exposure to malnutrition or stress on adult health and disease became fully appreciated. This was first identified through human cohort studies (such as those investigated by Barker or the Dutch 'Hunger Winter' studies), and was subsequently explored in detail in rodent models. The relatively recent discovery that some of these effects are present not only in offspring of the mother that experienced the dietary or stressful event but also in her grand-offspring, generated a great deal of interest concerning the mechanisms of inheritance.

4. **General issues:** Research in this area currently follows three lines of enquiry: (i) investigations (mostly in rodents) into the mechanisms (including epigenetic) by which *in utero* environmental impacts arise, and whether these effects can be reversed; (ii) studies of the longer term consequences of various forms of prenatal insult (generally in humans and rodents) directed towards non-communicable diseases and offspring behaviour, and (iii) theoretical considerations relating to the evolutionary nature of these mechanisms. This review focuses on research concerning the first two of these issues. It is noteworthy that the vast majority of studies which have investigated this phenomenon have tended to focus on the long-term consequences of negative environmental factors such as malnutrition and maternal stress.

5. **Epigenetics:** Every cell within the body retains a copy of the entire genetic code (i.e. the whole genome) of the organism, although not all this information is utilised by every cell. Different cell types are 'programmed' to use the genetic code selectively to achieve their functions. This cell-type specific 'programming' is established during normal development and involves epigenetic mechanisms. Epigenetic processes allow gene expression patterns to differ between cells

without alterations or mutations to the underlying DNA. Epigenetics can thus be described as: "*the study of mitotically and/or meiotically heritable changes in gene function that are not explained by changes in DNA sequence"*. Patterns of gene expression can be inherited across many cell cycles, with adult tissues carrying the 'memories' of 'modified genes' from embryonic development; or even from previous generations. Two classic memory systems exist in mammals that are based on epigenetic programming of the genome: (i) DNA methylation and (ii) associated higher-order chromatin & histone modifications. A detailed overview of our contemporary understanding of these mechanisms and others is provided in Section 2.

6. **Stage of pregnancy:** In recent times cohort studies in humans have tended to focus on early gestation, including the peri-conceptional period, which is a time when the mammalian genome is most sensitive to epigenetic modifications. In studies with ruminants, moderately severe undernutrition up to 30 days after mating does not affect birth weight or growth rate but produces offspring that show symptoms of 'metabolic syndrome' (e.g. hypertension, insulin resistance). In some studies with sheep offspring also have behavioural disturbances and reduced survivability. In contrast, mild undernutrition during this period is associated with increased placental development and enhanced embryo survival. Poor nutrition in late gestation is reliably associated with reduced birth weights which, through impacts on offspring behaviour, thermoregulation and body reserves are associated with increased mortality. Shearing the housed pregnant ewe increases lamb birth weight by increasing dietary intakes, although improved lamb survival and post-natal growth rates are not always evident. The impact of gestational nutrition on calf weight has been less convincingly demonstrated. However, calf weight is reduced when cows experience either heat or cold stress which, in the case of heat stress, may be associated with reduced feed intakes. In addition, birth weight is reduced in cases where mothers experienced ill health (of various forms) during pregnancy.

7. **Stress during pregnancy:** Pregnant farm animals may be exposed to many factors that can elicit physiological stress responses (e.g. transport, human contact, predators, housing). In pregnant rodents and humans these exposures are known to cause permanent and long lasting impacts on the developing offspring, particularly influencing behaviour, and stress reactivity. Very few studies have considered the impact of stress during pregnancy on offspring responses in cattle and sheep. In general, studies suggest that offspring behaviour is altered by exposure of the mother to stressful events, particularly if this occurs during early to mid-pregnancy; and that male offspring are more affected than females. Somewhat paradoxically, exposure to stress during late pregnancy may have positive impacts such as increasing offspring birth weight.

8. **Immunity:** As the immune system develops largely *in utero* in farm livestock, immune function is likely to be susceptible to the effects of the maternal environment. In lambs the absorption of immunoglobulins from colostrum is affected by maternal intake of micronutrients (e.g. cobalt, vitamin E) as well as macronutrients (e.g. protein). A recent and largely unexplored concept in farm animals relates to the hologenome (i.e. the genome of the host plus all microorganisms associated with the host). In mammals microbial symbionts are vertically transmitted to offspring initially via the birth canal and subsequently from milk and the surrounding environment. This has been shown to affect the development of the immune system in humans (associated with allergies, cancer and inflammatory bowel disease). In ruminants this is thought to affect the population of microbes that inhabit the rumen.

9. **Muscle development and carcass composition:** Muscle mass, is an important potential target for epigenetic mechanisms as, in cattle and sheep, the proliferation of muscle fibres occurs *in utero*. Lambs and calves are, therefore, born with a fixed number of muscle fibres, with subsequent growth occurring by hypertrophy (increase in fibre size). When nutritional insults on the pregnant ewe occur during early gestation (a critical period for muscle development) then effects on muscle fibre number and type can be detected in young offspring, but these effects tend to be lost (or are too difficult to detect) in older sheep. Such studies, however, are few and offspring were often subsequently placed on high-planes of nutrition, which probably induced an element of compensation; although the nature (i.e. mechanisms, including epigenetics) of how such compensation may come about has not been explored.

10. **Body fat, appetite and feed efficiency:** In humans and rodents, poor prenatal dietary intakes of energy, protein and micronutrients are associated with increased risk of adult obesity in offspring. In cattle and sheep there also appears to be some evidence of long-term programming of adiposity although, perhaps surprisingly, the development of adipose tissue in ruminants is less well understood than that of muscle. In sheep, nutritional restriction in early gestation, or low birth weight, is associated with increased adiposity, particularly in older (i.e. over 6 months) male offspring. Unlike muscle fibres, there is no evidence to suggest that the number of adipocytes (or precursor cells) is set at a specific stage of life. There is certainly considerable scope to explore this area further, and also how muscle and lipid metabolism can influence residual feed intake and overall feed efficiency. In this context it is noteworthy that although nutritional challenges *in utero* can alter the developing hypothalamic appetite-regulatory circuits in fetal cattle and sheep, there is no evidence that these changes result in alterations in subsequent food intake in current animal production systems. However, emerging data that epigenetic changes in anorexigenic genes could be of lasting significance for appetite drive deserves further study in livestock species.

11. **Reproduction and fertility:** In female cattle and sheep, lifetime supply of potentially fertilizable oocytes (eggs) is established before birth and cannot be replenished thereafter. In males new spermatozoa are produced continually after puberty, but the number of Sertoli cells which are the primary determinant of sperm production and testes size in adult life is determined by proliferation during the fetal, neonatal and peripubertal periods. There certainly appears to be effects of malnutrition *in utero* on development of both male and female gonads. However, there is little evidence for an effect of prenatal nutrition on the onset of puberty in sheep or cattle, and the main impact appears to be on the number of ovarian follicles. There is some evidence of a reduction in ovulation rate and litter size in ewes malnourished during pregnancy, but larger scale studies are required to confirm these observations and their significance in commercial practice. Likewise in cattle, there is some evidence of effects of early pregnancy malnutrition on ovarian follicle reserve in offspring leading to poor subsequent fertility; but here the evidence is even more limited. There is also limited evidence for a negative impact of prenatal undernutrition on fertility of males, although very few long-term follow-up studies have been conducted in this area.

    Environmental chemicals, including so called ‘endocrine disrupting compounds’ (EDCs) have the potential programme various components of the reproductive axis (i.e. brain- pituitary-gonad-uterus) to malfunction in later life, and so affect fertility. There is certainly evidence in rodents to support such effects. Cattle and sheep grazing sewage-sludge treated pastures are exposed to higher than normal levels of such compounds, and so are potentially most at risk. To date the most worrying implication of EDC research relates to the high incidence of

spermatogenic abnormalities in male offspring. Effects on female fertility are less evident, but there is a distinct lack of long-term follow-up studies for both sexes in both cattle and sheep. Given the well-known phenomenon of 'bioaccumulation' is there an increased risk for humans consuming milk and meat products from ruminants grazing sludge-treated pastures?

12. **Advanced reproductive technologies (ART):** The potential of these technologies to enhance reproductive rate of beef cattle and sheep, either within genetic improvement programmes or in commercial herds and flocks, has not been fully realised in the UK. CAP associated structural problems within the beef and sheep sectors are partly to blame for the lack of technical innovation and industry uptake in the past, but there have been issues with regard to success rates and fetal development leading to 'Large Offspring Syndrome'. The main issues pertained to early pregnancy losses and large calves/lambs at birth with associated obstetrical complications and morbidity. The available evidence indicates that there are no obvious long-term effects on animal production and health, although there have been few studies in this area. Subsequent refinements to methods of *in vitro* embryo production seem to have mitigated these adverse effects (although the situation requires monitoring). Developments in the use of sexed semen (e.g. for single-sexed once-bred heifer systems) and genomically evaluated sexed embryos offer huge potential advantages for livestock improvement programmes, recognised and practiced in various countries across the world; none more so than Brazil where it seems that these technologies work better in *indicus* than *taurine* breeds of cattle. The successful uptake of these technologies within the UK beef and sheep sectors requires improvements in the general level of reproductive management and on-farm facilities for handling livestock.

13. **Industry relevance and recommendations:** There are a number of commercially relevant traits that have not been considered in beef cattle and sheep, and aspects of normal agricultural practice that haven't been investigated. These omissions primarily reflect the nature and level of research funding in the past, which has primarily been research council and charity based, and where there has been a clear biomedical slant. The limited data that does exist pertains mostly to sheep.

    In this article we have considered the following traits that have been investigated to a greater or lesser extent: (a) neonatal survival, (b) growth rate and feed conversion, (c) whole-body and carcass composition, (d) animal behaviour, and (e) reproductive potential and fertility. Prenatal risk factors that can influence these traits include: (i) parental nutrition, (ii) gestational stress, (iii) environmental chemicals, and (iv) breeding technologies.

    Consequently, key take-home messages and recommendations include:

    A. *Nutrition during pregnancy*: Adherence to existing standard dietary recommendations for macro- and micro-nutrients should avoid suboptimal *in utero* development that could have negative long-term effects on offspring growth and health. However, there is a lack of information for beef cattle and sheep to predict effects on carcass composition. EBLEX funded studies, therefore, could establish KPIs on commercial herds and flocks to validate/refine these recommendations, and to quantify the extent to which early life development may impact on long-term performance (both physical and financial performance). Data collection should include ewe/cow body condition at key stages of the annual production cycle and birth weight, ultimately with corresponding data on carcass yields. Another key trait to monitor is fertility across successive parities.

B. *Gestational stress*: This is an area that has been under investigated in both beef cattle and sheep. Evidence from rodent and human studies indicates that these effects are real. Factors such as housing, stocking density and handling during pregnancy are all worthy of further investigation.
C. *Environmental chemicals*: As around 73% of sewage sludge is dispensed on agricultural land, so there is a need to assess the effects that this may have on grazing livestock. The available evidence indicates effects on the development of male reproductive organs in sheep, but long-term consequences for ram fertility have not been properly ascertained; and effects in beef cattle have not been established. There is also the issue of bioaccumulation and, consequently, effects in humans consuming meat from animal grazing sludge-treated pastures.
D. *Advanced breeding technologies*: A watching brief on 'Large Offspring Syndrome' is recommended should activity in this area pick up again. These technologies have much to offer for livestock improvement, but the UK lags behind other countries, particularly those in North and South America. Improved standards of reproductive management (i.e. for sperm/egg/embryo donors and recipients) in both beef herds and sheep flocks are required. Improved handling facilities are needed as well as an improved awareness of factors that affect fertility. There is scope also to develop our understanding of why these technologies are so much more successful in *Bos indicus* and opposed to *Bos taurine* cattle. This extends to establishing a better understanding of their underlying fertility, which also differs between these two sub species.

Introduction

The biology of 'fetal programming' is complex, but experimental studies in rodent models and large animals, as well as human epidemiological studies, have clearly demonstrated that they can have a profound effect on subsequent development and life-long impacts on health. Whilst relatively few studies have investigated epigenetic mechanisms in farm animal species, a number have considered the longer-term consequences and impacts of early life events on animal production. Consequently, there is evidence in farm animals that maternal nutrition, stress or ill-health during pregnancy can affect how animals develop before birth, with implications for their later health and productivity. Similarly, there is evidence that offspring development from birth to the onset of puberty can have long-lasting effects for adult traits of economic importance.

However, whilst research conducted to date has generated a basic understanding of the biology underlying prenatal effects in mammals, including farm animal species, the relevance of such effects for livestock production has been poorly explored. As such, the extent to which farmers, industry advisors and other stakeholders should devote effort and resources to modulating prenatal and pre-pubertal development within livestock management systems is unclear. The time, therefore, is right for a comprehensive appraisal of the scientific knowledge on such effects in livestock species, and to integrate such information as exists into farming practice as appropriate. It is clear that elucidating the degree to which maternal state during gestation alters fetal biology, with later implications for productivity, health and welfare outcomes, requires a joint consideration of both the science and the commercial realities of routine farm management. Also, beyond establishing the general principle that maternal state may impact upon progeny during their postnatal lifetime, it is necessary to consider the specific sources of such effects within normal farming systems, and use the available scientific knowledge to suggest possible practical solutions. The ultimate goal of research in this area is to improve the efficiency and competitiveness of beef and sheep production, with associated benefits for individual farmers, the industry as a whole, and for farm animals themselves.

The purpose of the current report, therefore, is to provide for the first time a comprehensive and critical account of our current state of knowledge of how early life events, particularly those that occur in utero, can impinge on long-term growth, development, productivity and health of offspring in cattle and sheep. Given the extent of work conducted in humans and laboratory animals, reference to these species will be made where they provide insights into underlying mechanisms or effects not yet reported in farm animals. In this context it is worth noting that the UK Scientific Advisory Committee for Nutrition' (SACN) subgroup on maternal and child nutrition recently undertook a similar project where they reviewed evidence of how early-life nutrition can influence growth and development in children, and the risk of developing chronic, non-communicable diseases in adulthood (SACN, 2011). Although this review focussed mainly on human nutrition, it considered evidence from animal (mostly rodent) studies where these provided insights into some of the underlying mechanisms, including epigenetic mechanisms. With respect to farm animal species, it is also worth noting that there have been some previous attempts to review aspects of this topic, including the effect of intra-uterine growth restriction on post-natal productivity (Wu *et al.*, 2006), and peri-natal programming of lifetime fecundity (Gardner *et al.*, 2008). However, no previous study in farm animals has undertaken such a comprehensive review of the topic, with implications for industry, as the one now presented. In this review factors influencing early life events in addition to parental nutrition, such as stress and exposure to environmental chemicals, will be considered.

1. Developmental Origins of Health and Disease: the 'Barker Hypothesis' and beyond

Contemporary interest in fetal physiology and development can trace its origins to the pioneering studies of Sir Joseph Barcroft and colleagues at Cambridge (UK) between the early 1920s and mid1960s. Barcroft is described as having been a 'broad-ranging integrative and comparative physiologist', who helped to cultivate an environment in Cambridge at that time where other leading reproductive/developmental physiologists such as Robert McCance and Elsie Widdowson, as well as Sir John Hammond, could flourish (Boyd and Boyd, 2010). Many of their pioneering studies were conducted in ruminant species, particularly sheep, which was utilised as a model to investigate fetal physiology and responses to a variety of environmental stimuli, including nutrition. A number of other leading investigators completed their doctorates at Cambridge during this period, including Lindsay Wallace, later to become Director of the Animal Research Station at Ruakura, New Zealand. Whilst Wallace was destined to develop interests in dairy-cow nutrition, he is best remembered by many for his landmark studies on fetal/neonatal development in sheep (e.g. Wallace, 1948), where mathematical concepts of growth allometry previously developed by Sir Julian Huxley, and later championed by Samuel Brody (Missouri) on concepts of bioenergetics and growth in domestic animals, were applied.

However, the concept that developmental processes *in utero* can predispose offspring to certain chronic diseases in later life, including cancer and various metabolic and cardiovascular diseases, only came to light following publication of the pioneering retrospective cohort studies on human subjects conducted by David Barker and colleagues at the University of Southampton. Initial studies associated the incidence of infant mortality to deaths in adults attributable to bronchitis, stomach cancer and rheumatic heart disease (Barker and Osmond, 1986). These authors proposed that 'poor nutrition in early life increases susceptibility to the effects of an affluent diet'. In a letter to the *British Medical Journal* two years later, however, they presented their first evidence that an adverse intra-uterine environment, culminating in low birth weight, was associated with hypertension in children (Barker and Osmond, 1988). Their findings on death by coronary heart disease in adult men were published in the *Lancet* the following year (Barker *et al.*, 1989). These and related observations gave rise to what is known as 'The Barker Hypothesis', now more commonly referred to as the Developmental Origins of Health and Disease or DOHaD.

Sadly, David Barker passed away on 27th August 2013. However, a plethora of studies have been conducted in the 25 years since his initial findings were published, both in humans and in a variety of animal-model species, including ruminants. These studies have been the subject of extensive review and meta-analysis (McMillen and Robinson, 2005; Gluckman *et al.*, 2008; Fowler *et al.*, 2012; Thayer *et al.*, 2012; Langley-Evans, 2013) and only brief reference will be made in this article to those that don't involve ruminants. Important issues to emerge during this period, and which will be addressed next and later in this report, include (i) nature of environmental exposure (e.g. maternal stress, parental nutrition, environmental chemicals and assisted reproduction), (ii) stage of development at time of exposure (e.g. early vs late pregnancy and infancy), (iii) developmental legacy (e.g. non-communicable chronic diseases, cognitive abilities, growth, fertility and ageing) and (iv) underpinning mechanisms and the likelihood of trans-generational inheritance.

1.1. Human epidemiological studies

In addition to the studies of Barker and colleagues, large-scale retrospective cohort studies in humans include those that investigated the legacy of the 'Dutch Hunger Winter' of 1944-45, where the population of Northwest Holland, including pregnant women, received as little as 400-800 calories per day for a period of 5 months. An extensive body of data has since been published which shows that famine exposure during pregnancy can impair glucose tolerance, increase obesity and atherogenic lipid profiles together with the incidence of coronary heart disease in adult offspring (Roseboom *et al.*, 2006). Females conceived during the famine also had an almost five times increased risk of developing breast cancer in adulthood. The incidence and severity of these various ailments was influenced by the stage of gestation and relative proportion of carbohydrates to protein in the restricted diet. In general, exposure to famine during early gestation resulted in a broader range of adverse effects, which tended to be more severe (Painter *et al.*, 2005). Famine exposure during pregnancy also affected cognitive function and stress sensitivity in adult offspring, and increased the incidence of schizophrenia and anti-social personality disorders (Hoek *et al.*, 1996; Neugebauer *et al.*, 1999). Somewhat paradoxically, women exposed to famine *in utero* were reproductively more successful than women not exposed; they started reproducing at a younger age, had more offspring, a higher proportion of twins and were generally less likely to remain childless (Painter *et al.*, 2008). In contrast, for females, famine exposure during childhood decreased the chances of childbirth and increased the risk of having a medical reason for having fewer children than desired (Elias *et al.*, 2005). In these studies there appear to be no effects of *in utero* famine exposure on male fertility.

Other human epidemiological studies have made use of disasters, either manmade (such as pregnant women living under the threat of rocket attacks; (Wainstock *et al.*, 2013a; Wainstock *et al.*, 2013b) or natural (particularly Project Ice Storm which is mapping the impacts of the Quebec ice storm of 1998; (King *et al.*, 2012), to examine the effect of psychological stress in pregnancy on child outcomes. These cohort studies currently only have data until adolescence for exposed mothers/children but report strong and persistent adverse child outcomes, similar to those seen following severe maternal food restriction. Birth weights are reduced (Dancause *et al.*, 2011; Wainstock *et al.*, 2013a; Wainstock *et al.*, 2013b), and insulin secretion in adolescence, and incidence of obesity in young children, are increased if mothers are exposed to stress in pregnancy (Dancause *et al.*, 2012; Dancause *et al.*, 2013). In particular these studies report detrimental impacts on child cognitive and motor development of maternal stress in pregnancy, with the greatest cognitive deficits occurring when mothers were exposed to stress in early pregnancy and graded by the severity of the subjective stress (Laplante *et al.*, 2004; King and Laplante, 2005; Laplante *et al.*, 2008; Cao *et al.*, 2014).

1.2. Insights from laboratory animals

Retrospective epidemiological cohort studies in humans may be in the species of clinical interest for some but they are open to a number of criticisms, including their ability to deal with confounding environmental factors that span the life-course, and indirect assessments of stress or malnutrition during pregnancy and infancy. Animal models overcome many of these limitations and have been instrumental in developing many of our contemporary theories (see Section 1.6) and in providing mechanistic insights. One of the most extensively studied models of developmental programming has been the maternal low-protein diet (mLPD) in rodents. Studies in rats and mice have shown that protein-restricted diets fed either throughout or during specific periods of pregnancy and lactation lead to hypertension and 'metabolic syndrome' (see Section 3) in offspring observed from weaning onwards (Langley-Evans, 2013). Importantly, variability in the nature and magnitude of

effects between studies can be attributed to the composition of diets (transpires that it's the nutrient balance with respect to provision of sulphur amino acids and oil and not low protein *per se* that's key), stage of pregnancy, species (i.e. rat vs mouse) and genetic strain within species. The same basic model has been used to demonstrate intergenerational programming of nephrogenesis and hypertension in rats (to F2 but not F3; (Harrison and Langley-Evans, 2009)) (see Section 1.5), and paternally-induced epigenetic programming of metabolism in mice (Carone *et al.*, 2010). A number of other rodent-based models exist that have investigated the effects of global-nutrient restriction, obesity, maternal diabetes, dietary sugars, fat and salt during various stages of pregnancy. By way of example, in one such study the feeding of paternal high-fat diets led to β-cell dysfunction in Sprague-Dawley female, but not male, offspring leading to impaired glucose tolerance and insulin secretion, which was mediated at least in part by epigenetic modifications to genes involved in pancreatic function (Ng *et al.*, 2010). Later in this article reference will be made to genomic imprinting and the consequences of environmentally-induced epigenetic alterations to these developmentally important genes. However, it is worth noting that in a murine model of dietary restriction, where pregnant females were fed 50% that of controls, imprinted genes as a class were found to be neither more nor less susceptible than non-imprinted genes to epigenetic regulation of expression (Radford *et al.*, 2012). However, those genes that were altered are known to play important roles in conceptus response to undernutrition.

Effects on offspring health and behaviour of prenatal stress or exposure to excess glucocorticoids have also been extensively investigated in both the rat and mouse (discussed next). However, for all of these studies the mouse in particularly is powerful because of the ability to analyse the effects of single-gene mutations, to conduct linkage analysis in crossbred strains and carry out gene targeting in order to establish disease phenotypes associated with specific genes or alleles. Orthologous genes in humans or other species can then be tested, either in linkage studies in families or in genome-wide association studies (GWAS), for effects on phenotype.

With respect to models of pre- and post-natal stress, in 1985 a paper detailing the impact of postnatal handling of rat pups on hippocampal glucocorticoid receptors (Meaney *et al.*, 1985) suggested a neurological basis to the altered stress reactivity seen in handled vs non-handled pups. Although several hypotheses were proposed, the induction of altered maternal care induced by handling the offspring has subsequently been shown to play a very significant role in subsequent development. Elevated maternal licking and grooming behaviour, either occurring as part of natural variation in behaviour or induced by pup handling, alters the pup epigenome at the hippocampal glucocorticoid receptor (which plays an important role in regulating stress reactivity) in comparison to low maternal licking (Weaver *et al.*, 2004; Weaver *et al.*, 2006). Maternal care in the rat thus influences offspring stress reactivity, and the expression of maternal behaviour in the female offspring (and so can have a transgenerational impact), and has been shown by cross-fostering to be related to epigenetic rather than genetic mechanisms (Champagne, 2008). As rats are born at a much earlier stage of development than farmed livestock, whether similar mechanisms may operate in farm animals is not known although there is evidence for similar responses to maternal care in humans. A second well-studied rat model is that of prenatal restraint stress (PRS; reviewed by (Darnaudery and Maccari, 2008)). These studies demonstrate hyperactivation of the hypothalamic-pituitary-adrenal stress axis in offspring of PRS mothers and enduring behavioural differences compared to offspring of unstressed mothers. These studies have also received considerable attention as they highlight often marked differences in impact in male and female offspring (Darnaudery and Maccari, 2008; Bale, 2011). These rodent studies are starting to inform thinking and research in farm animal species, and the impact of pregnancy stress is beginning to be explored more widely (see section 3.2)

The guinea pig has also been used as a more precocious model animal species than rodents, which makes it attractive as a model for farmed livestock. Studies have investigated prenatal nutritional impacts (e.g. Dwyer *et al.*, 1995), social stress in pregnancy (e.g. reviewed by Sachser *et al.*, 2013), other forms of prenatal psychosocial stress such as exposure to strobe lighting (e.g. Schopper *et al.*, 2012a; Schopper *et al.*, 2012b) and use of exogenous glucocorticoids in pregnancy (e.g. Owen and Matthews, 2007a and 2007b). As with rat models, psychological stress in pregnancy is associated with altered fetal brain development, dysmasculinisation of male offspring, and modified stress responses in adulthood in guinea pigs, which has been shown to persist into the second generation (Schopper *et al.*, 2012a; Schopper *et al.*, 2012b). Research in guinea pigs has also focused on adolescence as a second sensitive period, in addition to prenatal, when behavioural profiles may be modified (Sachser *et al.*, 2013).

1.3. Other farm animal models

Passing reference to studies conducted in the pig is made throughout this article, but it is worth pausing to reflect on the significant contribution that this species has made to biomedical research in general and specifically investigations into MetS. The value of porcine models for these types of study was comprehensively reviewed by (Litten-Brown *et al.*, 2010), who also considered the value of mini pigs. These authors noted important metabolic/physiological differences between domestic breeds (i.e. between Large White, Meishan and Prietran). The Large White is often favoured for these types of study due to the great variability that exists in body-weight among littermates, which serves as a natural model of intra-uterine growth restriction (IUGR).

As a litter bearing species the pig presents unique challenges to production processes and provides novel insights into within-litter interactions and competition. For example, both IUGR piglets and within-litter variability in piglet birth weights limit effective piglet management. The consequences of IUGR for traits of commercial importance in pigs (e.g. neonatal survival, muscle development) were considered by (Foxcroft *et al.*, 2006), who raised concerns about introducing hyperprolific females into the breeding herd. Alterations to the composition of the diet consumed by pregnant female pigs have been shown to increase average birth weight and reduce the incidence of IUGR piglets. These include feeding a diet low in vitamin A before mating and during the first month of pregnancy (Antipatis *et al.*, 2008), supplementing gilt diets with L-arginine from day 30 to day 144 (Mateo *et al.*, 2008) or 1% L-glutamine between days 90 and 114 of gestation (Wu *et al.*, 2011). L-Arginine supplementation, which has also been shown to prevent fetal growth restriction in undernourished ewes (Lassala *et al.*, 2010), is of particular interest as arginine is the immediate precursor of both the production of nitric oxide which is an important vasodilator and the production of polyamines which are critical for normal cell and tissue growth.

Female pigs that experience stressful situations during pregnancy give birth to piglets exhibiting a wide range of altered phenotypes, in the absence of effects of prenatal stress on litter size or piglet birth weight. For example, in studies prompted by the need to understand the consequences of group-housing pregnant pigs throughout gestation, pigs born to sows that were stressed by being mixed with an unfamiliar older sow for two 1-week periods during mid-pregnancy had heightened pain perception (Rutherford *et al.*, 2009), reduced post-weaning growth rates, altered immune status (as measured by concentrations of acute-phase proteins) and poorer reproductive development, particularly in male piglets (Ashworth *et al.*, 2011). Similarly injections of ACTH, which mimic a stress-induced increase in cortisol, during mid-pregnancy were associated with a shorter anogenital distance in male piglets at birth (Lay *et al.*, 2008), suggesting that pre-natal stress reduces the degree of masculinisation of male fetuses.

In contrast there have been few large-scale studies assessing possible associations between maternal diet during pregnancy and offspring development in dairy cattle. One study of 988 heifers from a controlled genetic selection trial found no significant relationships between the feeding system (high or low concentrates) and the reproductive performance of daughters in two genetic lines (Pryce *et al.*, 2002).

1.4. Inter-(trans)-generational inheritance

Considerable excitement and much debate has surrounded this topic in recent years, particularly in the context of epigenetic inheritance, as this this is often touted as the mechanism by which traits acquired in one generation are passed onto the next (reviewed by (Grossniklaus *et al.*, 2013; Aiken and Ozanne, 2014)). The concept of inheritance of acquired characteristics was first proposed by the French naturalist Jean-Baptiste Lamark (1744-1829), who subsequently became discredited for entertaining such a notion. However, our contemporary understanding of biological processes across a wide range of species that include plants, invertebrates and mammals broadly supports this concept and the involvement of epigenetics. It is known, for example, that heritable silencing of repetitive DNA sequences that constitute much of the heterochromatic regions of the genome occurs trans-generationally, and involves epigenetic mechanisms such as those described in Section 2 of this article. A related example involves inheritance of epigenetic modifications at the agouti locus in mice. In viable yellow (A^{vy}/a) mice a retrotransposon inserted upstream of the agouti gene causes ectopic expression of agouti protein which has pleiotropic effects, influencing coat colour, metabolism and health. The distribution of phenotypes is related to the methylation status of this retrotransposon which is incompletely erased when passed through the female germline, thus leading to inheritance of epigenetically induced modifications (Morgan *et al.*, 1999). However, as discussed in the next section, most acquired epigenetic 'marks' that involve modifications to nuclear DNA and associated proteins are erased or 'reset' in the germ line, and so are not passed on directly to subsequent generations. This would also appear to be the case for the A^{vy} locus, for maternal methyl-diet-induced epigenetic modifications at this region are not trans-generationally inherited (Waterland *et al.*, 2007).

At this point in the discussion it is worth noting that prions represent a class of protein that can be inherited across generations independently of chromosomes. Pioneering studies with prion proteins (PrP) in lower eukaryotes (i.e. yeast and filamentous fungi) by Susan Lindquist and others have revealed that they can act as 'epigenetic' elements and can at least partially account for non-Mendelian patterns of inheritance for a number of traits (Hofmann *et al.*, 2013). Mammalian prions share many common features with their counterparts in yeast, but their function and patterns of inheritance are less well known. They have, nevertheless, been shown to regulate pluripotency in mouse embryonic cells, and contribute to their differentiation into neural progenitor cells (Peralta *et al.*, 2011; Miranda *et al.*, 2013). Importantly, prion proteins (PrP^{C}) are also present in bovine oocytes and pre-elongation embryos (Peralta *et al.*, 2012), although again their function is poorly understood. Understandably, prions have received bad press over the last two decades due to the involvement of a misfolded form of this protein (designated PrP^{Sc}) in bovine spongiform encephalopathy (BSE) and variant Creutzfeldt-Jakob disease (vCJD) (Ironside, 2012). Intergenerational inheritance (i.e. vertical transmission) of these aberrant variants that lead to scrapie in sheep can occur across the placenta from around mid-gestation in genetically susceptible dams and fetuses (Wrathall *et al.*, 2008). The consensus formed from results of artificial insemination and embryo transfer experiments in both cattle and sheep, however, suggests that transmissible SEs are unlikely to be spread by semen or the pre-hatching embryo (i.e. < Day 7).

The foregoing discussion, nevertheless, highlights the importance of inheritance of cytoplasmic factors (that include mitochondria) in addition to nuclear chromatin at the point of fertilisation. It also indicates that the maternal environment can influence fetal development in a manner that leads to the inter-generational transmission of phenotypes, and that this may be independent of epigenetic effects. Jirtle and Skinner (2007) noted that for epigenetic modifications to chromatin to be considered a plausible mechanism for inheritance of phenotypic change then effects need to persist to at least the F_3 generation. The reason is that when an F_0 gestating female is exposed to environmental stimuli, both the F_1 embryo and F_2 generation germ-line are also directly exposed. For this reason neither parental nor indeed grandparental effects need have an epigenetic basis; although pup-licking and grooming behaviour in rats can lead to epigenetic modifications at the glucocorticoid gene-promoter in offspring (Weaver *et al.*, 2004). Jirtle and Skinner (2007) went on to summarise some of their own work in rats where exposure to environmental toxicants (e.g. the agricultural fungicide vinclozolin; see Section 5) led to germ-line alterations to the epigenome and phenotypic defects that were present in F_3 progeny. These defects included male infertility, breast cancer and immune abnormalities.

Other examples of trans-generational inheritance of phenotypic traits in rodents are emerging, although notably few extend to or beyond F_3; and these have been reviewed in detail elsewhere (Aiken and Ozanne, 2014). Not surprisingly, evidence for similar effects occurring in farm animals is scarce. However, some recent tantalising (i.e. not quite statistically significant) data in the pig indicate that F_0 boars, fed diets enriched in one-carbon metabolites (including methionine, choline, vitamin B_{12} and folate), sired F1 boars that in turn sired F2 pigs which produced leaner carcasses associated with global changes in gene expression and epigenetic modifications to at least one of these genes (Braunschweig *et al.*, 2012). Additionally, albeit in an avian model, evidence of transgenerational transmission of attenuated stress reactivity, due to early life stress, to male offspring has been demonstrated in domestic chickens (Goerlich *et al.*, 2012).

1.5. Theoretical considerations

The previous section on trans-generational inheritance raises theoretical considerations as to why such effects may arise. Over the years a number of paradigms have been advanced as a means to frame developmental programming within a scientific context. In 1986, it was proposed by Professors Nick Hales and David Barker that fetuses exposed to a less than optimal nutritional environment adopted and 'thrifty phenotype' (Hales and Barker, 1992). That is, in order to balance energetic supply and demand, fetuses down-regulated energy i.e. nutrient, consuming processes (e.g. growth) as a short-term reductive adaptation that ensured survival through any nutritionally restrictive period. Subsequent fixation of such a phenotype into adult life when nutrients may be more abundant can determine their intake. However, this has negative consequences; for a given energy intake fetuses rendered thrifty have greater fat deposition (particularly ectopic), skeletal muscle insulin resistance and other indices of metabolic syndrome. Furthermore, such term infants upon exposure to adequate nutrients not constrained by maternal supply, have a greater tendency towards 'catch-up growth' – itself an independent risk-factor for later deleterious metabolic outcomes (Barker *et al.*, 2005). Thus the thrifty phenotype hypothesis evolved into the 'mismatch hypothesis' (Gluckman *et al.*, 2005b) in that metabolic sequalae were exacerbated when the pre-natal/juvenile and adult environments were 'mismatched'. That is, fetuses experiencing poor nutrition prenatally but excess nutrition postnatally, perhaps as adolescents or adults. The 'mismatch' hypothesis perhaps explains a large proportion of the patients with maturity-onset diabetes in India that have experienced a 'thin-fat' nutrition transition (Yajnik, 2004). However, babies born to overweight and/or diabetic mothers are invariably large or 'macrosomic' but also

have an increased risk of diabetes, especially in Westernised countries (Catalano *et al.*, 2003). Here, both the developmental and adult environments are nutritionally excessive and therefore 'matched' and thus in accord with the 'mismatch' hypothesis should have reduced disease risk. This is clearly not the case and thus the thrifty or mismatch hypotheses required some revision.

As research into developmental programming progressed it was increasingly found that the thrifty phenotype and mismatch hypotheses tended to account for only those individuals exposed to a limited proportion of the full range of developmental experience and that certain individuals had excess risk of disease despite no obvious early compromise (e.g. they were not born low birth weight). As a result, Gluckman and Hanson sought to explain developmental programming in an evolutionary context where fetal adaptation to a poor early environment did not necessarily provoke an immediate 'survival response' leading to, for example, low birth weight, but rather they proposed that subtle adaptations only became evident and advantageous later in life (i.e. a 'predictive adaptive response' (PAR) (Gluckman *et al.*, 2005a). A PAR may be induced early in life, remain asymptomatic, but result in improved or maximized fitness at a later stage of development (i.e. adulthood, should the developmental and adult environments be similar). To support the hypothesis disparate examples of a PAR from the biological sciences were found. Only recently was a study conducted in human populations that sought to test this hypothesis (Hayward and Lummaa, 2013). Contrary to predictions of the PAR hypothesis, individuals that had experienced low early-life nutrition had lower survival and fertility during subsequent famines (i.e. low later-life nutrition) relative to individuals that experienced high later-life nutrition. Thus alternative theoretical models are still required to provide a framework of understanding for the epidemiology of metabolic disease.

Nevertheless, in regard to transgenerational inheritance, most human studies have only studied developmental programming effects within the first generation offspring (F_1). Even if such female offspring become pregnant and passed on a deleterious phenotype to their offspring (F_2) the effect cannot be truly considered transgenerational since how F_1 adapt to the anabolic and metabolic challenge of pregnancy has been shown to be influenced by the early life environment (Yinon *et al.*, 2010; King *et al.*, 2013). Very few studies have considered developmental programming effects through to the F_3 generation – a response that must evoke epigenetic programming of the germ-line, as discussed elsewhere in this review.

1.5.1. The hologenome concept and development

The hologenome concept in the context of developmental programming states that environmental factors, such as diet, can alter the microbiota in such a way as to not only benefit the holobiont (host plus all microorganisms) in the short term, but through transmission to offspring, have long-lasting multi-generational effects (Rosenberg and Zilber-Rosenberg, 2011). This line of thinking is comparatively new and, in mammals, largely untested. Under normal conditions the cooperation between the microbiota and host generally leads to improved fitness of the holobiont. For the host this includes protection against infectious disease, development and function of innate and adaptive immune systems (particularly in the gut), vitamin synthesis (including B vitamins such as cobalamin and folate), and protection against certain cancers and 'metabolic syndrome' (Kau *et al.*, 2011). Indeed, Ross *et al.* (2013) used metagenomic data derived from high-throughput deep sequencing to predict inflammatory bowel disease status and body mass index in humans. However, they also used the same approach to predict enteric methane production in cattle.

In mammals microbial symbionts are initially vertically transmitted to offspring through the birth canal (note reported differences in human infant microbiota between vaginal and caesarean

deliveries), subsequently from milk (note differences between breast vs formula fed infants), and from close physical contact with mum and the surrounding environment (Kaplan *et al.*, 2011; Kau *et al.*, 2011). In human medicine current interests mainly concern developmental programming of the immune system, whereas in ruminants the current primary driver for research into microbiota-host interactions lies in methanogenesis and greenhouse gas emissions (Morgavi *et al.*, 2010), where emerging evidence in sheep and goats indicate that the population of methanogens in the rumen may be acquired from a very young age (Gagen *et al.*, 2012; Abecia *et al.*, 2013). There is clearly considerable scope to extend these emerging ideas and data in all species to investigate long-term developmental programming in offspring, adaptive responses to changing environments and associated inter-generational inheritance.

2. Epigenetic mechanisms of embryonic and fetal programming

Cloning of “Dolly the sheep” demonstrated that virtually every cell within an adult mammal retains the entire genetic information (i.e. the whole genome) of the organism. It also made clear that not all of the genetic code is utilised by the many different cell types of an animal. That is, a mammary cell and a neuron are ‘programmed’ to selectively retrieve different genetic information encoded within the genome. Such cell type specific ‘programming’ is established during normal development and involves epigenetic mechanisms. A contemporary description of epigenetics states that it is “*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*” (Riggs, 1996). Epigenetic processes program gene expression patterns and thereby uphold cell identity without altering or mutating the DNA. Such states in gene activity can be inherited through many cell divisions. Cells in adult tissues have the capacity to carry memories of embryonic development (Hon *et al.*, 2013) or even from past generations.

Two classic memory systems exist in mammals that are based on epigenetic programming of the genome: i) DNA methylation and ii) chromatin & histone modifications.

2.1. DNA methylation

DNA methylation in mammalian cells is predominantly targeted to cytosines of the palindromic CpG dinucleotide sequence. ("p" refers to the phosphodiester bond that connects the bases "C" and "G"). DNA is duplicated prior to cell division by semi-conservative DNA replication to ensure that the daughter cells receive a full copy of the genome. Following DNA replication, DNA methyltransferases (Dnmts) copy the methylation pattern of the parent DNA strand onto the newly synthesized daughter strand; the methylation status of a ‘parent CpG’ serves as template for the ‘daughter CpG’. Such ‘maintenance methylation’ is therefore a mechanism that transmits epigenetic information ‘on the back’ of DNA to descendants of a given cell.

Mammalian DNA has millions of CpG dinucleotides. These potential methylation sites are unevenly distributed throughout the genome. Regions of high CpG-density speckle a genome that is otherwise characterised by a relative depletion of this dinucleotide. Approximately 70% of gene promoters are CpG-rich (Saxonov *et al.*, 2006). The majority of CpG-rich promoter regions remain completely unmethylated throughout development and adult life. Biologically important exceptions are CpG-rich sequences of imprinted genes and gene promoters present on the inactive X chromosome in somatic cells of females. Dense promoter methylation is generally associated with gene inactivity.

We still do not fully understand how cell type specific DNA methylation patterns emerge. The genotype exerts a strong influence and provides a blueprint for DNA methylation patterns found in adult tissues (Silva and White, 1988; Gertz *et al.*, 2011). However, evidence suggests that these DNA methylation patterns are subtly altered during the life-course of an animal by environmental, physiological and stochastic events (Jaenisch and Bird, 2003; Whitelaw and Whitelaw, 2006). Plasticity and modulation of DNA methylation patterns in response to environmental signals, likely processes involved in fetal programming, are thought to have particular impact during critical periods of development when cell fates are specified.

Thus, measuring differences in DNA methylation has become an important approach to explain phenotypic differences observed, for example in monozygotic twins and inbred animals. Measurements, however, are complicated by the presences of additional cytosine-modifications. The TET family of enzymes oxidise methylated cytosines to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito *et al.*, 2011). At present, routine epigenetic screens fail to efficiently distinguish between the different types of cytosine modifications in DNA. TET-oxidised cytosines may represent intermediate steps of a demethylation process that removes the epigenetic mark from DNA. These cytosine modifications may also play other, as yet unidentified roles in gene expression and DNA metabolism. Whatever their purpose, current findings indicate that there is a cross-talk between DNA methylation/modifications and the second epigenetic memory system, which is based on chromatin structure and histone modifications.

2.2. Histone modifications

Chromosomal DNA of eukaryotic cells is always in contact with certain nuclear proteins; this type DNA-protein complex is called chromatin. The core unit of chromatin is the nucleosome (see **Figure 2.1**). It is a structure that consists of a 146 base-pair DNA sequence that is wound around an octamer-complex composed of two histone proteins each (H2A, H2B, H3 and H4). Chromatin is a dynamic structure and its configuration ranges from open, transcriptionally active ‘euchromatin’, to condensed transcriptionally silent ‘heterochromatin’. Large portions of the genome are packed and organised into heterochromatin in differentiated cells. Genes poised for expression in a given cell-lineage are thought to emanate from ‘euchromatic’ chromosomal loops that provide access to transcription factors. Multi-subunit protein complexes are capable of remodelling the chromatin structure by repositioning of nucleosomes, leading to changes in established, lineage-specific gene expression patterns. Chromatin and gene expression are further influenced by posttranslational modifications of histones.

N-terminal tails of histones protrude from the nucleosome-core and extend beyond the associated DNA. This structural property permits communication with surrounding nuclear factors. A host of enzymes has been identified that can either add - or remove - an ever-expanding list of modifications to histone tails (reviewed by Bannister and Kouzarides, 2011; Arnaudo and Garcia, 2013). Many of these post-translational histone modifications promote or inhibit gene transcription and influence the general chromatin structure. The numerous possible combinations of histone modifications add to the complexity of epigenetic gene regulation. For most of these combined modification patterns the biological function(s) remain to be decoded. Generally, genomic regions with modification-rich histone tails are associated with gene regulation and expression. For example, histone H3 usually has three methyl-groups added to the fourth lysine (H3K4me3) in promoters of transcriptionally active genes. An overview of presently known histone modifications is provided in **Figure 2.1**. Unlike DNA methylation, epigenetic inheritance of region-specific

histone modifications from mother to daughter cells is only rudimentarily understood. Likewise, we are only beginning to unravel signalling pathways, environmental cues and cellular factors that determine how histone modifications are laid down.

2.3. Non-coding RNAs

Non-coding RNAs have emerged as functional molecules that can initiate and guide epigenetic changes in both DNA and histones (reviewed by Sabin *et al.,* 2013). Small RNAs and long-noncoding RNAs (lncRNAs) are the two broad classes of biological ribonucleic acids known to participate in epigenetic processes such as transcriptional silencing, chromatin remodelling and DNA methylation. For instance, methylation and inactivation of transposable genetic elements can be mediated by piRNAs, a class of small (26-31 nucleotides), non-coding RNAs. These piRNAs bind specialised protein-complexes and are thought to recruit Dnmts to repetitive elements present within the genome (Carmell *et al.*, 2007).

lncRNAs were first identified to play prominent roles in epigenetic phenomena such as X-inactivation and genomic imprinting. With the advent of new sequencing technologies that allow profiling of a cell's entire transcriptome it became apparent that thousands of genomic loci express lncRNAs (Ulitsky and Bartel, 2013). Thus, we are only starting to understand the specific roles of these non-coding RNAs in epigenetic regulation. The lncRNA *HOTAIR,* for example, associates with the Polycomb repressive complex 2 (PRC2) and is necessary to promote methylation of histone H3 at lysine 27 in certain chromosomal domains (Rinn *et al.*, 2007). Intriguingly, a recent study demonstrated that oestradiol induces transcription of the lncRNA *HOTAIR* (Bhan *et al.*, 2013). It is therefore reasonable to speculate that non-coding RNAs are mechanistically linked with environmental programming of the reproductive system.

2.4. Gender differences mediated by epigenetics

Differences in gene expression have been observed between male and female pre-implantation embryos (Kobayashi *et al.*, 2006; Bermejo-Alvarez *et al.*, 2010; reviewed by Gardner *et al.*, 2010). This type of sexual dimorphism appears to be a hormone-independent cell phenotype and affects both autosomal and X-chromosome-linked genes. For example, one-third of transcribed protein-coding genes analysed (~2,900 transcripts) show sex-specific differences in *in vitro*-generated bovine blastocysts (Bermejo-Alvarez *et al.*, 2010). Paternal imprinting of the bovine X chromosome could partly explain the up-regulated expression of X-linked genes in normal female blastocysts, as parthenogenetic embryos, which carry two maternal X chromosomes, were found to have lower transcript levels of representative X-encoded genes, such as *BEX1, CAPN6, BEX2, SRPX2, and UBE2A* (Bermejo-Alvarez *et al.*, 2010). Moreover, the activity of the two X-chromosomes in female blastocysts also appears to influence the expression of autosomal genes, leading to gender-specific transcript differences (reviewed by Wijchers and Festenstein, 2011) Female mouse ES cells with a deficiency of the DNMT3-like methyltransferase (*DNMT3L*$^{-/-}$), lose genomic DNA methylation patterns more rapidly than their male *DNMT3L*$^{-/-}$ ES counterparts (Ooi *et al.*, 2010). Altered nutrition during gametogenesis and pre-implantation development, shown to modulate DNA methylation patterns (e.g. Sinclair *et al.*, 2007), may augment sexual dimorphism of gene expression patterns and thereby contribute to more pronounced gender differences in adult animals.

2.5. Recent advances in epigenetics

Although cytosine modifications in mammalian genomes are generally thought to occur in a DNA sequence context of CpG dinucleotides, there are notable exceptions to this 'rule'. Prior to the high-throughput sequencing era, prevalent non-CpG methylation of cytosines had been detected in mouse embryonic stem cells at CpA and, to a lesser extent, at CpT sites (Ramsahoye *et al.*, 2000). More recent data confirm and extend this finding, demonstrating that non-CpG methylation is also present during male germ-cell development (Ichiyanagi *et al.*, 2013), in oocytes (Shirane *et al.*, 2013) and is enriched within gene bodies of highly transcribed genes in both fetal and adult mouse brain (Lister *et al.*, 2013). As mammals appear to lack enzymes that copy asymmetric non-CpG marks, it is currently not clear how this type of modification could contribute to the propagation of epigenetic states established as a result fetal programming events.

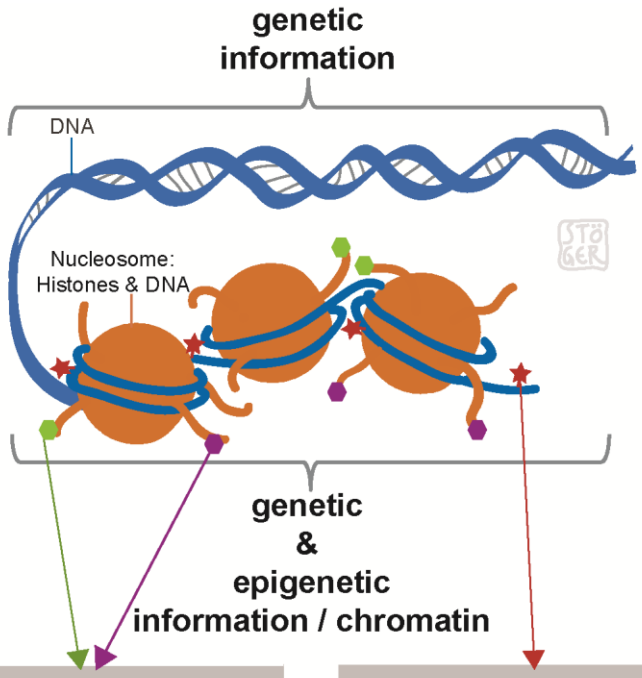
The study of RNA methylation is also an emerging field related to 'traditional' epigenetics and may prove relevant for our mechanistic understanding of fetal programming. Two modifications on bases located internally of RNA molecules - N6-methyladenosine (m6A) and 5-methylcytosine (5-mC) are now considered to have important roles, albeit their specific biological functions are only starting to be determined. For example, m6A is a reversible base modification which can be removed by FTO, a m6A-demethylase genetically associated with obesity and the control of energy homeostasis. How such RNA modifications might be able to contribute to heritable epigenetic phenotypes remains to be shown (reviewed by Liu and Jia, 2013).

2.6. Resetting the epigenome during mammalian development

Two major epigenetic reprogramming events take place during early embryo development. The first event occurs right at the onset of development (**Figure 2.2**). Soon after fertilization sperm and the egg DNA undergo extensive chromatin remodelling in a process that begins by the formation of two pronuclei containing highly decondensed DNA. The open chromatin configuration resulting from the decondensation of the sperm DNA facilitates the assembly of new nucleosomes in the male pronucleus and entails the replacement of protamines by histones. These newly incorporated histones acquire specific modifications during the first cleavage divisions. These modifications include marks indicative of transcriptional activation (e.g. histone H3K9ac and H3K4me3) as well as other enriched in transcriptionally inactive regions. This array of new histone marks establishes a chromatin landscape that will ensure the timely expression of developmental genes when the major zygotic genome activation takes place after several cell divisions. Likewise, the maternal genome undergoes remodelling of chromatin marks, however these follow a different kinetics to that of the male pronucleus (Morgan *et al.*, 2005). One of the best characterized epigenetic marks is DNA methylation. The sperm DNA, which is more methylated than oocyte DNA (Kobayashi *et al.*, 2012; Smallwood and Kelsey, 2012) undergoes active demethylation during the first cell cycle. This process is in part driven by TeT3 enzyme which catalyses the conversion of methylated cytosines into hydroxymethyl cytosines (Gu *et al.*, 2010; Iqbal *et al.*, 2011) before the start of DNA replication (Wossidlo *et al.*, 2010) Paternal DNA demethylation is an essential step in early development, as most TeT3 mutant embryos do not survive development to term (Gu *et al.*, 2010). The maternal genome, however, undergoes passive DNA demethylation by dilution during mitotic divisions and by the concurrent exclusion of *de novo* Dnmts from the nucleus of early blastomeres (Carlson *et al.*, 1992). The global DNA demethylation observed in the preimplantation embryo however excludes certain regions of the genome. Indeed, imprinted genes are protected from this

DNA demethylation activity, and recent evidence shows that the maternal and paternal imprints are protected by different mechanisms (Nakamura *et al.*, 2012). The extensive demethylation between the zygote and the morula stage prepares the chromatin of the totipotent blastomeres for the segregation of the lineages that will contribute to the formation of the conceptus. Although the complexity of these changes is just beginning to be understood, it is well known that this process is indispensable for ensuring normal embryo development. This is demonstrated by experiments where mutation of histone modifying enzymes or Dnmt in embryos leads to severe abnormalities or death (Li *et al.*, 1992; Peters *et al.*, 2001; Posfai *et al.*, 2012). This indicates that remodelling during early stages of development is of critical importance for resetting the epigenome in preparation for establishment of new programs of differentiation during lineage commitment. Importantly, the kinetics described above for rodents have also been observed in embryos of different domestic animals, including cattle, suggesting that these mechanisms are conserved across mammals (Lepikhov *et al.*, 2008; Maalouf *et al.*, 2008).

The second major wave of epigenetic reprogramming takes place in the germline (**Figure 2.2**). The embryonic precursors of the mature gametes, or primordial germ cells (PGC) are first located at the base of the allantois from where they will initiate their migration to their final destination, the gonadal ridges. In large mammals this period extends between two and eight weeks of development. It is here that environmental perturbations can have long lasting effects on offspring. Indeed, during this period PGC undergo extensive reprogramming of their epigenome, characterized by dynamic changes in histone modifications (loss of H3K9me1/2 and gain of H3K27me3 and H3K4me2), genome wide DNA demethylation (including imprinted genes), and reactivation of the X-chromosome in females (Saitou and Yamaji, 2012). Recent investigations however show that some retrotransposable elements (such as IAPs or intracisternal A-type particles) escape reprogramming in germ cells (Popp *et al.*, 2010), a mechanism that probably evolved to prevent parasitic sequences moving within the genome. Importantly, the resistance to reprogramming by these sequences can lead to phenotypic inheritance between generations (Morgan *et al.*, 1999; Daxinger and Whitelaw, 2012). This initial reprogramming resulting in the resetting of chromatin marks if followed by the differential re-establishment of imprints in male and female gametes. In males, paternal imprints are re-established in mitotically arrested gonocytes before birth. In females, however, imprints are re-established after birth during follicle growth. The mechanisms of germline reprogramming have been primarily characterized in rodents, however, studies in large mammals (i.e. human and pig) show that the overall equivalent kinetics is similar, although some of the changes occur in a more protracted manner, consistent with slower development compared to rodents (Hyldig *et al.*, 2011; Gkountela *et al.*, 2013).



histone modifications	
modification type	**modified amino acid**
Acetylation	Lysine
ADP-ribosylation	Lysine
Biotinylation	Lysine
Butyrylation	Lysine
Crotonylation	Lysine
Formylation	Lysine
Glutathionylation	Cysteine
Hydroxylation	Tyrosine
Malonylation	Lysine
Methylation	Lysine, Arginine
O-glcNAcylation	Serine
Phosphorylation	Serine, Threonine
Propionylation	Lysine
Succinylation	Lysine
Sumoylation	Lysine
Ubiquitylation	Lysine

cytosine modifications	
modification type	**modified base**
Methylation	Cytosine (5-mC)
Hydroxylation	Cytosine (5-hmC)
Formylation	Cytosine (5-fC)
Carboxylation	Cytosine (5caC)

Figure 2.1. Epigenetic mechanisms program and regulate gene expression patterns and thereby influence the phenotype without changing the DNA sequence (genetic information) of a cell. Well-defined epigenetic mechanisms include DNA modifications of the cytosine base and post-translational modifications of histone proteins which, together with around 146 base pairs of DNA, form the nucleosome, a core unit of chromatin.

It is thought that this highly complex and extensive remodelling of the PGC epigenome is critical for preventing the inheritance of epimutations acquired by the parental DNA. Having a detailed

understanding of the dynamic changes that occur during the epigenetic reprogramming of the germline in large mammals will inform into the periods of increased susceptibility of PGC to environmental effects and their potential effects in the offspring.

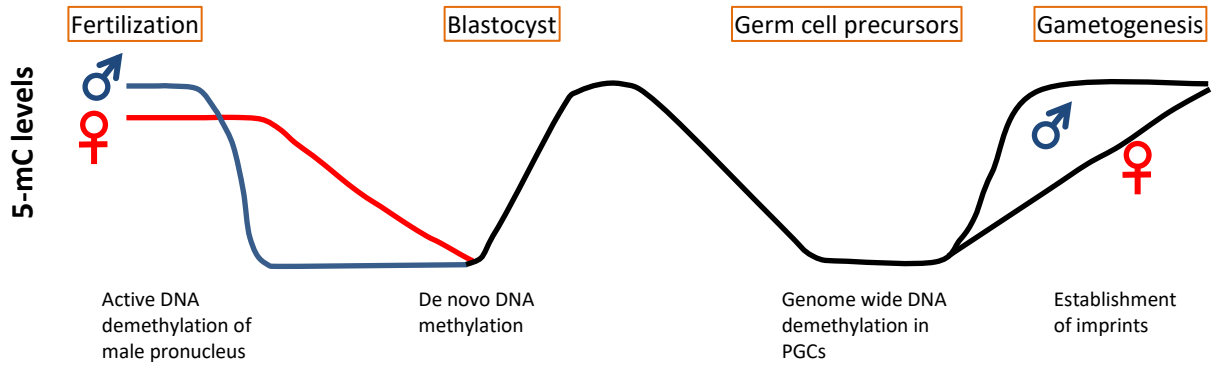


Figure 2.2. DNA methylation in pre-implantation embryos and germ cells. Removal of DNA methylation marks during embryo development prevents the transmission of epimutations between generations. Two major waves of methylation reprogramming take place during development: 1- rapid demethylation of the paternal genome takes place after fertilization. The maternal DNA is demethylated gradually during cleavage divisions. De novo methylation is established in a tissue specific manner during germ layer differentiation; 2- germ cell precursors undergo genome wide demethylation and erasure of imprinted loci during fetal development. During gametogenesis the germ cells acquire new imprinted methylation marks in a parent-of-origin specific manner.

2.7. Epigenetics and developmental programming

Given that the genetic code doesn't vary between cell types it follows that epigenetic mechanisms evolved in multicellular organisms to allow cell-lineage specific gene expression (Jablonka, 1994). How these mechanisms combine to facilitate cellular differentiation is incompletely characterised but, with the advent of contemporary deep-sequencing and related technologies, developmental epigenetics has become a highly active field of biology, so that our understanding of these processes is likely to improve rapidly in the very near future.

Much attention to date has focussed on the role of tissue-specific differentially methylated regions of DNA, particularly those that reside within CpG islands (CGIs). These may be associated with annotated gene transcription start sites, or lie within or between genes. (Illingworth *et al.*, 2008) demonstrated tissue specific methylation in a number of CGIs associated with developmentally important genes including homeobox (*HOX*) and paired box (*PAX*) family members in humans. More recently these authors showed that DNA methylation was more likely to occur at CGIs within gene bodies during the early stages of lineage specification, and to be associated with gene silencing (Deaton *et al.*, 2011) . Such regions may be potential targets for environmentally-induced epigenetic regulation and, as such, form the mechanistic basis of programming of lifelong health, productivity and fertility in animals.

3. Programming of lifelong health and wellbeing

3.1. Metabolic syndrome

Any 'syndrome' is defined by having a set of symptoms which consistently occur together. The constellation of metabolic symptoms that present more regularly than would occur by chance in individuals with cardiovascular disease and/or Type II diabetes (T2D) led to a working definition of 'metabolic syndrome (MetS)' (Eckel *et al.*, 2005; Zhang *et al.*, 2011). A human subject diagnosed with MetS must have either 1) glucose intolerance (defined as elevated fasting or postload (75g) glucose) and/or 2) insulin resistance (defined as a fasted insulin value in the upper quartile for a non-diabetic population) plus 2 or more of the following; 3) dyslipidaemia (elevated plasma triglyceride concentration and/or reduced high density lipoprotein (HDL) cholesterol), 4) elevated blood pressure, 5), central obesity and 6) microalbuminuria. In the Westernised world, MetS is prevalent (~7 to 24% population) and the incidence is higher in males than females, and rises with age (e.g. prevalence rose in US males from 7% to 44% between the ages of 20-29 years and 60-69 years).

3.1.1. Developmental Programming of MetS: What do we know?

Human cohort studies using retro- and prospective experimental designs have illustrated how variation in the developmental environment with or without associated changes in a phenotypic outcome such as growth may increase susceptibility to one or more elements of the metabolic syndrome and predispose toward cardiovascular or metabolic disease (Adair *et al.*, 2013). A number of recent narrative reviews have summarised the area (Victora *et al.*, 2008; Rinaudo and Wang, 2012) and, broadly speaking, the relationship appears to be reverse J- to U-shaped. That is, a poor or abundant early environment (i.e. nutritionally) increases risk of metabolic disease later in life, despite average or median growth thereafter. As an illustrative example of attributable risk due to poor early nutrition, Thurner and colleagues retrospectively examined the developmental history of the entire Austrian population that were currently receiving treatment for T2D (325,000 individuals; (Thurner *et al.*, 2013)). They determined the excess risk of developing T2D as a consequence of early malnutrition to be 40%. At the opposite end of the spectrum, exposure to nutritional excess early in life (e.g. marked by high birth weight) exacerbates your lifetime risk of being overweight later in life (Curhan *et al.*, 1996; Dabelea *et al.*, 2000). Excess body fat significantly increases your risk of MetS, especially when experienced early in life (The *et al.*, 2010). Importantly therefore, across the whole spectrum of developmentally-programmed risk, evidence of abundance experienced post-natally either as postnatal growth acceleration (i.e. centile crossing (Singhal *et al.*, 2010)) or excess fat deposition (becoming overweight or obese; (Law and Shiell, 1996; Franco *et al.*, 2009; Magnussen *et al.*, 2010)) is detrimental but markedly exacerbates any residual prenatally programmed risk of metabolic disease.

3.1.2. Use of cattle and sheep as models for human metabolic disease

With the exception of the non-human primate (Zhang *et al.*, 2011), for which experimental research is limited, there are no animal models that spontaneously develop MetS. Furthermore, very few animal models recapitulate >5 individual symptoms that comprise MetS. Less commercial, relatively feral breeds of pig, when exposed to commercial (or a westernised equivalent diet), rapidly develop many aspects of MetS (Spurlock and Gabler, 2008) but this is not observed in

artificially selected, commercial breeds, which are distinctly resistant to the syndrome. The metabolism of ruminants, being largely based on generation of short-chain fatty acids (particularly propionic) by the gastric microbiome, rather than on gastrointestinal glucose absorption means that one of the key symptoms of MetS – glucose intolerance – is unlikely to be observed. As a result they are also relatively insulin resistant *per se*. Nevertheless, aspects of developmental programming of other risk factors for MetS (e.g. elevated blood pressure, central obesity, dyslipidaemia) have, to some extent, been reproduced in ruminant models (for review see Sinclair *et al.*, 2010) but to a much greater extent in laboratory animal models (for narrative reviews see Armitage *et al.*, 2004; McMillen and Robinson, 2005; McMullen and Mostyn, 2009).The important point that is relevant for a consideration of the effect of developmental programming in cattle and sheep is the extent to which such programmed metabolic effects, however small, may actually translate and impact productive traits (i.e. offspring growth to culling, deposition of lean/fat mass to influence body composition, and fertility).

The reported effect size of programming of the cardiovascular system (e.g. an increase in blood pressure of 5-10 mm Hg), or renal system (e.g. evidence of microalbuminuria in young sheep), is unlikely to be of any consequence for farmers. Equally, relatively small effects on glucose tolerance, peripheral insulin resistance or dyslipidaemia when considered alone are unlikely to be of any significance. However, for many reasons as discussed in the highlighted reviews above, such work is important to be demonstrated in larger animals in their home environments and with very different life history strategies to contrast with laboratory animal models. Further, when considered as individually programmed parts of a whole affected system, which may be exacerbated by artificial selection for certain traits of interest in agricultural animals, then some trade-off may be expected that impacts productive traits of interest. For example, dairy cows are an extreme example of selection for differential nutrient partitioning to support mammary growth and function. They also demonstrate marked insulin resistance with concomitant ectopic fat deposition (i.e. in organs and not peripheral tissues) (Sinclair *et al.*, 2010) which may explain in part their decline in fertility (Royal *et al.*, 2000).

3.1.3. Critical periods of development

Passing reference was made previously (Section 1.1) to differential effects of gross nutrient restriction during early, mid or late gestation in women subjected to the Dutch hunger-winter of 1944-45. *In utero* exposure to famine during the first trimester of pregnancy did not affect birth weight but led to adult offspring that exhibited the most striking health-related effects. These individuals were the most obese and exhibited a 3-fold increase in the incidence of coronary heart disease compared to those not exposed to famine (Painter *et al.*, 2005). In contrast, famine exposure during late gestation reduced birth weight and impacted more on intermediary metabolism, in particular, glucose-insulin homeostasis. Nutrient sensitive periods during *in utero* development and infancy have been identified in a number of mammalian species (reviewed by Sinclair and Singh, 2007; Fowden *et al.*, 2010) and knowledge of these stages offers the prospect of remedial dietary interventions (Vickers, 2011). Perhaps not surprisingly most reports of dietary interventions are in rodents fed mLPD. These interventions typically involved supplementation with various one-carbon metabolites (i.e. methionine, folate, choline or taurine). However, neonatal leptin treatment of rats born following maternal under-nutrition has been shown to prevent the onset of diet-induced obesity and metabolic syndrome in later life. Similarly, post-natal leptin therapy can partially reverse naturally-occurring litter associated intra-uterine growth

restriction and metabolic sequelae in piglets by correcting growth rate, body composition and the development of organs involved in metabolic regulation (Attig *et al.*, 2008).

Studies in sheep have also investigated effects of nutrient restriction at different stages of gestation on aspects of MetS. For example, Gopalakrishnan *et al.* (2004) fed pregnant ewes to either 100% AFRC (1993) recommended intake from conception to term, or 50% recommended intake to Day 95 and 100% thereafter. In the absence of differences in birth weight and subsequent growth, they observed increases in mean arterial blood pressure and heart rate in 3-year old offspring exposed to nutrient restriction during early- to mid-pregnancy. Encouraged by these observations a follow-up study by the same group limited the period of nutrient restriction to the first 30 days of gestation and assessed cardiovascular function in offspring at 1 year of age. Once again, neither birth weight nor post-natal growth was influenced by treatment. However, evidence of cardiovascular dysfunction, including a blunted baroreflex function and heightened sensitivity of the renin-angiotensin system (both of which are predictive of late-onset hypertension) were evident in these young animals (Gardner *et al.*, 2004).

The peri-conceptional period has attracted particular attention in recent years, not least because it is acknowledged as the period when the mammalian genome is most sensitive to environmentally induced epigenetic dysregulation (Sinclair and Watkins, 2013) (see also Section 2). Defining this period in humans (Steegers-Theunissen *et al.*, 2013) proposed a 24-week period from 14 weeks prior to mating (when ovarian follicles commence their growth phase) to 10 weeks following mating, coincident with closure of the secondary palate of the embryo. As discussed by (Sinclair and Singh, 2007), nutrient restriction during early pregnancy is of interest to clinicians as up 80% of women encounter symptoms of nausea and vomiting (termed morning sickness) leading to modest weight loss between weeks 4 and 12 of gestation. Somewhat paradoxically, mild forms of this condition are associated with positive pregnancy outcomes in terms of increased placental development and reduced risks of miscarriage, low birth weight and perinatal death. The chronology of these developmental processes in ruminants is strikingly similar to that of humans, where modest nutrient restriction in mature ewes of good but not poor body condition can also enhance placental development (Robinson *et al.*, 2000).

Studies in rodents fed mLPD for variable periods, spanning conception ± 4 days, reported increased systolic blood pressure and other specific features of MeS in 6 to 12 month-old adult offspring (Kwong *et al.*, 2000; Watkins *et al.*, 2008; Watkins *et al.*, 2011). In ewes, physiologically relevant reductions in specific dietary B-vitamins (i.e. B_{12}, folate) and methionine from 8 weeks prior to 1 week following mating led to genome-wide epigenetic modifications to DNA methylation in their progeny, which as 2-year old adults were hypertensive and exhibited additional signs of 'metabolic syndrome' including insulin resistance; effects most pronounced in male offspring (Sinclair *et al.*, 2007).

3.2. Animal welfare

In animal welfare terms, the responses of interest are those that alter the ability of the animal to respond appropriately to the environment in which they are managed, either through changes in behavioural adaptations, stress physiology and responsiveness or immune responses, and hence disease susceptibility. These effects are of relevance to the animal itself, but also to the farmer as they can affect mortality and morbidity, disease susceptibility, reactiveness of animals to common on-farm practices (e.g. vaccine responses can be reduced in animals that find handling very stressful), and ease of handling. The prenatal, and early postnatal, period is of critical importance in defining how individuals respond to their environment throughout life. This has been the subject

of several recent reviews in farm livestock demonstrating the important role that variation in maternal state can have on progeny health and welfare (Braastad, 1998; Bell, 2006; Greenwood *et al.*, 2010; Rutherford *et al.*, 2012; Merlot *et al.*, 2013). Prenatal stress or sub-optimal maternal nutrition have both been shown to affect how well offspring cope with their social, physical and infectious environment. To date, work in cattle investigating such effects has been limited. However, studies do show that maternal health, nutrition and experiences of stress in pregnant dairy cattle can affect their progeny (Arnott *et al.*, 2012). More extensive studies exist in sheep, particularly investigating the impact of prenatal nutrition on subsequent responses, but also more recently considering the impacts of different stress paradigms.

3.2.1. Impact of the prenatal environment on birth weight and mortality

Perhaps the best described, and unambiguous, impacts on welfare (and productivity) are the effects of early life events on the survival of the offspring. Many studies have investigated the causes of offspring mortality, particularly in sheep but also in cattle, and demonstrate that preterm delivery, low birth weight, a difficult or prolonged birth process, poor behavioural development in early life, and an inability to adjust to postnatal life (e.g. impaired ability to thermoregulate) all contribute to an increased risk of mortality. Many of these risk factors have their origins in prenatal development of the fetal lamb or calf.

The single greatest contributor to lamb mortality is birth weight (Dwyer *et al.*, 2003), although the relationship is not linear with very low and very heavy lambs having high mortality (Wu *et al.*, 2006, Sawalha *et al.*, 2007). Numerous studies consistently show that maternal under-nutrition of the ewe in late pregnancy (after day 100) reduces lamb birth weight (e.g. Dwyer *et al.*, 2003, Corner *et al.*, 2008, Hammer *et al.*, 2011). Under-nutrition prior to day 100 has variable effects across different studies: severe early under-nutrition has a marked impact on birth weight (e.g. reduction to 15% requirements for the first 60 days of gestation, (Vincent *et al.*, 1985)) and an equivalently large impact on mortality. Moderate under-nutrition in early to mid-gestation generally does not affect birth weight except in particularly circumstances: young and growing females (Munoz *et al.*, 2009) or ewes selected or adapted for a well fed environment (Burt et al., 2007; Rooke *et al.*, 2010). In these studies mortality was generally not affected if birth weight was not influenced, although the study of (Rooke *et al.*, 2010) reports increased lamb mortality with early under-nutrition (to d90 of gestation), even in the absence of an impact on birth weight. Conversely maternal over-nutrition of ewe lambs results in a severe reduction in lamb weight, as the mother parturitions nutrient towards growth of their own tissues to a level greater than is achieved by under-nutrition (Wallace *et al.*, 2011). Low body weight is associated with slower behavioural development (Dwyer *et al.*, 2003), with delays in reaching the udder and sucking which contributes to the higher mortality of low birth weight lambs (Dwyer *et al.*, 2001).

Shearing pregnant ewes has consistently been reported to increase lamb birth weight (Kenyon *et al.*, 2003; Kenyon *et al.*, 2005; Corner *et al.*, 2007b; Banchero *et al.*, 2010), particularly if conducted during early to mid-gestation (**Figure 3.1**). Associated with this response are increases in maternal feed intake, gestation length and maternal plasma glucose concentrations (Symonds *et al.*, 1988; Morris *et al.*, 2000; Keady and Hanrahan, 2009; Banchero *et al.*, 2010). Although shearing is associated with a robust stress response in the ewe, these data suggest that its primary impact on birth weight is through increased feed intake and hence nutrition of the developing lamb. This is supported by studies that mimicked the handling associated with shearing but did not remove the fleece, which report no increase in birth weight (Corner *et al.*, 2010). However, although

shearing affects birth weight, there is no evidence that this is associated with improved lamb survival (Corner *et al.*, 2006; Keady and Hanrahan, 2009).

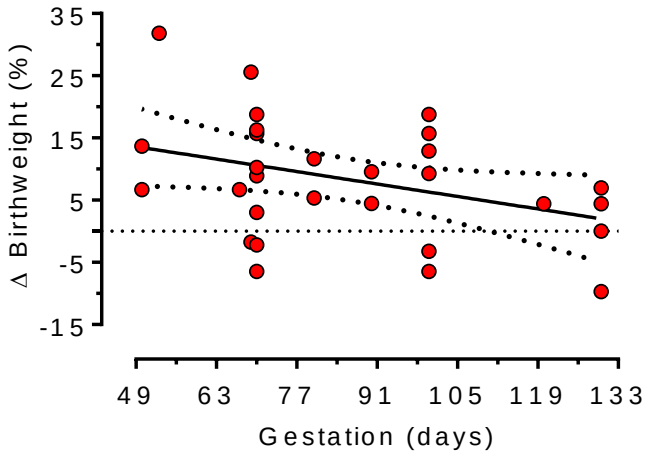


Figure 3.1. Birth-weight responses to shearing (increase (%) relative to unshorn ewes) at different stages of gestation, showing 95% confidence intervals. R^2 = 0.13 (P = 0.042). Mean responses are combined for male and female twin and single lambs from the following studies: (Morris *et al.*, 1999; Morris *et al.*, 2000; Revell *et al.*, 2000; Cam and Kuran, 2004; Corner *et al.*, 2006; Kenyon *et al.*, 2006; Corner *et al.*, 2007b and 2007a; Jenkinson *et al.*, 2009; Banchero *et al.*, 2010; Mousa-Balabel and Salama, 2010; Sphor *et al.*, 2011)

Other late gestation treatments of the ewe which are associated solely with stress (social isolation or aversive handling by humans) have also reported an increase in birth weight (Roussel *et al.*, 2004; Hild *et al.*, 2011). In addition, shearing studies in late gestation that did not elicit an increase in birth weight, have been reported to improve lamb behavioural progression to sucking (Banchero *et al.*, 2010), although none of the maternal stress studies report changes in lamb survival. The mechanisms underlying these effects are largely unknown.

In cattle, the major contributor to calf mortality is dystocia, although birth weight and other factors are also very relevant. Dystocia is also a risk factor for calf morbidity and mortality in later life (Henderson *et al.*, 2011; Arnott *et al.*, 2012; Barrier *et al.*, 2012). The main cause of calving difficulty seems to be fetal-maternal disproportion (Arnott *et al.*, 2012), which is likely to be related at least partially to prenatal nutrition, although studies of stillborn calves suggest that prenatal factors also contribute to the likelihood that calves will not survive the birth process (Barrier *et al.*, 2013). Studies of maternal nutrition in cattle are far less numerous than in sheep and generally, the impact of maternal nutritional restriction and calf weight are variable. The effects differ dependent upon timing, duration and severity of the dietary insult as well as parity of the dams. Some studies have found no effect (Carstens *et al.*, 1987, Hough *et al.*, 1990, Martin *et al.*, 1997, McGee *et al.*, 2006, Long *et al.*, 2010). These studies were characterised as having a number of shortcomings (e.g. low numbers per treatment, small differences between dietary treatments, short dietary exposures, or were conducted only during the first trimester. In contrast, other studies have reported a reduction in calf birth weight as a consequence of maternal undernutrition during the second and/or third trimester of pregnancy (Warrington *et al.*, 1988; Houghton *et al.*, 1990; Freetly *et al.*, 2000; Micke *et al.*, 2010a; Micke *et al.*, 2010b). In general, reducing calf birth weight by means of maternal dietary restriction does not reduce the incidence of dystocia or calf morbidity. This is due to effects on both maternal physiology as well as altering humoral immune status in

the calf (McGee *et al.*, 2006). In a few studies, however, cow nutrition has been linked to calf mortality (e.g. when cows were kept on an energy restricted diet in late gestation – led to reduced birth weight and increased neonatal morbidity (diarrhoea) and mortality in crossbred beef calves (Corah *et al.*, 1975).

Calf birth weight and survival can be influenced by environmental conditions, independent of the nutritional status of the mother. Both heat (Collier *et al.*, 1982) and cold exposure (Andreoli *et al.*, 1988) in gestation are reported to reduce calf birth weight, and this is associated with increased calf mortality and morbidity (Azzam *et al.*, 1993). Gestation length is also often reduced by heat stress (**Table 3.1**), but (Tao *et al.*, 2012) calculated that the reduction in gestation length accounted for only around one third of the reduction in birth weight as a consequence of heat stress. Drought exposure of pregnant cattle can profoundly influence offspring development and survival (Arnott *et al.*, 2012). In particular this has been associated with a condition in beef calves termed congenital chondrodystrophy of unknown origin (CCUO; White *et al.*, 2010a), where failure in long bone growth results in disproportionate dwarfism, breathing difficulties and perinatal death (McLaren *et al.*, 2007). This condition seems to occur as a result of maternal malnutrition in early gestation as a consequence of severely reduced rainfall (White *et al.*, 2010b).

Table 3.1. Effect of heat stress in cattle on gestation length and birth weight (data from Tao and Dahl, 2013)

Gestation (days)		Fetus / birth weight (kg)		Reference
Heat stress	**Control**	**Heat stress**	**Control**	
281	281	36.6*	39.7	(Collier *et al.*, 1982)
		40.6	43.2	(Wolfenson *et al.*, 1988)
		33.7*	37.9	(Avendaño-Reyes *et al.*, 2006)
274	278	40.8*	43.6	(Adin *et al.*, 2009)
		31.0*	44.0	(do Amaral *et al.*, 2009)
		39.5*	44.5	(do Amaral *et al.*, 2011)
274	277	41.6*	46.5	(Tao *et al.*, 2011)
272	276	36.5*	42.5	(Tao *et al.*, 2012)

* Statistically significant reduction relative to control calves.

In both cattle and sheep, maternal health in pregnancy contributes to the survival and growth of the offspring. Treating sheep for footrot was associated with an increase in the number of lambs reared (Wassink *et al.*, 2010), whereas an outbreak of sheep scab during pregnancy led to a decrease in lamb birth weight (Sargison *et al.*, 1995). In a Swedish study on dairy cattle (Lundborg *et al.*, 2003) found that calf birth weight was reduced if the gestating mother had had mastitis during the 49 day period to calving. Several studies have also reported decreased calf growth rate if the mothers experienced disease during pregnancy (reviewed by Arnott *et al.*, 2012). Health treatment decisions during pregnancy can also impact on calf outcomes. For instance, (Lents *et al.*, 2008) found that calf growth was improved when beef cows were treated with dry cow antibiotic therapy. Alternatively, a failure to treat cows for parasite infection during pregnancy reduced calf

birth weight and subsequent weaning weight (Loyacano *et al.*, 2002). The mechanism for these effects is unclear. It may be a consequence of direct immune communication between mother and her developing offspring, or occur indirectly via maternal stress or pain, or a reduction in feed intake that often accompanies ill health.

3.2.2. Impacts of the fetal environment on stress responsiveness

Common farm husbandry practices, such as restraint, handling and transportation, are associated with stress responses indicating activation of the hypothalamo-pituitary adrenal (HPA) axis (e.g. Roussel *et al.*, 2006; Roussel-Huchette *et al.*, 2008). This axis represents a hormonal cascade where increased expression of corticotropin-releasing-hormone (CRH) in the paraventricular nucleus of the hypothalamus, stimulates release of adreno-corticotropic-hormone (ACTH), from the pituitary. ACTH subsequently stimulates the release of glucocorticoids (cortisol or corticosterone in some species) from the adrenal gland. Cortisol plays a role in numerous biological systems within the body, but its primary role within the stress response system of the body is to mobilise energy reserves.

In rodent models, prenatal stress (classically induced by restraining the mother regularly over the last half to third of pregnancy) is known to cause long-lasting changed in the HPA axis of the offspring associated with an increased reactivity (Henry *et al.*, 1994). This is associated with behavioural disturbances characterised by high anxiety and depressive-like behaviour, and impaired memory for hippocampus-dependent tasks, e.g. spatial tasks (Darnaudery and Maccari, 2008). However, many studies report sex-dependent impacts with males typically showing an increase in anxiety after prenatal stress and dysmasculinised behaviours, whereas females show converse behavioural responses (Zuena *et al.*, 2008; Bale, 2011). The release of glucocorticoids by the stressed mother, which can cross the placenta to influence the developing offspring, appear to mediate these responses (Mesquita *et al.*, 2009; Harris and Seckl, 2011) and alteration of over 700 genes, in a region- and sex-specific manner have been reported (Mychasiuk *et al.*, 2011). Epigenetic alterations in the offspring brain are known to follow prenatal stress (Gudsnuk and Champagne, 2012), accompanied by changes in the development of neurogenesis, particularly in the hippocampus (Korosi *et al.*, 2012). Very recently, evidence has also emerged for an impact of paternal stress on the stress reactivity of his offspring in rats (Mychasiuk *et al.*, 2013). Stress of males during spermatogenesis before conception reduced learning responses and reduced stress responsiveness male offspring, and had sex-specific impacts on DNA methylation patterns.

In farm animals a detailed mechanistic understanding of the effects of maternal stress is lacking. However, the clear impact of maternal, and paternal, stress on the subsequent behaviour and stress responsiveness of the offspring in rodents suggests that similar mechanisms may operate in cattle and sheep. To date experimental studies have focussed on assessing HPA axis function (such as release of cortisol and/or ACTH) or the altered behavioural stress responsiveness of the offspring either in response to pharmacological challenge or when exposed to an environmental or social stressor. **Table 3.2** details the effect on HPA axis function in lambs that has been observed following maternal undernutrition in pregnancy, or after maternal stress. Responses are variable (partly related to inconsistency between studies in time at which the offspring were assessed), but do suggest some sex-specific variation as seen in rodents (e.g. Gardner *et al.*, 2006), and differences in responses when lambs are younger or older. Studies in cattle are very scarce, but (Lay *et al.*, 1997) showed that repeated transportation of Brahman cows during gestation increased the cortisol response of the progeny to an acute restraint stress.

Table 3.2. Studies investigating progeny stress responses as a consequence of maternal stress or under-nutrition in sheep

Study	Gestation day	Effect on Progeny
A. Under-nutrition		
(Bloomfield *et al.*, 2003)	105 to 115	Increased ACTH response to CRH/AVP challenge, and increased baseline concentrations of cortisol and ACTH
	105 to 125	No effect on ACTH response to CRH/AVP challenge, or baseline concentrations of cortisol and ACTH.
(Gardner *et al.*, 2006)	0 to 30	CRH/AVP challenge produced a lower ACTH and cortisol response in female, but not male, UN lambs.
(Chadio *et al.*, 2007)	30 to 100	No effect on ACTH and cortisol response to CRH at 2 months old.
	1 to 30	Increased ACTH and cortisol response to CRH at 2 months old.
(Hernandez *et al.*, 2010)	2 to 30	No effect at 4 months of age on cortisol response to isolation.
		Reduced cortisol response to isolation at 18 months of age.
(Long *et al.*, 2010)	28 to 105	No change in response to CRH/AVP or ACTH. Reduced ACTH and cortisol response to environmental stressors.
B. Maternal nutrition		
(Roussel *et al.*, 2004)	110 to 150	No effect on cortisol response to isolation.
(Roussel-Huchette *et al.*, 2008)	110 to 150	No effect on cortisol response to isolation
(Fisher *et al.*, 2010)	135 to 138	Reduced febrile and cortisol responses to endotoxin challenge

3.2.3. Impacts of the fetal environment on aspects of offspring behaviour

Unlike the impacts on stress physiology, which frequently report no effect, impacts on offspring behaviour are more frequently reported with studies of gestational under-nutrition and maternal stress in pregnancy inducing altered behavioural reactivity (**Table 3.3**). However, some studies report an increased behavioural reactivity (e.g. Erhard and Rhind, 2004; Roussel *et al.*, 2004) which, in keeping rodent studies, was more pronounced in males than females. Other studies suggest that lambs are less reactive to stress (e.g. Roussel-Huchette *et al.*, 2008; Hernandez *et al.*, 2010).

Table 3.3. Sheep studies investigating progeny behaviour as a consequence of maternal stress or under-nutrition.

Study	**Maternal Treatment** (Stage of gestation)	**Effect on Progeny**
(Erhard and Rhind, 2004)	UN 0 to 95	Higher activity during restraint in male, but not female, lambs. Longer approach latency to novel object. Male UN lambs more active in response to startle than controls. In a maze test male lambs from UN ewes showed reduced learning speed. Behavioural laterality of lambs was also altered
(Hernandez *et al.*, 2010)	UN 0 to 30	Lambs born to UN ewes made fewer escape attempts during a five minute isolation test at 4 months of age.
(Simitzis *et al.*, 2009)	UN 31 to 100	No effect on response to isolation with a novel object at 2, 3, 4 and 5 months of age.
(Hernandez *et al.*, 2009)	UN 0 to 30	Behavioural laterality of lambs was altered
(Roussel *et al.*, 2004)	Stress 110 to 150	At 8 months of age: increased jumping during isolation; increased activity after exposure to novel object; increased exploration of novel object; higher frequency of changes between light and dark compartment.
(Roussel-Huchette *et al.*, 2008)	Stress 110 to 150	At 3 months of age: males spent increased time close to novel object; males reduced jumping in novel arena test. No effect on female lambs.
(Coulon *et al.*, 2011)	Stress 110 to 145	Reduced activity in human approach test.

3.2.4. Prenatal influences on welfare: summary and interim conclusions

Across both species it is difficult to draw firm conclusions about specific effects which could be communicated to farmers (with the exception of the already well known effects of poor nutrition in late gestation in sheep). Yet the collected studies do provide evidence that variations in the prenatal environment, as dictated by the management of the pregnant mother, can contribute to animal welfare outcomes in both sheep and cattle. In particular the impact of maternal ill-health or disease in pregnancy on offspring birth weight and growth is important.

Research in cattle is limited with respect to health and welfare outcome of maternal challenges during gestation. Negative effects on birth weight may suggest possible postnatal problems, but direct demonstrations of welfare deficits are rare. Periods of severe under-nutrition during gestation can have very obvious negative effects on health and welfare (chondrodistrophy studies; White *et al.*, 2010a; White *et al.*, 2010b), but little is known about more subtle and commercially relevant effects.

In some instances, in sheep, experience of maternal stress during pregnancy can lead to apparently improved welfare status in offspring. Shearing during pregnancy has well known beneficial effects on birth weight for example (Kenyon *et al.*, 2003). Other repeated stress treatments (Roussel *et al.*, 2004; Roussel-Huchette *et al.*, 2008) have also been shown to cause changes in progeny that can be interpreted as being positive.

3.3. Immune function and health

Opportunities to manage pregnant livestock in ways that increase the likelihood that their offspring have attributes that promote animal well-being, productivity and consumer acceptance raise the question of whether immune function can be prenatally programmed. Immune competence can be assessed in numerous ways, including the ability of the individual to mount an effective immune response, functionally mature immune organs and cells and by appropriate concentrations of key cytokines, immunoglobulins, acute phase proteins and other molecules. In the context of livestock production the most relevant and therefore frequently studied parameters include the acquisition of passive immunity, as measured by concentrations of immunoglobulins in offspring, parasite resistance and the development of key organs of the immune system, such as the thymus. Alterations in maternal nutrient quantity and composition, and both social and thermal stresses during pregnancy affect various measures of livestock offspring immune status, although few comprehensive studies have been reported in ruminants. In addition, as most of the perturbations imposed during pregnancy affect the hypothalamic-pituitary-adrenal (HPA) axis and the metabolic status of the maternal body, programming effects on offspring immune function could occur either directly or be mediated by an up-regulation of the HPA axis or alterations in the metabolism of glucose, a major immune system substrate.

Although most studies addressing fetal programming of immune competence have focussed on laboratory species, there are compelling reasons to believe that the fetuses of livestock species may be more susceptible to the effects of the maternal environment. The immune systems of mammals that give birth to precocious offspring, such as sheep, cattle and pigs, develop predominantly in utero and hence represent a potential target for fetal programming. For example, in sheep the development of the thymus commences in early gestation and CD5 T cells appear by day 35 and are soon followed by CD8 and CD4 T cells (Cronje, 2003). Circulation of lymphocytes begins between days 70 – 75 of fetal life and by day 80, the cellular and

immunohistological appearance of the ovine thymus is identical to the post-natal thymus (Cahill *et al.*, 1999). By parturition, the central and peripheral lymphoid systems of the ovine fetus are at an advanced stage of development (Cunningham *et al.*, 1999).

Passive immunity in neonates of ruminant species is predominantly acquired by the uptake and absorption of immunoglobulins in colostrum, rather than placental transfer. Alterations to both the quantity (Hammer *et al.*, 2011) and the micronutrient composition (reviewed by Rooke *et al.*, 2008) of the diet offered to the pregnant ewe can affect the serum immunoglobulin G (IgG) content in her lambs. Such effects could be mediated by altered composition of colostrum and/or the amount ingested by the neonate or the ability of the neonatal gut to absorb immunoglobulins. For example, low periconception levels of cobalt/Vitamin B12 reduced serum IgG in 2 and 4 week-old lambs (Fisher and Macpherson, 1991), whereas some studies report an increase in lamb plasma IgG concentrations following vitamin E supplementation before lambing (Gentry *et al.*, 1992). Excess intakes of minerals during pregnancy do not alter colostrum IgG content, but impair immunoglobulin absorption in lambs, and this appears to be largely attributable to the iodine component of the mineral mix (Boland *et al.*, 2005). Generally, under-nutrition during early- (Munoz *et al.*, 2009) or late- (Hammer *et al.*, 2011) pregnancy increased lamb plasma IgG concentrations at birth and 24 hours of age, respectively. The study of (Hammer *et al.*, 2011) is of particular interest, as lambs were removed from their mothers before suckling and fed colostrum replacer and so the differences in lamb IgG absorption were independent of maternal colostrum production. An increase in serum IgG levels in lambs born to under-nourished ewes appears counter-intuitive, but speculation about under-lying mechanisms is difficult given the absence of data on colostrum composition, intake or lamb plasma volume.

In cattle, heifers born from cows exposed to natural summer heat stress during the last 45 days of pregnancy had lower serum concentrations of IgG after colostrum consumption than heifers born to mothers receiving a cooling treatment, despite similar colostrum IgG concentrations and feeding levels (Tao *et al.*, 2012). It has been suggested that alterations in the profile of maternal glucocorticoids following maternal stress may accelerate neonatal gut maturation (Merlot *et al.*, 2013), leading to reduced immunoglobulin absorption.

Measures of acquired immune function of offspring whose mothers were subjected to different pregnancy regimens are varied; with differences between breeds and breeding seasons and it is difficult to consolidate current findings. For example, in one year of a study conducted over 2 consecutive years in which Scottish Blackface and Suffolk ewes received either maintenance or 75% maintenance rations during days 1 to 90 of pregnancy the offspring of under-fed Suffolk, but not under-fed Scottish Blackface, ewes had higher Strongyle fecal egg counts at weaning age (Rooke *et al.*, 2010). The differences between breeding seasons could be attributed to a range of factors including differences in lamb postnatal growth and grazing behaviour. It is of interest that the breed by maternal nutrition interaction affected lamb spleen and thymus weight (Ashworth, Hogg, Matheson, Dwyer and Rooke, unpublished data) as the thymus has been proposed as a possible mediator of the effect of pre-natal nutrition on immune competence in later life (Cronje, 2003). In cattle, heat stress during late gestation reduced blood lymphocyte proliferation in female offspring until 56 days of age (Tao *et al.*, 2012), providing evidence that a specific prenatal treatment can affect both passive and acquired immune function in offspring. This had previously been observed in sheep where a maternal periconceptional diet deficient in elemental cobalt and sulphur altered both the innate and acquired immune function of offspring at 12 months of age (Sinclair *et al.*, 2007). Relative to matched controls, both the acute-phase (serum haptoglobin) and adaptive (serum IgG) responses to purified ovalbumin in Quil A adjuvant were increased in 12-

month old lambs from ewes fed a cobalt and sulphur deficient diet prior to and around the time of mating.

4. Programming of body composition

To understand how nutritional and epigenetic factors during early development can impact upon future body composition of the offspring, it is necessary to consider the origins and developmental trajectories of the cells that form the functional tissues, particularly muscle and adipose tissue. The processes that drive muscle formation, myogenesis, have been well studied in a number of systems, whereas adipogenesis, the formation of fat cells (adipocytes), is much less well characterised.

4.1. Evidence for programming of skeletal muscle

Myogenesis is the term used to describe the processes whereby pluripotent embryonic cells become committed to the muscle cell lineage and subsequently proliferate and fuse together (i.e. differentiate) to form large multinuclear cells called myotubes (in *in vitro* studies) or muscle fibres (in *in vivo* studies). Evidence to date suggests that the processes and regulatory factors involved are very similar across mammals, birds and fish, but that the timings for when they occur can be quite different. There are numerous reviews (e.g. Brameld and Daniel, 2008; Rehfeldt *et al.*, 2011b) describing this process and the factors that regulate it, including cross-species comparisons (Rehfeldt *et al.*, 2011a).

In all vertebrates the muscles of the trunk and limbs are derived from segmented embryonic structures known as somites that also produce vertebrae, ribs, tendons and dermis. Within somites muscle progenitor cells are specified and begin to differentiate into the primary myotome. These early proliferative progenitors, known as myoblasts, subsequently differentiate and fuse to form the multinucleated functional adult muscle cell (the muscle fibre). Several genes have been shown to be required for this process. Specification and proliferation of myoblasts depends on expression of the transcription factors, Pax3 and Pax7, while Myogenic Regulatory Factors, a group of related muscle-specific transcription factors, are involved in both the later stages of myogenic differentiation as well as specification and proliferation of the myoblasts (Buckingham, 2007; Mok and Sweetman, 2011; Sweetman, 2012).

Within the embryo, different muscle groups follow distinct developmental routes (Buckingham and Vincent, 2009). Trunk and back muscles are derived directly from the primary myotome, which extends into the regions where the adult muscles will be located and is then able to grow and form the adult muscular pattern. In contrast, other muscles such as the limb muscles come from myoblasts which delaminate from somites and then migrate into the developing limbs (**Figure 4.1**). This also includes some muscles of the pelvic and pectoral girdles that develop from limb muscle cells which, having migrated into the limbs, then migrate out again to populate these regions (**Figure 4.1**). This is known as the ‘in-out mechanism’ (Evans *et al.*, 2006; Valasek *et al.*, 2011). Understanding the different origins and developmental processes that generate various muscle types will be important in designing interventions that target these muscles.

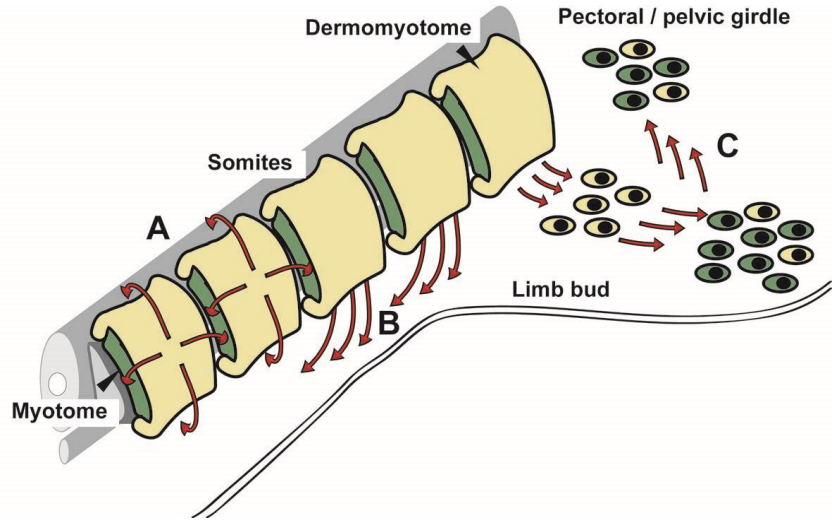


Figure 4.1. Cells of the dorso-medial region of somites form the dermomyotome (DM, yellow). The myotome (green) is formed when DM cells migrate around the edges to form an underlying layer of cells where the first muscles of the embryo begin to differentiate (A). Trunk muscles are formed when the myotome extends ventrally into the body wall (B) while limb muscles are derived from myoblasts which delaminate from the DM and migrate into the limb. Some of these cells then subsequently migrate back into the trunk to form some of the muscles of the pectoral and pelvic girdles (C).

Before fibre formation, the mononuclear myoblasts continue to proliferate and thereby increase in number, whereas fibre formation involves the myoblasts exiting the cell cycle and fusing together (i.e. differentiation) to form the multinuclear fibres containing all the contractile proteins and structures required for muscle function. Dependent upon the species of interest, this fibre formation occurs in 2 or 3 phases. The initial phase of differentiation and fusion of myoblasts generates the primary muscle fibres. These provide the scaffold on which the adult musculature is built and so are responsible for generating the mature muscle pattern. Following formation of primary fibres further rounds of differentiation and fusion occur, such that secondary muscle fibres form around each primary fibre, with tertiary fibres described as forming between the secondary fibres in some larger mammals, including sheep (Wilson *et al.*, 1992). The main difference between species is the time at which these phases of fibre formation take place, with fibre formation in most large mammals (including sheep, cattle and humans) believed to be completed by the middle of gestation, whereas poultry and small mammals (e.g. rats) continue fibre formation for a limited period after hatch/birth (see Brameld and Daniel, 2008; Rehfeldt *et al.*, 2011b).

Hence, the number of muscle fibres in sheep and cattle is believed to be set at the time of birth and subsequent muscle growth is driven by increases in fibre size (i.e. hypertrophy) rather than fibre number (Buttery *et al.*, 2000). Because of this it is critical to understand how changes during development can influence muscle fibre number, as changing this may directly affect meat quality and yield. The central question underlying approaches to maximise muscle production is how to control the switch from the early, proliferative myoblast to the differentiated muscle fibre. Methods to increase the numbers of myoblasts by increasing their proliferation rate or delaying their differentiation have the potential to lead to increased muscle fibre numbers and therefore increased muscle mass in adult animals.

Another important consideration in muscle fibres is the distinction between fast and slow fibres. It is thought that primary fibres initially become slow fibres, whereas secondary and tertiary fibres initially become fast fibres. In adult muscle, fast fibres tend to be larger, especially if they are frequently used, but this size difference is not clear in younger animals. It appears that this initial relationship between primary/secondary and slow/fast fibres is lost as the animal develops, probably because the fibres are able to change fibre type and that the proportions of fast and slow fibres can impact on muscle mass. Unlike rodents where individual muscles are classified as fast or slow, in large mammals all muscles are mixed fibre types and the relative proportions of the fibre types can be altered during development or adult life.

4.1.1. Muscle fibre number (MFN)

A number of studies have shown that the numbers of muscle fibres that form in various animal species can be altered via genetic (e.g. double muscling in cattle) or environmental (e.g. maternal nutrition or administration of hormones) factors, but only if the environmental insults take place at specific times during gestation (see Brameld and Daniel, 2008; Rehfeldt *et al.*, 2011b and 2011a). **Table 4.1** summarises the studies published to date investigating the effects of maternal nutrition on muscle fibre formation in sheep. It is clear that effects of nutritional insults on the pregnant ewe during the critical period of muscle development (early gestation) can be detected in young offspring (late gestation fetuses or neonates), but these effects tend to be lost (or are too difficult to detect) in older sheep. The main effect observed in the young lambs/fetuses is a change in the numbers of secondary fibres formed and/or the ratio of secondary fibres to primary fibres (often determined as fast:slow ratio). Since fibre formation is thought to be complete at this stage in sheep muscle development, it might be predicted that the changes in numbers of fibres would be permanent and may therefore impact upon subsequent carcass quality, particularly lean muscle mass. Studies to date would appear to suggest this is not the case, but this may be due to the capacity for skeletal muscle to adapt during postnatal growth via changes in fibre type and metabolism or the difficulty with the measurements in larger muscles from older animals. It is worth mentioning that the few studies to date that have taken the lambs to market weight or beyond have all provided good quality diets during the postnatal growth period. It is not known whether the animals would still be able to compensate/adapt if they were on a relatively poor diet or were challenged in some other way.

4.1.2. Muscle mass or size

Although there appear to be no long term effects of maternal nutrition on muscle fibre numbers and/or diameters (in sheep), there are suggestions that pre-natal environmental factors can affect certain measures of lean muscle mass and/or carcass composition. The effects on various measures of adiposity will be the focus of the next section, but some studies have observed differences in muscle related carcass measurements. For example, some studies have observed differences in cross-sectional area (CSA) of individual muscles as measured using ultrasound or following muscle dissection after slaughter. However, these effects tend to be largely dependent upon the sex of the animals, with effects only seen in males or females at a particular stage of growth. It is therefore unclear as to how permanent these effects are or the mechanisms involved, although sex steroids (oestrogens and/or androgens) may obviously play a role. A number of studies have been carried out on cattle in Australia, as part of the Australian Beef Cooperative Research Centre (see Robinson *et al.*, 2013). Effects specific to maternal nutrition during pregnancy are difficult to pick up in these studies since they also manipulate postnatal nutrition as

well, but importantly they suggest that the effects of maternal nutrition during pregnancy and lactation are additive and the authors appear to suggest that a life-time approach should be taken rather than simply investigating the effects at a particular stage of development (Robinson *et al.*, 2013).

4.1.3. Genetic control of muscle growth

As muscle formation and growth is a complex process involving numerous biological processes there are, unsurprisingly, many genes involved. One area that has been particularly interesting is the investigation of the genetics relating to signalling molecules involved in myogenesis. Experiments in various model systems, such as mice and chickens, have uncovered a range of signals that can influence the rate of myoblast proliferation, differentiation and fibre type (Duprez, 2002). However, the exact mechanisms that leads to muscle growth, even for very well characterised signals with long established roles, such as the IGF family (Schiaffino and Mammucari, 2011), remain to be established.

One of the most important examples of this type of molecule is myostatin, a secreted member of the transforming growth factor-β (TGFβ) family that negatively regulates muscle growth. Myostatin mutations have been identified in many animal lines selected for high muscle growth (Lee, 2004), such as Belgian blue cattle (McPherron and Lee, 1997), elite sheep (Tellam *et al.*, 2012), as well as high growth chickens (Bhattacharya and Chatterjee, 2013). A number of mutations in myostatin have been shown to lead to loss of functional protein, resulting in increases in both muscle fibre number and size. Interestingly, at least some of these effects take place in the developing fetus and are associated with increased rates of muscle cell proliferation and delayed differentiation (Gerrard and Grant, 1994), resulting in increased numbers of muscle fibres at birth. These are the same mechanisms as those proposed for the effects of maternal nutrition and environmental factors on MFN in the developing fetus (see above). In Texel sheep a mutation has been identified in the 3'UTR of the myostatin mRNA that creates a binding site for the muscle specific microRNA miR-1/206. This leads to muscle-specific down-regulation of myostatin protein levels and increased muscle mass (Clop *et al.*, 2006). A transgenic sheep line has recently been generated with artificial RNAi which mimics this effect and these sheep also show increased muscle mass (Hu *et al.*, 2013a). However there are also some breeds of cattle (Smith *et al.*, 2000) and pigs (Jiang *et al.*, 2002a; Jiang *et al.*, 2002b) with similar or other mutations in the myostatin gene that do not show the double muscling phenotype, suggesting that other factors are also involved.

Another example of a signalling pathway with direct relevance to animal production is seen in Callipyge sheep. These sheep have a complex genotype with a mutation in an imprinted regulatory region which leads to increased expression of DLK1, part of the Notch/ Delta signaling pathway. Overexpression of DLK1 in skeletal muscle leads to increased muscle mass (Davis *et al.*, 2004) and is also up-regulated in broiler chickens (Shin *et al.*, 2009), suggesting that it might be a good target for intervention in various species. It appears that the callipyge phenotype relates mainly to changes in post-natal, rather than pre-natal, muscle growth, but it does have a complex inheritance pattern (see Georges *et al.*, 2003). However, Callipyge sheep produce tough meat (Koohmaraie *et al.*, 1995), which is thought to relate to decreased protein degradation pre- and post-mortem due to increased levels of calpastatin, the endogenous inhibitor of the calpain proteolytic enzymes.

4.1.4. Epigenetic programming of muscle development

There appear to be no studies to date investigating whether the effects of environmental factors on muscle fibre formation are associated with changes in epigenetics (e.g. DNA methylation patterns). The main hypothesis investigated in this area relates to genetic mutations or environmental insults altering the rates and/or timings for muscle cell proliferation and differentiation (see Brameld and Daniel, 2008), so it is unclear as to whether epigenetic mechanisms are involved.

Although there are limited data from large animals there have been many studies on epigenetic control of muscle development in cell culture systems and model animal species. Expression of myogenin is a key step in myogenic differentiation and changes in DNA methylation patterns at this locus have been identified as differentiation proceeds and this gene is induced (Fuso *et al.*, 2010; Palacios *et al.*, 2010). The ability of MyoD to bind DNA and induce expression of muscle determining genes is also regulated by epigenetic changes to binding sites in the promoters of its target genes (Fong *et al.*, 2012). Current work is beginning to identify the dynamic changes in DNA modification that underlie the different stages of myogenic commitment and differentiation (Tsumagari *et al.*, 2013) and global changes in DNA methylation patterns have been mapped in fast and slow growing strains of chicken, providing evidence for a direct epigenetic influence on muscle growth (Hu *et al.*, 2013b). It is also becoming apparent that long non-coding RNAs play an important role in regulating muscle growth, at least in part by controlling muscle specific promoter activity (Mousavi *et al.*, 2013). The interplay between muscle specific gene transcription, epigenetic regulation and chromosomal dynamics is at the forefront of current research in myogenesis and is likely to have profound effects on animal production as these results are translated to sheep and cattle.

4.1.5 Interim conclusions

The basic molecular mechanisms that drive muscle formation are fairly well understood and it is clear that different muscles use specific variations of this developmental programme. Of particular interest is determining how maternal effects can influence this process, especially in terms of how proliferation versus differentiation is determined, how primary and secondary muscle fibre formation is altered by these cell fate decisions, how fibre type is regulated and whether these processes can be influenced to enhance production. The data to date would appear to suggest relatively small (if any) long-term effects of maternal nutrition on MFN or % fibre types in sheep, but all studies have provided good quality diets during the post-natal growth. Whether the same would be true if lambs were subjected to poor(er) quality diets or other challenges during postnatal growth is not known.

4.2. Evidence for programming of body fat

In contrast to muscles the developmental processes leading to the formation of mature adipose tissue cells (adipocytes) are poorly understood; however some recent work has given insights into this process (Billon *et al.*, 2008; Berry *et al.*, 2013). In general, adipocytes can be divided into two types, white and brown. Brown adipose tissue (BAT) adipocytes contain numerous small lipid droplets, have large numbers of mitochondria and provide the main mechanism for maintaining body temperature via heat production in cold exposed rodents. White adipose tissue (WAT) adipocytes contain a single large fat droplet and are the classical fat cell type used for long term storage of excess energy in the form of triacylglycerol (TAG). Although brown fat has been

predominantly associated with young animals (e.g. new born lambs), recent work has also identified BAT deposits in adult animals as well (Billon and Dani, 2012). This division into WAT and BAT has been questioned recently and it has been shown that animals raised in cold conditions also have extensive brown-like adipocytes in their WAT fat depots, with such cells now referred to as either BRITE or Beige adipose cells (Wu *et al.*, 2012) It is unclear if these cells are white cells that have changed their phenotype or if they are brown cells that form in the WAT from a separate stem cell population or a mixture of both (Liu *et al.*, 2013).

Perhaps surprisingly the development of adipose tissue is far less well characterised than that of muscle. BAT, but not WAT, has been shown to derive from early myoblasts (Seale *et al.*, 2008), so shares an origin with muscle cells, but the source of WAT adipocyte precursor cells remains largely unknown. One recent study has shown that some neck WAT depots are colonised by neural crest cells (Billon *et al.*, 2007), migratory multipotent cells from the dorsal neural tube, but very little is known about the origins and signals that regulate WAT adipocyte formation. As a result there are also fewer well established molecular markers of specific developmental stages of adipocyte cell formation available to inform studies of how maternal influences can affect adipose development. Indeed those molecular markers that have been identified as transcriptional regulators of adipogenesis (e.g. CEBPα and β, PPARγ) are common to both BAT and WAT adipocytes. In all cases, these factors are involved in the differentiation of the proliferative precursor cells called preadipocytes into terminally differentiated (non-proliferative) adipocytes. The main differences identified to date (mainly in rodent studies) are that BAT adipocytes tend to have higher expression of genes relating to mitochondrial biogenesis and oxidative metabolism (e.g. PGC1α), with the only BAT-specific protein being Uncoupling protein-1 (UCP-1), the key mitochondrial protein involved in the heat generating properties of BAT.

4.2.1. Body fat/adiposity

As a general observation, measures of adiposity tend to go in the opposite direction to measures of lean or muscle mass. For example, double-muscling in Belgian blue cattle is associated with reduced body fat, as well as increased muscle mass and numbers of muscle fibres. A number of studies have investigated the effects of maternal nutrition on various measures of adiposity, including back fat thickness, individual adipose tissue depot weights, carcass and/or muscle lipid content and total body fat. **Table 4.2** summarises the studies published to date in sheep. It appears that the magnitude and direction of the effect observed is dependent upon the age of offspring being studied, but also the timing of the nutritional insult during gestation. In relatively young (late fetal or early neonatal) offspring (up to about 77d), the adiposity tends to go in the direction you would expect, with reduced nutrition resulting in reduced adiposity and vice versa (Muhlhausler *et al.*, 2006; Luther *et al.*, 2007). Then there appears to be an age period that would include normal market weight lambs in the UK (up to 4 or 5 months), where little or no effects are observed, although females consistently have higher adiposity measures than males. There are then a few studies (Daniel *et al.*, 2007; Ford *et al.*, 2007; Sinclair *et al.*, 2007; Jaquiery *et al.*, 2012) where maternal food restriction, particularly during early gestation (conception to 80 days), results in increased measures of adiposity in older (>6 months) offspring, particularly in males. These latter studies would appear to suggest that there may indeed be long-term “programming” of adiposity, particularly in the normally leaner males, and that this is not associated with differences in birth weight. In contrast, there are a number of studies

Table 4.1. Effects of altered fetal nutrition on muscle fibre formation in sheep (Fahey

Time of challenge	Nutritional challenge (% ME requirements)	Fetal/postnatal offspring age (d)	Muscle(s) studied	Fibre effects	Reference
A. In utero					
-18 to 6d	50% vs 150%	75	ST	Decreased total no. 2º fibres No change total no. 1º fibres Decreased 2º:1º fibre ratio No change in diameters	(Quigley *et al.*, 2005)
28-78d	50% vs 100%	78	LD	Decreased 2º:1º fibre ratio	(Zhu *et al.*, 2004)
B. Post-natal					
30-70d	50% vs 100%	14	LD, VL, ST[a]	Fast fibres: decreased density, increased diameters Slow fibres: increased density Decreased fast: slow ratio	(Fahey *et al.*, 2005)
55-95d	50% vs 100%	14	VL	Fast: increased diameter Slow: No effects	(Fahey *et al.*, 2005)
55-95d	50% vs 100%	14	LD, ST	Fast: No effects Slow: No effects	(Fahey *et al.*, 2005)
85-115d	50% vs 100%	14	LD, VL, ST	Fast: No effects Slow: No effects	(Fahey *et al.*, 2005)05
30-85d	50% vs 100%	119	ST (No effects in LD, VL)	IIb/IIx: Increased density (no./μm^2) I, IIa: No effects	(Daniel *et al.*, 2007)
30-70d	50% vs 100%	168	LD (No effects in ST, VL)	Fast: increased density (no/μm^2), decreased diameter Slow: No effects	(Daniel *et al.*, 2007)
-30 to 100d	70% vs 100%	203 vs 185	ST	No change in total no. fibres	(Nordby *et al.*, 1987)
28-78d	50% vs 100%	240	LD	Increased total no. fibres (P<0.1) Increased % IIb, decreased %IIa No effects on % I and IIx	(Zhu *et al.*, 2006)

et al., 2005) **ME** metabolisable energy; **LD** *longissimus dorsi*; **ST** *semitendinosus*; **VL** *vastus lateralis*; 1º Primary muscle fibres; 2º Secondary muscle fibres; I Slow oxidative (SO) fibres; IIa Fast oxidative glycolytic (FOG) fibres; IIx/IIb Fast glycolytic (FG) fibres.

(Louey *et al.*, 2005; De Blasio *et al.*, 2007; Wallace *et al.*, 2011; Hancock *et al.*, 2012) suggesting that low birth weight, often as a consequence of placental insufficiency, is associated with increased adiposity in both young and old offspring. There is also one cattle study (Long *et al.*, 2010) suggesting that over-nutrition throughout gestation can also result in increased adiposity in older offspring (at 22 compared with 19 months). One of the main problems in trying to draw conclusions from these various studies is the variability in the timing of the nutritional insult and the age at which the offspring are studied.

4.2.2. Energy balance

Although there does appear to be some evidence of long-term programming of adiposity, the mechanisms for how this might occur are far from clear. Whether this apparent programming is via a direct effect on adipocytes and their development is not known. Unlike muscle fibres, there is no evidence to suggest that the numbers of adipocytes (or precursor cells) might be set at some stage of life. Indeed it would be counter-intuitive that this would occur, since the main function of WAT adipocytes is to store excess fatty acids from the blood, since high levels of circulating free fatty acids are toxic. The mechanism(s) for effects on body fat are therefore more likely to involve long-term changes in energy balance, involving changes in whole body energy expenditure (e.g. Basal Metabolic Rate, BMR) and/or appetite regulation. The effects of environmental insults on appetite regulation in the offspring are the focus of the next section.

There are very few (if any) studies in this area that have directly measured energy expenditure, BMR or heat production. However there is one study (i.e. Daniel *et al.*, 2007) showing increased adiposity of adult offspring in response to maternal undernutrition, with no significant changes in food intake, implying that a difference in energy expenditure might be involved. Interestingly, a very similar study (George *et al.*, 2012) observed no effect of maternal undernutrition on whole body fat or peri-renal (PR) and Omental (OM) depot weights in 6 year old ewes, despite increased body weight, food intake and feed efficiency, suggesting that energy expenditure might be altered. Whether such changes in metabolism/energy expenditure relate to skeletal muscle (which accounts for a major proportion of BMR due to its mass), brown adipose tissue (a highly metabolic, heat generating tissue in rodents) and/or other tissues (e.g. the gut has a very rapid turnover), is not known. It is interesting to note, that a recent study comparing high and low feed efficiency or residual feed intake (RFI) in sheep (Sharifabadi *et al.*, 2012) indicates reduced mitochondrial respiration in the muscles from the more efficient animals. This appears to involve reduced BMR and is associated with genes that encode for mitochondrial proteins, suggesting that oxidative metabolism and/or efficiency of ATP synthesis might be key. Once again, the important tissues would seem to be skeletal muscle and BAT, but more work is needed to investigate this further.

4.2.3. Epigenetic programming of body fat

As for the work on programming of skeletal muscle, there appear to be few studies to date that investigate the role of epigenetics in programming adiposity in sheep or cattle. A recent study of male rat offspring of overfed, obese mothers reports up-regulation of lipogenic pathways and adipogenic regulators in WAT, associated with changes in DNA methylation at key sites (Borengasser *et al.*, 2013). It is also known that adipocyte differentiation is regulated by the transcription factors CEPB and PPARγ and that recruitment and activity of these molecules to chromatin requires epigenetic changes to histones and DNA methylation patterns (Cristancho and Lazar, 2011). This is strongly suggestive of altered adipocyte commitment and differentiation via epigenetic mechanisms and deserves further study in livestock species, particularly in mapping

how these changes occur during normal adipocyte formation and determining their functional significance in executing the adipocyte transcriptional programme.

4.2.4 Interim conclusions

As with muscle development, the central question is how adipose tissue development is affected by *in utero* influences. However, since adipogenesis is not as well understood as myogenesis, there is still a need to clarify the underlying biology to identify markers and cellular processes that can be used to study this system *in vivo*. We know that different depots grow at different stages of development, with some real contrasts observed between different species. For example, in sheep the PR depot grows fairly early, being present in young neonatal lambs, whereas the subcutaneous (SC) depot only really grows much later in adult sheep, but the opposite is observed in pigs (SC first, PR much later). Since the factors that regulate adipogenesis, lipogenesis and lipolysis appear to be the same in all adipose tissue depots, we still have no real understanding of how this differential in fat depot growth/ development is regulated or whether it might be altered by pre-natal environment/ nutrition.

4.3. Evidence for programming of appetite regulation

Food intake is fundamental to animal productivity. The central nervous system, in particular the hypothalamic region of the brain, plays a pivotal role in the control of voluntary food intake and appetite drive in mammals, whether ruminant or non-ruminant. The activity of these neural pathways is modulated by factors circulating in the bloodstream that provide information on the body's nutritional status. Under normal conditions, adequate nutritional intake is thereby achieved for basal metabolic requirements, growth, reproduction and appropriate deposition of energy stores as fat. Since these neural and feedback pathways develop in early life, it is pertinent to examine the extent to which the adult phenotype may be altered or programmed by early life challenges.

The mature hypothalamic arcuate nucleus produces both appetite stimulating (orexigenic) neuropeptides, primarily neuropeptide Y (NPY) and agouti-related peptide (AGRP), and appetite-suppressing (anorexigenic) neuropeptides, primarily pro-opiomelanocortin (POMC) gene product and cocaine- and amphetamine-regulated transcript (CART). Output of these neuropeptides is able to respond appropriately to a range of peripheral nutrient and hormonal metabolic signals, most notably the adipose-derived hormone leptin, and neuronal projections from the arcuate nucleus to other hypothalamic regions such as the paraventricular nucleus (PVN) are important in mediating their effects (Schwartz *et al.*, 2000). Central regulation of appetite has been largely studied in the context of human obesity (e.g. Dhillo, 2007) rather than livestock.

Table 4.2. Effects of fetal nutrition on adiposity in sheep

Time of challenge	Nutritional challenge	Effect on growth	Fetal/postnatal offspring age	Effect on adiposity	Reference
A. In utero					
0-130d	Maternal food restriction70%	Fetal weight decreased	130 d	Decreased perirenal fat and total carcass fat	(Luther *et al.*, 2007)
Throughout	Overnourished adolescent dam – placental insufficiency IUGR	Fetal weight decreased	130 d	Increased relative perirenal fat weight	(Matsuzaki *et al.*, 2006, Redmer *et al.*, 2012)
28 -80d	Maternal food restriction 60%	Fetal weight decreased	140 d	Increased perirenal fat mass	(Bispham *et al.*, 2003)
B. Post natal					
115 – 124d	Maternal overnutrition 160%	No effect	30 d	Increased subcutaneous fat	(Muhlhausler *et al.*, 2006)
Throughout	Placental restriction by carunclectomy IUGR	Low birth weight	45 d	Increased visceral fat	(De Blasio *et al.*, 2007
Throughout	Overnourished adolescent dam - placental insufficiency IUGR vs control-fed dams	Low birth weight	77 d	Increased total body fat (DXA)	(Wallace *et al.*, 2011)
Throughout	Overnourished adolescent dam - placental insufficiency IUGR	Low vs normal birth weight	77 d	No effect, but female>male	(Wallace *et al.*, 2013)
Embryo donor	Overnutrition 170-190% for 5 months	No effect	120 d	Females fatter, males no effect	(Rattanatray *et al.*, 2010)
Embryo donor	Overnutrition for 4 months then food restriction 70% for 1 month	No effect	120 d	No effect	(Rattanatray *et al.*, 2010)
28 – 78d	Maternal food restriction 50%	No effect	120 d	Increased backfat (males)	(Ford *et al.*, 2007)
30 – 70d	Maternal food restriction: 50%	No effect	120 d	No effect subcutaneous backfat depth, omental fat mass and perirenal fat mass; but females>males	(Daniel *et al.*, 2007)
-	-	Range	150 d	Positive correlation between total body fat (DXA) vs birth weight	(Muhlhausler *et al.*, 2008)

Table 4.2. (Cont…)

Time of challenge (gestation)	Nutritional challenge	Effect on growth	Fetal/postnatal offspring age	Effect on adiposity	Reference
30 – 70d	Maternal food restriction: 50%	No effect	180 d	Increased intramuscular fat (LD and ST muscles), particularly in Males. No effect subcutaneous backfat depth, omental fat mass and perirenal fat mass; but females>males.	(Daniel *et al.*, 2007)
Throughout	Overnourished adolescent dam - placental insufficiency IUGR vs control-fed dams	Low birth weight	240 d	No effect, but female>male (DXA)	(Wallace *et al.*, 2011)
28 – 78d	Maternal food restriction 50%	No effect	270 d	Increased kidney-pelvic fat (males)	(Ford *et al.*, 2007)
0 – 30d	Maternal food restriction 50%	No effect	12 months	No effect	(Gardner *et al.*, 2005)
110d - term	Maternal food restriction 50%	No effect	12 months	Increased perirenal and omental fat mass	(Gardner *et al.*, 2005)
Throughout	Maternal overnutrition 150% - obese dam	No effect	19 months	No effect (DXA)	Long et al 2010
Throughout	Maternal overnutrition 150% - obese dam	No effect	22 months	Increased total body fat (DXA)	(Long *et al.*, 2010)
-	Twins vs singles	Low birth weight	2 years	Increased total body fat (DXA)	(Hancock *et al.*, 2012)
Throughout	Twinning and placental embolization	Low birth weight	2.3 years	Increased abdominal fat mass	(Louey *et al.*, 2005)
Periconception, -61– 0d, -61 – 30d or 2 – 30d	Maternal food restriction: ~50%	No effect	3 – 4 years	Increased total body fat (DXA) and increased perirenal fat mass – in males; no effect in females.	(Jaquiery *et al.*, 2012)
28-78d	Maternal food restriction: 50%	No effect	6 years	No effect (DXA and perirenal and omental fat mass), but plasma leptin increased	(George *et al.*, 2012)

4.3.1. Hypothalamus

In cattle and sheep, these neural pathways develop early in fetal life, with the hypothalamus being morphologically distinct by the end of the first third of gestation. Gene expression for the primary appetite-regulating hypothalamic neuropeptides is seen in the fetal sheep arcuate nucleus from early (50d, Adam et al unpublished) to mid-gestation (81d, Adam *et al.*, 2008) onwards (110d - 130d, (Muhlhausler *et al.*, 2004); term = 145d), and evidence is emerging that expression levels may be affected by alterations in the prenatal nutritional environment. The postnatal persistence of such changes in gene expression may contribute to the programming of an altered adult appetitive phenotype, and this hypothesis forms the basis for many investigations into the fetal origins of human obesity (Muhlhausler and Ong, 2011). However the majority of such investigations use laboratory rodents in which the hypothalamus is relatively immature at birth and the extrapolation of findings to larger mammals needs to recognise the temporal differences in development between altricial (rodent) and precocious (livestock) species. This overview will therefore focus on findings from sheep.

The fetus relies passively on transplacental transfer of nutrients (primarily glucose) from the maternal circulation for its nutrition, and fetal nutritional status can affect the developing hypothalamic appetite-regulating circuitry (**Table 4.3**). Hypothalamic NPY (orexigenic) is increased in late gestation sheep fetuses of undernourished mothers (Warnes *et al.*, 1998) and anorexigenic CART gene expression is decreased in late gestation intra-uterine growth restricted (IUGR) sheep fetuses in overnourished adolescent mothers (Adam *et al.*, 2011b). Conversely, late gestation intra-fetal glucose infusion increased anorexigenic POMC gene expression (Muhlhausler *et al.*, 2005). In mid-gestation, POMC gene expression correlated positively with fetal glycemia (Adam *et al.*, 2008) but maternal overnutrition/obesity had no effect on hypothalamic levels of orexigenic or anorexigenic neuropetides in ovine foetuses (Breton *et al.*, 2011).

The foregoing suggests that relative expression levels of appetite-regulatory hypothalamic neuropeptides are sensitive in sheep to prenatal nutrition, but the key question is whether these changes persist to affect their appetite-regulatory actions postnatally (**Table 4.3**). Maternal overnutrition in late gestation resulted in increased POMC gene expression in the arcuate nucleus of lambs at postnatal day 30 (Muhlhausler *et al.*, 2006), whereas maternal food restriction in early gestation decreased hypothalamic NPY expression at postnatal day 7 (Sebert *et al.*, 2009). However, no effects on hypothalamic gene expression levels were seen in obese 1 year-old offspring following early gestation maternal food restriction (Sebert *et al.*, 2009) or in 11 week-old low birth weight lambs following IUGR (Adam *et al.*, 2013). Importantly, however, this latter study highlighted a major effect of gender, with orexigenic genes predominating in males and anorexigenic genes predominating in females, linked closely to the sex differences in body composition (adiposity) and consequent metabolic hormone status (leptinaemia) (Adam *et al.*, 2013; Wallace *et al.*, 2013).

Metabolic hormones that regulate the hypothalamic appetite circuits in adults also control their development (Bouret, 2013). Notably leptin determines patterns of neurogenesis, axon growth and synaptic plasticity in the developing hypothalamus, especially during a discrete developmental period soon after birth in rodents (Bouret and Simerly, 2007). It is not known exactly when this developmental period occurs in more precocious larger mammals like sheep and cattle, but it is likely to be prenatal given the greater maturity of the hypothalamus at birth (Grayson *et al.*, 2010). Leptin secretion is initiated in the later stages of gestation in sheep and cattle following significant adipose tissue deposition and therefore fetal nutrition and growth will be critical in this regard. Late

gestation sheep fetuses with increased adiposity had both increased leptinaemia and increased hypothalamic expression of the leptin receptor (Adam *et al.*, 2011a), and indeed the adipose-hypothalamic axis is thought to be critical to the developmental programming of hypothalamic feeding circuits (Horvath and Bruning, 2006; Breton, 2013). Thus, leptin plays an important neurotrophic role in early life and elevated circulating leptin in lambs soon after birth does not appear to be anorexigenic (De Blasio *et al.*, 2010) However, by 5-6 months of age adult-like anorexigenic actions of leptin are seen in sheep given leptin administered into the hypothalamus, irrespective of birth weight or gender (Adam et al., 2011b)

4.3.2. Epigenetic programming of appetite regulation

There are very limited published data on epigenetic changes in central appetite-regulating pathways in sheep or cattle. Periconceptional undernutrition led to hypomethylation of the POMC promoter, though no change in POMC or NPY gene expression, in the late gestation fetal sheep hypothalamus (Stevens *et al.*, 2010); this was further exacerbated by twinning and the consequent additional nutritional challenge of placental restriction (Begum *et al.*, 2012). Since the rodent hypothalamic POMC promoter region is a key target of epigenetic changes following perinatal nutritional manipulations (Coupe *et al.*, 2010), this clearly warrants further investigation in livestock species.

4.3.3. Food intake and appetite

Studies of appetite (voluntary food intake) in offspring from nutritionally perturbed ovine pregnancies have produced variable results depending on the age at study, postnatal management and nature of the perturbation (**Table 4.3**). Following late gestation maternal overnutrition, lambs had increased appetite for the first 3 weeks but not at 4 weeks of age (Muhlhausler *et al.*, 2006); whereas IUGR lambs from pregnancies characterised by placental insufficiency (carunclectomy) also had increased feeding activity at 2 weeks of age (De Blasio *et al.*, 2007). Lamb birth weight was unaltered in the foregoing studies, whereas there was no effect on suckling activity in 3 week old IUGR lambs with low birth weight from overnourished adolescent placentally-insufficient pregnancies (Adam *et al.*, 2013). Others have reported no effect of low birth weight on food intake in the first five weeks of life (Vilette and Theriez, 1981; Villette and Theriez, 1983). However, low birth weight lambs consume more food to achieve a given live weight because it takes them longer to achieve it; thus low birth weight lambs consumed 13% or 20% more than normal birth weight lambs when artificially reared rapidly or slowly, respectively, to 20 kg (Greenwood *et al.*, 1998). Nonetheless, Vilette and Theriez (1981) reported that food intake from weaning to 35kg was not related to birth weight, Sibbald and Davidson (1998) found that food intake from weaning to 2 years of age was not affected by moderate maternal nutrient restriction in late gestation and consequent low birth weight, and Daniel *et al.* (2007) describe no effect on food intake by lambs up to 17 and 24 weeks of age after severe maternal food restriction in early gestation. In longer term studies, following early gestation maternal food restriction, (Sebert *et al.*, 2009) report no effect on appetite in obese 1 year-old offspring but (George *et al.*, 2012) report increased appetite drive in obese female 6 year-old offspring. On the other hand, (Long *et al.*, 2010) report increased appetite at 19 months of age in the offspring of overnourished obese mothers.

Table 4.3. Effects of altered fetal nutrition on appetite regulation in sheep

Time of challenge	Nutritional challenge	Fetal/offspring age	Hypothalamic neuropeptide changes	Appetite/Food intake	Reference
A. In utero					
Throughout	Maternal overnutrition: 150%	75 d	No effect CART, POMC, NPY, AGRP	-	(Breton *et al.*, 2011)
Throughout	Overnourished adolescent dam - placental insufficiency IUGR	130 d	Decreased CART No effect POMC, NPY, AGRP	-	(Adam *et al.*, 2011b)
130 – 140d	Fetal glucose infusion	140 d	Increased POMC No effect CART, NPY, AGRP	-	(Muhlhausler *et al.*, 2005)
115-145d	Maternal food restriction: 50%	145 d	Increased NPY	-	(Warnes *et al.*, 1998)
B. Post natal					
30 – 80d	Maternal food restriction: 50%	7 d	Decreased NPY No effect POMC, AGRP	-	(Sebert *et al.*, 2009)
Throughout	Low birth weight, increase fetal no.	7 d	-	No effect	(Villette and Theriez, 1983)
Throughout	Placental restriction by carunclectomy IUGR	15 d	-	Increased	(De Blasio *et al.*, 2007)
Throughout	Overnourished adolescent dam - placental insufficiency IUGR	21 d	-	No effect	(Adam *et al.*, 2013)
115 – 124d	Maternal overnutrition 160%	30 d	Increased POMC	Increased 1-3wks No effect at 4wks	(Muhlhausler *et al.*, 2006)
Throughout	Low birth weight, increase fetal No.	35 d	-	No effect	(Vilette and Theriez, 1981)
Throughout	Low birth weight - Overnourished adolescent dam - placental insufficiency IUGR	77 d	No effect CART, POMC, NPY, AGRP	-	(Adam *et al.*, 2013)
30 – 70d	Maternal food restriction: 50%	17 wks	-	No effect	(Daniel *et al.*, 2007)
30 – 70d	Maternal food restriction: 50%	24 wks	-	No effect	(Daniel *et al.*, 2007)
30 – 80d	Maternal food restriction: 50%	12 months	No effect POMC, NPY, AGRP	No effect	(Sebert *et al.*, 2009)
-60d - term	Maternal obesity: 150%	19 months	-	Increased	(Long *et al.*, 2010)
105d - term	Maternal food restriction: 75%	2 years	-	No effect	(Sibbald and Davidson, 1998)
28-78d	Maternal food restriction: 50%	6 years	-	Increased	(George *et al.*, 2012)

CART, cocaine- and amphetamine-regulated transcript; **POMC,** pro-opiomelanocortin; **NPY,** neuropeptide Y; **AGRP,** agouti-related peptide

Data from cattle report no differences in food intake (at 26-30 months of age) attributable to wide differences in prenatal growth and birth weight (Greenwood and Cafe, 2007). Low birth weight cattle at a given age eat less than normal birth weight counterparts but not when intakes are adjusted for current body weight, and likewise twin cattle tend to eat less food than singletons at a given age by virtue of their smaller size (de Rose and Wilton, 1991).

4.3.4. Interim conclusions

Nutritional challenges *in utero* alter the developing hypothalamic appetite-regulatory circuits in fetal cattle and sheep, but there is a lack of evidence for the persistence and functional significance of such alterations for food intake in current animal production systems. However, emerging data on sensitivity to epigenetic changes by the promoter of the anorexigenic POMC gene could be of lasting significance for appetite drive and deserves further study in livestock species.

5. Programming of fertility

Successful reproduction and fertility are central to the financial success of most livestock enterprises and have their origins firmly in fetal life. Crucially, in female sheep and cattle, the resting reserve of primordial follicles that will determine their lifetime supply of potentially fertilizable oocytes (eggs) is established definitively before birth and cannot be replenished thereafter (Erickson, 1966a and 1966b; McNatty *et al.*, 1995). In contrast, males continuously produce new spermatozoa after puberty, but the number of Sertoli cells which are the primary determinant of sperm production and testes size in adult life is determined by proliferation during the fetal, neonatal and peripubertal periods (Sharpe *et al.*, 2003). Thus in both sexes, the developing reproductive axis and its hormonal control systems are potentially susceptible to the range of environmental programming stimuli detailed in the preceding sections of this review.

5.1. Impact of early life nutrition

5.1.1. Female offspring: sheep

The impact of early life nutrition on the developing reproductive axis and on adult fertility in sheep is summarized in **Table 5.1**. In sheep (gestation length ~145 days) the overwhelming body of evidence relates to maternal undernutrition in adult ewes, typically 0.5 to 0.7 x maintenance compared with a control group nourished to meet the needs for fetal growth. Exposures during pregnancy are either limited to the known key periods of gonadal development, or span the entire prenatal period. Where endpoints were assessed in fetal life, the consensus seems to be one of delayed germ cell degeneration or delayed ovarian follicular development as measured by elaboration of the granulosa cell layer (Borwick *et al.*, 1997, Rae *et al.*, 2001). Altered proliferation and apoptosis within the developing ovary may be the root cause (Lea *et al.*, 2006; Grazul-Bilska *et al.*, 2009). In all cases the effects of maternal undernutrition were independent of the growth rate of the fetus *per se*. In contrast, compelling reductions in primordial follicle number (80% less) were evident in fetuses destined to be growth restricted at birth (Da Silva *et al.*, 2002 and 2003). Although in this instance the adolescent dams were overnourished (~2 x maintenance), in this well-established paradigm competition for nutrients between the growing mother and her conceptus results in restricted placental development, and a major reduction in uteroplacental blood flow and fetal nutrient supply from mid-gestation onwards (Wallace *et al.*, 2011).

Accordingly, by late gestation, the size of the fetal ovarian follicle population was positively correlated with both placental and fetal weight.

None of these aforementioned prenatal nutritional manipulations appear to influence the onset of puberty in spring-born females, at least when fed *ad libitum* after birth to ensure they exceed the critical weight required to respond to the appropriate photoperiodic cues at the start of their first breeding season (Da Silva *et al.*, 2001; Kotsampasi *et al.*, 2009b). Similarly, there is little evidence of a robust effect of prenatal nutrition on the postnatal function of the hypothalamic –pituitary axis in that baseline and GnRH- stimulated gonadotrophin secretion are largely unperturbed at puberty and later in adult life (Borwick *et al.*, 2003; Kotsampasi *et al.*, 2009b). This suggests that the prenatal nutritional programming of lifetime fertility in females primarily has its origins within the ovary and / or uterus. At its simplest this could be manifest as (a) a reduction in ovulation rate directly reflecting a diminished ovarian follicle reserve, (b) a reduction in embryo survival variously reflecting poor oocyte quality, fertilization failure, inability to progress beyond the maternal recognition of pregnancy stage and / or (c) a reduction in litter size due to a failure to implant or limited uterine capacity. There is some supporting evidence to substantiate these various possibilities but more than one factor is likely to be involved. Maternal undernutrition from conception to 95 days of gestation was associated with a modest (20%) reduction in the natural ovulation rate of female offspring expressed at a single time point within their second breeding season (Rae *et al.*, 2002a). While ovulation rate clearly sets the upper limit of eggs shed by the ovary in any one cycle it is unknown whether this effect on ovulation rate was sustained throughout the life course or indeed translated into a reduction in litter size. In contrast when maternal undernutrition was limited to the first 35 days of gestation, there was no effect on the naturally occurring ovulation rate of the female offspring measured on seven occasions during the first two breeding seasons or following mild ovarian stimulation with pregnant mare's serum gonadotrophin on one occasion during the second (Parr *et al.*, 1986). Similarly ovulation rate of female offspring at the end of a three year breeding life (corrected for current pre-mating adiposity) was not influenced by maternal nutrient supplementation throughout the second two thirds of pregnancy (Gunn *et al.*, 1995). Effects on ovulation rate may not limit litter size until relatively late in a female's reproductive life when her ovarian reserve may become exhausted. Furthermore, theoretically the ovarian follicle population may become limiting earlier in life in pedigree females repeatedly superovulated as part of genetic improvement programmes involving multiple ovulation and embryo transfer, but this has not been tested experimentally.

On sheep farms, fertility is most often recorded in terms of pregnancy rate and litter size. Again the available evidence is apparently contradictory. When assessed on a single occasion following a synchronised mating in the first breeding season, pregnancy rate and litter size of female offspring were not influenced by diverse nutritional exposures (under versus overnutrition) during early or mid-pregnancy (Munoz *et al.*, 2009). In contrast, a major reduction in pregnancy rate following hand mating and a 45-day breeding period were reported in a small study of two year old females who had been exposed to maternal undernutrition between 28 and 78 days gestation (Long *et al.*, 2010). A more robust assessment of the impact of early life nutrition on fertility is provided when female offspring are studied over several breeding seasons and in large numbers. Accordingly, Gunn *et al.* (1995) studied the effect of supplementing maternal nutrition during the last 100 days of pregnancy or during the first 100 days of lactation on female offspring fertility over three lambing seasons. Relative to the unsupplemented control group, both periods of supplementation were associated with a higher lifetime incidence of multiple births, with lactation > pregnancy due to a reduced incidence of barrenness and ewe mortality. Similarly, there were fewer lambs born to female offspring over a period spanning up to eight pregnancies, when stocking density was high (low available nutrition) from conception to weaning but only when

stocking density was also high in adult life (Langlands *et al.*, 1984). This interaction suggests that negative effects of prenatal undernutrition may not be revealed unless nutrient availability at the time of breeding is also marginal. Within genotypes, birth weight is a useful indicator of *in utero* fetal nutrient supply that can be readily measured on farm. Intriguingly, data from performance recorded Suffolk flocks reveals that females born at both birth weight extremes (i.e. 2 standard deviations above or below the mean birth weight) had a lower number of lambs per litter during a median of three pregnancies (Gardner *et al.*, 2009). This effect was independent of offspring post-natal growth rate from birth to 8 weeks age but growth rates in these pedigree females were relatively high across the board reflecting the intensive nutritional inputs common in such flocks. In contrast, a lower lifetime incidence of multiple births has been reported for female offspring who along with their mothers were exposed to poor pasture from birth to weaning to restrict offspring growth (Rhind and McNeilly, 1998). These lifetime fertility studies were low intensity and generally not designed to investigate mechanisms but reductions in litter size are likely in part to involve increased embryo or fetal mortality. In the absence of experimental assessments of litter size in relation to ovulation and fertilisation rates, embryo quality and pregnancy rates within a single study it is perhaps pertinent that maternal undernutrition during mid-pregnancy increased markers of DNA damage in fetal oogonia (Murdoch *et al.*, 2003) and that blastocyst production *in vitro* from pre-pubertal ewe lambs was highest when they had been exposed to high rather than low maternal nutrition during mid-late pregnancy (Kelly *et al.*, 2005). Conceptus survival is also dependent on appropriate uterine development and capacity (Vallet *et al.*, 2013). While under normal circumstances it is generally assumed that the capacity of the uterus and placentae of ruminants to provide support for fetuses exceeds the number of fetuses present, it is intriguing to note a modest but significant reduction in uterine caruncle number, and hence potential placentomes, in low birth weight female lambs (Aitken *et al.*, 2003). However the possible impact on litter size remains untested.

5.1.2. Female offspring: cattle

The effect of early life nutrition on aspects of reproductive performance in beef and dairy cattle are summarized in **Table 5.2**. In cattle (gestation length ~285 days), there is a scarcity of data relating prenatal nutritional exposure to altered fetal ovarian development. Nevertheless, it is now well established that the number of antral follicles present at all stages after birth is a direct reflection of the ovarian follicle reserve established during fetal life (Evans *et al.*, 2012). Thus birth weight (as a proxy for fetal nutrient supply), was positively associated with the antral follicle count (AFC) in a large population of neonatal beef calves who died due to dystocia, and in adult heifers of various ages examined by ultrasound (Cushman *et al.*, 2009). Reduced ovarian weight and large follicle diameter at 30 months of age has been measured following slow prenatal growth rates (Wilkins *et al.*, 2006), while a general reduction in all follicle types was evident following exposure to a low then high protein diet during the first two thirds of gestation (Sullivan *et al.*, 2009). A direct effect of maternal undernutrition during the first third of gestation on the ovarian follicle reserve has recently been documented using serial ovarian ultrasound on five occasions during pre-pubertal and adult life (Mossa *et al.*, 2013). The robust decrease in follicle number reported appears to be associated with increased maternal testosterone during dietary restriction and was independent of calf birth weight and postnatal growth rate. Furthermore, recent *in vitro* data suggests that fetal ovarian steroids are potent negative regulators of follicle formation and as such environmental factors that alter steroid production in the dams and/or fetus may influence the size of the ovarian follicle reserve at birth (Fortune *et al.*, 2013).

Similar to sheep, prenatal maternal nutrition did not impact the onset of puberty in beef cattle (Martin *et al.*, 2007; Mossa *et al.*, 2013) but higher offspring pregnancy rates were observed following protein supplementation in late pregnancy (Martin *et al.*, 2007), and when the AFC pre-breeding was high (Cushman *et al.*, 2009). In contrast, manipulation of the post-weaning diet in beef heifers did not impact AFC or overall pregnancy rate (Eborn *et al.*, 2013) In cattle there is a surprising lack of data linking prenatal nutrition and offspring reproductive performance. Although dairy cows with a high AFC are three times more likely to become pregnant by the end of the breeding period (Mossa *et al.*, 2012), there are no data directly linking birth weight with the AFC in dairy breeds. Moreover, size at birth did not impact fertility in the first lactation and low birth weight was in part protective against abnormal ovarian cycles in the second service period (Swali and Wathes, 2006). In contrast, growth data obtained from 17 UK dairy farms suggests that low postnatal growth rates increase age at first breeding and age at calving (Brickell *et al.*, 2009).

5.1.3. Male offspring: sheep and cattle

Relative to the female, there is a dearth of published information on the impact of early life nutrition on male offspring fertility in ruminants (**Tables 5.1** and **5.2**). The single bovine study shows FSH levels and testis volume were increased in the bull calf by first trimester protein restriction (Sullivan *et al.*, 2010). In sheep when endpoints were assessed in mid-pregnancy the consensus is that neither maternal undernutrition nor reduced fetal nutrient supply impact Sertoli cell number, number of seminiferous cords or basal pituitary gonadotrophin secretion (Rae *et al.*, 2002b; Da Silva *et al.*, 2003; Andrade *et al.*, 2013). However in the newborn lamb, the number of seminiferous cords and Sertoli cells were reduced following maternal undernutrition from mid-pregnancy onwards and was associated with a modest reduction in birth weight (12% reduction) relative to the adequately nourished control group (Bielli *et al.*, 2002). In addition, male lambs that were severely growth restricted in utero (47% reduction) as a result of overnourishing their adolescent dams exhibited slower absolute postnatal growth rates, delayed age at puberty, lower testosterone concentrations and reduced testicular volume per unit live weight between 28 and 35 weeks of age (Da Silva *et al.*, 2001). As Sertoli cells set the ceiling for sperm production, and continue to proliferate until puberty, it is likely that poor prenatal growth followed by a delayed attainment of the pubertal live weight threshold could well impact ram libido and sperm production and quality, particularly if rams are used in their first breeding season. However, this has not been directly tested. By contrast in studies where maternal nutrition is restricted during the first two thirds of gestation and lamb birth weight and postnatal growth are unaffected, there is no evidence of a long term effect on the onset of puberty (Kotsampasi *et al.*, 2009b) or on indices of semen quality in adult life (Rae *et al.*, 2002a).

5.2. Impact of environmental chemicals

Environmental chemicals, including the so called ‘endocrine disrupting compounds’ (EDCs) can potentially programme various components of the reproductive axis (brain- pituitary-gonad-uterus) to malfunction in later life, altering important aspects of fertility and causing financial loss to sheep and cattle producers (Rhind *et al.*, 2003; Rhind, 2005). These chemicals come from a variety of sources including industrial processes, domestic effluents, and agricultural practices. Much of the current data relating to the reproductive actions of EDCs is derived from epidemiological studies of wildlife species and from rodent studies involving supra-environmental exposures. There is a comparative lack of information in farm animal species and most of our current knowledge comes

from sheep studies involving relatively small numbers of subjects. Detailing the effect of these EDCs is further complicated because they do not necessarily comprise one chemical substance but mixtures, in which each individual component may carry a low level of risk but may cause significant physiological disruption when combined in mixtures found in the real-life environment (Bellingham *et al.*, 2009). These mixtures include sewage sludge, a by-product of the treatment of waste water from domestic, industrial and agricultural sources which is commonly disposed of by spreading it on pastures that may in turn be grazed by domestic ruminants (Rhind *et al.*, 2011). As such sewage sludge is arguably one of the most relevant EDCs in the context of this review. The fetal and neonatal stages are particularly sensitive to EDCs and exposure during these critical windows of development can impact the reproductive axis in a sexually differentiated manner as will be discussed in the following sections. This review will concentrate on five important EDCs; Bisphenol-A (BPA), Octylphenol (OP), Methyoxychlor (MXC), Polychlorinated biphenyls (PCB) and sewage sludge (SS). Main effects are summarized in **Table 5.3**.

5.2.1. Effects of EDCs in the hypothalamus

Reproduction in animals is ultimately controlled by approximately 2000 neurones in the hypothalamic-preoptic area of the brain that synthesise and secrete gonadotrophin releasing hormone (GnRH: (Dees and McArthur, 1981; Lehman *et al.*, 1986). These neurones are, in turn, regulated by neurotransmitter and neuropeptide systems that convey information about the animals' internal and external environment including its steroidal status (**Figure 5.1**). GnRH neurones themselves do not possess nuclear hormone receptors (Herbison *et al.*, 1993) and therefore information must be relayed by steroid-receptive neural systems including Kisspeptin and Galanin. Further, it is known that many of the EDCs act as steroid mimetics exerting their actions via classical steroid receptors in reproductive tissue. EDCs can reduce the population of both GnRH neurones and GnRH receptors (Bellingham *et al.*, 2010) (SS); (Mahoney and Padmanabhan, 2010) (BPA and MXC)). Whether these reductions in GnRH synthetic capacity impact on reproduction in the females used in these studies is unclear as relatively small amounts of GnRH are needed to trigger ovulation (Bowen *et al.*, 1998). However, a lack of receptors for GnRH may alter signalling pathways and reduce pituitary LH and FSH secretion with downstream effects on fertility. EDCs also alter oestrogen receptor alpha and beta expression in the female sheep hypothalamus (ESR1, ESR2: (Mahoney and Padmanabhan, 2010)). Specifically, female fetuses exposed to BPA have increased ESR1 gene expression in adulthood while ESR2 gene expression is reduced by exposure to both BPA and MXC. Changes in steroid receptor abundance may alter the feedback mechanisms responsible for ovulation potentially altering mating behaviour and fertility (Mahoney and Padmanabhan, 2010), but this hypothesis has not been directly tested. In addition, specific oestrogen receptive neural populations that regulate GnRH secretion have also been shown to be altered by EDCs, namely Kisspeptin, its receptor and Galanin receptors 1-3 (Bellingham *et al.*, 2009; Bellingham *et al.*, 2010) (SS)). Reductions in these neural populations were observed in 110 day old male and female fetuses, although whether these neural networks remain perturbed after birth and into adulthood is currently unexplored. After birth, both Kisspeptin and Galanin are important for the timing of puberty, ovulation and receptivity in farm animals (Caraty *et al.*, 2012) and, therefore, reductions in these neurotransmitter systems may potentially reduce reproductive performance in sheep.

Table 5.1. Summary of impact of early life nutrition on fetal gonadal development, hypothalamic – pituitary - gonadal function and adult fertility in sheep

Nutritional exposure	Period of exposure	Litter size	Effect on fetal / birth wt.	Life-stage 1^0 endpoints measured	Main effect(s) reported[c]	Study size and gender	Reference
Maternal UN[a]	0 to 62d GA[b]	Singletons, twins	None	Fetal d62	Delayed ovarian follicular development	11 females	(Borwick *et al.*, 1997)
Maternal UN[a]	0 to 30, 31 to 50, 31 to 65, 65 to 110d GA[b]	Singletons, twins	None	Fetal d50, 65 or 110	Delayed ovarian follicular development & stage specific effects on markers of apoptosis	130 females	(Rae *et al.*, 2001, Lea *et al.*, 2006)
Maternal UN[a]	0 to 30, 31 to 50, 31 to 65, 65 to 110d GA[b]	Singletons, twins	None d50 or 65, 15%↓at d110	Fetal d50, 65 or 110	No effect on testes mass, transient (d50) effect on steroidogenic capacity. No effect on Sertoli cell number or markers of apoptosis (d110)	113 males	(Rae *et al.*, 2002b, Andrade *et al.*, 2013)
Maternal UN[a]	28 to 78d GA[b]	Singletons, twins	None	Fetal d78	Increased oxidative DNA damage in oogonia	12 females	(Murdoch *et al.*, 2003)
Maternal ON[d] /reduced fetal nutrient supply	4 to 103d GA[b]	Singletons[e]	None	Fetal d103	Reduced primordial & total follicle number; No effect on seminiferous cord or Sertoli cell no.	11 females 17 males	(Da Silva *et al.*, 2002)
Maternal ON[d] /reduced fetal nutrient supply	4 to 131d GA[b]	Singletons[e]	31% ↓	Fetal d131	Reduced primordial & total follicle number. Higher pituitary LHβ mRNA	19 females	(Da Silva *et al.*, 2003)
Maternal UN[a]± high selenium	50 to 135d GA[b]	Singletons	None	Fetal d135	Variable effects of UN and selenium on proliferation in ovarian follicles and blood vessels	32 females	(Grazul-Bilska *et al.*, 2009)
Maternal UN[a]	70d GA[b] to term	Singletons	12% ↓	Neonatal d2	Reduced Sertoli cell number	25 males	(Bielli *et al.*, 2002)

Maternal UN/ON[f]	-82 to 70, 71 to 100, 100 to 126d GA[b]	Singletons, twins	None	Pre-pubertal (2 months)	Blastocyst production in vitro highest in females exposed to ON mid-late pregnancy	36 females	(Kelly *et al.*, 2005)
Maternal ON[d] /reduced fetal nutrient supply	4d GA[b] to term	Singletons[e]	31% ↓ female 47% ↓ male	Pre-adult (10 months)	No effect on age at puberty, normality or number of ovarian cycles; Delayed onset of puberty, lower testosterone, reduced testes volume	28 females 14 males	(Da Silva *et al.*, 2001)
Maternal UN[a]	0 to 30 (UN1), 31 to 100d GA[b] (UN2)	Twins (artificially reared)	None	Pre-adult (10 months)	No effect on onset of puberty. Higher FSH post GnRH challenge & lower Sertoli cell number- UN2	19 males	(Kotsampasi *et al.*, 2009a)
Maternal UN[a]	0 to 30 (UN1), 31 to 100d GA[b] (UN2)	Twins (artificially reared)	None	Pre-adult (10 months)	No effect on onset of puberty or LH surge parameters. Higher FSH post GnRH challenge-UN1	17 females	(Kotsampasi *et al.*, 2009b)
Maternal UN[a]	0 to 95d GA[b]	Singletons, multiples	None	Adult (20 months)	Reduction in ovulation rate No effect on testes size or semen quality	49 females 32 males	(Rae *et al.*, 2002a)
Maternal UN or ON	1 to 39d, 40-90d GA[b]	Singletons, multiples	Not reported	Adult (mated at 8 months)	No effect on conception rate, litter size or number of lambs weaned	60 females	(Munoz *et al.*, 2009)
Maternal UN[a]	100d GA[b] to 14 weeks postnatal age	Twins	18% ↓	Pre-pubertal (7months), Adult (18months)	No effect on hypothalamic -pituitary function at either stage	2 cohorts of 28 females each	(Borwick *et al.*, 2003)
Maternal UN[a]	28 to 78d GA[b]	Singletons	Not reported	Adult (12 & 24 months)	Lower progesterone in one cycle - both years. Reduced pregnancy rate in year 2	14 females	(Long *et al.*, 2010)

Maternal UN[a]	0 to 35d GA[b]	Singletons	None	Adult (18 & 30 months)	No effect on natural ovulation rate (7 measures) or after PMSG[g]	~170 females	(Parr *et al.*, 1986)
Maternal supplementation	50d GA[b] to term, term to 100 days postnatal	Singletons, multiples	Pregnancy supplemented ↑14%	Adult (3 pregnancies)	No effect on ovulation rate. Higher lifetime incidence of multiple births in supplemented groups (lactation > pregnancy)	450 females	(Gunn *et al.*, 1995)
Variable fetal nutrient supply[h]	Pregnancy	Singletons	Not applicable – as per study design	Adult (median of 3 pregnancies)	Reduced average number of lambs per litter in females born at both birth weight extremes	2427 females	(Gardner *et al.*, 2009)
High stocking density/ low available nutrition[i]	0d GA[b] to 3months, 3 to 15months, >15months postnatal age	Singletons, twins	None	Adult (up to 9 years & 8 pregnancies)	Fewer lambs born if stocking density high from conception to weaning but only if also high in adult life	283 females	(Langlands *et al.*, 1984)
Undernutrition	2 to 15 weeks postnatal age	Singletons	Not reported	Adult (up to 7 years, 4-6 pregnancies)	Lower lifetime incidence of multiple births	499 females	(Rhind and McNeilly, 1998)

[a]UN, undernutrition (typically 0.5-0.7 x maintenance in adult ewes); [b]GA, gestational age; [c]where effects reported, minimum P<0.05 relative to optimally nourished reference control group; [d]ON, overnutrition (typically 2 x maintenance in adolescent ewes); [e]singleton pregnancies derived by embryo transfer using a single sire; [f]UN/ON, undernutrition 0.7x maintenance and overnutrition 1.5 x maintenance in adult ewes, 2x2x2 factorial design; [g]PMSG, pregnant mares serum gonadotrophin; [h]birth weight as a proxy for variable fetal nutrient supply, lambs categorised as relatively small or large at birth if 2 standard deviations below or above the mean birth weight, respectively; [i]High versus low stocking density during three periods, 2x2x2 factorial design.

Table 5.2. Summary of impact of early life nutrition on reproductive function in cattle

Nutritional exposure	Period of exposure	Type	Effect on fetal / birth wt.	Life-stage 1[o] endpoints measured	Main effect(s) reported[c]	Study size and gender	Reference
Variable fetal nutrient supply[a]	Pregnancy	Beef cattle	Not applicable – as per study design	Neonatal Adult (12-14 months)	Birth weight positively associated with AFC (neonatal and adult life). Decreased pregnancy rate when AFC is low.	181 females 406 females	(Cushman *et al.*, 2009)
Maternal UN (0.6 x maintenance)	-11 to 110d GA	Beef cattle	None	Pre-pubertal (7, 18, 35 weeks) and adult (56, 86 weeks)	No effect on age at puberty. Diminished ovarian reserve; lower AFC at 7, 18, 56, 86 weeks, lower AMH and higher FSH	23 females	(Mossa *et al.*, 2013)
Maternal low/ high protein (2 x 2 factorial)	0 to 93, 93-180d GA	Beef cattle	Not reported	Pre-pubertal and adult (5, 23 months)	Reduced primordial, primary and AFC after low-high protein in first two thirds of gestation	36 females	(Sullivan *et al.*, 2009)
Maternal protein supplementation / improved pasture (2 x 2 factorial)	Late gestation, early lactation	Beef cattle	None	Adult (up to start of second breeding season)	No effect on age at puberty. Earlier first calving and higher pregnancy rates following supplementation (protein) in late gestation	170 females	(Martin *et al.*, 2007)
Slow or rapid growth by varying maternal nutrition	30-90dGA to term, birth to weaning	Beef cattle	24% ↓	Adult (30 months)	Reduced ovarian weight and large follicle diameter after prenatal growth restriction. No effect of postnatal growth	162 females	(Wilkins *et al.*, 2006)

High or low weight gain	Weaning to 15months	Beef cattle	Measured but not reported	Adult (15months)	Weight gain category did not impact AFC or overall pregnancy rate	212-300 females	(Eborn *et al.*, 2013)
Variable fetal nutrient supply[b]	Pregnancy	Dairy cattle	24% ↓ low<high	Adult (spanning two service periods)	Low birth weight did not impact fertility in first service period, protective against abnormal ovarian cycles in second.	65 females	(Swali and Wathes, 2006)
Variable postnatal growth (on- farm data)	30 to 180, 181-450d postnatal age	Dairy cattle	Not reported	Adult (first calving)	Suboptimal growth increased age to first breeding and age at calving	392 females	(Brickell *et al.*, 2009)
Maternal low/ high protein (2 x 2 factorial)	0 to 93, 93-180d GA	Beef cattle	Not reported	Pre-pubertal (5 months)	Baseline (but not GnRH stimulated) FSH higher after low dietary protein in first two thirds gestation.	33 males	(Sullivan *et al.*, 2010)

UN, undernutrition; GA, gestational age; d, day; AFC, antral follicle count; AMH, anti-mullerian hormone; FSH, follicle stimulating hormone; [a]birth weight as a proxy for variable fetal nutrient supply, neonatal follicle parameters determined after calves died as a consequence of dystocia at <31d of age, adult follicle data obtained by ultrasound prior to breeding. [b]birth weight as a proxy for variable fetal nutrient supply, 3 equal sized groups based on lowest, average and highest birth weight.

Table 5.3. Summary of impact of specific endocrine disrupting chemicals on different aspects of reproductive axis function in sheep.

Chemical exposure	Period of exposure	Stage 1^{o} endpoints measured	Main effect(s) reported	No. of exposed & control animals (gender)	Reference
Bisphenol A	30 to 90d GA	21 months postnatal	Hypothalamic GnRH & ESR2 mRNA ↓, ESR1 ↑	12 (females)	(Mahoney and Padmanabhan, 2010)
Bisphenol A	4 to 11 weeks postnatal age	11 weeks postnatal	Pulsatile LH secretion ↓	12 (females)	(Evans *et al.*, 2004)
Bisphenol A	2 to 4 months postnatal age	4 months postnatal	Basal LH & LH pulse frequency ↓	18 (females)	(Collet *et al.*, 2010)
Bisphenol A	30 to 90d GA	6 to 40 weeks postnatal	Duration of first breeding season ↑, LH surge ↓at induced cycle	26 (females)	(Savabieasfahani *et al.*, 2006)
Bisphenol A	30 to 90d GA	Fetal,65 and 90d GA	Ovarian steroidogenic genes x 2 ↑ (d65), microRNA ↓(45 at d65, 11 at d90)	19 (females)	(Veiga-Lopez *et al.*, 2013)
Bisphenol A	4 to 11 weeks postnatal age	11 weeks postnatal	Uterine weight ↑, altered uterine ESR1 & 2 distribution	12 (females)	(Morrison *et al.*, 2003)
Sewage Sludge[a]	0 to 110d GA	Fetal, 110d GA	Hypothalamic GnRH, GnRHR & GALRs mRNA ↓, pituitary GALRs ↓	18 (10 female, 8 male)	(Bellingham *et al.*, 2010)
Sewage Sludge[a]	0 to 110d GA	Fetal, 110d GA	Hypothalamic & pituitary kisspeptin mRNA ↓, pituitary kisspeptin, LHβ & ERα↓	39 (not reported)	(Bellingham *et al.*, 2009)
Sewage Sludge[a]	0 to 110d GA	Fetal, 110d GA	Perturbed ovarian development	23 (females)	(Fowler *et al.*, 2008)
Sewage Sludge[a]	0 to 110d GA	Fetal, 110d GA	Testis weight, gonocytes, Sertoli & Leydig cell no. ↓, plasma inhibin & testosterone ↓	19 (males)	(Paul *et al.*, 2005)
Sewage Sludge[a]	Conception to 7 months postnatal	19 months postnatal	Spermatogenic abnormalities in 42% of exposed males	24 (males)	(Bellingham *et al.*, 2010)
Methoxychlor	30 to 90d GA	21 months postnatal	Hypothalamic GnRH & ESR2 mRNA ↓	11 (females)	(Mahoney and Padmanabhan, 2010)

Methoxychlor	30 to 90d GA	40 weeks postnatal	LH surge delayed at induced cycle	26 (females)	(Savabieasfahani *et al.*, 2006)
Octylphenol	70d GA to birth	At birth	Pituitary FSH mRNA & protein, testis weight, Sertoli cell no. ↓	Not reported	(Sweeney *et al.*, 2000)
Octylphenol	70d GA to birth or weaning, birth to weaning	I year postnatal	Morphologically abnormal sperm ↑ (birth to weaning group only)	22 (males)	(Sweeney *et al.*, 2007)
Octylphenol	70d GA to birth or weaning, birth to weaning	Up to 10 months (end of first season)	All OP groups, onset of puberty ↑, duration of first breeding season ↑	19 (females)	(Wright *et al.*, 2002)
Polychorinated biphenyl (2 types)	Conception to birth	60 days postnatal	GnRH induced LH ↑, advanced follicle dynamics	26 (females)	(Kraugerud *et al.*, 2012)

[a]dams maintained on plots fertilised with sewage sludge (SS) throughout their breeding lives (typically at least 3 years) prior to mating. SS, by product of waste water treatment from domestic, industrial and agricultural sources (Stevens et al. 2003). Bisphenol A, used in the production of polycarbonated plastic and epoxy resins (vom Saal & Hughes, 2005); Methoxychlor, a pesticide (ATSDR, 2002); Octylphenol, non-ionic surfactant used in the production of detergents (White et al., 1994); Polychlorinated biphenyls, industrial pollutants now banned but abundant in environment (Lindenau & Fischer, 1996)

5.2.2. Effects of EDCs on pituitary gonadotrophins

The gonadotrophins LH and FSH are important in the female for ovarian development and function and to promote ovulation, and in the male to promote testicular development and spermatogenesis. Exposure of the male sheep fetus to OP from day 70 until birth suppressed FSHβ gene expression and the percentage of FSH immunoreactive (FSH-ir) cells in the pituitary gland while LH gene expression and cell numbers were unaffected (Sweeney *et al.*, 2000). These pituitary effects were associated with altered testicular function (see later section). In the female fetus populations of pituitary gonadotrophs have been shown to be reduced following exposure to SS (Bellingham *et al.*, 2009) as well as the percentage of cells double labelled for Kisspeptin and LHβ and those labelled for ESR1. In relation to gonadotrophin secretion *per se*, FSH release is suppressed by OP exposure in fetal animals (Sweeney *et al.*, 2000) while episodic LH has been shown to be inhibited by both short and long-term administration of BPA to pre-pubertal ewe lambs (Evans *et al.*, 2004; Collet *et al.*, 2010). Conversely, GnRH stimulated LH release was higher in pre-pubertal female lambs exposed to PCB 118 (but not PCB 153) during gestation, indicative of selective PCB modulation of the responsiveness of the pituitary gland to hypothalamic stimulation (Kraugerud *et al.*, 2012). Both PCBs altered follicular dynamics in these ewe lambs (see following section) which may be an indirect effect of altered gonadotrophin release or a direct effect on the ovary.

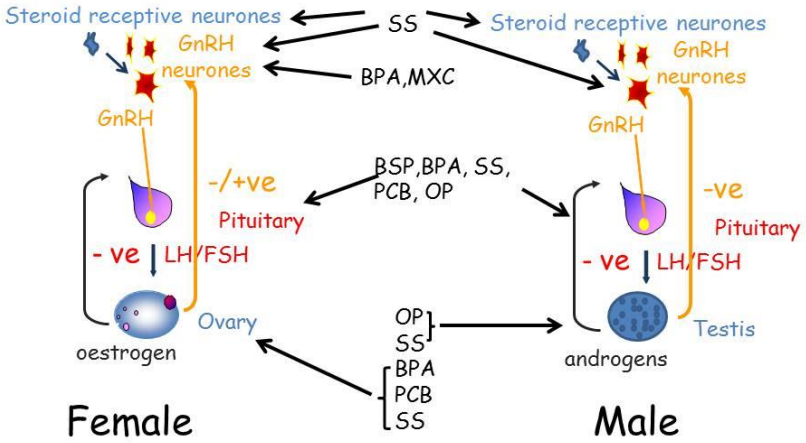


Figure 5.1. Impact of environmental disrupting chemicals on different components of the reproductive axis of female (left) and male (right) ruminants. The black arrows indicate published work that supports effects of EDCs on hypothalamic steroid receptive neurones, GnRH neurones, the pituitary gland, or the gonads. BPA (Bisphenol A), MXC (Methoxychlor), OP (Octylphenol), PCB (Polychlorinated biphenyls), SS (sewage sludge).

5.2.3. Effects of EDCs on ovarian and uterine function

Perturbations induced by EDCs during germ cell formation may cause a permanent reduction in reproductive function in the adult. Importantly, these changes in the germ line could be passed to later generations as has been documented in rodents (Skinner *et al.*, 2010). There are well documented actions of EDCs on the morphology and development of the sheep ovary

predominantly during fetal and early postnatal life. SS exposure alters ovarian dynamics in 110 day old sheep fetuses reducing oocyte density, advancing follicle development and increasing a pro-apoptotic protein (Bax) key to normal ovarian development (Fowler *et al.*, 2008). Similarly, exposure to PCB118 and 153 throughout gestation altered follicular dynamics in pre-pubertal animals at 60 days after birth. PCB 153 increased the number of primary follicles at this age, while PCB 118 increased the sum of secondary, early antral and antral follicles (Kraugerud *et al.*, 2012). While the long-term consequences of such enhanced development are unknown, these authors suggest that increased recruitment from the primordial pool of follicles could lead to a more rapid depletion of mature follicles and premature ovarian failure. Fetuses exposed to BPA from day 30 of gestation until necropsy at day 65 or 90 exhibit reduced ovarian steroidogenic gene and micro RNA expression for a number of genes central to successful gonadal differentiation and folliculogenesis (Veiga-Lopez *et al.*, 2013). These changes in gene expression may also partly underlie the altered reproductive endocrine function reported in prenatally BPA exposed ewes during their first breeding season (Savabieasfahani *et al.*, 2006). The onset of puberty was not impacted by prenatal BPA or MXC exposure in the latter study. In contrast when females were exposed to OP for varying periods from 70 days gestation to weaning at 4 months, the onset of puberty was advanced by 3 weeks with an associated increase in the duration of the first breeding season (Wright *et al.*, 2002). As well as EDC-induced changes in ovarian function, changes have also been noted at the uterocervical level. Specifically, BPA (but not OP) exposure of ovariectomised ewe lambs for 6 weeks from 4 weeks of age increased uterine weight (in part due to endometrial oedema), increased keratinisation of the cervical epithelium and altered the distribution of oestrogen receptors in the uterine endometrium. Further work will be necessary to determine if these pathological changes impact on the ability of ovary-intact ewes to carry healthy and viable lambs (Morrison *et al.*, 2003).

In cattle, a number of studies demonstrate negative impacts of organochorines, OPs and PCB on the developmental competence of the oocyte when added to the culture media during *in vitro* maturation procedures (Tiemann *et al.*, 1996; Alm *et al.*, 1998; Pocar *et al.*, 2001a; Pocar *et al.*, 2001b). However, there is no corresponding data following EDC exposure *in vivo* and this is clearly required if the relevance to beef and dairy fertility is to be ascertained. Indeed for both cattle and sheep there is a complete dearth of information on the programming effects of EDCs on crucial aspects of female offspring reproductive function including ovulation and fertilisation rates, embryo quality, pregnancy rates and lifetime litter size.

5.2.4. Effects of EDCs on testicular function

OPs have been shown to variously disrupt testicular development and function in sheep following both prenatal and early postnatal exposure. Maternal exposure of dams to OP from day 70 of gestation until birth resulted in decreased testicular weight and a reduction in Sertoli cell number at birth (Sweeney *et al.*, 2000) but an equivalent exposure did not impact semen quantity, quality or IVM/IVF characteristics at 12 months of age (Sweeney *et al.*, 2007). In contrast when males were exposed to OP from birth to weaning (but not from day 70 of gestation to weaning) the number of morphologically abnormal sperm was increased by 12% (Sweeney *et al.*, 2007). The impact of this small increase in the proportion of abnormal sperm on ram fertility in field conditions has not been assessed. Pre- and early postnatal exposure to SS has also been shown to disrupt testicular function. In fetal life (day 110), long-term maternal SS exposure resulted in a major attenuation of fetal testicular development associated with reduced testicular hormone secretion (Paul *et al.*, 2005). When exposure was continued until weaning at 4 months and tissues

subsequently collected at 19 months of age, about half of the exposed rams had testes that appeared to be morphologically normal, while the other half had major reductions in germ cell number and a greater number of Sertoli cell- only tubules (Bellingham *et al.*, 2012). This observation is of particular interest because it indicates that specific individuals may be more susceptible to EDC disruption of the reproductive axis than others and reinforces that population data may underestimate the problems associated with EDC exposure. Moreover it suggests that identifying at risk or affected individuals on farm could be a challenge.

5.3. Evidence of epigenetic involvement

Our understanding of the molecular mechanisms that putatively underlie the prenatal programming of domestic livestock fertility is in its infancy. Epigenetic marks are candidates for bearing the memory of early life exposure and both primordial germ cells (the direct progenitor of sperm or oocytes) and the pre-implantation embryo are subjected to intense epigenetic modifications (Seisenberger *et al.*, 2013). As discussed earlier (Section 2), DNA methylation in germ cells is erased and new DNA methylation is acquired during multiplication of spermatogonia in the male (fetal event) while in females it primarily takes place during follicle/oocyte growth and maturation (after puberty and prior to each ovulation). Thus the key times when offspring are potentially susceptible to epigenetic modification is likely to be gender specific with males theoretically being more sensitive than females during fetal life (Dupont *et al.*, 2012).

Variations in the maternal, placental and fetal hormonal milieu during intrauterine development are postulated as epigenetic signals (Fowden *et al.*, 2010). As the glucocorticoids and reproductive steroids are highly sensitive to maternal nutrition (Fowden *et al.*, 2010; Wallace *et al.*, 2011) and EDCs have major oestrogenic properties (Fowler *et al.*, 2012) they are clearly important potential candidates in this respect. However to date there is no direct link between these putative environmental cues and epigenetic modifications that relate to a fertility phenotype in farm species. This is primarily because these mechanisms have not been investigated to any great extent in farm animals. In contrast there is compelling evidence from rodent studies that at least some of the effects of environmental chemicals such as DES (Alworth *et al.*, 2002) and the isoflavonoid phytoestrogen Genstein (Tang *et al.*, 2008) are mediated via changes to DNA methylation. (Anway *et al.*, 2005) went further to demonstrate male-germline mediated epigenetic transgenerational effects of the antiandrogenic compound vinclozolin (an agricultural fungicide) and the oestrogenic compound methoxchlor on gonadal sex determination, which persisted to the F3 generation in the rat.

5.4. Summary and interim conclusions

There is reasonable evidence to support a role for early life nutrition in the programming of specific aspects of female offspring fertility. In sheep the most robust effects to date relate to the negative impact of low fetal nutrient supply on the number of ovarian follicles. However even this may not limit litter size until relatively late in a female's breeding life or until she experiences a second environmental challenge such as poor nutrition or repeated superovulation in adult life. A similarly robust impact of poor prenatal nutrition on the ovarian follicle reserve in beef cattle is also emerging and in both species low birth weight may be a useful proxy marker of the individuals most likely to be perturbed. In contrast, in male offspring the negative impact of poor prenatal nutrient supply is likely to be confined to the first breeding season and of little consequence to

fertility thereafter. To date the most worrying implication of EDC research relates to the higher incidence of spermatogenic abnormalities in male offspring. Further, while early life exposure to EDCs clearly impact the reproductive axis at the brain, pituitary and ovarian levels, the impact on female offspring fertility and fecundity has simply not been examined in the relevant farm species.

6. Impact of advanced (assisted) reproductive technologies (ART)

Artificial insemination (AI) was developed for livestock during the late 1930s and, since the advent of semen cryopreservation pioneered during the 1940s, has completely revolutionised global cattle breeding. Around 20% of the estimated 550 million breeding cattle and buffalo that populate the planet are AI (Thibier and Wagner, 2002). Uptake in sheep breeding is limited by comparison. In part this is attributable to the more extensive systems of husbandry associated with this species, but uptake has also been constrained by biological factors associated with cervical insemination that require the use of more invasive laparoscopic techniques (Anel *et al.*, 2006). In the current article AI with conventional non sex-sorted semen is not considered to be an 'advanced' reproductive technology. That accolade is reserved for the use of sex-sorted semen, and particularly *in vivo* derived (IVD) and *in vitro* produced (IVP) embryos. Compared to AI, the transfer of IVD and IVP embryos is largely restricted in cattle to breeding within elite/nucleus herds. Previous large-scale studies that sought to increase calf and/or carcass output in commercial beef cattle through the induction of single and/or twin pregnancies by embryo transfer, whilst successful, proved technically challenging and only marginally beneficial compared to AI (Sinclair *et al.*, 1995a; Sinclair *et al.*, 1995b). Such experiences subsequently limited industry uptake to that directly associated with genetic improvement. Nevertheless, recent estimates place the number of embryos transferred globally at just under 1 million (IETS, 2012). The number of IVP embryos transferred has quadrupled over the last decade and now constitutes 40% of total embryos transferred in cattle. One driver for the current surge in interest in IVP embryos is the prospect of genomic selection of Day 7 embryos (Lauri *et al.*, 2013). This can significantly reduce the breeding interval (and so increase response to selection) but can also reduce wastage and associated costs that arise from the production of unwanted calves when sex-sorted semen is used during in vitro fertilisation (IVF).

The first calf (named Virgil) born following the transfer of IVP embryos was reported by Benjamin G Brackett and colleagues at the University of Pennsylvania in 1982 (Brackett *et al.*, 1982). Further developments and refinements to bovine IVP arose during the 1980s leading to the global commercialisation of this technology by the early 1990s. However, the first reports of developmental anomalies leading to the birth of large calves and associated obstetrical complications following the transfer of IVP embryos arose soon thereafter. These observations were later reported in sheep, and collectively became known as the 'Large Offspring Syndrome' (LOS). It is beyond the scope of the current article to review this topic in detail. This has been done elsewhere (e.g. Young *et al.*, 1998; Sinclair *et al.*, 2000; van Wagtendonk-de Leeuw *et al.*, 2000; Farin *et al.*, 2006). However, the study of *in utero* and post-natal development following the transfer of IVP and cloned embryos (which undergo a period of *in vitro* culture) has served to highlight the sensitivity of the periconceptional period, and the earliest stages of mammalian development, to environmental influences. Given that from fertilisation to hatching (at around Day 7/Day 8 of gestation) ruminant embryos contain a large population of pluripotent cells, it follows that many of **t**he altered phenotypes described in offspring (discussed next) may have arisen, at least in part, as a consequence of epigenetic alterations to DNA and associated proteins in a number of developmentally important genes (discussed later).

6.1. Long-term post-natal consequences of ART

Differences in birth weight between calves/lambs conceived naturally or by AI, and calves/lambs derived from IVP embryos, normally disappear at around 6 to 12 months of age (Wilson *et al.*, 1992; Walker *et al.*, 1996), indicating that *in utero* overgrowth is transient and doesn't persist post-natally; although in the study of (McEvoy *et al.*, 1998) oversized calves at birth derived from IVP embryos had abnormally large hearts when slaughtered at just over 1 year of age. Anecdotal evidence based on a limited number of observations from some of the early studies indicated that IVP derived offspring of the same genotype may be more muscular. Subsequent studies in both cattle and sheep confirmed that primary muscle fibre cross-sectional area was increased as was the ratio of secondary to primary muscle fibres in late gestation foetuses (Maxfield *et al.*, 1998; Crosier *et al.*, 2002). These effects in sheep were associated with a shift in the temporal expression of *Myf-5*, a member of the *MyoD* gene family responsible for inducing mesodermal precursor cells to differentiate into myoblasts and to proliferate, both under the influence of Sonic hedgehog and Wnt-1 (Maltin *et al.*, 2001). In contrast, there was no effect of embryo source on expression of *Myf-5*, *MyoD* or Myogenin (*MYOG*) in skeletal muscle of Day 222 bovine fetuses; instead there was a reduction in expression of mysotatin (*MSTN*) (Crosier *et al.*, 2002). Loss of function of this transforming growth and differentiation factor-beta (TGF-β) family member is known to lead to muscle hypertrophy in cattle and sheep (Rodgers and Garikipati, 2008), the origins of which occur pre-natally. These observations are remarkable because the initiating factors during IVP would have had to act on the population of pluripotent cells that constitute the pre-implantation embryo; reinforcing earlier statements in this article that the earliest stages of mammalian development are particularly sensitive to environmental perturbation.

Few studies have formally evaluated carcass and muscle characteristics of offspring conceived by IVP, and none of these were designed specifically to address the issue of whether or not the IVP process itself altered these traits. Patterson et al. (1993) assessed carcass characteristics of single and twin beef calves derived from IVP embryos. They concluded that, following ET, viable twins have similar beef producing potential to single-born calves. Similarly, (Amen *et al.*, 2007) used IVP and ET to produce reciprocal crosses of *Bos indicus* and *Bos taurus* cattle in order to assess carcass and meat traits, but no assessment of independent effects of ART could be made. The study of (Sinclair *et al.*, 1995a) is often cited (wrongly) as providing evidence that the process of IVP itself leads to increased carcass weights with greater yields of saleable meat. Semen from a single beef sire was used to inseminate Hereford x Friesian cows (to produce three-quarters beef cross offspring) and to fertilise oocytes derived from three-quarters crossbred beef heifers (predominantly Charolais and Simmental x Hereford x Friesian) in order to produce seven-eighths beef cross calves. The objective was to demonstrate that, in so doing, IVP-ET could be used to produce better quality calves. Carcass and saleable meat yields were greater for IVP-ET derived offspring than for AI Controls, but this was almost certainly due to the selection/breeding process and not the IVP *per se*.

Similarly, until very recently no studies have assessed long-term effects of IVP-ET on subsequent offspring fertility and milk yield. However, a large study (comprising 426 ET recipients) in Florida, involving the transfer of fresh or frozen-thawed and sexed IVP embryos vs AI in Holstein cows, found no effects of ART on pregnancy rates to first service or daily milk yields during first lactation in resultant female offspring (Bonilla *et al.*, 2014). Consequently, it is unlikely that these traits would be affected in beef cattle.

6.2. Long-term effects of reproductive cloning

Pre-natal losses, obstetrical complications and post-natal morbidity associated with reproductive cloning generally fall under the heading of LOS, have been reviewed elsewhere (e.g. Young *et al.*, 1998; Chavatte-Palmer *et al.*, 2000) and won't be considered further except to state that the incidence and severity of pregnancy losses, obstetrical complications and neonatal morbidity are usually greater for pregnancies generated from embryonic-cell nuclear transfer embryos (Garry *et al.*, 1996), but particularly from somatic-cell nuclear-transfer (SCNT) embryos (Hill *et al.*, 1999; Chavatte-Palmer *et al.*, 2004), than with either IVP of IVD embryos. Attention instead is focussed on the health and productivity of cloned offspring. Here again this topic has been extensively reviewed elsewhere driven largely by statutory requirements of various government agencies (e.g. US Federal Drug Agency, European Food Standards Authority) to ensure that cloning does not compromise animal welfare and that food products from cloned animals are safe for human consumption. The available evidence indicates that SCNT-cloned offspring that survive to puberty are generally healthy and that the composition and nutritive value of milk and meat products from cloned (non-transgenic) livestock does not differ from that of animals conceived naturally (Norman and Walsh, 2004; Takahashi and Yoshihiko, 2004; Tome *et al.*, 2004, Heyman *et al.*, 2007; Laible *et al.*, 2007; Rudenko and Matheson, 2007; Rudenko *et al.*, 2007; Watanabe and Nagai, 2008). There does appear, however, to be subtle differences in muscle fibre contractile types (i.e. more slow-twitch oxidative relative to fast-twitch glycolytic fibres) in young cloned heifers (at around 8 months of age) but again, as with general issues regarding animal health, these compositional differences in muscle subsequently disappear following the onset of puberty and are not evident in cattle > 12 months age (Jurie *et al.*, 2009).

6.3. Epigenetic programming of long-term development

It became apparent early on that many of the LOS features resembled that of naturally occurring overgrowth syndromes in humans (e.g. Beckwith-Wiedemann syndrome (BWS)) which are associated with errors in an imprinted cluster of genes on human chromosome 11 (Sinclair *et al.*, 2000). This led to the discovery that LOS in sheep was due, at least in part, to a loss of imprinting and expression of the gene encoding the type 2 insulin-like growth factor receptor (*IGF2R*) in a range of tissues, but particularly those emanating from the mesodermal lineage (Young *et al.*, 2001). This loss of imprinting arose as a consequence of loss of DNA methylation in the second-intron differentially methylated region (DMR2) of that gene. Loss of methylation at this DMR, and a conserved DMR located upstream of the ovine *H19* gene, was prevalent among SCNT-cloned lambs (Young *et al.*, 2003) and SCNT-cloned calves (Smith *et al.*, 2012), leading to biallelic expression of these imprinted genes.

Germ-line epigenetic marks are established in a parent-specific manner in a small subset of genes (current maximum best estimates in the mouse are between 300 to 400; (Kelsey and Bartolomei, 2012) that facilitate tissue and developmental stage-specific monoallelic expression of affected genes following fertilisation. These processes are best understood in the mouse (Cedar and Bergman, 2009; Tomizawa *et al.*, 2012) with only limited data available in ruminants (Thurston *et al.*, 2008). The dynamics of imprint establishment during gametogenesis and early embryogenesis (coincident with procedures used in ART) are such that, depending on the precise timing and nature of the procedural insult, it is highly probable that different combinations of imprinted genes may be affected to a greater or lesser extent. Thus, Bebbere *et al.* (2013) failed to detect

differences in allelic expression ratios and *IGF2R*-DMR2 methylation in the few (n = 4) overgrown IVP fetuses studied compared to normal weight IVP and in vivo conceived fetuses at Day of 80 of gestation in the cow. Instead, (Chen Cárdenas *et al.*, 2013) found a loss of imprinting leading to biallelic expression of *KCNQ1OT1* (the gene most misregulated in BWS) in bovine LOS fetuses derived from IVP embryos, and that this is associated with a loss of methylation at the KvDMR1 on the maternal allele. This observation confirmed an earlier report of abnormal hypomethylation of KvDMR1 and expression of *KCNQ1OT1* in 2/7 SCNT-cloned calves and 1/2 IVP-derived calves (Hori *et al.*, 2010). Although generally highly conserved there are also recognisable differences in imprinting within eutherian mammals that could account for many such differences between species; differential imprinting at the *IGF2R* locus being a case in point (Das *et al.*, 2009; Renfree *et al.*, 2013).

The emerging picture is further complicated by the fact that many of these imprinted genes are polymorphic. In taurine cattle, for example, single-nucleotide polymorphisms (SNPs) are known to exist in at least seven imprinted genes (including *IGF2R*) and to be associated with a number of commercially important traits including those associated with fertility (e.g. gestation length, calving difficulty, perinatal mortality), milk yield (e.g. protein percentage, somatic cell counts) and growth (e.g. carcass weight, conformation, rump depth) (Magee *et al.*, 2010; Berkowicz *et al.*, 2011). There is also some evidence of allelic switching of imprinted *IGF2R* in SCNT-cloned bovine fetuses where the paternal allele is imprinted in one tissue whilst the maternal allele is imprinted in another tissue (Suteevun-Phermthai *et al.*, 2009). These potentially confounding factors could account for at least some of the discrepancies and apparent stochastic effects observed in aberrant genomic imprinting patterns between studies that frequently report effects in only small numbers of animals.

Finally, whilst the focus of most research has understandably been directed towards errors in genomic printing following ART in both animals and humans, it is highly likely that the epigenetic status, and possibly expression, of many more non-imprinted genes are also affected (Grace and Sinclair, 2009). Surprisingly, this hypothesis has never fully been tested. Some groups (e.g. (Santos *et al.*, 2010) have used immunoflorescent techniques to visually quantify global 5-methyl-cytidine staining to assess effects of ART procedures on DNA methylation in embryos, but this method lacks sensitivity, cannot identify locus-specific changes in methylation and possesses other methodological limitations (Li and O'Neill, 2012). However, in a microarray analysis of 1,536 CpG sites in just over 700 genes, (Katari *et al.*, 2009) found that imprinted loci were no more or less likely to be differentially methylated than non-imprinted loci. DNA in that study was extracted from human cord blood and placentae from term pregnancies established naturally or following IVF. More recent data in humans and mice, where genome-wide DNA methylation was assessed by array of immunoprecipitated DNA (MeDIP-array), confirm epigenetic differences in promoter methylation of non-imprinted genes between offspring conceived naturally or by IVF (Li *et al.*, 2011; Oliver *et al.*, 2012). These studies are somewhat preliminary in that they were limited by scale and/or tissues sampled (e.g. peripheral blood in the human study). The platforms used are also somewhat insensitive compared to contemporary deep-sequencing approaches that can provide single-base pair resolution.

6.4. Interim conclusions

Sweeping changes to DNA methylation and chromatin remodelling take place in gametes and in the preimplantation embryo during the normal course of development, rendering these cells

particularly vulnerable to environmentally induced epigenetic modifications to DNA as can occur during ART, leading to problems such as LOS. It is very likely that a broader but more subtle range of aberrant phenotypes, than have been described to date, manifest following the use of these technologies, but that under normal conditions of commercial livestock production these go unnoticed. The major adverse phenotypes include pregnancy failure following ET, and an increased but variable incidence of obstetrical complications during parturition. The latter effects occur less frequently and more sporadically these days for reasons that are not fully understood. There is scant information on the longer-term effects of ART on farmed livestock. The available evidence indicates that the vast majority of ART offspring that reach puberty are for the most part normal. However, in beef cattle at least, genomic imprinting significantly contributes to the genetic variance of a number of commercially important traits, with estimated proportions of between 8 and 25% of total additive genetic variance (Neugebauer *et al.*, 2010). It remains to be determined if procedures used in ART might affect these traits to any great extent.

7. Industry Relevance and Recommendations

The previous sections of this report provide a detailed contemporary overview of our understanding of factors that affect early development and the consequences that this can have for life-long health, wellbeing, productivity (including growth, body composition and carcass composition) as well as fertility. The aforementioned discussion considered evidence from other farm animal species, humans and model organisms such as rodents, where the burden of evidence resides for many traits. The current section draws conclusions on those effects most likely to be of relevance to beef cattle and sheep. In so doing it is important to acknowledge a number of caveats:

1) There are a number of traits that have not been considered fully in the target species and aspects of normal agricultural practice that could have an impact on prenatal development but have never been assessed. In particular those relating to animal management and potential for psychological stress, such as aspects of housing, social stress from interactions with conspecifics (shown to be very influential in pigs for example but rarely assessed in sheep and never in beef cattle), and the impact of weaning of the present calf on the subsequent offspring in the pregnant beef cow.

2) Studies are almost invariably carried out under controlled experimental conditions and some of the treatments may only have a minor bearing on beef cattle and sheep production and health.

3) Many of the aforementioned studies have focussed on developing our mechanistic understanding of the underlying biology (including epigenetics) in farm animals rather than clearly establishing commercially relevant adult phenotypes. This reflects the source and nature of funding over the past two decades, which has largely come from research councils (i.e. BBSRC, MRC), charities (e.g. BHF and Cancer Research UK), and from overseas (e.g. EU, NIH), and which have a clear biomedical slant.

4) In experimental studies frequently only single factors were assessed, with other influences being tightly controlled. In reality animals will be exposed to multiple, concurrent stressors (e.g. undernutrition, high stocking density and thermal challenge) which can have a greater impact than when applied individually.

With these caveats in mind the following traits in offspring which are of commercial importance for English beef and sheep producers and which can be influenced by fetal development during include:

- Dystocia and neonatal survival
- Growth rate and feed conversion
- Offspring health and disease susceptibility
- Saleable meat yield and meat quality
- Behavioural traits associated with ease of handling
- Reproductive potential including fertility and litter size

7.1. Maternal nutrition and body-condition score (BCS)

Of the risk factors impacting on commercially relevant traits, the vast majority of studies have focussed on nutritional impacts, elicited through experimental manipulations of total feed, protein level or dietary fat, via pasture management (e.g. sward height) or through assessing or manipulating BCS. There is more evidence available for sheep than cattle, but the evidence that exists for both species is summarised below.

7.1.1. Sheep

*7.1.1.1. Lamb birth weight and survival (**Section 3.2.1.**):* Maternal undernutrition during late pregnancy (after day 100) is reliably reported to reduce lamb birth weight, whereas the effects of undernutrition during early gestation are more variable, with reports of both decreases (although genotype specific; Rooke *et al.*, 2010) and increases (Holst *et al.*, 1986) in birth weight. Other studies suggest that maternal BCS at conception may be an important determinant of birth weight (Wallace *et al.*, 2010; Wallace *et al.*, 2011) although nutritional intake during late gestation seems to be more important than BCS (Thompson *et al.*, 2011). Some of these apparent discrepancies between studies may arise as a consequence of differences in placental development, as almost two-thirds of the variation in birth weight is attributable to placental mass (Robinson *et al.*, 2000). In this comprehensive review of nutritional effects on fetal growth, these authors explained how during the critical period of placental growth (i.e. days 35 through to 80) the degree of ewe maturity, BCS and plane of nutrition interact to affect pregnancy outcome and birth weight. Thus, for mature ewes of good, but not poor, BCS at mating, modest undernutrition from Days 30 to 90 of gestation can enhance placental growth. In contrast over-nutrition, particularly of young ewes, can reduce both placental and fetal growth leading to reduced birth weights (Wallace *et al.*, 2010; Wallace *et al.*, 2011). As lamb birth weight is an important risk factor for neonatal mortality it is not surprising that lamb deaths are influenced by pre-natal nutrition. Some studies also report increased neonatal mortality, in the absence of differences in birth weight, when ewes are severely undernourished around conception or during early to mid-pregnancy (Vincent *et al.*, 1985; Heasman *et al.*, 2000; Rooke *et al.*, 2010). Similarly nutritional supplementation during mid-gestation can increase lamb survival to weaning (Kleemann *et al.*, 1993; Encinias *et al.*, 2004; Mulvaney *et al.*, 2008).

Take-home messages: For mature ewes producers should strive to attain optimal BCS (i.e. a score of 3.5 using the recognised EBLEX standard) and allow a modest (i.e. 0.5 units) loss of condition up to the end of the 3rd month of pregnancy. Subsequently, dietary allowances for pregnant ewes should be tailored to match that of fetal number (i.e. litter size). Young growing pregnant ewes should be fed to allow a modest rate of growth of around 80g/day throughout gestation. There is compelling evidence that for housed ewes shearing during the 3rd month of

pregnancy can increase birth weight of lambs. Evidence of longer-term benefits, in terms of lamb growth and finishing weights, is limited.

Recommendations for research: Current EBLEX funded studies developing KPIs for commercial flocks will generate important data linking BCS in ewes to pregnancy outcomes and neonatal viability. However, thought should be given as to how these studies could record birth weights. Scope exists on monitor farms to formally assess and quantify long-term benefits of shearing housed ewes during pregnancy.

*7.1.1.2. Carcass traits (**Sections 4.1. and 4.2.**)*: Mid pregnancy undernutrition has been reported to result in increased lamb growth and weight at 63 and 120 days postnatal, associated with a tendency for heavier carcass weights (Ford *et al.*, 2007). However, this seems to be achieved through increased kidney and pelvic fat and reduced muscle weights. Additionally, ewes fed high energy diets during mid to late gestation produce offspring with more carcass fat and a lower percentage lean tissue in comparison to ewes fed lower energy diets (Long *et al.*, 2010). Over-feeding of mothers during pregnancy also results in more fat in the carcasses of female lambs, but this can be reversed by short period of underfeeding (Rattanatray *et al.*, 2010). It appears that effects of maternal diet during pregnancy on muscle fibre number and muscle mass are transitory and can be modified subsequently.

Take-home messages: It appears that in all but the most extreme cases of maternal malnutrition during pregnancy, there is little evidence that muscle development can be manipulated *in utero* in a manner that would lead to permanent and measurable differences in carcass and saleable-meat yields in slaughtered offspring. Subtle alterations in muscle fibre type and in muscle mass can be induced at various stages during both pre- and post-natal development, so that systems of management should consider the life-course of the animal destined for slaughter. Effects on organoleptic properties of meat that arise as a direct consequence of *in utero* mediated modifications to muscle development are not known but are likely to be minimal. In contrast, although less is known about the developmental processes that lead to the formation of mature adipose tissues, it appears that these depots in offspring may be influenced by maternal diet during pregnancy to a much greater extent than is the case for muscle. At present, however, it is difficult to predict the long-term consequences of either restricted or excessive feeding during pregnancy on final carcass composition and meat quality.

Recommendations for research: If one could record birth weight of lambs on current EBLEX sponsored monitor farms, and information was available on ewe BCS during pregnancy, then it should be possible to generate a large body of data within just a couple of seasons on how this might affect whole-body composition and saleable meat yields. Such information would help guide future research endeavours to more directly investigate the effects of *in utero* development on post-natal growth, body composition, saleable meat yields and overall production efficiency.

*7.1.1.3. Appetite regulation (**Sections 4.3 and 1.5.1**):* In an era where reducing emissions of greenhouse gases (GHG) from ruminants are of paramount importance, and where there is a need to improve residual-feed intake (RFI) in order to reduce feed costs, the significance of appetite regulation cannot be overestimated. Yet, in the context of developmental programming, this is the least-well researched and understood trait in ruminant livestock. Most of what we know is limited to rodents. The hologenome concept in the context of developmental programming is a relatively

recent development but merits consideration both in the context of immunological protection afforded to neonates and putative long-term implications for improving RFI and reducing GHG.

Take-home messages: There is insufficient evidence to indicate whether or not appetite regulation can be programmed permanently *in utero*. Small lambs at birth will often eat more over the life course to attain slaughter weights comparable to their normal birth-weight contemporaries, but this apparently is only by virtue of their small size at birth.

Recommendations for research: The huge gap in our understanding of how basic mechanisms regulating appetite in both sheep and cattle can be programmed in utero, and the implications that the hologenome concept could have for reducing GHG and improving RFI, identify appetite regulation as a future research priority; but one led by research councils such as BBSRC in the first instance.

*7.1.1.4. Welfare traits (**Sections 3.2.1, 3.2.2, 3.2.3**):* Low birth weight reduces lamb neonatal behavioural development and sucking behaviours (Dwyer *et al.*, 2003), and supplemental fat in pregnancy increases lamb activity (Capper *et al.*, 2006; Pickard *et al.*, 2008) as does shearing in late pregnancy (Banchero *et al.*, 2010); all of which contribute to lamb survival (see section 7.1.1.1). Furthermore, undernutrition of the late pregnant ewe can reduce the expression of maternal behaviour (Dwyer *et al.*, 2003), increasing the incidence of rejection, affecting ewe-lamb bonding, colostral antibody transfer to the neonate, and susceptibility to infectious disease. Maternal fish oil supplementation, however, reduces colostrum yield, and fat concentration, and has variable impacts on proportions of different types of fats in colostrum (Capper *et al.*, 2006, Annett *et al.*, 2008). Similarly, colostrum yield and composition (IgG) is reduced in high intake ewes in comparison to ewes fed a maintenance diet (Wallace *et al.*, 2010). The overall impact of supplemental nutrition on welfare traits in lambs (survival) is therefore positive for ewe and lamb behaviour but may negatively influence the acquisition of passive immunity through its impact on colostral antibodies; although this remains to be confirmed. Intriguingly, early or late maternal undernutrition reportedly increases the ability of lambs to absorb colostral IgG (Hammer *et al.*, 2011), which may serve as an adaptation to the poor prenatal environment.

Specific nutrients and minerals in the diet have been associated with lamb adequate behavioural development at birth, particularly cobalt, iodine, selenium and vitamin E. For each, deficiency in pregnancy leads to adverse neonatal outcomes (reviewed by Rooke *et al.*, 2008). However, there is little evidence that supplementation above requirements has any beneficial effects, and in some cases may have a detrimental impact due to the known-toxicity of some of these nutrients.

There are some reports that maternal undernutrition, particularly if severe and early in gestation, causes an increase in stress reactivity (either assessed in terms of behavioural reactivity or physiological responses of the offspring), although other studies show opposite effects (see **Tables 3.2 and 3.3**). There is also some evidence for sexually dimorphic responses in sheep as has been frequently reported in the rodent literature. Reaching a definitive conclusion in this area is hampered by the relatively low number of studies, the different nutritional paradigms assessed and the lack of consistency in outcome measures used. In addition, in many of these experimental studies ewes are fed in a rather artificial way to achieve the undernutritrion treatment, which may compound the nutritional effects with psychological stress (e.g. feeding animals housed in individual pens) and does not mimic normal farm practice.

Only two papers (Erhard and Rhind, 2004; Gutleb *et al.*, 2011) have considered whether maternal grazing of sewage sludge treated pastures impacts on the behaviour of offspring. The evidence suggests that a dysmaculinisation of male lamb behaviour may occur, which may reflect in altered development of reproductive organs as described in section 5.1.1.

Take-home messages: Maternal nutrition affects the expression of ewe and lamb behaviours at parturition, with young and inexperienced ewes being particularly vulnerable to behavioural disturbance. Feeding regimes as described in 7.1.1.1, therefore, will also contribute to improved expression of ewe and lamb behaviours at birth. In areas where there are deficiencies in pasture cobalt, iodine, selenium or vitamin E, supplementation will be beneficial for improving lamb behaviours and colostral uptake. However, supplementation of an adequate maternal diet is not recommended as at best this may have no benefit, and at worse can lead to detrimental effects. At present no convincing picture is emerging of a detrimental impact of nutrition on subsequent behavioural reactivity, although several studies have reported alterations in responsiveness. Whether this is associated with poorer welfare, or increased difficulty in handling, has not been clearly demonstrated.

Recommendations for research: Evidence for a link between maternal undernutrition and behavioural reactivity likely to impact on welfare or ease of handling might be obtained by incorporating measures of responses to standard husbandry activities (weighing, restraint for vaccination, yarding) to those studies proposed above for monitor farms (see sections 7.1.1.1 and 7.1.1.2). This could yield a comprehensive picture of the impact of maternal nutrition on offspring outcomes that could, for example, be used in whole-farm modelling to determine optimal nutritional strategies. Studies to determine practical methods to boost colostral antibody transfer between ewes and lambs, and characterise the health outcomes, would also be beneficial.

*7.1.1.5. Offspring fertility (**Section 5.1.1.**)*: There is limited evidence for lasting effects of undernutrition during pregnancy on fertility of offspring leading to poorer pregnancy rates and/or reduced litter sizes. Effects reported (e.g. Rae *et al.*, 2002a, Long *et al.*, 2010) are modest and would be difficult to detect under commercial conditions without the use of powerful statistical techniques which are beyond the scope of most producers. Two studies suggest a decrease in litter size and lifetime lamb production in ewes undernourished as fetuses or as lambs (Langlands *et al.*, 1984; Gunn *et al.*, 1995). Undernutrition was achieved in these studies either by restricting herbage allowance, through increases in stocking density, or by preventing access of pregnant ewes and/or lambs to supplementary feed. However, these adverse effects on offspring fertility were only expressed when suboptimal nutritional conditions persisted into adulthood. There is also some evidence for reduced lifetime productivity and litter size in ewe lambs of low birth weight, and in ewe lambs experiencing poor growth rates during early life (Rhind and McNeilly, 1998; Gardner *et al.*, 2009), although high birth weight lambs are also reported to have smaller litters (Gardner *et al.*, 2009).

Sewage sludge (a treated by-product of human domestic, agricultural and industrial waste water) contains a complex mix of organic and inorganic pollutants, which is deemed non-hazardous when applied to agricultural land, as it results in low to modest increases in environmental chemicals within soil (Rhind *et al.*, 2002; Rhind *et al.*, 2010). However, it has been demonstrated that pregnant ewes grazing treated pastures give rise to offspring with subtle defects in development of their reproductive organs (Fowler *et al.*, 2012). Male offspring appear to be most affected (Bellingham *et al.*, 2012), but as this species has only ever been used as a 'model system' to gain insights into the likely consequences of exposure to environmental chemicals in humans, so long-term consequences on offspring fertility have never fully been explored.

Take-home messages: Although difficult to fully quantify the benefits for offspring fertility *per se*, nutritional strategies during pregnancy designed to optimise birth weight and neonatal viability (Section 7.1.2.1) are likely to have positive knock-on effects for subsequent offspring fertility. There is compelling evidence that exposure to environmental chemicals (as can occur on sewage-sludge treated pastures) during *in utero* development can affect components of the reproductive axis. Male fetuses are more sensitive than female fetuses, with defects in testis development and sperm production being detected in ram lambs at 20 months of age. Consequences for subsequent ram fertility remain to be ascertained.

Recommendations for research: Extending currently funded KPI studies to include flocks that breed their own replacements would allow a full and quantitative assessment of pregnancy nutrition on offspring fertility and reproductive rate. If such studies were allowed to run for several years on selected monitor farms, representing different breeds, then robust quantitative assessments could be made over successive parities in offspring under commercial conditions. As around 1.6 million tonnes of sewage sludge (i.e. 73% of that produced) is dispensed on agricultural land in England and Wales there is an urgent need to formally assess its effects on fertility in grazing ruminants. The fertility of rams born and reared on such pastures is most at risk. Effects in cattle are likely to be similar but have not been investigated.

7.1.2. Beef cattle

*7.1.2.1. Calf birth weight and survival (**Section 3.2.1**):* Calf birth weight has been shown to be either decreased or unaffected by maternal undernutrition (e.g. Long *et al.*, 2010; Micke *et al.*, 2010) or fat supplementation in late gestation (Petit and Berthiaume, 2006); and likewise calf mortality is reported to be increased with undernutrition (e.g. Corah *et al.*, 1975) or unaffected (% calves weaned: Freetly and Cundiff, 1998). The variability in these studies may be related to differences in the timing of undernutrition, and/or the severity of nutritional restriction. This has not been explored in detail in cattle for birth weight, although there is some evidence for an effect on calf growth (e.g. low nutrition in early gestation reportedly does not affect calf growth rate or growth rate (Freetly and Cundiff, 1998; Long *et al.*, 2010)). In contrast, late gestation protein supplementation increases calf weaning weight and weight at breeding or at first pregnancy (Martin *et al.*, 2007; Larson *et al.*, 2009). There are no reported effects of protein supplementation or restriction in late gestation on the incidence of calving difficulty or abnormal presentations (Carstens *et al.*, 1987; Funston and Deutscher, 2004; Martin *et al.*, 2007), which are the major risk factors for neonatal mortality in cattle.

Take home messages*:* The impacts of nutrition in pregnancy on calf growth and productivity are highly variable. There is good evidence that undernutrition in late pregnancy impacts on calf birth weight, survival, growth rate and carcass conformation, but evidence for early or mid-pregnancy impacts are extremely scarce. This may be due to a genuine lack of impact, but could equally be related to the dearth of research in this area.

Recommendations for research: In comparison to sheep, there is a scarcity of data on beef cattle responses to pregnancy nutrition. There is a need for a more comprehensive assessment of the impacts of early and mid-gestation nutritional treatments on calf birth weight and survival. The variability between different studies currently makes it hard to reach firm conclusions on the potential impacts for producers without further study.

7.1.2.2. Carcass traits: Few studies report impacts of prenatal nutritional treatments on carcass traits except for evidence of larger individual muscle fibres in underfed steers (Long *et al.*, 2010), and a tendency for greater fat in the carcass, greater marbling scores and better grading scores from animals protein supplemented in late gestation (Larson *et al.*, 2009). A greater muscle area has also been reported in offspring resulting from pregnancies where cows were offered high dietary intakes in mid gestation (Micke *et al.*, 2010b).

7.1.2.3 Appetite regulation ***(Section 4.3.3)***: The very few studies in cattle that have addressed appetite or feed intake suggest there are no impacts of birth weight or early growth rate on appetite.

*7.1.2.4. Welfare traits (**Section 3.2.1**):* There are almost no studies of the impact of prenatal nutrition on subsequent calf behaviour but some evidence exists for negative impacts of undernutrition on calf serum immunoglobulin-G (IgG) at 48 hours old (Houghton *et al.*, 1990; McGee *et al.*, 2006), and positive impacts of dam crude protein intake and IgG at 36 hours old (Blecha *et al.*, 1981), which may have been partially mediated by calf-sucking behaviour. Epidemiological studies in Australia have also shown some evidence of maternal feed restriction caused by drought on progeny health: drought experienced five months before birth was associated with an increased incidence of congenital chondrodystrophy of unknown origin (White *et al.*, 2010b).

Take home messages: The impacts of nutrition in pregnancy on welfare traits in beef cattle are virtually unknown as almost no studies have considered suitable outcome measures. The evidence that does exist supports positive benefits for calves of good maternal gestational nutrition in promoting calf health after birth. The existence of relationships between maternal pregnancy nutrition and welfare traits in other animals, and the potential exposure of calves to nutritional restriction in utero (see below) does suggest that calf responses will be affected by prenatal undernutrition.

Recommendations for research*:* For most beef producers a planned reduction in cow body condition over the winter prior to spring calving is the norm. The impact of this reduction during an important period of fetal calf development is uncertain. A more pressing issue is the relatively high prevalence of very low body condition, as revealed in recent on-farm research (Defra project AW0509: 12% of all observed cows were observed to be Very Lean; 32.4% of observed farms have ≥10% of lean cows). Further research into the relationship between body condition and offspring outcomes in beef production systems would help clarify the extent to which poor dam body condition, or loss of maternal body condition over winter, contributes to offspring health, growth and profitability. A more specific issue relating to feeding is temporary feed restriction after weaning. A recent survey of UK beef farmers (Defra project AW0509) found that 20% of farms fed cows on a restricted diet for more than two days after weaning). Such an acute feed restriction may have impacts on the nutritional supply received by the fetus, although it is unknown how such sudden yet short-term dietary restrictions affects fetal development.

7.1.2.5 *Offspring fertility (**Section 5.1.2**):* Data on the impacts of nutrition on cattle fertility is patchy and incomplete but suggest that pregnancy rate in beef and dairy cows may be impaired by slow

early growth rates (Wilkins *et al.*, 2006; Brickell *et al.*, 2009). Under nutrition during pregnancy may reduce the ovarian reserve of eggs (there is a tentative association with fertility) and advance the onset of reproductive senescence, although this will be of less relevance to commercial producers.

Take home messages: Unfortunately current knowledge of the impacts of prenatal nutrition on any aspect of subsequent calf outcomes is incomplete. The available data suggest that calves that grow slowly in early life are likely to have reduced productivity in terms of the number of calves they themselves produce. However, whether this is related to epigenetic factors, or early management, is not clear.

Recommendations for research: There is a need for research in this area to more completely understand the impact of current feed management strategies on future fertility, and whether altered nutrition in pregnancy could have relevant production outcomes.

7.2. Behaviour and stress

7.2.1. Sheep

*7.2.1.1. Lamb birth weight and survival (**Section 3.2.1**):* From the few studies that have considered whether maternal gestational stress influences lamb birth weight or subsequent growth, the emerging picture suggests that psychological stress (i.e. isolation, transport, exposure to dogs, aversive handling) experienced during late gestation increases lamb birth weight and/or weaning weight. However, studies that attempt to mimic maternal stress through the administration of glucocorticoids in late pregnancy have the opposite effect and reduce lamb birth weight (Moss *et al.*, 2001; Miller *et al.*, 2009). Furthermore, our very recent research has shown that mid-pregnancy stress, induced by aversive handling, in goats results in reduced placental transport capacity and increased fetal mortality (Baxter, Hall, Zanella and Dwyer, unpublished).

Take-home messages: The possibility to increase lamb birth weight through the use of maternal stress in pregnancy needs to be approached with caution, particularly as the mechanisms underpinning this effect are unknown. Furthermore, the potential to reduce litter size through fetal absorption or abortion from gestational stress, as has been shown in rodents, is a significant risk. Thus advice to farmers to reduce stress in pregnancy remains most appropriate at the present time.

Recommendations for research: Our knowledge of the impact of relevant psychological stressors on fetal growth, development and survival is extremely sparse. However, the rodent, primate and human literature demonstrates that these factors can have a very significant and detrimental impact on offspring outcomes. There is a need for research that considers the impact of relevant stressors (such as factors relating to housing, stocking density, handling etc.) on production outcomes.

7.2.1.2. Carcass traits and *7.2.1.3. Appetite regulation:* No research exists that attempts to link carcass traits or appetite regulation with exposure to prenatal stress in sheep. Studies in the rodent and human literature suggest that early life exposure to stress can alter appetite and anhedonic responses (i.e. reduced seeking of pleasurable stimuli). Additionally, in the human literature it has been proposed that maternal stress in pregnancy affects offspring body composition (Entringer *et*

al., 2012) and in rodents prenatal or early postnatal stress has been shown to increase offspring adiposity (Purcell *et al.*, 2011; Haley *et al.*, 2013). However, whether this is also true of farm animals is completely unknown.

Take-home messages: At present it is not possible to provide guidance on whether prenatal stress will impact on body composition or appetite regulation in sheep. That these effects have been reported in rodents and humans suggest this is a possibility but no data in farm animals exist.

Recommendations for research: Our knowledge in this area is almost non-existent and, as with section 7.2.1.1, a programme of work, particularly focussing on production-relevant stressors and outcomes measures in sheep and cattle, is required.

*7.2.1.4. Welfare traits (**Sections 3.2.2** and **3.2.3)***: As above reaching a definitive conclusion on the impact of pregnancy stress on welfare outcomes is hampered by the low number of studies and variation in stress paradigms and outcome measures. In general, most studies report altered behavioural responsiveness in the offspring of ewes subjected to some form of psychological stress in late pregnancy (e.g. isolation, transport, exposure to dogs, aversive handling). Whereas some studies might be suggestive of increased reactivity to normal handling as part of routine husbandry (e.g. Erhard *et al.*, 2004), others just report effects of prenatal stress which may have no practical or welfare impact on the animal. However, lambs born to ewes that were cold-stressed (i.e. ewes shorn and exposed to 6 ºC) during the last 2 weeks of pregnancy were better able to mount a metabolic response to cold challenge after birth than those born to control ewes or those exposed to higher temperatures *in utero* (26 ºC; Slee and Stott, 1986). This suggests that there may be some beneficial impacts to offspring of some forms of maternal stress. Finally, a single study has investigated the impact of maternal disease in pregnancy (modelled using endotoxin challenge; Fisher *et al.*, 2010). When lambs were tested at 5 or 18 months, female offspring of challenged ewes had a reduced febrile response to the endotoxin, although the longer term health impacts of maternal infection are not clear.

Take-home messages: The available data suggest that stress to the pregnant mother can result in altered behaviour in offspring, and some studies suggest increased stress reactivity. Whether these changes are negative, neutral or positive for welfare and/or practical sheep handling have not been determined. At present, the best advice would be to reduce maternal exposure to stress and disease in pregnancy, as this does result in altered offspring responses which could potentially be detrimental for health and welfare. Research is currently underway to investigate the impact of housing management on offspring development, which may help form more definitive conclusions.

Recommendations for research: As with other sections in 7.2.1, our knowledge of the impacts of maternal stress or disease on offspring is inadequate, in particular with regard to husbandry-relevant stressors and outcome measures. However, increased intensification of production, in some areas, means that the impacts of housing, stocking density, human handling and social stress on ewes and their offspring is urgently needed to develop suitable housing environments for sheep. The impact of maternal health status on subsequent lamb disease susceptibility is also required, although this might be best directed to the research councils (e.g. BBSRC) in the first instance.

7.2.1.5. Offspring fertility ***(Section 5)****:* No studies have investigated the impact of psychological stress on fertility in sheep. In the rodent literature there is some evidence of impaired fertility (e.g. reduced testicular weight and apoptosis) in male offspring whose mothers were subjected to restraint in late pregnancy (Chen Cárdenas *et al.*, 2013), and in female offspring whose mothers were given exogenous glucocorticoids in late pregnancy (Piffer and Pereira, 2004). Thus, it remains a possibility that stress can reduce male and female fertility in sheep too.

Take-home messages: At present it is not possible to provide any guidance on whether prenatal stress will impact on fertility in sheep. That these effects have been reported in rodents suggest this is a possibility but no data in farm animals exist.

Recommendations for research: Our knowledge in this area is almost non-existent and, as with other sections in 7.2.1, a programme of work, particularly focussing on production-relevant stressors and outcome measures in sheep and cattle, is required.

7.2.2 Cattle

There has been so little work in this area that all impacts are considered together here.

7.2.2.1 Offspring outcomes: The only study to investigate the impact of maternal psychological stress in pregnant cattle found that transportation stress in mid-pregnancy increased calf birth weight, and also caused an increased stress response in the offspring (Lay *et al.*, 1997).

Limited evidence of an impact of other more physical forms of stress (e.g. heat, cold, disease) also exist. Cold stress through exposure to winter weather (Andreoli *et al.*, 1988) or to heat stress in beef and dairy cattle (Collier *et al.*, 1982; Tao and Dahl, 2013) reduce the birth weight of calves. For beef calves such an effect would represent a risk to survival, and also could have detrimental effects on performance through to slaughter. Heat stress can also affect offspring immune function (Tao *et al.*, 2012), which may impact on later disease susceptibility. Various studies have highlighted the negative impact of disease state during pregnancy on offspring health outcomes (Loyacano *et al.*, 2002; Lundborg *et al.*, 2003; Lents *et al.*, 2008).

7.2.2.2 Potential but unexplored prenatal impacts on offspring: Weaning stress is an interesting example of a maternal stressor in cattle that could be investigated from a prenatal perspective. Although much of the research focus has been on the stress for the calf, the dam also experienced an acute stress response in reaction to calf removal (Lefcourt and Elsasser, 1995; Lynch *et al.*, 2010; Ungerfeld *et al.*, 2011). As mentioned in section 7.1.2.5, weaning also often involves temporary feed restriction for the cow, and this could increase weaning stress through experiences of hunger. More generally, the impact of the social environment during pregnancy on progeny performance has not been studied in cattle. Research in other species, however, has clearly demonstrated that social stress can be a particularly potent form of challenge to animals. Spring calving gestating beef cows are generally housed for around six months over the winter, and this period may be a source of social stress for some cows.

Finally, much of the fetal programming literature has tended to focus on negative outcomes for progeny. There are, however, also opportunities to increase offspring health, welfare and production by supplementing prenatal conditions. For beef cattle, one possible area of investigation is whether the provision of grooming brushes for housed cows has a beneficial impact on their progeny. A recent dairy cow study found that provision of a mechanical grooming brush before calving increased the time that cows spent licking their newborn calf (Newby et al., 2012).

Take home message: Very little work has investigated non-nutritional stressors in cattle during gestation, and the possibility of prenatal stress effects as a consequence of various aspects of cow management has been largely overlooked. However, research conducted does at least demonstrate that negative effects of maternal cow status during pregnancy can affect offspring in ways that are important for their health, welfare and production. Disease also represents a possibly commercially relevant challenge to beef production, which could have impacts on prenatal development. Accurate and up-to-date prevalence information on cattle disease is generally lacking. Whilst minimisation of disease in breeding stock already forms part of best practice management, the additional impact of disease on progeny performance could provide additional incentives to farmers to devote resources to disease control. For instance, a full appraisal of the economic impact of disease should include performance deficits in progeny. This may currently be a hidden cost of various disease states in suckler cows.

Research recommendations: The limited findings available (summarised by (Arnott *et al.*, 2012)) suggest that future work should be targeted at areas of normal management that are under farmer control, and which represent the highest risk of negative prenatal stress effects. In many cases, calf outcomes could be added to research projects relating to cows, as a way of adding value and maximising the information gained. Research is also required to identify whether aspects of housing (such as high stocking density, social mixing, or reduced space to feed), or the presence of highly aggressive individuals in a group, could generate social stress in cows, and subsequently whether this affects their offspring.

The potential positive benefits of providing positive stimuli for pregnant mothers (in both cattle and sheep) suggests that future research could consider ways to improve animal performance by such means, in addition to identifying areas of possible negative outcomes which farmers should avoid.

7.3. Breeding technologies

The impact of advanced breeding technologies in cattle greatly surpasses that in sheep. This is best exemplified for artificial insemination which is used in around 20% of the global population of cattle and buffalo (see Section 6). Here in the UK, recent developments in the production of sex-sorted semen offer the realistic prospect of establishing the much heralded single-sexed once-bred heifer system (SSBH; Sinclair and Webb, 2005), long-recognised as being the most efficient means of producing beef; with predicted efficiencies of food utilisation approaching that of pig-meat production (Taylor *et al.*, 1985). The principal barrier to the successful uptake of this and other advanced breeding technologies lies in the variable but generally poor levels of reproductive management on commercial farms. For example, pundits frequently quote pregnancy rates following fixed-time AI of between 40 and 50% in cattle and blame these meagre results on the technology but, as can be clearly seen from **Figure 7.1**, pregnancy rates in excess of 70% are achievable under good systems of husbandry. Furthermore, whilst there is considerable scope to improve systems for *in vitro* production (IVP) of both cattle and sheep embryos in a manner that would enhance pregnancy rates following transfer, the very fact that the transfer of IVP embryos leads to greater pregnancy rates than can be achieved by either natural mating or AI in regions of the world where cattle encounter heat stress (e.g. Stewart *et al.*, 2011), highlights the importance of cow (i.e. donor/recipient) management. Identifying and modifying the management factors that underlie differences in the successful use of advanced breeding technologies both within and between farms will be of great future benefit to the industry.

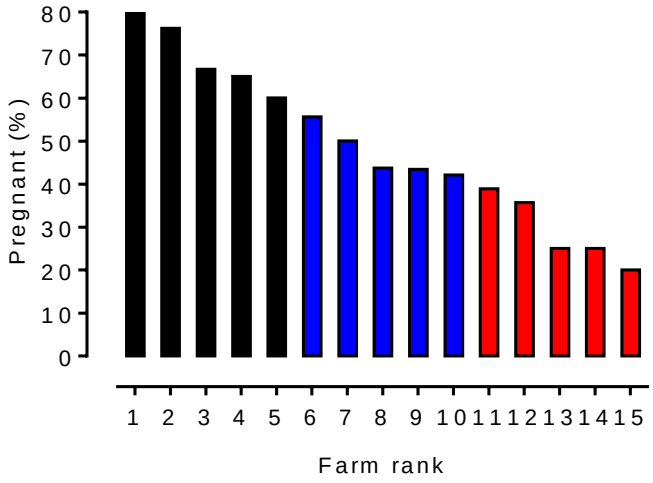


Figure 7.1. Pregnancy rates (determined by ultrasound between Days 69 and 87 of gestation) following a standard oestrous synchronisation and AI protocol (involving the same technicians) in 323 crossbred beef cows across 15 commercial farms (data derived from (Sinclair and Broadbent, 1996)). Mean pregnancy rates for top (black), average (blue) and bottom (red) third herds were 70%, 47% and 29% respectively.

7.3.1. 'Large-offspring syndrome' (LOS)

This phenomenon was first reported in cattle and later in sheep, but there is evidence of related phenomena in mice and other model species, as well as in humans (***Section 6***). Currently, the main issues following ET pertain to early pregnancy losses and obstetrical complications associated birth weight and extended gestations. There are no systematic, long-term follow-up studies in beef cattle and sheep to indicate effects (either positive or negative) on adult offspring health and productivity. Some of the imprinted genes known to be affected by LOS, however, are polymorphic and associated with traits of commercial importance (***Section 6.3***), and so this merits further investigation. The few reports of LOS in recent years may reflect improvements in systems of IVP (that now generally don't include somatic support cells and serum), but equally may reflect the lack of funding and research activity in this area, and the relatively low level of commercial activity in sheep and taurine cattle in Europe and North America (**Figure 7.2**).

A similar situation exists with respective to reproductive cloning. In dairy cows autologous somatic-cell nuclear transfer (SCNT) has been shown to significantly increase the efficiency of bovine cloning leading to higher pregnancy rates and fewer developmental anomalies (Yang *et al.*, 2006). This procedure has never been explored in either beef cattle or sheep, nor has autologous embryonic-cell nuclear transfer been assessed in any species. In theory, this technique could be as efficient as standard IVP. It could, therefore, be used in contemporary breeding schemes to improve the efficiency of IVP of sexed embryos destined for biopsy and genomic evaluation prior to transfer.

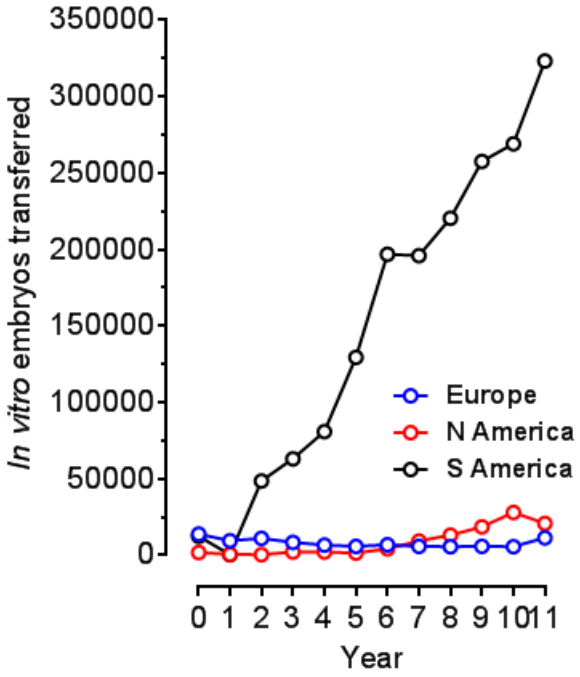


Figure 7.2. Number of bovine *in vitro* produced (IVP) embryos transferred between 2000 and 2011 in Europe, North and South America (IETS, 2012). During this decade the number of IVP embryos transferred globally quadrupled. In 2011 an estimated (conservative) 374,000 IVP embryos were transferred of which 86% were in South America (mostly Brazil), 6% in North America and 3% in Europe. There were virtually no IVP embryos transferred in the UK.

Take home message: Barriers to the increased use of advanced reproductive technologies by both the beef and sheep sectors of the livestock industry arise primarily as a consequence of their perceived low levels of efficiency (i.e. pregnancy rates) and associated costs. However, IVP offers a number of advantages over multiple ovulation and embryo transfer (MOET) including increased numbers of transferrable embryos (i.e. OPU/IVP produces 12-times more embryos per year than AI (assuming one embryo/one calf), and 3-times more embryos than MOET). The use of sexed semen for AI in nulliparous, let alone multiparous, cows is not without its challenges (DeJarnette *et al.*, 2008, Mallory *et al.*, 2013). This is particularly so for reverse-sorted sexed semen (Morotti *et al.*, 2014). In contrast, sex-sorted and reverse-sorted sexed semen could be used to greater effect in the laboratory to produce sexed embryos for subsequent transfer. Furthermore, these embryos can be biopsied for genomic evaluation and to simultaneously confirm sex. Such a scheme, sponsored by the TSB and involving the University of Nottingham, has recently been initiated in the UK.

Variable pregnancy outcomes are more likely to arise as a consequence of poor egg/embryo donor and recipient management, rather than suboptimal laboratory procedures *per se* (although there is scope for improvement here also). Key aspects of donor/recipient management that affect pregnancy outcomes include animal handling facilities, cow/ewe nutrition, oestrous synchronisation and ovarian stimulation, and heat detection. The few reports of LOS in recent years should not lull industry into a false sense of security. Systems (both on-farm and nationally) should be put in place to record pregnancy outcomes, including obstetrical complications and neonatal mortality, following embryo transfer. That said, reference to **Figure 7.2** indicates the

extent to which Europe (and in particular the UK) languishes behind South America (and particularly Brazil) in the use of these technologies and the advantages they offer.

Research recommendations: In the context of beef cattle and sheep breeding/genetic improvement in the UK, there is considerable merit in producing two separate but related contemporary reviews on (a) the costs and benefits of advanced reproduction technologies (ARTs) in livestock improvement within the UK and (b) technical challenges for their successful use. Such articles would help guide future direction, including research, in these fields but would also help devise the framework needed to consider the application of ART outside genetic improvement programmes – for example their use in establishment and running of SSBH systems, or in conventional beef herds and sheep flocks. Such articles might culminate in the establishment of blueprints and technical guidelines for the successful management of egg/embryo donors and AI/embryo transfer recipients.

There is a need to develop improved systems for gamete/embryo donor and recipient management to enhance pregnancy rates following AI or embryo transfer. Such studies would investigate nutritional management, underlying fertility, synchronisation and stimulation protocols.

There are a number of structural and legislative reasons for why ARTs have not been adopted by the cattle and sheep industries in the UK to the extent that they have elsewhere, and in the longer term these need to be addressed. However, important genetic differences exist between *Bos indicus* and *Bos taurus* cattle which mean that Brazilian operators enjoy much greater success when it comes to egg collection, zygote culture and embryo transfer using standard protocols. An understanding of these genetic differences would be of great benefit to beef and dairy cattle fertility and breeding in the UK.

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