THE DEVELOPMENT AND GROWTH OF SKELETAL MUSCLE IN FETAL AND NEONATAL LAMBS

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Animal Science at Massey University.

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1998

GENERAL ABSTRACT

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Massey University/ AgResearch Grasslands 1998.

The objective of these studies was to identify and investigate factors involved in the regulation/control of fetal growth and development in the sheep, with particular emphasis on cellular development of skeletal muscle.

Two models with the potential to impose growth-restriction on the developing fetus without invasive manipulation of the fetal environment were used in this series of studies. First, ewes mated out-of-season generally give birth to offspring with lower body weight than comparable offspring born to ewes mated in their natural breeding season. Fetal growth restriction in this situation is associated with impaired placental development in the out-of-season ewes which is evident by 84 days of gestation. Despite impaired placental growth, ewes mated out-of-season did not, in this study, consistently give birth to low-birth-weight offspring. Although differential effects on myofibre morphology were observed between fetuses from each group, the lack of differences in muscle weights and inconsistent effects on body weight indicated that this comparison proved an unreliable model with which to study fetal muscle growth and development.

The second model involved the comparison of twins versus singles. Twin lambs are consistently lighter than single lambs as a result of maternal constraint characterized by restricted placental size per fetus. Coupled with low birth weights, the growth-restricted twin lamb also had smaller hindlimb muscles compared to singles. Maternal constraint in this situation not only had a negative influence on body and muscle weight, but myofibre hypertrophy was also retarded as indicated by smaller myofibre cross-sectional area. The *adductor* muscle DNA content, and total nuclei

number in selected hindlimb muscles, were lower in twins than in singles. Myofibre number did not differ between ranks. An immunohistochemical technique involving the muscle-specific regulatory factor MyoD allowed the identification of myogenic precursor cell nuclei, which are likely to be satellite cell nuclei in muscles from fetuses in late gestation or early postnatal lambs. Bromodeoxyuridine proved to be unsatisfactory as a marker of actively dividing cells because it did not cross the feto-placental barrier in sheep. However, MyoD was a useful marker of active satellite cells. MyoD-positive nuclei were less abundant in hindlimb muscles of twins than in singles suggesting differential effects of growth restriction on cell cycle activity. The pattern of expression of this factor during development suggests that MyoD may also have an important role in late fetal and postnatal muscle growth.

These results illustrate that growth restriction during late gestation can have important consequences for birth size and skeletal muscle hypertrophy. The observation that myofibre number is not affected suggests that the full complement of fibres has been reached prior to any major nutritional impact which results in growth restriction. The delayed myofibre hypertrophy observed in twin lambs as compared to singles, coupled with lower total DNA content and fewer myogenic precursor nuclei, suggest that the late fetal developmental period is important for muscle growth and the attainment of an adequate birth weight. This result also shows that this period of development has important implications for postnatal muscle growth and may be important in determining ultimate mature muscle mass and postnatal growth potential.

ACKNOWLEDGMENTS

I wish to express my sincere thanks to my supervisors Dr Sam W. Peterson (Institute of Veterinary, Animal and Biomedical Sciences, Massey University), Professor Stuart N. McCutcheon (Assistant Vice Chancellor (Research) Massey University), Dr Warren McNabb (AgResearch Grasslands), Dr Patricia M. Harris (Corporate Manager, Science and Technology, AgResearch, Hamilton), and Dr Charlotte Maltin (Rowett Research Institute, Aberdeen, Scotland) for their interest, advice and inspiration throughout the course of the thesis.

I wish to thank Mr Mervyn Birtles and Mr Guy Hessell for their expert advice and assistance in the histology laboratory, and thanks also to Ms Jane Naoro for her assistance with digital analysis. The advice and assistance in the molecular biology laboratory provided by Ms April Shannon and Mr Graeme Attwood is greatly appreciated. The constructive criticism and advice provided by Associate Professor Roger Purchas, Associate Professor Alex Davies, Mr Graeme Naylor, Professor John Harris (Department of Physiology, University of Otago), Dr John McEwan (Invermay Agricultural Centre, AgResearch) and Dr Paul L. Greenwood (Armidale, Australia) is gratefully acknowledged. I also thank Mr Bob Fletcher (AgResearch Grasslands), Professor Dorrian Garrick, Dr Patrick Morel, for their advice on statistical analysis.

I wish to extend thanks to Mr Tim Harvey (Director of Agricultural Services, Massey University), and the farm staff Mr Kerry Kilmister and Ms Lynley Free for their help with daily management of animals used in the experiments described in the thesis.

Sincere thanks are due to Dr Charlotte Maltin, Ms Margaret Delday, Dr Emily Maxfield, Mr Rob Molan, Ms Wendy Johnson, Mr Nik Harris (Rowett Research Institute, Scotland), Dr John J. Robinson, Dr Peter Broadbent and Dr Kevin Sinclair (Scottish Agricultural College, Scotland) and Mette Christensen, for their

inspiration, friendship and hospitality which made my stay in Scotland a pleasurable and valuable experience.

The generous help of Mr Jason Peters, Ms Catriona Jenkinson, Ms Kate Cooper, Mr Ben Frecklington, Mr Barry Parlane, Mrs Debbie Chesterfield, Ms Margaret Scott, Ms Wigati Sri, Mr Chandana Herath, Mr Mason Stewart, Mr Ken Greaney, Mr John Williamson, Mr John Campbell, Ms Yvette Cottam, Ms Penny Back and Ms Baukje Vlemick during the course of experiments in this PhD is gratefully acknowledged.

The friendship, advice and motivation provided by Associate Professor Duncan Mackenzie, Associate Professor Colin Holmes and Mrs Jill Jonasen, is greatly appreciated.

I would like to thank the Foundation for Research, Science and Technology (FRST) for their extensive financial support for these studies. I wish to express my sincere thanks to the Agricultural and Marketing Research and Development Trust (AGMARDT) for their generous PhD Scholarship and funding of tertiary fees and for their interest, support and friendship extended to me during my studies. I thank the Palmerston North Medical Research Foundation for the provision of a Travel Grant which made my visit to the Rowett Research Institute, Scotland, possible. The personal research expenses grant provided by the Leonard Condell Farming Scholarship Trust is also greatly appreciated. The Graduate Research Fund of Massey University also deserve thanks for generous funding support.

Finally, I wish to extend my sincere thanks to my parents, Peter and Nita, for educating me and providing both financial and moral support. I also thank my brother Mark and his wife Louise, and Jean and Frank Stewart, for their friendship and support. I would also like to thank Neville for this companionship.

Special thanks are due to my fiancee Mason Stewart for his patience, understanding and encouragement.

To Mason

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1.1. INTRODUCTION

One of the basic goals of studying growth in farm animals is to improve animal productivity, efficiency and/or the quality of animal products available for human consumption. Research in this area is driven by both economic and theoretical imperatives. Production of animals with a greater mass of muscle in order to increase productivity per animal is one goal of animal breeding and husbandry. Increased awareness of the implications of excessive intakes of saturated fats for human health has also led to new objectives for manipulation of animal production. This is particularly true for the meat industry. This has created a demand for the manipulation of animal production to incorporate not only the production of animals with increased mass of muscle, but also the production of lean meat.

Traditionally, most research has focused on increasing the gross efficiency of meat animal production, which is based on nutrient supply (e.g., provision of the optimal mix of dietary nutrients to the animal), digestion (e.g., efficiency of utilisation of absorbed nutrients) and nutrient partitioning/metabolic control (e.g., the role of hormones). As a result of the work done in these areas, a good understanding of how muscle tissue grows during postnatal life has been gained. Such research however, does not provide sound knowledge of the cellular and molecular aspects of muscle growth during fetal life, during which muscle develops.

Farm animals are classed as precocial species due to their relative maturity at birth. Precocial species, including the sheep, are able to run and stand soon after birth, which suggests that considerable functional development of skeletal muscles has occurred prior to birth. Therefore, to better understand the cellular and molecular factors underlying the control of muscle development and growth, prenatal investigation is necessary.

1.2. FETAL GROWTH AND DEVELOPMENT

Mammals are viviparous, i.e., embryonic, and fetal development occurs within the uterus. This intrauterine developmental period is terrned "pregnancy" or "gestation", and spans the period from fertilization through to parturition. The length of gestation is largely genetically determined (e.g., species, breed and fetal genotype) however, maternal (e.g., age of the dam), fetal (e.g., litter size and sex) and environmental factors (e.g., season, nutrition or temperature) can modify its duration.

The prenatal development of sheep can be divided into three main periods. The period from fertilisation until the initial attachment of the blastocyst to the uterine wall before the establishment of intraembryonic circulation, is termed the "ovum period" and overlaps the "embryonic" period. The "embryonic" period extends from day 12 to about day 34. Rapid tissue growth and differentiation occurs during the embryonic period and it is during this time that the major tissues, organs and systems (e.g., circulatory system) are established and the external features of the body can be identified. Embryo losses at this stage would be unlikely to have an adverse effect on growth of any remaining embryo(s) as they are relying on nourishment from amniotic fluid rather than a direct nutrient supply from the maternal circulation.

Implantation of the embryo occurs at about day 28-34 of gestation. A proportion of embryos are wasted around implantation, possibly to reduce the number of conceptuses to a level equal to uterine capacity (Robinson, 1993). Placentome number is thought to be fixed at implantation, and each fetus develops its own placenta and can grow independently from its littermates. Thus, embryo losses at this stage could have a detrimental effect on the surviving fetus(es), as the proportion of placentomes available for each fetus declines. Since the remaining fetus(es) are unable to utilize the placentomes of the aborted fetus, the surviving embryos are restricted to a smaller proportion of placentomes resulting in subsequent reduction in fetal growth and birth weight (Rhind *et al.,* 1980).

Rapid growth and changes in the form of the fetus occur during the fetal period that follows implantation around day 34 of gestation, and continue through until birth (Jainudeen and Hafez, 1993). Fetal weight increases slowly in early gestation, then increases rapidly towards term, with about 90% of the birth weight gained in the last 40% of gestation in sheep. Fetal growth usually follows an exponential growth curve and, at term, the weight of the fetus contributes approximately 60% of the total weight of the conceptus.

1.3. PLACENTAL DEVELOPMENT

The uterus is a highly specialized organ that nourishes and protects the blastocyst/embryo/fetus from the time of conception through until parturition. Approximately 60-150 endometrial thickenings (caruncles) are found in the non-pregnant uterus (Alexander, 1964, 1974). From about day 15 of gestation, the chorion, which fills most of the uterine cavity, begins to attach to these caruncles on the maternal membrane (Robinson, 1982). The fetal cotyledons fuse with these caruncles to form placentomes (Ferrell, 1989) that, with the fetal membranes, form the placenta. This process of implantation is complete by about day 34 of gestation in the sheep. Generally 70-80% of the "available" caruncles become occupied.

Placentome numbers vary between species (Amoroso, 1952) and between individuals. Differences may be due to littersize, parity or season (Jenkinson *et al.,* 1994) and, in general, placentome numbers per fetus decrease as littersize increases (Mellor, 1983). This decrease in placentome number is not fully compensated for by an increase in the weight of the individual placentomes (Rhind *et al.,* 1980). Thus, the number of placentomes plays an important part in determining overall rate of fetal growth.

By 70-80 days of gestation in the sheep, maximum placentome size and placental weight is attained (Kelly, 1992; Ehrhardt and Bell, 1995). Placental growth rate far exceeds the growth rate of the fetus(es) during this period. From this time until the end of gestation, placental weight may decline by up to 50%, however, the dry weight of the placenta remains constant (Vatnick and Bell, 1992).

1.3.1. Placental Function

The placenta provides a mechanism for exchange of gas, metabolic substrates and waste products between the conceptus and its external environment. and is also an important endocrine organ (Anthony *et al.*, 1995; Reynolds and Redmer, 1995; Robinson et al., 1995). The placenta also acts as a barrier against pathogens and the maternal immune system, thus ensuring the survival and development of the conceptus in mammals. The blastocyst and the early embryo are nourished by endometrial fluid and the fetus is nourished by a constant supply of nutrients from the maternal circulation across the placenta (Reynolds and Redmer, 1995). During gestation the fetus has priority over available nutrients, even when maternal nutrition is insufficient, and in this way the fetus could be considered a "parasite".

1.4. FACTORS AFFECTING FETAL GROWTH

Fetal growth and development is primarily determined by the fetal genome, but superimposed on this genetic drive to grow are two opposing influences. One involves factors which impose constraint on fetal growth, such as the supply of nutrients to the fetus, determined mainly by maternal supply (Robinson and McDonald, 1989; Robinson and Symonds, 1995) and placental transfer capabilities (Carter *et al.*, 1991; Robinson *et al.*, 1995). The other involves factors which stimulate fetal growth such as hormones and growth factors, e.g., the insulin-like growth factors (Owens *et al.*, 1994; Robinson *et al.*, 1994; Harding *et al.*, 1994; Anthony *et al.*, 1995; Kuhn-Sherlock *et al.*, 1995). Thus, fetal growth represents a balance between constraining and stimulating forces influencing the genetically determined drive to grow.

1.4.1. Placental Factors

Placental size and fetal growth rate are well correlated in late gestation, indicating that the placenta plays a key role in the regulation of fetal growth. Since there is a high correlation between placental and fetal size, it has been assumed that placental size is a major determinant of intra-uterine growth retardation (IUGR) (Bassett, 1991). Growth-

retardation can be experimentally induced by a variety of methods, e.g., alteration of maternal substrate availability, interference with uteroplacental circulation, interference with umbilical circulation, and restriction of placental growth by surgical removal of caruncles prior to implantation of the embryo. These procedures all lead to reduced placental growth, and thus, smaller placental size and subsequent fetal growth retardation.

Small placentas have reduced functional transport capacity which results in restricted placental transport of oxygen and nutrients from the maternal uterine circulation to the fetal umbilical circulation (Owens *et al.,* 1987 ab). Since an adequate supply of oxygen and nutrients is essential to support anabolism in the fetus, a reduction in placental size with a subsequent decrease in uterine blood flow can lead to fetal hypoxaemia, hypoglycaemia and growth retardation (Owens *et al.,* 1987 ab).

When placental size and function are restricted, the fetal:placental (F:P) size ratio is often seen to increase, suggesting that compensatory mechanisms operate to increase placental efficiency to maintain fetal growth. One mechanism that may occur is an increase in the size of the cotyledons, which is observed following attempts to restrict placental size or growth in sheep (Robinson *et al.,* 1979; Caton *et al.,* 1984). This compensatory growth is most obvious during the first half of gestation when placental tissue is growing rapidly (Owens *et al.,* 1989). A larger fetus than expected from a small placenta may be due to increased placental transport and efficiency allowing a small placenta to support a disproportionately larger fetus (Owens *et al.,* 1989).

1.4.2. Fetal Factors

Factors influencing fetal development and growth are present from early gestation, before implantation of the embryo. These regulatory mechanisms are present and develop in the outer layer of the developing blastocyst, the trophectoderm (or trophoblast), even before implantation occurs and before development of the membrane layers and the vascular system joining the fetal circulation with the maternal circulation

(Bassett, 1991; Reynolds and Redmer, 1995). If these mechanisms are impaired in early life, subsequent fetal growth retardation may occur (Rivera *et al.*, 1996).

As the conceptus grows, substrate supply and transport to the embryo must also change to supply adequate nutrients to the developing embryo. It has been suggested that there is a critical time window during which the conceptus can cause changes in placental development to enable increased glucose and oxygen transfer to the fetus necessary to support a higher rate of growth and development. This is supported by the observations that fetal losses around implantation can result in bigger placentas, e.g., in rats up to 14 days of gestation (Ogata and Finley, 1988) and in the rabbit (Fletcher *et al.,* 1982). The fetus may also regulate placental growth by altering concentrations of nutrients and hormones transported to the placenta via umbilical cord blood and/or by alteration of the rate of umbilical blood flow (Bassett, 1986). The fetus may also expand the vascular network of the endometrium around implantation (Bassett, 1991) and in conjunction with the important role that the fetus plays in placental metabolism (Bassett, 1994), may dictate its own future growth.

1.4.3. Maternal Factors

Maternal genotype has a large influence on fetal growth and development (Gluckman and Liggins, 1986). In addition, growth can be constrained by a variety of non-genetic factors that are mainly derived from the dam. This situation has been termed "maternal constraint". The maternal constraint may operate in various ways including limitations in the availability of nutrients, and/or maternal placental diffusion and placental growth limitations. The inverse linear relationship between litter size and mean fetal weight demonstrates that fetal growth is normally constrained by maternal factors, except when dams have a high circulating level of IGF-1 which reduces or abolishes the maternal constraint, e.g., in mice (Morel *et al.,* 1994).

Fetal weight can determine the amount of maternal tissues that are mobilised or deposited. Ewes that are well fed throughout gestation show normal fetal growth rates until day 120 of gestation and then growth rate gradually declines (Mellor and

Matheson, 1979; Mellor and Murray, 1982). Moderate to severe maternal under-nutrition during the first 90 days of pregnancy can restrict placental weight in sheep (Everitt, 1964; Alexander and Williams, 1971; Mellor, 1983) and retard subsequent fetal growth (Mellor and Murray, 1982). For example, when previously well-fed ewes are severely underfed during the last 40-50 days of gestation, fetal growth rate declines within 3 days to 30-70% of normal (Mellor and Matheson, 1979; Mellor and Murray, 1982). There is also some indication that the weight of placentas in twin-bearing, but not single-bearing ewes is reduced by underfeeding during late pregnancy (Thomson and Thomson, 1948; Mellor and Murray, 1981). In contrast, under-nutrition during mid-pregnancy can either decrease or increase placental weight (Wallace, 1948ab; Faichney and White, 1987; McCrabb *et al.*, 1991). This effect probably depends on the nutritional status of the ewe around the time of conception (DeBarro *et al.*, 1992). Despite the differential effects that maternal nutrition appears to have on placental size, the impact of the nutritional regulation of placental growth should not be ignored, as placental size is an important determinant of fetal growth (Mellor and Murray, 1981; Bassett, 1991; Kelly, 1992).

The rapid decline in fetal growth rate during severe maternal under-nutrition shows that maternal body reserves fail to meet the demands of the growing fetus. During maternal fasting, glucose supply to the fetus falls dramatically and, with decreasing substrate availability, fetal growth rate declines, and in extreme cases may cease (Mellor and Matheson, 1979) in order to conserve energy. Therefore, maternal nutrition plays an important role in the regulation of fetal growth, especially later in gestation. The greater the total fetal weight, the more impact maternal under-nutrition during pregnancy has on fetal growth, with triplets being most susceptible and singles least susceptible. Therefore, it is thought that one component of maternal constraint is limitation of substrate supply in late gestation.

Maternal constraint can limit fetal growth as described above, but pathological conditions can have a larger impact on fetal growth, e.g., poor placentation, placental disease or maternal disorders affecting uterine substrate delivery. Maternal cardiovascular disease can also affect uterine blood flow and may contribute to

decreased substrate supply to the fetus. Other factors that decrease uterine blood flow include increased temperature, some chemicals (e.g., prostaglandin E and alpha-adrenergic agonists), posture, exercise and mechanical obstruction (reviewed by Gluckman and Liggins, 1986).

1.5. MODELS OF FETAL GROWTH RETARDATION

Much of our current knowledge of factors involved in the regulation of fetal growth is derived from studies in growth-retarded animals, in particular, studies of pregnant sheep (Carter, 1994; Robinson *et al.,* 1994). Fetal growth restriction in this species has been achieved by experimental manipulation involving extreme or surgically invasive models. Such models may include chronic undernutrition particularly in late gestation (Mellor, 1983), resulting in fetal growth restriction regardless of whether the supply of energy or protein or both is restricted. Changes in maternal nutrition can also have detrimental effects on embryo survival (see reviews by Robinson, 1990 and Ashworth, 1994). However, the extent of the reduction in fetal growth rate and birth weight depends on the timing and severity of the nutrient deprivation. Experimental reduction of placental mass, such as carunclectomy, or surgical removal of caruncles prior to mating, results in fewer placentomes being formed, which subsequently results in a reduction in fetal size (Alexander, 1964; Robinson *et al.,* 1979). Uterine artery ligation also results in a reduction in fetal size (Emmanoulides *et al.,* 1968).

The external environment can also have detrimental effects on fetal growth. Heat stress, for example, can result in fetal growth retardation (Alexander and Williams, 1971; Bell *et al.,* 1987). An environment which can also severely alter the growth potential of some fetuses, is the culture environment during embryo transfer procedures. It has been reported that exposure of early cleavage-stage *ovine* and *bovine* embryos to differing culture conditions, can result in prolonged gestation, increased birth weight and increased perinatal death rates (Walker *et al.,* 1992; Bishonga *et al.,* 1994; Farin *et al.,* 1994; Thompson *et al.,* 1995; Holm *et al.,* 1996; Young *et al.,* 1996; Thompson, 1997). The average birthweight increase is a major concern for the health and wellbeing of the fetus and dam, as individual lambs can weigh, in extreme cases,

greater than twice the average (11 kg versus 4.8 kg). Similar effects have been observed in cattle (Behboodi *et al.,* 1995; Farin and Farin, 1995). The factors responsible for this effect on fetal size and viability are not well understood. However, these results suggest that factors or mechanisms which act during the first few days following fertilization are likely to have important implications for subsequent embryonic and fetal development and growth.

All of the above procedures that influence the size of the resulting offspring involve invasive or extreme maternal/placental manipulation and do not mimic natural restriction of fetal growth. Two situations in which fetal growth restriction is observed under natural or relatively non-invasive circumstances have been described. When ewes are mated outside of their normal breeding season (i.e., mated in December rather than March in New Zealand) the resulting offspring are growth-restricted compared to their spring-born counterparts. Litter size also affects birth weight (singles are generally larger than twins which are, in turn, larger than triplets).

1.5.1. Seasonal Effects on Fetal Growth in Sheep

Recent studies have indicated that the season in which a ewe lambs can have a large influence on the growth of the offspring. In brief, ewes that lamb outside of their normal breeding season, i.e., ewes mated in summer (December to February, in New Zealand) to lamb in the autumn (April to June) rather than being mated in autumn (March to May) to lamb in the spring (July to September), can give birth to offspring which have birth weights reduced by 0.5 to 1 kg compared to their spring-born counterparts (Reid *et al.,* 1988; Peterson, 1992; Morris *et al.,* 1993; Jenkinson *et al.,* 1994). This effect is not always observed, however. Rumball (1980) reported no differences in birth weight of lambs born in either spring or autumn. In addition, McQueen (1986) reported small, but not statistically significant, differences in lamb birth weight between seasons and between years.

The nature of this seasonal effect on fetal growth is largely unclear. The effect is not a consequence of differences between seasons in the nutritional status of the pregnant

ewe, at least as measured by ewe liveweight change (Jenkinson *et al.,* 1994). The difference in birth weight observed in that study was associated with a difference in placental development (Jenkinson *et al.,* 1995). Specifically, although caruncle number was the same between seasons, placentome number was significantly lower, resulting in a considerably smaller placenta in autumn- than in spring-lambing ewes. Since placentomes are formed early in pregnancy and placental weight reaches a maximum prior to 100 days of gestation in the sheep, the factors involved in this seasonal effect on placental growth are likely to act early in pregnancy while the placenta is still forming, however this has not yet been confirmed by experimental evidence.

This is an important finding since it is one of the few situations in which large differences (0.4-1 kg) in birth weight can be produced without gross nutritional manipulation of the pregnant dam. Despite the implications that this growth restriction of lambs born out-of-season may have on the introduction of year-round lamb production systems (Andrewes and Taylor, 1986), the seasonal effect on birth weight provides a unique model with which to study fetal growth without severe manipulation of the ewe or fetus.

1.5.2. Influence of Litter Size on Fetal Size

It is well established that litter size has a significant impact on birth weight of lambs, with mean fetal weight decreasing as litter size increases and on average, singles tend to be about 0.5 kg heavier than twins (Starke *et al.,* 1958). This reduction in fetal weight as litter size increases was originally presumed to be an effect of differences in growth rate late in pregnancy, since a single maternal nutrient source must be shared between littermates. However, the mathematical model which describes the growth of the fetus between 55 and 145 days of gestation in prolific ewes (Robinson *et al.,* 1977), suggests that these differences in birth size originate early in pregnancy. In addition, Rhind *et al.* (1980) and Dingwall *et al.* (1987) reported that very small lambs present in some large litters are not a consequence of differential growth-rates in late pregnancy.

A decrease in the number and weight of placentomes per fetus as litter size increases has been noted (Rhind *et al.*, 1980). Although a degree of compensatory growth of the placentomes from placentas of twins and triplets does occur in late pregnancy, this increase in weight is not large enough to compensate for the lower number of placentomes per fetus associated with large litters (Rhind *et al.*. 1980). Therefore, as litter size increases, concomitant increases in competition for placental attachments result in a decrease in the number of attachments per fetus, subsequently resulting in lower birth weights of multiple offspring compared to singles.

1.5.3. Potential Use of the Seasonal and Littersize Models for Studying Fetal Muscle Growth and Development

The implications of restricted fetal size for tissue development following the influence of season have not been investigated in individual tissues from fetal sheep. Studies on the effect of season on fetal size, have reported that fetuses born out-of-season have lower overall body weight without a concomitant restriction in skeletal size, as measured by crown-rump length (Jenkinson *et al.*, 1995). Since muscle represents a large proportion of the total carcass weight of sheep (Kempster *et al.*, 1982), it is possible that muscle development and growth may be differentially affected between autumn- and spring-born lambs, which may contribute to the difference in overall size observed between seasons. Recently Hunt *et al.* (1996) reported that changes in body weight as a result of increased litter size can influence muscle development and growth. Therefore, the effect of litter size may also be a good model with which to study how muscle develops and the factors and mechanisms by which myogenesis is controlled or regulated. Although there is a good understanding of how muscle develops anatomically in the sheep fetus (Ashmore *et al.*, 1972; Swatland and Cassens, 1973; Maier *et al.*, 1992; Wilson *et al.*, 1992), the factors involved in the regulation of muscle development and growth in the fetal lamb are poorly understood, as most current understanding of muscle development and growth has been generated either from *in vitro* studies, or from studies in laboratory animals.

Myogenesis (muscle development and growth) is a complex process involving the integration of several regulatory systems. The investigation of factors which are involved in myogenesis is necessary before this process can be manipulated to benefit the animals' survival and wellbeing, as well as meat production potential. The two models described above offer an opportunity to investigate the development and growth of skeletal muscle, as well as the factors that control or influence its development and/or growth, under natural conditions.

1.6. MUSCLE DEVELOPMENT AND GROWTH

Muscle is a highly specialized tissue which is required for survival, being essential for every movement of the body and for postural stability. There are three main classes of muscles in the body, cardiac, smooth and skeletal, which differ in either their organization as seen under the light and electron microscopes, or in their specialized functions. Skeletal muscle quantitatively makes up the largest of the three classes, contributing 50-60% of the carcass weight in sheep. Due to the large contribution of this tissue to overall body mass it is likely that muscle growth and/or development is adversely affected in the growth-restricted fetus. Thus, skeletal muscle is the tissue of interest in this study.

1.6.1. Gross Muscle Structure

1.6.1.1 Skeletal Muscle Connective Tissue

Muscle connective tissue is an essential anatomical structure within a muscle, as it provides support, a medium through which nutrients and waste products pass between muscle fibres and capillaries, and a means for transmitting movements and forces produced by the fibres to the tendon. Muscle connective tissue also plays an important role in the development of muscle (Sivachelvan and Davies, 1986). There are three anatomical parts to muscle connective tissue; the epimysium, a heavy sheath of connective tissue surrounding the entire muscle and separating it from other muscles, the perimysium which surrounds the fascicles, and the endomysium which envelopes each individual fibre (Plate 1.1.).

1.6.1.2. The Muscle Fibre

The muscle fibre (Plate 1.1.) is the fundamental organizational unit of muscle. In isolation it can contract if stimulated, and perform all the functions of muscle, although it generally operates in larger functional units termed "fascicles". Each muscle fibre is long, cylindrical, and multinucleate, with the nuclei (Plate 1.1) normally being on the surface of the fibre, just beneath the plasmalemma in the mature fibre. The large number of nuclei and their location on the outside of the fibre are distinguishing characteristics of skeletal muscle fibres. Each fibre is an independent structure and varies from 1-14 mm in length and from 10-100 μm in diameter. Some fibres extend the length of the muscle; this is common in small animals, e.g., rats. However, in other muscles, especially those of large animals, individual fibres begin and terminate within the muscle (intrafascicular terminations; see Swatland and Cassens, 1972 and Gans and Gaunt, 1992).

Plate 1.1. Photomicrograph of fetal sheep skeletal muscle illustrating the endomysium, perimysium, and epimysium connective tissue layers, myofibres and myonuclei. (Mag x500).

epimysium perimysium endomysium myofibre myonucleus

Each fibre is composed of smaller subunits, the most obvious of which are myofibrils, which are responsible for fibre contraction and relaxation. Myofibrils are about 1-2 μm in diameter, and are separated by mitochondria and the sarcoplasmic and transverse tubular systems. Each myofibril contains a number of smaller, long, thin protein filaments, termed myofilaments, that account for 80-87% of the muscle fibre volume. There are two types of protein filaments, actin (thin filaments) and myosin (thick filaments), which are responsible for the contractile capabilities of a muscle fibre (Guyton, 1991).

1.6.1.3. Types of Muscle Fibre

There are three main types of muscle fibre, which can be classified by their speed of contraction. "Fast-twitch" fibres, as the name suggests, contract quickly and are used for fast movement, e.g., running. Conversely, "slow-twitch" fibres contract slowly and are responsible for slow movements, e.g., postural control. Intermediate fibres contract at an intermediate rate between "slow-" and "fast-twitch" fibres. Muscles always comprise a heterogeneous mixture of all fibre types when examined histologically. Several muscle-specific contractile proteins are synthesized within each muscle fibre from gene families which encode multiple isoforms. There are many different combinations of these isoforms expressed in muscle fibres and this gives rise to many muscle fibre phenotypes (Staron and Pette, 1987). Transformations in the myosin isoforms occur during development. For example, the transformation from the fast to slow myosin heavy chain phenotype, at least for some fibres in the fetal lamb, is not complete until about 20 postnatal days (Maier *et al.,* 1992).

1.6.2. Muscle in Large and Small Animals

1.6.2.1. Altrical Species

Species such as humans, cats and rats are termed "altricial" (unable to walk for a period of time following birth). At birth, the limb muscles from these species are not fully differentiated, either functionally or structurally, and differentiation into the mature

phenotype occurs during early postnatal life. The fast and slow contractile properties of skeletal muscle develop after birth and involve myogenic, hormonal, nutritional and behavioural factors. The final development of each muscle, and the fibre types that comprise it, is also influenced by the gradual emergence of specific motor patterns and exercise of the muscles, effects mediated by load-bearing exercise and the stretch imposed by bone growth, and by the different types of myosin contained within each fibre (Walker and Luff, 1995).

1.6.2.2. Precocial Species

Precocial species, such as sheep and goats, are relatively mature at birth and are able to stand and run, which suggests that considerable functional development of skeletal muscles has occurred prior to birth. The prenatal development of fibre phenotype in precocial species is quite well understood (Ashmore *et al.*, 1972; Maier *et al.*, 1992; Wilson *et al.*, 1992), however, the relative importance of neural and hormonal factors in allowing this functional specialisation to occur in the presumed absence of significant load-bearing exercise in the uterine environment is unclear.

1.6.2.3. Differences in Muscle Growth and Development Between Large and Small Animals

Earlier studies were conducted on the assumption that muscle development in larger animals progresses along the same path as muscle development in small animals. However, recent studies have suggested that this is not a valid assumption. Instead, these studies have identified three main areas in which muscle growth and development in larger animals is different to that in small animals; first, fibres in muscles from large animals do not run from one end of the muscle to the other, instead they form intrafascicular terminations (Huber, 1916; Loeb *et al.*, 1987; for review see Gans and Gaunt, 1992). Therefore, estimates of fibre number from a single region of a muscle do not account for a large number of intrafascicularly terminating fibres, and thus, can greatly underestimate the total number of fibres in the adult muscle. Samples taken in this way can only represent an estimate of the total number of fibres in the actual cross-

section under study. Second, muscles from large animals have a different innervation pattern: whereas innervation in a rat muscle is restricted to a single endplate, multiple bands of endplates along the length of the muscle are apparent in muscles from large animals (Coers and Woolf, 1959; Wilson *et al.,* 1992). Third, large muscles from large animals have greater total fibre numbers (Duxson and Sheard, 1995).

1.6.3. Muscle Fibre Differentiation and Growth

The development of muscle is a highly ordered process comprising a series of interrelated events. During gastrulation, the mesoderm is induced from presumptive ectoderm, following which a portion of the mesoderm becomes committed to skeletal muscle development. These committed cells within the mesoderm (myogenic cells) then proliferate and differentiate and this process is followed by fusion of these myogenic cells to form fibres. The body plan, e.g., outgrowth of limb buds, is then determined and the assembly of muscles follows. These processes will be discussed in more detail below.

1.6.3.1. Mesoderm Induction from Ectoderm

Following conception, the fertilized egg divides repeatedly to form a multicellular blastula. In response to inductive signals, the blastula divides into three main parts, the ectoderm, endoderm and mesoderm.

1.6.3.2. Commitment of Mesodermal Stem Cells

The totipotent mesodermal stem cells become committed to one of three main lineages: adipoblasts, which form fat tissue; chondroblasts, which form bone and associated tissue; or myoblasts, which form muscle tissue (Olson *et al.,* 1991, also see Figure 1.1.). The area of the mesoderm that gives rise to all skeletal muscle is called the somite (Kenny-Mobbs, 1985). Determination of that part of the somitic mesoderm which will become muscle tissue, rather than one of the other tissues, is made prior to the expression of one or more of the muscle-specific regulatory factors (Tajbakhsh and Buckingham, 1994).

The mesodermal stem cells that are committed to the myogenic lineage become myogenic progenitor cells (presumptive myoblasts) which are located in the blastodiscs. Presumptive myoblasts are cells which replicate within the myogenic lineage but which cannot fuse or synthesize myofibrillar contractile proteins. However, these cells do contain the cytoskeletal proteins that are required for cell motility, endocytosis, exocytosis and other cellular activities (Pearson and Young, 1989). The mesodermal marker Pax-3 (Goulding *et al.,* 1994) regulates the migration of those myogenic progenitor cells (Daston *et al.,* 1996) to the limbs where they differentiate and fuse to form fibres.

1.6.3.3. Myogenic Cells

Myoblasts (myogenic cells) are mononucleated cells that can express muscle specific genes and can, under appropriate conditions, assemble myofibrillar contractile proteins into functional myofibrils (Pearson and Young, 1989; Stockdale, 1992). Myoblasts can be divided into three main types; embryonic, fetal and adult (satellite cells), which differ in their myogenic fate.

1.6.3.3. Embryonic Myoblasts

Embryonic myoblasts are the first cells in the somite and limb buds of mammalian embryos (Vivarelli *et al.,* 1988). Mammalian embryonic myoblasts are generally thought to form primary fibres (the first fibres seen) in the absence of innervation (Stockdale, 1992). There is no direct evidence for this assumption, however, the only myoblasts which can be isolated from the limb bud when primary fibres first form are of the embryonic type (White *et al.,* 1975; Hauschka *et al.,* 1979; Miller and Stockdale, 1986 ab). The embryonic myoblasts can be distinguished from other myoblasts throughout all other stages of development (Vivarelli *et al.,* 1988; Cossu *et al.,* 1989; Smith and Miller, 1992). Embryonic myoblasts, in developing muscles of mammals (and birds), dominate the limbs of the embryo until morphogenesis is nearly complete.

1.6.3.3.2. Fetal Myoblasts

The predominant type of myoblast which can be isolated during the fetal period of development, is the fetal myoblast. Fetal myoblasts differ from the embryonic type in many ways including myosin heavy chain (MHC) expression (Cerny and Bandman, 1986; Vivarielli *et al.*, 1988), response to the tumor-promoting agent, 12-O-tetradecanoylphorbol-13-acetate (TPA) (Cossu *et al.*, 1982, 1985) and expression of surface antigens and desmin (Kaufman *et al.*, 1991). Fetal myoblasts are the predominant myoblast type during the early fetal period. However, by midfetal life, adult myoblasts are the most abundant myoblast type (Feldman and Stockdale, 1990; Hartley *et al.*, 1992).

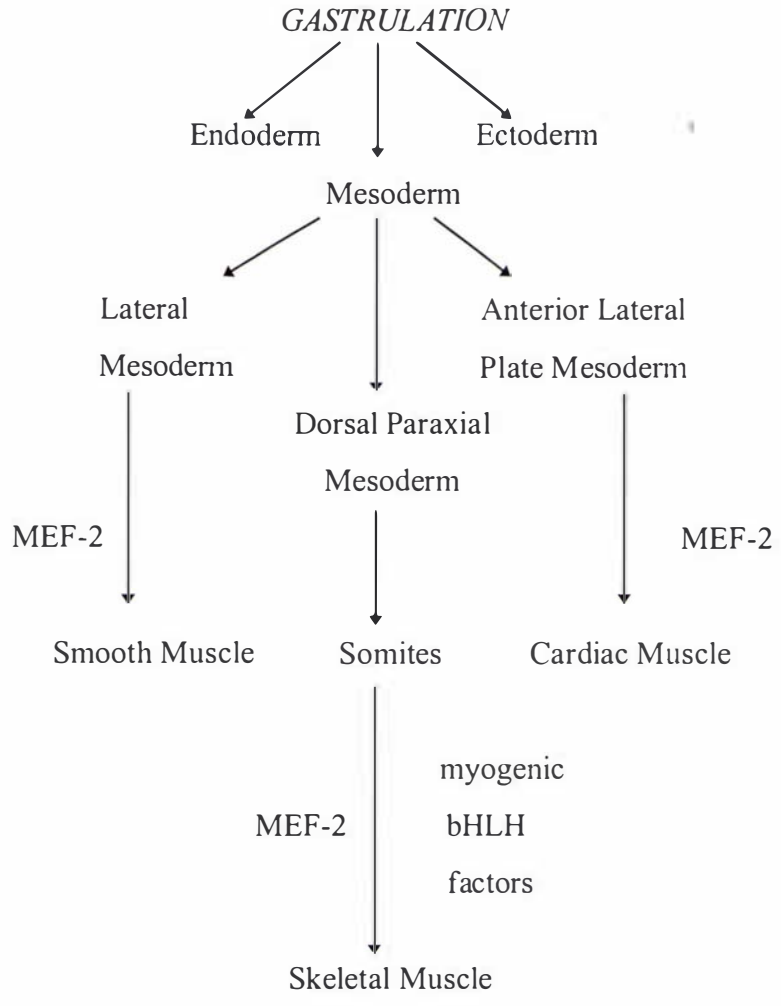

Figure 1.1. Schematic diagram of vertebrate myogenic lineages. (from Olson *et al.*, 1995).

1.6.3.3.3. Adult Myoblasts (Satellite cells)

Adult myoblasts, which are more commonly known as "satellite cells", are mononucleated cells that are found between the basal lamina and cell membrane of muscle fibres. These satellite cells have many functional similarities to myoblasts at other stages of development (Stockdale and Feldman, 1990). Satellite cells appear during midfetal life and are the only myoblast type that persists into adulthood. Therefore, they are the sole source of nuclei for postnatal growth and they are also important for the regeneration of muscle following injury (Campion, 1984).

Satellite cells differ from embryonic and fetal myoblasts in many ways. However, it is still unclear how satellite cells and fetal and embryonic myoblasts are lineally related, which factors contribute to their diversity, and which factors limit their proliferation and distribution within muscles *in vivo* (Bischoff, 1990a). The control of the proliferation of satellite cells is important to our understanding of the myogenic lineage, but the mechanisms which control satellite cell proliferation are not yet known (Bischoff, 1990b). This is in part, due to the lack of adequate markers for them in muscle (Grounds and Yablonka-Reveni, 1993). When muscle matures, satellite cells become quiescent, only becoming active again during regeneration following injury. Quiescent satellite cells can be identified by their anatomical location (Zhang and McLennan, 1994), but once they become active and begin to migrate, there is currently, no reliable method for identifying them. Desmin and NCAM have been used as satellite cell markers, but these proteins are expressed by many cell types making it difficult to distinguish between satellite cells and other cell types (Grounds and Yablonka-Reveni, 1993). The MyoD protein (this and other members of the MyoD family will be discussed in section 1.7.1.) has been reported as a marker of active satellite cells in neonatal mouse muscle (Koishi *et al.*, 1995), but may also label some recently incorporated myonuclei in adult mouse tissue (Hughes *et al.*, 1997). This finding should enable further investigation into the role of satellite cells, and mechanisms regulating their proliferation and differentiation, at least in early life.

1.6.4. Muscle Fibre Hyperplasia

Myoblasts which arise from mesoderm have two possible fates. They can remain in a determined but undifferentiated state, in which they remain in the cell cycle and continue to proliferate. Alternatively, they can enter the differentiation pathway, resulting in the cessation of cell division accompanied by the expression of various muscle-specific genes (e.g., those coding for actin and myosin) and the fusion of these mononucleated differentiated myoblasts to form multinucleated myotubes. Activation of the differentiation pathway is controlled through a repression-type mechanism in which specific peptide growth factors (e.g., Fibroblast Growth Factor (FGF) and Transforming Growth Factor-β (TGF-β) block the expression of muscle-specific genes. While the levels of these peptide growth factors are high, differentiation is inhibited. When these myoblasts are exposed to an environment lacking growth factors, the myoblasts cease proliferation and subsequently differentiate and fuse to form multinucleated myotubes, and express their muscle-specific phenotype.

The fusion process is relatively brief, taking about 8-10 minutes (Bischoff and Holtzer, 1969). It involves alignment of differentiated myoblasts, which then fuse to form myotubes. The nuclei of unfused differentiated myoblasts line up with nuclei in the centre of existing myotubes and subsequently fuse to the existing myotube. This fusion process continues, resulting in the length of the myotube increasing. Following fusion, muscle-specific gene expression is activated, followed by the synthesis of myofibrillar proteins (e.g., actin and myosin). Myofibrils can be seen within a few days after fusion. As the myotube enlarges, more myofilaments are formed and eventually the nuclei move to the outside of the cell and rest beneath the plasmalemma. The myotube is now a muscle fibre.

The fate of a myoblast to proliferate or differentiate is determined by a balance of opposing cellular signals. Peptide growth factors play an important role and will be discussed in greater detail in section 1.7.2. The MyoD family of muscle-specific transcription factors which can activate the muscle differentiation programme and inhibit cell proliferation, are also important in the control of myoblast proliferation and differentiation (see section 1.7.1.).

The first fibres that form during embryogenesis are primary fibres. These fibres form by the fusion of embryonic myoblasts around the time that the premuscle masses split. In the sheep fetus, primary fibres can be seen as early as day 32 of gestation and reach their maximum number by day 38 of gestation (Wilson *et al.*, 1992). Primary fibre formation ceases 1-2 days before secondary fibres form (Ontell, 1982).

Following formation of primary fibres, secondary fibres form by the fusion of mononucleated fetal myoblasts in the region of innervation of the supporting primary fibres (Duxson *et al.*, 1989) and grow in length towards the tendons (Kelly and Zacks, 1969; McLennan, 1983; Ross *et al.*, 1987), using the primary fibres as a framework for their growth. In small animals (e.g., rats), primary fibres extend from tendon to tendon. The primary fibres of muscles in larger animals do not extend from tendon to tendon, instead they overlap. They also have several innervation zones that act as a generative centre for secondary fibre formation (Duxson and Sheard, 1995). Secondary fibres form near the innervation zones of the primary fibres and, due to the large number of generative zones in primary fibres, many more secondary fibres are produced in the muscle of large animals compared to small animals. The way in which the secondary fibres form around the primary fibres creates a rosette pattern of primary fibres surrounded by secondary fibres (Kelly and Rubenstein, 1980). The secondary fibres eventually form the main bulk of muscle.

A third type of fibre (tertiary fibre) has been described in large muscles of large animals (sheep, Wilson *et al.*, 1992; human, Draeger *et al.*, 1987). Tertiary fibre formation uses the secondary fibres as a structural framework for development in much the same way that secondary fibres form around primary fibres. In sheep, the presence of tertiary myotubes has been observed by day 62 of gestation (Wilson *et al.*, 1992).

The maximum number of fibres is reported to be reached by 100 days of gestation in the sheep fetus (Ashmore *et al.*, 1972) and the reported increase in fibre number (per unit cross-sectional area) observed after this time is thought to be due to increasing length of the overlapping fibres (Swatland and Cassens, 1973).

1.6.5. Postnatal Muscle Growth

The development of muscle during fetal life is characterized by an increase in muscle fibre number as a result of myoblast hyperplasia during the embryonic and fetal developmental periods. Postnatal muscle growth, however, results from cellular hypertrophy, characterized by increased DNA and protein content (Moss, 1968; Kang *et al.,* 1985) without an increase in muscle fibre number (Smith, 1963).

Protein accretion is an increase in the number of both actin and myosin filaments in myofibrils. In turn, the myofibrils within each muscle fibre split to form new myofibrils resulting in both longitudinal growth of the fibres by the addition of new sarcomeres at the ends of growing fibres (Williams and Goldspink, 1971) and an increase in the diameter of the fibres. Thus, it is mainly the increase in the number of additional myofibrils that comprises muscle fibre hypertrophy. Since myosin and actin are major muscle proteins (Guyton, 1991), constituting over 50% and 10% of all myofibrillar proteins, respectively, and are important in myofibrillar assembly, these proteins have been used as markers to investigate skeletal muscle protein synthesis. For example, Helferich *et al.* (1990) reported that an increase in skeletal muscle hypertrophy is accompanied by an increase in the fractional synthesis rate of alpha-actin in pigs. An increase in the abundance of alpha-actin mRNA was also observed.

Muscle fibre hyperplasia only occurs during fetal life, whereas hypertrophy may occur throughout all stages of growth and requires proliferation of satellite cells in late fetal and postnatal life. Although satellite cell proliferation and incorporation into existing muscle fibres does not result in an increase in muscle fibre number, due to the subsequent fusion of the cells with existing muscle fibres, this process is still considered a hyperplastic event because cell proliferation and an increase in nuclei number are involved. Addition of new nuclei from satellite cells results in the increase in DNA content of the fibres that is necessary for growth, as DNA synthesis stops in terminally differentiated myoblasts (Moss and Leblond, 1970, 1971). Thus, DNA content can be used to indicate changes in cell proliferation in muscle tissue, e.g., satellite cell proliferation. Although tissue DNA content is often used as an index of

cell number, this index must be used with caution if estimating changes in skeletal muscle fibre number due to the multinucleated nature of skeletal muscle cells.

1.7. FACTORS INFLUENCING MUSCLE GROWTH AND DEVELOPMENT

1.7.1. Myogenic Regulatory Factors (MRFs)

Skeletal muscle is formed by fusion of myogenic cells that originate in the dorsomedial region of each developing somite (Chevallier *et al.,* 1977; Christ *et al.,* 1977; Kenny-Mobbs, 1985; Buckingham, 1992; Christ and Ordahl, 1995). The formation of skeletal muscle involves not only commitment of these myogenic cells to the skeletal muscle lineage, but also withdrawal of myoblasts from the cell cycle, and the transcriptional activation of a battery of muscle structural genes. The cellular and molecular mechanisms that are involved in the formation and diversification of myogenic cells are unknown. A family of muscle-specific transcription factors, the myogenic regulatory factors (MRFs), however, are likely to play a major role.

Four members of the MRF family of transcription factors have been identified to date in mammals: MyoD (Davis *et al.,* 1987), myogenin (Wright *et al.,* 1989; Edmondson and Olson, 1989), Myf-5 (Braun *et al.,* 1989) and Myf-6/MRF-4/herculin (Rhodes and Konieczy, 1989; Braun *et al.,* 1990; Miner and Wold, 1990). These factors are DNA-binding proteins and form a basic helix-loop-helix (bHLH) family of proteins, which can activate skeletal muscle gene expression in a wide variety of both muscle and non-muscle cell types (reviewed by Olson, 1990; Weintraub *et al.,* 1991; Rudnicki and Jaenisch, 1995). The MRFs are localized in the nucleus and contain a bHLH motif which mediates dimerization and DNA binding, which has about 80% amino acid homology (Murre *et al.,* 1989 ab; Davis *et al.,* 1990). Homologues of each of the myogenic factors have been identified in numerous mammals, birds and frogs (Olson, 1993). Remarkable conservation among these factors suggests that the mechanisms which regulate muscle-specific gene expression are highly conserved.

The myogenic bHLH proteins activate the expression of skeletal muscle genes by binding to a consensus DNA sequence (CANNTG). This DNA sequence, known as an "E-box", is found in the control region of many, but not all, skeletal muscle genes (Hauschka, 1994) and is necessary for transcriptional activation of many skeletal muscle genes. However, the E-box cannot function alone: it depends on the adjacent binding sites of other factors, such as MEF-2 binding sites (Olson *et al.,* 1995), to activate transcription of muscle-specific genes. Thus, these factors do not act alone. Instead they interact with components of the cell cycle machinery to control the withdrawal of the myoblasts from the cell cycle, and then act in combination with other transcription factors to activate transcription of muscle-specific genes.

Early reports (see Olson, 1990 for review) suggested that the MRFs may play a role in the commitment of precursor cells to the myogenic lineage. However, Tajbakhsh and Buckingham (1994) reported that the MRFs are not expressed at this stage of development. Although the MRFs are able to activate skeletal muscle differentiation in cultured muscle and non-muscle cells (Rudnicki *et al.,* 1992), they each exhibit distinct expression patterns *in vivo*, suggesting that they have unique roles in muscle differentiation. The first member of the family to be expressed in the mouse is Myf-5 which is first seen at embryonic day 8 (E8) (Ott *et al.,* 1991; Smith *et al.,* 1994). Half a day later (E8.5) myogenin expression is observed (Sassoon *et al.,* 1989; Wright *et al.,* 1989; Cheng *et al.,* 1993; Yee and Rigby, 1993) followed by the expression of Myf-6 and MyoD at E9.5 and E10.5, respectively (Bober *et al.,* 1991; Hinteberger *et al.,* 1991). In the limb buds, Myf-5 expression is observed at E10.5 and the expression of MyoD and myogenin is observed half a day later. This order of expression during development varies according to muscle origin and the species being studied (reviewed by Sassoon, 1993). This suggests that each member may regulate a different stage of development and the possibility that the order of expression, and/or the type of factor expressed at a given time during development, may give rise to the different muscle types.

Gene knockout experiments have investigated the roles of these factors during muscle development *in vivo* (see reviews by Olson and Klein, 1994 and Lassar and

Munsterberg, 1994). Mice lacking either the Myf-5 or MyoD gene develop normal skeletal muscle, but, if both genes are lacking, myoblasts fail to form (Braun *et al.*, 1992; Rudnicki *et al.*, 1992; Rudnicki *et al.*, 1993). This suggests that Myf-5 and MyoD have similar roles. However, it was recently reported that Myf-5 and MyoD each determine different muscle cell lineages from independently committed stem cells (Braun and Arnold, 1996). In contrast to MyoD- and Myf-5-null mice, mice lacking the myogenin gene exhibit normal myoblast formation, but there is a large reduction in the number of skeletal muscle fibres that form (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). These results suggest that myogenin is necessary for muscle differentiation. But, since myogenin can convert fibroblasts to myoblasts and induce the expression of MyoD (Edmondson and Olson., 1989; Wright *et al.*, 1989), an earlier role for myogenin during myogenesis is not impossible. However, a recent report demonstrates that the functions of myogenin do not overlap those of MyoD or Myf-5 (Rawls *et al.*, 1995) and that myogenin is particularly important for the late stages of embryogenesis while it is not required for the initial aspects of myogenesis including the appearance of myoblasts (Venuti *et al.*, 1995). It has recently been reported that, in addition to controlling transcription of muscle structural genes, myogenin may also control the extracellular environment in which myoblasts fuse (Myer *et al.*, 1997).

Mild alterations in skeletal muscle development and an imbalance of contractile protein isoform expression is observed following inactivation of the Myf-6 gene (Braun and Arnold, 1995). Myf-6 may also be able to substitute for myogenin in activating early muscle gene expression (Zhang *et al.*, 1995). In general, Myf-5 and MyoD act to establish the myogenic lineage by controlling myoblast determination, while myogenin and Myf-6 mediate the activation and maintenance of muscle gene expression (Weintraub, 1993; Rudnicki and Jaenisch, 1995; Venuti *et al.*, 1995; Braun and Arnold, 1995, 1996). All four MRFs are present in the early embryonic period, but by late fetal life, myogenin, MyoD and Myf-6 are more abundant (Bober *et al.*, 1991; Hinterberger *et al.*, 1991). Myf-6 is the major MRF detected in postnatal muscle, which suggests a possible role in the maintenance of muscle gene transcription (Rhodes and Konieczny, 1989). Myogenin and MyoD transcripts are also detectable postnatally, but at low

levels. Recently, MyoD has also been described as a good marker for active satellite cells (Koishi *et al.,* 1995) (see section 1.7.3.).

1.7.2. Peptide Growth Factors

The myogenic progenitor cells that form within the somites during vertebrate embryogenesis are committed to the myogenic fate. These cells require appropriate environmental signals before the phenotypic markers of muscle are expressed. The exact signals involved in this process *in vivo* are unknown.

Cultured skeletal myoblasts have been used to investigate the signals involved in activating differentiation. In this system, myoblasts remain in the proliferative phase of the cell cycle and cannot enter the differentiation pathway until the concentrations of certain peptide growth factors (e.g., TGF-β and FGF) are decreased below a critical threshold (Olson, 1992). Following growth factor withdrawal, myoblasts are able to exit from the cell cycle and irreversibly differentiate. This is followed by fusion of the differentiated myoblasts to form multinucleated myotubes and transcriptional activation of muscle-specific genes. Once myoblasts have fused, their nuclei lose their ability to synthesize DNA and muscle-specific genes become refractory to repression by exogenous growth factors (Nadal-Ginard, 1978). In contrast to these growth factors that have a negative influence on myoblast differentiation, insulin-like growth factors (IGFs) have been implicated as having a stimulatory effect on the myogenic differentiation pathway.

1.7.2.1. Insulin-like Growth Factors as Positive Regulators of Myogenesis

In addition to the role that the IGFs play in anabolism (in muscle cultures IGF-1 increases protein synthesis in both fetal and postnatal muscle), the IGFs are also important for muscle development and growth. IGF-1 and IGF-2 are thought to act on growth and development by autocrine and paracrine mechanisms (Daughaday and Rotwein, 1989; Baker *et al.,* 1993). Myoblast differentiation is accompanied by an increase in the levels of IGFs and induction of their cell surface receptors (Olson, 1992, 1993) supporting the idea that IGFs can stimulate myoblast differentiation via an autocrine loop.

The mechanisms by which IGFs stimulate muscle gene expression are largely unknown. IGFs have been shown to up-regulate myogenin expression (Florini *et al.,* 1991) and therefore the IGFs may act by targeting the myogenin gene. IGF-1 and -2 receptors are also up-regulated during differentiation in culture, providing a positive feedback loop through which amplification of the actions of IGFs on myogenic differentiation could be achieved (Olson, 1993).

Using cultured myoblasts to study the effects of IGFs on the myogenic differentiation pathway gave results which contrasted with those observed *in vivo.* For example, IGF-2 *in vitro* (Montarras, 1996) appears to be essential for muscle formation, but disruption of the IGF-2 gene *in vivo* does not seem to affect muscle formation (De Chiara *et al.,* 1990). This is likely to be due to complementation by IGF-1 (Liu *et al.,* 1993). IGF-1 is also required *in vitro,* while mice lacking IGF-1 are reported to have underdeveloped muscle tissue (Powell-Braxton *et al.,* 1993). Montarras (1996) reported a link between IGF-2 and MyoD expression using loss or gain of function experiments. The relationship between MyoD and IGF-2 *in vivo* has not been reported as MyoD- and IGF-2-deficient animals are yet to be produced. Therefore, the precise role of the IGFs in the regulation of myogenesis *in vivo* is unclear and requires further investigation. Eludication of the effects of IGFs on myogenesis in culture is further confounded by species differences in the action of these factors on proliferation and differentiation. It is outside the scope of this review to discuss these species differences in the myogenic responses to IGFs, however, these differences have been reviewed extensively elsewhere (Allen and Boxhorn, 1989; Florini and Magri, 1989; Allen and Rankin, 1990; Harper and Buttery, 1995).

1.7.2.2. Transforming Growth Factor-β (TGF-β) and Fibroblast Growth Factor (FGF)

In vitro, TGF-β appears to have a potential regulatory role in muscle development because it can inhibit the progression of myoblasts to the differentiated state even in the absence of proliferation (Massague *et al.,* 1986; Florini *et al.,* 1986; Allen and Boxhorn, 1989; Hathaway *et al.,* 1991). TGF-β acts by binding to specific receptors

(Massague, 1992), with the type 1 and type 2 receptors mediating most of its biological effects (Laiho *et al.*, 1990).

TGF-β blocks the expression of both MyoD (Vaidya *et al.*, 1989) and myogenin (Brennan *et al.*, 1991) and thereby prevents the expression of downstream muscle-specific genes. TGF-β has also been implicated in determining fibre type. The differentiation of embryonic myoblasts (which form primary fibres) and fetal myoblasts (which form secondary fibres) is differentially affected by TGF-β *in vitro* (Cussella-De Angelis *et al.*, 1994). These results suggest a possible mechanism by which TGF-β may influence the formation of primary and secondary fibres *in vivo*, although there is no experimental evidence for this. Recently, a new TGF-β superfamily member, growth/differentiation factor-8 (GDF-8) was identified and implicated as a potent negative regulator of skeletal muscle growth (McPherron *et al.*, 1997).

Fibroblast Growth Factors are signalling molecules that are important in patterning and growth control during vertebrate limb development, and FGF-8 has been reported to be a key signal involved in the initiation, outgrowth and patterning of the developing vertebrate limb (Vogel *et al.*, 1996). FGF also appears to advance the myoblasts through the cell cycle to a point beyond the stage at which differentiation occurs (Lathrop *et al.*, 1985). In contrast, FGF is reported to stimulate proliferation of satellite cells but to depress differentiation (Allen and Boxhorn, 1989). FGF has also been shown to inhibit DNA binding of myogenin (Li *et al.*, 1992) and possibly other myogenic regulatory factors. These differences in the action of FGFs may be due to differential responsiveness to FGF between species. Species differences in the myogenic responses to both FGFs and TGF-β have been extensively reviewed (Florini and Ewton, 1988; Allen and Boxhorn, 1989; Florini and Magri, 1989; Allen and Rankin, 1990; Hathaway *et al.*, 1994; Harper and Buttery, 1995) and discussion of these differential responses is outside the scope of this review.

1.7.3. Role of the MRFs and Growth Factors in the Regulation of Satellite Cell Proliferation and Differentiation

The mechanisms by which satellite cells are regulated are unclear, mainly because, until recently, there was no adequate marker for active satellite cells. Koishi *et al.* (1995) have identified MyoD as a marker for active satellite cells, which could enable mechanisms of satellite cell regulation to be eludicated. MyoD is a member of the basic helix-loop-helix transcription factor family and is expressed in myogenic cells in muscle-forming regions from early stages of (rodent) development, reaching a peak around birth, suggesting a role for MyoD in the formation and differentiation of myoblasts (Eftimie *et al.,* 1991; Witzemann and Sakmann, 1991). MyoD mRNA continues to be expressed in adult muscle, suggesting that it also functions to control gene expression in the adult (Hughes *et al.,* 1997). MyoD is expressed in proliferating (active) satellite cells, but its expression ceases following the fusion of the satellite cell to an existing fibre in neonatal mouse tissue (Koishi *et al.,* 1995), although some labeling of recently incorporated myonuclei in adult mouse tissue occurs (Hughes *et al.,* 1997). MyoD appears not to be expressed in quiescent satellite cells which are found in mature muscle once muscle mass becomes static (Koishi *et al.,* 1995).

By altering the concentration of TGF-β, IGF-1 and FGF, satellite cells can be induced to proliferate, differentiate or to remain quiescent. Since TGF-β can inhibit differentiation of myoblasts (and satellite cells), but cannot depress the mitogenic actions of IGF-1 and FGF (which stimulate proliferation), depression of the levels of TGF-β may cause MyoD expression to be elevated. This may result in differentiation of the satellite cells and trigger their subsequent fusion to existing fibres, in the same way that MyoD expression is regulated in fetal and embryonic myoblasts.

Since members of the myogenic regulatory factor family are expressed during muscle differentiation in embryonic and fetal development, and MyoD has been implicated as having a possible role in the maintenance of satellite cells in an active state in the myogenic lineage, the other members of the family (i.e., myogenin and myf-5 and -6) may also play a role in muscle fibre hypertrophy and hyperplasia which is as yet

undefined. Therefore, by studying the expression and localization of MyoD and the other members of this family, and the roles of TGF-β, IGF-1 and FGF in regulating satellite cell proliferation and differentiation, it may be possible to gain a better understanding of muscle hypertrophy, not only in the postnatal animal but also in the late stages of fetal development, an area which has received little attention in the past.

1.7.4. Hormones

Several hormones may have important effects on myogenesis. For example, thyroid hormones are thought to play a role in the development of the different muscle phenotypes (fast or slow) due to their differential effects on the myosin heavy chain (MHC), fast and slow isoforms. In altrical species (e.g., rats), thyroid hormones appear to have a direct effect on the development of the fast phenotype (Gambke *et al.,* 1983; Russell *et al.,* 1988). In contrast, the development of the slow phenotype occurs independently of thyroid hormones. Rather it is dependent on locomotor activity and the innervation received by the slow muscle. Similarly, in precocial species such as the sheep, fetal thyroidectomy illustrates that the functional maturation of fast-twitch muscles is influenced by the presence of an intact thyroid from at least 70 days of gestation (Finklestein *et al.,* 1991). Slow-twitch muscle development in these animals occurs independently of thyroid hormone influence.

Several other agents may have stimulatory or inhibitory effects on myogenesis such as beta-agonists (Grant *et al.,* 1990) including clenbuterol (Maltin *et al.,* 1990); glucocorticoids (Kayalia *et al.,* 1987) and insulin (Dodson and Mathison, 1988). These hormones will not be discussed in this review (see review by Florini, 1987; Harper and Buttery, 1995).

1.7.5. Innervation

Cross-innervation and chronic stimulation studies have shown that neural activity plays an important role in determining the contractile characteristics of fast- and slow-twitch muscle. There is evidence for both nerve-dependent and nerve-independent secondary

fibre formation (Condon *et al.,* 1990; Fredette and Landmesser, 1991) and evidence that secondary fibres differ from primary fibres in the manner in which myoblasts fuse to form them (Harris *et al.,* 1989). The specific patterns of neuronal activity are thought to be important, if not critical, for phenotypic development of muscles following birth (Navarette and Vrbova, 1983; Vrbova *et al.,* 1985). This is especially true for altricial species whose muscles are not phenotypically developed. In these animals, the development of muscles with fast or slow contractile characteristics depends on neural activity, associated with postural stability and movement. The functional development of muscles from precocial species is relatively complete at birth. Therefore, it must be assumed that fetal pandiculation (movement *in utero*) allows normal muscle development, that there are neural signals reaching muscles other than those that cause movement, or that phenotypic development of muscles of precocial species occurs independently of activity or any other neural growth signal (Walker and Luff, 1995).

There is some evidence that the MRFs may be involved in these processes. Following denervation in the rat, the expression of all four MRFs, especially myogenin, is up-regulated (Voytik *et al.,* 1993). The rapid increase in myogenin transcripts in denervated muscle is similar to the rapid increase in myogenin expression observed during the myoblast differentiation pathway *in vitro.* Therefore, it is possible that myogenin is involved in the initial differentiation pathway in myoblasts as well as a rapid response programme in adult denervated fast-twitch muscle, while the other factors may modulate specific aspects of individual muscle fibres. Future studies focusing on the role of each factor in establishing and maintaining the muscle phenotype will be required to answer these important questions.

1.7.6. Mechanical Activity

The type and pattern of mechanical activity plays an important role in the development of muscle, including passive stretch imposed by bone growth, shortening of muscle fibres during movement or exercise (Goldspink *et al.,* 1992) or chronic stretch imposed by limb immobilization (Williams and Goldspink, 1973), which each have differing effects on muscle development.

Limb immobilization experiments illustrate the importance of stretch for the development of muscle. Immobilization of a limb in the stretched position results in an increase in the muscle wet weight and the RNA content and can induce fibre type transformations. The increase in muscle length following chronic stretch is related to the formation of new sarcomeres and myofibrils within existing fibres, and the addition of new myotubes to the ends of these muscles (Dix and Eisenberg, 1990). Conversely, when a limb is immobilized in the shortened position, muscle length and mass are reduced as a result of the loss of sarcomeres and myofibrils from the ends of muscles (Williams and Goldspink, 1973).

In the sheep, where functional development of muscles is virtually complete at birth, limb movements during fetal life occur in the freedom of the amniotic sac. Since most of the functional development of the muscles is completed under these circumstances, activity may not be important for muscle development. However, as noted by Fraser (1989), fetal movements, which occur from early gestation, may play a part in the development of muscles, tendons and joints. Muscle and bone development are reported to be affected in abnormal conditions where the fetus is mechanically restricted *in utero.* Examples include, oligohydramnios, where the amniotic fluid volume is chronically reduced, and situations of fetal immobility, as in some cases of *spina bifida* and spinal atrophy. Moessinger (1988) also reported a reduction in muscle mass in the limbs following fetal immobilization. Therefore, the stretch imposed by bone growth and limb movement may be an important determinant of muscle growth. Whether differentiation of fibre type is affected under these conditions is yet to be determined.

1.8. INFLUENCE OF BODY SIZE ON MUSCLE GROWTH AND DEVELOPMENT

Despite the important relationship between muscle fibre number and meat content of an animal (Luff and Goldspink, 1967; Swatland and Kieffer, 1974) there are few quantitative studies of muscle development in agricultural animals. Myogenesis in the sheep fetus has been described previously (Ashmore *et al.,* 1972; Swatland and

Cassens, 1973; Maier *et al.,* 1992; Wilson *et al.,* 1992), and the effects of maternal nutrient deprivation during gestation on postnatal muscle development and growth of lambs has also been described (Nordby *et al.,* 1987). Muscle fibre formation occurs during the fetal period and is complete by birth in precocial species such as sheep (Ashmore *et al.,* 1972; Swatland and Cassens, 1973) emphasising the importance of the gestational period in the long-term growth potential of animals. However, the effect of perturbations on fetal size, such as litter size and seasonal effects on muscle development and growth during gestation, have received little or no attention in the past.

Skeletal muscle represents a large proportion of the total carcass of an animal (50-60% by weight in sheep). Therefore, any reduction in body size would imply that muscle development or growth has been impaired in some way, resulting in a proportionate or disproportionate decrease in the overall mass of muscle. Since the maximum number of fibres in a muscle from large animals is reached prior to birth, adverse prenatal conditions, such as maternal undernutrition (Wigmore and Stickland, 1983; Ward and Stickland, 1991) and denervation (McLennan, 1983) may reduce the number of muscle fibres which can impose permanent effects on postnatal growth rate (Hegarty and Allen, 1978) and size, as the normal fibre complement cannot be restored (Bedi *et al.,* 1982; Wilson *et al.,* 1988). Runting in pigs results in a decrease in the number of fibres in a muscle (Ward and Stickland, 1991; Dwyer and Stickland, 1992) as a result of a reduction in the secondary fibre complement (Wigmore and Stickland, 1983; Handel and Stickland, 1987; Wilson *et al.,* 1988; Ward and Stickland, 1991). In contrast, Hunt *et al.* (1996) compared lambs selected for high birth weight and low birth weight and found that although muscle weight was reduced, fibre number was not affected. Muscle fibre phenotype (fast-twitch or slow-twitch) was also not affected, however, the DNA content of the muscles from the high-birth-weight lambs was greater than the low-birth-weight lambs, suggesting a greater nuclei number. The effect of body size on muscle fibre cross-sectional area was not reported in that study. Swatland and Cassens (1973) also suggested that the decrease in body size caused by inadequate maternal nutrition and competition between fetal sheep, is associated with a restriction of the longitudinal growth of the intrafascicularly terminating fibres rather than an effect on fibre number *per se*. Coupled with lower birth weight, twins have proportionally

smaller muscles at birth (Sailer *et al.,* 1994) and tend to grow more slowly than singles postnatally, which can result in twin lambs reaching market weight up to 3 weeks later than singles (Nordby *et al.,* 1987) suggesting impairment of muscle development. These results suggest that body size at birth may be an indicator of alterations in muscle development and growth during fetal life.

The two models discussed earlier in the review, i.e., the seasonal model and the litter size model, illustrate that fetal growth can be altered during gestation without extreme manipulation of the ewe placenta or fetus. The difference in birth size also suggests that disruption of the normal uterine environment, could alter the processes involved in the regulation of fetal muscle development and growth. However, studies to date offer little indication as to what morphological changes, if any, take place within the muscles of the growth restricted *ovine* fetus. The precise timing of such alterations also remain unexplained and unexplored. Therefore, the objective of this series of studies is to use the seasonal and litter size models to study myogenesis in the sheep fetus in order to identify morphological characteristics and possible control mechanisms that explain or contribute to the difference in size observed at birth.

All of the procedures described in the following chapters have been approved by the Massey University Animal Ethics Committee.

Chapter Two

SEASONAL EFFECTS ON FETAL GROWTH IN SHEEP

This chapter has been published; McCoard, S.A.; Peterson, S.W.; Jenkinson, C.M.C.; Campbell, J.W. and McCutcheon, S.N. (1996). Seasonal effects on fetal growth in sheep. *Proc. N.Z. Soc. Anim. Prod.* **56**:297-300.

2.1. ABSTRACT

Previous studies have shown that autumn-born lambs are lighter at birth than spring-born lambs, an effect that is associated with impaired placental development in ewes destined to lamb in the autumn. As previous studies have focused on near-term fetal weights and birth weights, the objective of this study was to determine the stage of gestation at which this effect on fetal weight can first be observed. Mature Romney ewes were randomly assigned to groups mated in December (n=23) or March (n=28) and managed on pasture in both seasons for similar live weight profiles from mating to day 140 of gestation. Ewes were slaughtered at day 56, 84, 112 or 140 of gestation, and measures of fetal and placental weight were determined. The weight (kg) of the fetuses from December- versus March-mated ewes were: d56 (0.04±0.002 vs 0.04±0.002, P>0.10); d84 (0.43±0.02 vs 0.49±0.02, P<0.05); d112 (2.01±0.10 vs 2.10±0.08, P>0.10); d140 (4.77±0.29 vs 5.62±0.26, P<0.10). December-mated ewes exhibited lower cotyledon (221.47±22.26 vs 333.27±20.59g, P<0.001) and placentome (299.78±18.87 vs 368.35±18.06g, P<0.01), but not caruncle (137.46±14.44 vs 112.91±10.99g, P>0.10) weights, than March-mated ewes (pooled across gestational ages). There were no significant differences in caruncle or cotyledon numbers. These results suggest that the effect of season on fetal development is established early in pregnancy (by day 84 of gestation), and is associated with impaired placental development in the December-mated ewes, which is consistent with previous results.

2.2. INTRODUCTION

Earlier reports have indicated that autumn-born lambs have substantially (by up to 25%) reduced growth *in utero* and birth weights compared to spring-born lambs (Reid *et al.* 1988; Peterson, 1992; Morris *et al.*, 1993; Jenkinson *et al.*, 1994). This difference occurs in the absence of marked differences in maternal liveweight change and is associated with impaired placental development in lambs destined to be born in the autumn (Jenkinson *et al.*, 1994).

Since studies conducted to date have examined only seasonal effects on birth weight or on the weight of fetuses at day 140 of gestation, it is not known when in gestation the

difference in weight arises. This experiment was therefore designed to address that question: specifically, it sought to test the hypothesis that there is a gradual divergence in weight between fetuses destined to be born in the autumn versus spring and to further examine the seasonal differences in placental development first identified by Jenkinson *et al.* (1994).

2.3. MATERIALS AND METHODS

2.3.1. Animals and Treatments

Sixty-two Romney ewes aged five to six years were used in a 2 x 5 factorial design incorporating two seasons of conception (conceived to lamb in autumn or spring) and five stages at slaughter, days 0 (controls), 56, 84, 112, and 140 of gestation, with six ewes per cell. Ninety-six ewes in the autumn-lambing group and sixty-six ewes in the spring-lambing group were mated after being induced to cycle synchronously. Autumn-lambing (December-mated) ewes were synchronised using progesterone-impregnated controlled internal drug releasers (CIDRs; Eazi-breed CIDR type G, Carter Holt Harvey Plastic Products, Hamilton, New Zealand) plus 400 iu of pregnant mare serum gonadotrophin (PMSG Folligon, Intervet International B.V. Boxmeer, Holland), while only CIDRs were used in the spring-lambing (March-mated) group. Six ewes for the control groups (slaughtered on day 0 of gestation) were randomly chosen following CIDR removal. The same six Suffolk rams were introduced at CIDR removal in December and March and mating marks produced by the harnessed rams were recorded daily. Pregnancy status (single versus twin) was determined by ultrasound fifty-three days after mating (and again at day eighty-one in the autumn-lambing group to confirm previous results). This resulted in twenty three and twenty eight single-bearing ewes in the autumn-lambing and spring-lambing groups respectively. Ewes were assigned at random to slaughter dates.

Ewes within each seasonal mating group were grazed on pasture as one mob and monitored (via weekly weighing) so that the two seasonal groups had similar patterns of liveweight change throughout gestation. Controlled grazing was used to minimise seasonal difference in maternal nutrition (as measured by live weight) which could lead

to changes in fetal growth. The control animals were used only to generate live weight and carcass weight data.

2.3.2. Slaughter Procedure

Ewes were slaughtered by captive bolt pistol and exsanguination. Slaughter was conducted between 0800 h and 1300 h, and the data on each ewe (excluding caruncle and cotyledon weights) collected within forty-five minutes of slaughter. The uterus was removed from the ewe and ligated at the utero-cervical junction and the cervix, vagina, ovaries and excess tissue removed before the gravid uterus was weighed. The outer curvature of the gravid uterus was carefully opened to expose the intact amniotic and allantoic fluid sacs. The weights of amniotic and allantoic fluids were recorded separately. The fetus(es) were gently squeezed to remove amniotic fluid from the wool. The umbilical cord was ligated near the point of attachment to the fetal abdomen and approximately 5 cm distal to that point, and the umbilical cord severed between the two ligatures. The fetus was then removed from the uterus and fetal weight, sex, curved crown-rump length (with the fetus lying in a "relaxed" position) and chest girth measurements were recorded. Fetus(es) still alive at this stage (n=19) were euthanased using an overdose of sodium pentobarbitone (Pentobarb 500, Chemstock Animal Health, Christchurch, New Zealand). Uteri were stored at 4°C in sealed plastic bags until dissection on the afternoon following slaughter, except for the uteri from the December-mated ewes which were slaughtered on days 0, 56 and 112 of gestation. These uteri were immediately placed into sealed plastic bags following slaughter and frozen for no more than 3 months before dissection. The placentomes were separated into the caruncle and cotyledon portions by applying gentle pressure at the base of the caruncle portion which resulted in easy separation of the two portions. Although no histological analyses of the two portions were made to ensure distinct separation, each portion was of distinct colour and texture as described by Alexander (1964). Furthermore, in other studies, histological analyses of the two portions following separation showed that negligible amounts of caruncle tissue remained on the cotyledon and vice versa (K. Sinclair, Scottish Agricultural College, pers. comm.). Despite the fact that there may be small amounts of caruncle tissue remaining on the cotyledon and

vice versa, the amounts are negligible in relation to the observed differences in weights of the cotyledon and caruncle tissue between treatment groups. The number and weights of individual caruncles dissected from the non-pregnant uteri, and cotyledons and caruncles dissected from the pregnant uteri, were recorded. The weights of the fetal membranes and the myoendometrium were also recorded. Following dressing out of the ewes, the maternal carcass weight was recorded.

2.3.3. Statistical Analysis

All data were analysed using an Analysis of Variance for a 2 x 5 factorial to determine effects of season, stage of gestation and their interactions. Data are expressed as means±s.e.m. for ewes and their fetuses in both groups. Statistical analyses were conducted using the computer package Minitab (Release 10, 1994).

2.4. RESULTS

The autumn-lambing (n=17) and spring-lambing (n=22) ewes used in this trial all carried singles. In addition to these animals, 6 ewes in each season were slaughtered at day 0 (controls).

Table 2.1. shows the live weight and carcass weight profiles for autumn- and spring-lambing ewes from day 0 (mating) through to day 140 of gestation. There were no significant effects of season on live weight but spring-lambing ewes had significantly ($P< 0.05$) heavier carcasses than autumn-lambing ewes.

Spring-lambing ewes had a greater total gravid uterus weight (TGUW) than autumn-lambing ewes ($P<0.01$, Table 2.2.). This difference in TGUW between seasons was associated with a difference in amniotic fluid ($P<0.01$), myoendometrium ($P<0.01$) and fetal membrane ($P< 0.001$) weights although there was no effect of season on allantoic fluid weight.

Table 2.1. Live weight and carcass weight of Autumn- vs Spring-lambing ewes (mean± s.e.m.) at 0, 56, 84, 112 and 140 days of gestation, and the significance of Season, Stage of Gestation (Stage) and the Season x Stage (S x S) interaction.

	Season	Stage of gestation (days)					Significance		
		0	56	84	112	140	Season	Stage	S x S
Ewe Number	Aut	6	4	4	4	5			
	Spr	6	5	5	6	6			
Live weight (kg)	Aut	58.5 ± 2.3	57.3 ± 2.3	57.7 ± 1.5	57.7 ± 1.5	62.0 ± 2.2	NS	*	NS
	Spr	55.4 ± 2.1	57.9 ± 2.0	58.5 ± 2.0	61.6 ± 1.2	64.7 ± 2.0			
Carcass weight (kg)	Aut	26.1 ± 1.5	26.1 ± 1.6	23.8 ± 1.4	24.0 ± 1.9	21.5 ± 1.9	*	NS	NS
	Spr	27.2 ± 1.5	26.8 ± 1.5	27.6 ± 1.2	27.6 ± 1.6	27.4 ± 1.7			

NS, P>0.10; *, P<0.05; Aut = Autumn-lambing; Spr = Spring-lambing.

Table 2.2. Total gravid uterus weight and weights of placental components of Autumn- and Spring-lambing ewes (mean ± s.e.m.), at days 56, 84, 112 and 140 of gestation and the significance of Season, Stage of Gestation (Stage) and the Season x Stage (SxS) interaction.

	Season	Stage of gestation (days)				Significance		
		56	84	112	140	Season	Stage	S x S
Ewe Number	Aut	4	4	4	5			
	Spr	5	5	6	6			
TGUW (g)	Aut	623.7 ± 62.2	2083.0 ± 151.6	3610.0 ± 277.2	7593.2 ± 533.9	**	***	**
	Spr	683.8 ± 62.2	2347.0 ± 135.6	4260.0 ± 226.3	9504.7 ± 487.4			
Amniotic fluid wt(g)	Aut	145.2 ± 8.1	538.5 ± 61.1	521.5 ± 156.2	632.8 ± 137.7	**	***	*
	Spr	147.8 ± 7.3	581.8 ± 49.9	735.2 ± 127.6	1250.3 ± 125.7			
Allantoic fluid wt (g)	Aut	31.9 ± 13.9	75.3 ± 14.4	196.8 ± 36.7	908.4 ± 199.3	NS	***	NS
	Spr	41.6 ± 12.5	77.8 ± 11.7	234.0 ± 29.9	1033.2 ± 182.0			
Myoendometrium wt	Aut	123.2 ± 9.5	233.3 ± 21.5	337.1 ± 25.1	584.3 ± 41.3	**	***	NS
(g)	Spr	146.1 ± 8.5	269.1 ± 17.5	398.9 ± 20.5	668.9 ± 37.7			
Fetal membranes wt	Aut	42.1 ± 9.4	97.5 ± 22.3	142.2 ± 12.1	199.8 ± 13.8	***	***	NS
(g)	Spr	68.3 ± 8.4	119.5 ± 18.2	176.0 ± 9.8	274.5 ± 12.6			

NS, P>0.10; *, P<0.05; **, P<0.01; ***, P<0.001; TGUW= Total Gravid Uterus Weight; Aut = Autumn-lambing; Spr = Spring-lambing.

Spring-lambing ewes had significantly heavier placentomes ($P<0.01$, Table 2.3.), due to a significant difference in cotyledon weight ($P<0.001$) rather than an effect of season on caruncle weight. Despite differences in placentome weight, there was no effect of season on cotyledon or caruncle numbers (Table 2.3.).

Table 2.4. shows the weights of fetuses from autumn- and spring-lambing ewes, and their crown-rump length (CRL) and girth measurements. There was a significant overall effect of season on fetal weight ($P<0.05$) and a significant season by stage of gestation interaction ($P< 0.001$) reflecting the gradual divergence in weight (in favour of fetuses from spring-lambing ewes) as gestation advanced. There was no effect of season on CRL although fetuses of spring-lambing ewes had significantly larger girth measurements ($P< 0.01$) than those of autumn-lambing ewes.

Table 2.3. Caruncle, cotyledon and placentome weights and numbers of Autumn- vs Spring- lambing ewes (mean ± s.e.m.) at days 56, 84, 112 and 140 of gestation, and the significance of Season, Stage of Gestation (Stage) and the Season x Stage (SxS) interaction.

	Season	Stage of gestation (days)				Significance		
		56	84	112	140	Season	Stage	S x S
Ewe Number	Aut	4	4	4	5			
	Spr	5	5	6	6			
Placentome wt (g)	Aut	166.1 ± 19.8	561.9 ± 72.6	378.7 ± 40.7	366.7 ± 40.7	**	**	NS
	Spr	171.4 ± 17.7	691.4 ± 64.9	501.4 ± 28.5	512.2 ± 37.2			
Cotyledon wt (g)	Aut	70.3 ± 14.0	348.6 ± 80.2	150.7 ± 20.7	253.4 ± 31.3	***	***	NS
	Spr	96.0 ± 12.5	463.0 ± 71.8	356.2 ± 16.9	401.4 ± 28.6			
Caruncle wt (g)	Aut	95.8 ± 10.9	213.4 ± 57.8	227.9 ± 17.6	113.3 ± 20.0	NS	**	NS
	Spr	75.4 ± 9.8	228.4 ± 51.7	145.2 ± 14.3	110.8 ± 18.3			
Cotyledon no.	Aut	83.8 ± 7.4	97.0 ± 8.0	87.2 ± 8.9	97.4 ± 9.9	NS	NS	NS
	Spr	87.6 ± 6.6	95.5 ± 6.5	87.0 ± 7.3	87.8 ± 9.1			
Caruncle no.	Aut	126.2 ± 6.4	132.0 ± 11.3	87.0 ± 23.5	136.6 ± 12.4	NS	NS	NS
	Spr	129.6 ± 5.8	130.0 ± 9.2	118.8 ± 19.2	115.3 ± 11.3			

NS, P>0.10; **, P<0.01; ***, P<0.001; Aut = Autumn-lambing; Spr = Spring-lambing.

Table 2.4. Fetal weight, crown-rump length (CRL) and girth measurements of fetuses from Autumn- and Spring-lambing ewes (mean ± s.e.m.) at 56, 84, 112 and 140 days of gestation, and the significance of Season, Stage of Gestation (Stage) and the Season x Stage (SxS) interaction.

	Season	Stage of gestation (days)				Significance		
		56	84	112	140	Season	Stage	S x S
Fetal Number	Aut	4	4	4	5			
	Spr	5	5	6	6			
Fetal weight (g)	Aut	39.7 ± 2.3	431.6 ± 19.1	2009.0 ± 101.3	4772.0 ± 288.0	*	***	*
	Spr	38.4 ± 2.1	490.8 ± 17.1	2097.0 ± 82.7	5616.0 ± 262.9			
CRL (mm)	Aut	129.5 ± 1.9	282.5 ± 6.2	426.2 ± 5.8	605.0 ± 15.4	NS	***	NS
	Spr	126.3 ± 1.8	287.0 ± 5.5	449.2 ± 7.8	585.0 ± 14.1			
Girth (mm)	Aut	76.3 ± 1.9	166.3 ± 3.3	266.3 ± 5.3	362.0 ± 8.4	**	***	*
	Spr	71.4 ± 1.7	168.0 ± 2.9	282.5 ± 4.4	389.2 ± 7.6			

NS, P>0.10; *, P<0.05; **, P<0.01; ***, P<0.001; Aut = Autumn-lambing; Spr = Spring-lambing.

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2.5. DISCUSSION

Studies conducted to date have examined only seasonal effects on fetal weight at day 140 of gestation (Jenkinson *et al.,* 1994) or on birth weight (Reid *et al.,* 1988; Peterson, 1992; Morris et al., 1993). However, it is not known when in gestation the seasonal effect on fetal weight occurs. Therefore, the objective of this study was to determine the stage of pregnancy at which this difference in fetal weight occurs and to further examine the difference in placental development first identified by Jenkinson *et al.* (1994).

The magnitude of the seasonal difference in fetal weight at day 140 of gestation (and by inference in birth weight) was consistent with that observed in previous studies. These results suggest that this seasonal effect was associated with a gradual divergence in fetal weight from about day 84 of gestation through to day 140 of gestation in favour of fetuses destined to be born in the spring. However, the bulk of the fetal weight difference arose in the last month of gestation. This effect on fetal weight does not appear to be due to maternal live weight differences as the live weight profiles for both the spring- and autumn-lambing ewes were similar throughout gestation, with the greatest difference (3.9 kg) being observed at day 112 of gestation. However, there was a significant overall effect of season on carcass weight of the dam. This difference in carcass weight (up to 5.9 kg) is unlikely to have been the main cause of the fetal weight difference as live weight differences of up to 10-12 kg have been observed up to day 100 of pregnancy with no obvious adverse effect on birth weight (Parr *et al.,* 1986; Rattray *et al.,* 1987).

The reduced weights of fetuses from December-mated as compared to March-mated ewes were associated with impaired placental development in the December-mated ewes, which is consistent with the findings of Jenkinson *et al.* (1994). However, this impairment of placental development, as measured by a reduction in total placentome weight in December-mated ewes, was not associated with a reduction in placentome number as observed by Jenkinson *et al.* (1994). The difference in placentome weight was due to a difference in total cotyledon weight rather than a seasonal effect on

caruncle weight. Thus, cotyledon weight was the most significant factor contributing to the fetal weight difference, which is consistent with the observation of Alexander (1964) that birth weight was more closely correlated to cotyledon weight than to cotyledon number. Since the placentome is the site of nutrient transfer between the dam and the fetus, reduction in the amount of placentome tissue could restrict nutrient supply to the fetus and hence cause a restriction in fetal growth. Thus, it is likely that the early restriction of placentome weight in ewes due to lamb in the autumn is associated with their retarded fetal growth, although the fact that the reduced placentome weight is mainly a reflection of reduced cotyledonary growth suggests that the seasonal effect is mediated via the fetus rather than via the dam.

In conclusion, these results show that there is a gradual divergence in the weight of fetuses destined to be born in the autumn versus the spring. The weights of the fetuses from autumn-lambing ewes were substantially reduced as compared to fetuses from spring-lambing ewes, an effect which was established early in pregnancy (by day 84 of gestation). This effect of season on fetal growth was associated with impaired placental development which was due to reduced cotyledon weight in the autumn-lambing ewes. These results suggest that further studies on the cause of this seasonal effect on fetal weight should target stages earlier than 84 days of gestation, as the seasonal effect on fetal and placental growth appears to be established by this stage of gestation.

Chapter Three

MATERNAL CONSTRAINT INFLUENCES MUSCLE FIBRE DEVELOPMENT IN FETAL LAMBS.

Chapter 3 has been accepted for publication; McCoard, S.A.; Peterson, S.W.; McNabb, W.C.; Harris, P.M. and McCutcheon, S.N. (1997). Maternal constraint influences muscle fibre development in fetal lambs. *J. Reprod. Fert. Dev.*

3.1. ABSTRACT

The objective of these studies was to examine myogenesis in two situations expected to be characterised by maternal constraint. Experiment 1 was a comparison of myogenesis in autumn- and spring-lambing ewes, whilst in Experiment 2, single and twin fetuses were compared. In Experiment 1, mature Coopworth ewes were randomly assigned to groups mated in December (summer; autumn-lambing; n=10) or March (autumn; spring-lambing; n=10). In Experiment 2, 16 single-bearing and 10 twin-bearing ewes, mated in December, were randomly selected. Ewes in both experiments were managed on pasture from mating until sacrifice at 140 days of gestation, when measures of fetal and placental weight were determined. Fetal hindlimb muscles were weighed and histological characteristics of the *semitendinosus, plantaris* (Experiment 1) and *semitendinosus, plantaris*, medial *gastrocnemius* and *adductor* (Experiment 2) muscles determined. Total placentome weights per fetus were lower in both autumn-lambing compared to spring-lambing ewes (402.7±31.6 vs 534.2±39.4 g, $P<0.05$) and in twin-bearing compared to single-bearing ewes (338.3±26.0 vs 532.4±31.3 g, $P<0.05$), indicating the presence of maternal constraint. Despite the lower placental weight in autumn-lambing ewes, the weights of fetuses due to be born in the autumn or spring were not significantly different (4641±194 and 4725±216 g respectively). In contrast, the smaller placental size per fetus in twin-bearing ewes was associated with lower ($P<0.01$) fetal weights of twins (4478±141 g) compared to singles (5026±194 g). Although an effect on total fetal weight was not observed between seasons there was a reduced fibre number (3.3±0.4 vs 4.7±0.2 $x10^5$, $P<0.01$) and smaller cross-sectional area (32.6±0.9 vs 37.2±1.4 μm^2, $P<0.05$) in the *semitendinosus* muscle of the autumn compared to the spring fetuses. In contrast, neither *plantaris* muscle fibre number (2.9±0.2 vs 2.6±0.2 $x10^5$) nor cross-sectional area (28.6±1.0 vs 31.3±1.2, μm^2) differed between the autumn and spring fetuses. Similar differential effects were seen between muscles of the twin and single fetuses. Muscle fibre number ($x10^5$) was not affected in the *semitendinosus* (4.8±0.2 vs 4.2±0.3), *plantaris* (3.3±0.2 vs 3.0±0.1) or *adductor* muscle (6.4±0.5 vs 5.6±0.4), but fibre number was greater in the *gastrocnemius* muscle (2.3±0.1 vs 1.8±0.09, $P<0.05$) of twin compared to single fetuses. Muscle fibre cross-sectional area (μm^2) was smaller in the *semitendinosus* (66.8±4.3 vs 78.1±3.8, $P<0.05$),

plantaris (58.0±1.9 vs 71.6±2.6, P<0.001), and *gastrocnemius* (79.8±3.8 vs 107.3±10.4, P<0.01) muscle of the twins compared to singles. The fibre cross-sectional area of the *adductor* muscle (63.3±4.5 vs 68.3±7.5, μm^2) was unaffected by rank. The differences in muscle fibre morphology between spring- or autumn-born fetuses, suggest that muscle fibre development was influenced by maternal constraint in the absence of an effect on fetal weight. The differences in muscle fibre number and cross-sectional area in particular muscles from twin and single fetuses suggest that more severe maternal constraint, reflected in a lower placental size per fetus, not only influences fetal weight but can also affect muscle development.

3.2. INTRODUCTION

Muscle mass is determined primarily by the number of fibres in muscle and the size of those fibres. Fibre number has been related to meat content of animals (Luff and Goldspink, 1967; Swatland and Kieffer, 1974), growth rate of several species (e.g., mouse, Ezekwe and Martin, 1975; pig, Dwyer *et al.,* 1995), and postnatal growth potential (Stickland, 1995). Muscle fibre formation occurs during the fetal period and is complete by birth in precocial species such as sheep and cattle (Ashmore *et al.,* 1972; Swatland and Cassens, 1973; Russell and Onteruelo, 1981) which emphasizes the importance of the gestational period in the long-term growth potential of animals.

Fetal growth and development can be constrained by many non-genetic factors such as: maternal uterine capacity; limitations in the supply of nutrients and other substances required for fetal growth; competition between littermates; and restriction of placental size or function, which may result in reduced nutrient transfer from the mother to the fetus. This situation has been termed "maternal constraint". Two situations in which fetal growth restriction is observed as a result of maternal constraint, under relatively non-invasive circumstances, have been described. First, autumn-born lambs are generally lighter than those born in the spring (Reid *et al.,* 1988; Peterson, 1992; Morris *et al.,* 1993). Seasonal differences in fetal weight are evident by day 84 of pregnancy, and are associated with early restriction of placental development in autumn-lambing ewes (Jenkinson *et al.,* 1995; Chapter, 2). Second, as litter size increases there is a

decrease in the birth weight of individual offspring (Donald and Russell, 1970). The average ratio of individual twin lamb weight to the weight of single lambs is approximately 0.80 (Robinson *et al.,* 1977) reflecting their smaller placentas and competition for nutrients.

Coupled with reduced birth weight, twins have proportionally smaller muscles at birth (Sailer *et al.,* 1994; Hunt *et al.,* 1996) and tend to grow more slowly than single lambs postnatally, which can result in twin lambs reaching market weight up to 3 weeks later than singles (Nordby *et al.,* 1987). This suggests that factors which act *in utero* influence subsequent muscle development and growth in twin lambs. Similarly, fetuses nearing term in autumn have lower overall body weight without a concomitant smaller skeletal size, as measured by crown-rump length (Jenkinson *et al.,* 1995; see also Chapter 2), compared to fetuses completing development during spring. Since skeletal muscle represents a large proportion of the total carcass weight of sheep (Kempster *et al.,* 1982), retardation of muscle development and/or growth may have occurred in autumn-born lambs. These two models offer a unique opportunity to examine the development of muscle in the growth-restricted fetus without severe manipulation of the ewe, placenta or uterus.

Myogenesis in the sheep fetus has been described previously (Ashmore *et al.,* 1972; Maier *et al.,* 1992; Wilson *et al.,* 1992), but the effects of maternal constraint on this process have not been described. Thus, the objective of these studies was to examine myogenesis in two situations expected to be characterised by maternal constraint.

3.3. MATERIALS AND METHODS

3.3.1. Animals

Experiment 1. Lambs due to be born in spring and autumn were compared at 140 days of gestation. In both late December (n=40) and March (n=40), oestrus in Romney ewes shorn about one month previously was synchronized with progesterone-impregnated controlled internal drug releasers (CIDRs; Eazi-Breed CIDR type G; Carter Holt

Harvey Plastic Products, Hamilton, New Zealand). December-mated ewes were induced with 275 i.u. of pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet International BV, Boxmeer, Netherlands) to ovulate. The December-mated (autumn-lambing) ewes were artificially inseminated with fresh Suffolk ram semen (as part of an unrelated trial), whereas the March-mated (spring-lambing) ewes were naturally mated using the same rams which had earlier provided the semen. Only ewes mated in the first three days of the first oestrous cycle (in each season) were used. Pregnancy status was determined by ultrasound seventy days after mating, and ten single-bearing ewes were selected from each of the two seasonal groups. In both seasons, ewes were grazed on pasture in which perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) were dominant.

Experiment 2. Forty four-year-old Romney ewes were mated in late December, and thirty-one, four to six year-old Coopworth ewes were mated in early January. Oestrus in both groups was synchronized with CIDRs as described earlier. Ewes were induced with 275 i.u. of PMSG to ovulate. The same Suffolk rams were used for each group. Mating harnesses and crayons were used to identify mated ewes and only those mated in the first three days of the first oestrous cycle were used. Pregnancy status was determined at day seventy of gestation by ultrasound, and sixteen single-bearing ewes and ten twin-bearing ewes (balanced for age, breed and date of mating) were selected. Ewes were grazed on pasture as a single group following mating.

3.3.2. Slaughter Procedure

In Experiment 1 and 2, ewes were slaughtered by captive bolt pistol and exsanguination on day 140 of gestation. The intact uterus was removed, weighed and the fetus(es) exposed. The umbilical cord was ligated and severed, and the fetus(es) weighed and euthanased using sodium pentobarbitone via cardiac puncture. The crown-rump length (CRL) and chest girth were measured. Eight major hindlimb muscles (*biceps, gastrocnemius, plantaris, adductor, semitendinosus, semimembranosus, quadriceps, gluteus)* were dissected from the left hindlimb of each fetus, and their weight and length (using digital callipers) measured. The femur, tibia and humerus bones were also

dissected, and weights and lengths recorded. The placenta was dissected out and the number and weight of caruncles and cotyledons, and weights of maternal and fetal membranes, determined.

3.3.3. Histology Samples

From the eight hindlimb muscles dissected, the *plantaris* and *semitendinosus* muscles were selected for histological characterisation of muscle fibre number and cross-sectional (CS) area in Experiment 1. In Experiment 2, four muscles (*plantaris, semitendinosus, gastrocnemius* and *adductor*) were selected for muscle fibre number and CS area measurement. These muscles were selected on the basis of their size and ease of sectioning. The *gastrocnemius* muscle was divided into two as it is too large to section as a whole. Only the medial part of the *gastrocnemius* muscle was sectioned.

Within 5 minutes of dissection, muscles were frozen rapidly (whole) in isopentane chilled over liquid nitrogen. This resulted in the muscle being frozen near its physiological length. Frozen muscles were wrapped in aluminium foil and stored, initially in liquid nitrogen (up to eight hours), and then at -85°C until sectioning.

Prior to sectioning, the whole frozen muscles were held at -20°C for up to 15 minutes to allow the tissue to equilibrate to this temperature. A mid-belly block (approximately 1cm in length) of tissue was then cut from the whole muscle using a small oscillating bone saw. This technique allowed the inital freezing of whole muscles following dissection from the fetus to avoid confounding effects of freeze-shortening of the fibres (Chamberlain and Lewis, 1989; Roy et al., 1996). This technique also allowed accurate orientation of the muscles to ensure a true CS of tissue was sectioned. The *semitendinosus* muscle is unusual in that it has a fibrous band separating the proximal and distal regions of the muscle. This fibrous band is located approximately one third the length from the proximal end of the muscle (Greenwood, 1997) and thus, was avoided by taking a CS of muscle from the mid-belly region. In addition, no histological evidence of excessive amounts of connective tissue in the sections was evident.

Six 10 μm transverse sections were cut with a cryostat from the mid-belly region of each muscle. Sections were air dried and stained with Haematoxylin Van Geisen (HvG), which stains all fibres yellow, connective tissue red and nuclei blue/black (Plate 3.1.). Sections once stained, were mounted using DPX mountant, and stored at room temperature until analysis of muscle fibre number and CS area.

3.3.4. Estimation of Total Fibre Number

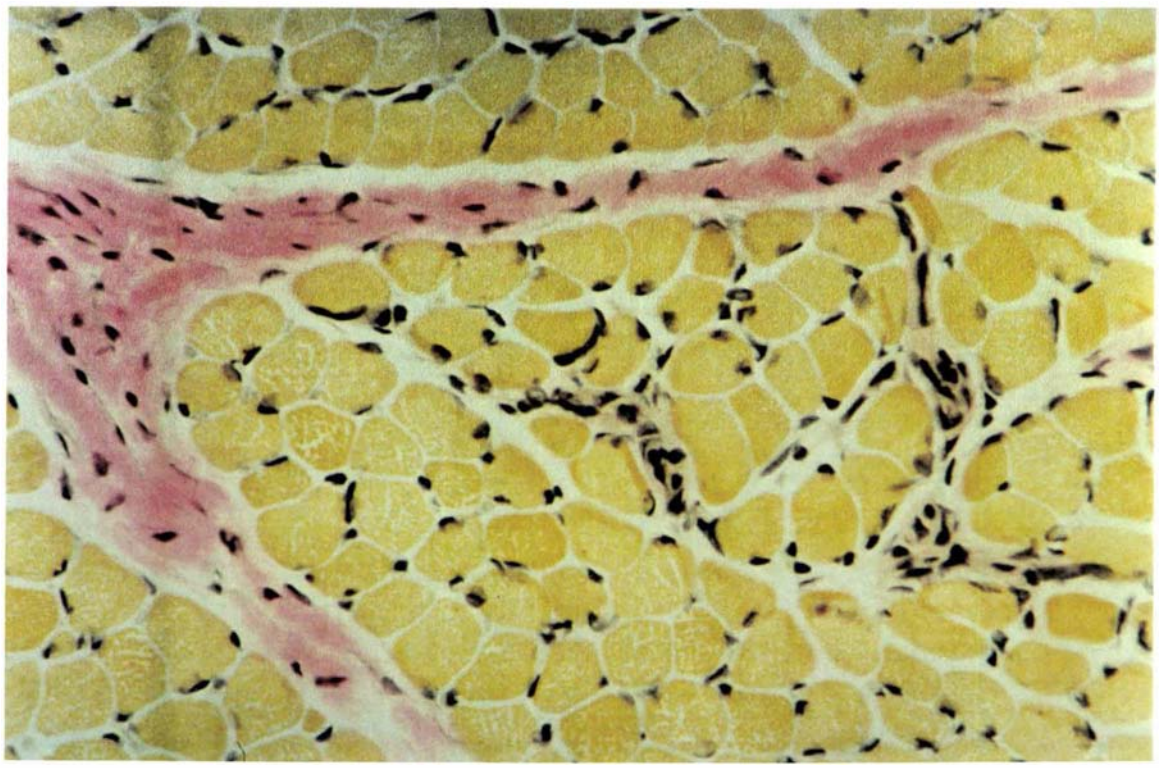
Photomicrographs of 10 randomly-selected areas were used to estimate the total number of fibres in a cross-section of each muscle. The photomicrographs were taken using a random stratified sampling method as follows. A photomicrograph of a portion of the CS at a random starting point on the edge of the section was taken. The subsequent photomicrographs were taken at standard intervals across the diameter of the muscle until 10 photomicrographs had been taken. If the required number were not achieved, then another random starting point on the edge of the muscle was found and the procedure repeated. The number of fibres per photomicrograph was counted according to the method of Gundersen *et al.* (1988). This technique involved counting fibres inside a "guard area" in order to correctly estimate the number of profiles. The total cross-sectional area of the section was determined by projecting the image of each section onto a screen, tracing its outline, and determining the area using a digitizer pad attached to a computer and the Sigma Scan Scientific Measurement Programme (Version 3.90, Jandel Scientific, 1988).

The area of each photo as a proportion of the actual section was determined by photographing a micrometer slide. This was done with each set of micrographs and provided an accurate measure of the magnification. All micrographs were photographed with the same camera, film and magnification and all photographs were processed identically. An estimate of the total number of fibres in each cross-section was determined from the following equation: estimated total fibre number = (muscle area / photographs area) x average number of fibres per photograph. Fibre counts were made from 2 to 5% of the total cross-sectional area of each section and represented approximately 8 to 10% of the total number of fibres present in the muscle cross-section. It must be emphasised that due to the overlapping nature of fibres from

muscles of large animals (Swatland and Cassens, 1972), this gives an estimate of total fibre number in a single cross-section, not an estimate of total fibre number in a muscle.

In order to validate this method of fibre enumeration, a comparison was made of within animal and between animal variation in estimated fibre number per cross-section. First, within animal variation was measured by making repeated estimates (n=12) of fibre number from a single mid-belly CS of the semitendinosus muscle from a single lamb. Each estimate was made using the fibre enumeration technique described above. Second, between animal variation was measured by estimating fibre number in a single mid-belly CS of the semitendinosus muscle from each of 10 single lambs using the above procedure. To determine whether the variability in the fibre number estimate was greater within or between animals, a statistical analysis of the data was made using an F-test (Vital, Version 3.05, B. Fletcher, AgResearch, pers. comm.). Comparison of the estimated and tabulated F-values showed that the between animals variation was larger than the within animal variation in fibre number ($P<0.05$), indicating that the fibre enumeration method was adequate.

Plate 3.1. Skeletal muscle tissue from the *gastrocnemius* muscle from a sheep fetus at 140 days of gestation. The tissue has been stained with Haematoxylin Van Geisen which differentially stains fibres yellow, connective tissue pink and nuclei blue/black (Mag x500).



3.3.5. Fibre Size

Average fibre size was determined from the same sections used to determine fibre number. Cross-sectional areas (CS areas) of individual fibres were traced using a digitiser pad and a computer attached to a microscope. Using the Sigma Scan Scientific Measurement Programme, the areas of the individual fibres were calculated. A total of 200 fibres, taken from 15 randomly-selected areas across each muscle section were measured and the average fibre CS area calculated. When determining fibre CS, the length of the muscle sample when frozen in preparation for histological analysis can have a significant impact on fibre CS area (Chamberlain and Lewis, 1989; Roy et al., 1996). The greatest degree of fibre shortening is observed when a portion of the muscle is removed and frozen as a block, while minimal shortening occurs when a muscle is frozen whole at a fixed length, such as when held with forceps. Since the present study was designed to compare the relative effects of birth rank on fibre CS area, muscles were frozen whole to minimize differences in freeze shortening between muscles.

3.3.6. Statistical Analysis

Data were analysed using a general linear model for Analysis of Variance using Minitab for Windows (Release 10 1994). Results are expressed as unadjusted means±s.e.m. Fetal data were adjusted for sex and maternal breed and these effects remained in the model when significant and are discussed in the text where relevant.

3.4. RESULTS

Experiment 1:. Maternal live weight and carcass weight did not differ between seasonal groups (Table 3.1.).

Total placentome weight was higher in spring- than in autumn-lambing ewes ($P<0.05$), which is a reflection of the differences in total caruncle weight and total cotyledon weight of 42% and 20% respectively (Table 3.2.). There were also more placentomes

in the placentas of the spring-lambing ewes compared to autumn-lambing ewes, and a greater percentage occupancy ((placentome number/ caruncle number) x100).

Fetal weight, CRL and girth measures were not different between seasons (Table 3.3.). There were also no differences between seasons in the individual weights of the eight hindlimb muscles and the femur bone (Table 3.3.).

In the *semitendinosus* muscle, fibre number and mean fibre CS area were greater ($P<0.05$) in spring than in autumn fetuses. In contrast, fibre number and mean fibre CS area of the *plantaris* muscle were similar in both seasons (Table 3.4.).

Experiment 2: There was no difference in maternal live weight at slaughter between twin- and single-bearing ewes, but twin-bearing ewes had lighter carcasses than the single-bearing ewes (Table 3.1.).

Table 3.1. Characteristics of spring- and autumn-lambing ewes and single- and twin-bearing ewes at 140 days of gestation (mean ± s.e.m.).

	Experiment 1		Experiment 2	
	Spring	**Autumn**	**Single**	**Twin**
Ewe Number	10	10	10	10
Live weight (kg)	62.0 ± 0.8	64.0 ± 1.6	65.5 ± 1.2	65.0 ± 1.2
Carcass weight (kg)	23.4 ± 2.3	23.0 ± 0.8	24.2 ± 0.8	20.2 ±0.9**

** $P < 0.01$.

Total placentome weight was higher in twin- than in single-bearing ewes (Table 3.2.) and there were more placentomes in the placentas of the twin- than single-bearing ewes. Despite their greater total placental weights, placentome weight and the number of placentomes per fetus were lower in twin- compared to single-bearing ewes.

Twin fetuses had lower body weights and smaller CRL than singles, but their girth measurements did not differ (Table 3.3.). The individual hindlimb muscles were lighter in the twin fetuses than in the singles (Table 3.3. - data not adjusted to a common fetal weight). However, following adjustment to a common fetal weight, the only muscles which remained heavier in the singles than the twins were the *biceps* and *gastrocnemius* muscles ($P<0.01$). This indicates that these two muscles were growing disproportionately slowly compared to body weight in twins while the other muscles were growing in proportion with body weight. The weight and length of the femur bone were not different as a result of fetal number, but the tibia and humerus bones were both lighter and shorter in the twin fetuses than in singles (Table 3.3.).

Although twin lambs had lighter muscles, the number of fibres per unit area of the *plantaris, semitendinosus* and *adductor* muscles did not differ between singles and twins, while the *gastrocnemius* muscle had more fibres per unit area in the twins than in the singles (Table 3.4.). These data were adjusted for fetal sex and maternal breed, but the effect of maternal breed was significant only for the *semitendinosus* muscle. Lambs born to Coopworth dams had a greater fibre number ($P<0.05$) in the *semitendinosus* muscle compared to lambs born to Romney dams. Therefore the data presented for this muscle are adjusted for maternal breed. The mean CS area of the fibres in the *semitendinosus, plantaris,* and *gastrocnemius* muscles were all lower in the twins than in the singles, but fibre CS area was not affected by fetal number in the *adductor* muscle (Table 3.4.).

Table 3.2. Placental characteristics of spring- and autumn-lambing ewes and single- and twin-bearing ewes at 140 days of gestation (mean ± s.e.m.).

	Spring	Autumn	Single	Twin
Ewe Number	10	10	10	10
Total gravid uterus weight (g)	8079 ± 293	7822 ± 259	8094 ± 274	11923 ± 1093**
Total placentome weight (g)	534.2 ± 39.4	402.7 ± 31.6*	532.4 ± 31.3	676.6 ± 53.4***
Total caruncle weight (g)	107.8 ± 6.2	62.5 ± 5.2***	67.4 ± 5.1	111.7 ± 7.2***
Total cotyledon weight (g).	426.4 ± 38.6	340.2 ± 32.9†	365.0 ± 27.7	564.9 ± 51.3**
Total placentome number	85.3 ± 4.8	69.6 ± 6.2*	74.2 ± 5.4	110.6 ± 5.7***
Placentome number per fetus	-	-	74.2 ± 5.4	55.3 ± 2.8**
Placentome weight per fetus (g)	-	-	432.4 ± 31.3	338.3 ± 26.7*
Caruncle occupancy (%)	91.2 ± 5.9	76.0 ± 4.9*	-	-

† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3.3. Characteristics of fetuses due to be born in spring and autumn and of single and twin fetuses at 140 days of gestation (mean ± s.e.m.).

	Experiment 1		Experiment 2	
	Spring	**Autumn**	**Single**	**Twin**
Fetal Number	10	10	10	20
Fetal weight (g)	4725 ± 216	4641 ± 194	5026 ± 194	4478 ± 141**
CRL (cm)	56.2 ± 1.2	57.4 ± 0.9	57.9 ± 0.8	51.7 ± 0.7***
Girth (cm)	35.7 ± 0.8	35.6 ± 0.6	36.5 ± 0.6	35.3 ± 0.5
Muscle weights (g)				
Biceps	23.92 ± 0.93	23.08 ± 0.87	24.7 ± 1.1	20.6 ± 0.7*
Gastrocnemius	10.86 ± 0.54	11.08 ± 0.56	11.5 ± 0.5	9.3 ± 0.3*
Plantaris	4.12 ± 0.19	4.00 ± 0.23	4.2 ± 0.2	3.7 ± 0.1*
Semitendinosus	8.02 ± 0.49	7.79 ± 0.51	8.2 ± 0.4	7.1 ± 0.3*
Semimembranosus	20.13 ± 0.98	19.54 ± 0.80	20.9 ± 0.9	17.7 ± 0.7**
Adductor	9.18 ± 0.45	8.42 ± 0.49	9.1 ± 0.5	7.5 ± 0.3*
Gluteus	14.33 ± 0.82	12.46 ± 0.69	13.2 ± 0.7	11.5 ± 0.4*
Quadraceps	33.55 ± 1.24	33.84 ± 1.50	36.2 ± 1.6	30.7 ± 1.4*
Lower mass	14.29 ± 0.74	15.53 ± 0.72	16.6 ± 0.7	14.2 ± 0.5**
Total muscle	144.00 ± 6.16	140.20 ± 6.15	149.3 ± 6.5	126.9 ± 4.5***
Bone weights (g) and lengths (cm)				
Femur weight	34.1 ± 1.8	31.2± 1.5	31.7 ± 1.3	29.4 ± 0.9
Femur length	-		92.7 ± 1.2	87.2 ± 2.8
Tibia weight	-		31.8 ± 1.4	28.6 ± 0.9*
Tibia length	-		110.3 ± 1.4	105.4 ± 1.2*
Humerus weight	-		27.6 ± 2.0	23.8 ± 1.8*
Humerus length	-		87.7 ± 1.7	81.9 ± 1.0*

† P < 0.10; * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 3.4. Estimated total fibre number (Fibre No.) and mean fibre cross-sectional (CS) area (μm^2) of the *semitendinosus* and *plantaris* muscles from fetuses due to be born in the spring and autumn, and the *semitendinosus, plantaris, gastrocnemius* and *adductor* muscles from single and twin fetuses, at 140 days of gestation (mean ± s.e.m.).

	Experiment 1		Experiment 2	
	Spring	Autumn	Single	Twin
Fetal Number	10	10	10	20
Semitendinosus muscle:				
Fibre No. ($x10^5$)	4.7 ± 0.2	3.3 ± 0.4**	4.2 ± 0.3	4.8 ± 0.2
Fibre CS area (μm^2)	93.0 ± 3.5	81.5 ± 2.3*	78.1 ± 3.8	66.8 ± 4.3*
Plantaris muscle:				
Fibre No. ($x10^5$)	2.6 ± 0.2	2.9 ± 0.2	3.0 ± 0.1	3.3 ± 0.2
Fibre CS area (μm^2)	78.2 ± 3.5	71.6 ± 2.5	71.6 ± 2.6	58.0 ± 1.9***
Gastrocnemius muscle:				
Fibre No. ($x10^5$)			1.8 ± 0.1	2.3 ± 0.1*
Fibre CS area (μm^2)			107.3 ± 10.4	79.8 ± 3.8**
Adductor muscle:				
Fibre No. ($x10^5$)			5.6 ± 0.4	6.4 ± 0.5
Fibre CS area (μm^2)			68.3 ± 7.5	63.3 ± 4.5

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.5. DISCUSSION

Maternal constraint can operate in various ways including limitations in nutrient availability and/or limitations in placental growth and diffusion. For example, severe maternal undernutrition can restrict placental weight in sheep (Everitt, 1968; Alexander and Williams, 1971; Mellor, 1983) and retard subsequent fetal growth (Mellor and Murray, 1982). Caruncle ctomy results in growth-retarded fetuses with reduced viability after birth (Alexander, 1964; Robinson *et al.,* 1979; Harding *et al,.* 1985); this also emphasises the importance of the placenta for fetal growth (Robinson *et al.,* 1995). Although the models used in this trial influence the development and/or growth of the resulting fetus(es), they do not involve severe manipulation of the ewe, placenta or fetus.

Restricted placental growth in December-mated (autumn-lambing) ewes was evident as total placental weight was significantly different between seasons, reflecting the difference between spring-lambing and autumn-lambing ewes in the weight of both the cotyledons and caruncles. The number of placentomes and percentage caruncle occupancy were also higher in spring- than in autumn-lambing ewes. This is consistent with previous results (Jenkinson *et al.,* 1995; see also Chapter 2) and may reflect reduced circulating concentrations of progesterone in autumn-lambing ewes during early pregnancy (Rhind *et al.,* 1978). In contrast to previous studies (Reid *et al.,* 1988; Peterson, 1992 Morris *et al.,* 1993; Jenkinson *et al.,* 1995; see also Chapter 2), and despite the difference in placental size between seasons, fetal weight was not restricted in the autumn in this trial. This implies that placental efficiency was improved in some way in the autumn-lambing ewes, thus compensating for the smaller placental size.

Although the placentas from twin-bearing ewes contained more placentomes of greater weight (as a result of compensatory overgrowth of individual cotyledons) than placentas of single-bearing ewes, lower placental mass per fetus was observed in twin-bearing ewes. Small placental size associated with a small fetus is consistent with previous studies (Alexander, 1974; Owens *et al.,* 1987 ab; Bassett, 1991) and with the high correlation observed between placental weight and birth weight (Kelly, 1992). In

association with reduced placental size per fetus, twin fetuses had lower fetal weights, indicating the presence of maternal constraint.

Since there was no effect of season on overall fetal weight, and given that muscle weight is closely correlated to body weight, the lack of difference in muscle weight between seasons is not surprising. In contrast, the lower fetal weight of twins was also associated with reduced weights of the individual hindlimb muscles examined. The *gastrocnemius* and *biceps* muscles appeared to be growing disproportionately slowly compared to body weight in twins than in singles, while the remaining muscles studied showed growth rates proportional to body weight, consistent with the results of Sailer *et al.* (1994) and Hunt *et al.* (1996). Muscle mass is determined mainly by the number of fibres within a muscle and the sizes of those fibres. Therefore, the difference in muscle weight as a result of fetal number suggests that morphogens acting during gestation can influence muscle fibre morphology.

Although the smaller placentas of autumn-lambing ewes did not lead to differences in fetal weight or muscle weight, differential effects on muscle fibre number were observed between autumn and spring fetuses. Fibre numbers were greater in the *semitendinosus* muscle from spring fetuses than autumn fetuses. However, fibre numbers did not differ between seasonal groups in the *plantaris* muscle, suggesting that maternal constraint in this situation may influence muscle development in a differential manner between muscles without influencing overall fetal size. Autumn-lambing ewes are grazed on pasture that may be suboptimal compared to that grazed by spring-lambing ewes as a result of dry summer conditions. However, ewes mated in either season show differences in fetal weight even when they are managed for similar patterns of liveweight change during gestation (Jenkinson et al., 1994; see also Chapter 2) with no observable detrimental effect to lamb birth weight. Maternal live weight was also similar between seasons in the present study (data not shown). Thus, while the possibility that differences in the quality of nutrition may have contributed to the reduction in fibre number in the *semitendinosus* muscle of fetuses due to be born in the autumn compared to spring, this seems unlikely.

Despite the relationship between muscle mass (Luff and Goldspink, 1967; Swatland and Kieffer, 1974) and growth rate (Ezekwe and Martin, 1975; Dwyer *et al.,* 1995) in a variety of species, there was no effect of fetal number on fibre number for the *plantaris*, *semitendinosus* and *adductor* muscles observed in Experiment 2. Results from these muscles are compatible with studies which indicate that fibre number is not consistently related to body weight at birth in lambs (Hunt *et al.,* 1996), though in pigs small littermates have 17% fewer fibres than large littermates (Wigmore and Stickland, 1983). In contrast, the *gastrocnemius* muscle of twin fetuses had significantly more fibres than that of the single fetuses. This differential effect on muscle growth and development in the hindlimb is also supported by the observation that this muscle appeared to be growing disproportionately slower than body weight in the twins. Since fibres are formed early in gestation, this suggests that the environment of twins is altered in early pregnancy and has affected muscle development. Alternatively, the difference in fibre number between singles and twins may not be real but rather a result of more advanced development of this muscle in singles than twins. The *gastrocnemius* muscle is a complex muscle and as these types of muscle grow there is often an apparent loss of fibres (Swatland and Cassens, 1972). Perhaps this phenomenon explains the difference observed between singles and twins. Although a greater fibre number was observed in the *gastrocnemius* muscle in the twins, there are no reports on the postnatal growth of this muscle. Therefore, it is yet to be determined whether the postnatal growth of the *gastrocnemius* muscle is influenced by its fibre complement.

Differential effects on fibre hypertrophy were also observed in both experiments. The *semitendinosus, plantaris* and *gastrocnemius* muscles of twin fetuses all exhibited fibres of lower CS area than muscles in the single fetuses. By contrast, fibre CS area in the *adductor* muscle was not affected by fetal number. Differences were also observed between seasons as autumn fetuses had lower fibre CS areas in the *semitendinosus* muscle but not the *plantaris* muscle compared to spring fetuses. Fibre hypertrophy is characterised by increased protein and DNA content, an indicator of myonuclei number (Moss, 1968; Kang *et al.*, 1985). Skeletal muscle consists of multinucleate muscle fibres that gain additional nuclei (DNA) as a result of postmitotic satellite cell fusion

(Moss and Leblond, 1971), facilitating postnatal muscle hypertrophy (Allen *et al.*, 1979). Therefore, smaller fibres may be a result of differences in satellite cell proliferation and subsequent fusion. Muscle fibre size can be influenced by several factors including age (Joubert, 1956), nutrition (Stickland and Goldspink, 1975) and hormones/growth factors (Buttery *et al.*, 1990). The mechanisms leading to differences in fibre size in this study are unknown However, since differences were apparent prior to birth, and either a lower placental mass per fetus (for twin fetuses) or restricted placental size (for autumn-lambing ewes) was observed, differences in muscle fibre development during fetal life were likely to be the result of maternal constraint.

It is possible that "single-site" fibre number and fibre CS area determination, as used in this study, may not be representative of the whole muscle. It is emphasised, as mentioned earlier, that the estimates of fibre number in this study are not estimates of total fibre number in a muscle but rather of fibre number in a given mid-belly cross-section. The total number in a whole muscle would be expected to be much greater due to intrafascicularly terminating fibres (Swatland and Cassens, 1972). Similarly, since the average fibre CS areas were determined from fibres in a "single-site" and not fibres throughout the whole muscle, average fibre CS area in the whole muscle may differ. Bias as a result of "single-site" sampling has been addressed in our subsequent studies. In addition, an attempt to minimise bias in estimates of fibre number was made by employing stereological techniques outlined by Gundersen *et al.* (1988).

The differences in muscle fibre characteristics between seasons suggest that maternal constraint operating through limitation of placental size, can influence muscle fibre morphology of certain fetal hindlimb muscles, without affecting the whole body development of the fetus. Lower placental size per fetus observed between singles and twins, can also influence both muscle fibre morphology and whole body development of the fetus. These results further emphasise the importance of the gestational period for muscle development in the sheep. In addition, these models may be useful for studying factors that influence muscle fibre development and growth during gestation, and which may have important implications for the long-term growth potential of lambs.

Chapter Four

ONTOGENY OF MYOGENESIS IN SINGLE AND TWIN FETAL LAMBS

4.1. ABSTRACT

Lower body weights and muscle weights of twin fetuses compared to singles near birth suggests possible differences in muscle development during gestation. The objective of this study was to compare myogenesis between single and twin fetuses during late gestation in order to identify possible morphological differences that may contribute to the overall difference in muscle size near birth. Twenty-six single-bearing and nineteen twin-bearing mature Coopworth ewes were naturally mated and randomly assigned to one of four slaughter groups (80, 100, 120 and 140 days of gestation). Following slaughter, fetal weight, crown-rump length and girth were measured. Fetal hindlimb muscles were weighed and histological characteristics of the *adductor*, *semitendinosus*, *gastrocnemius* and *plantaris* muscles determined. Single fetuses had greater body weights (g) compared to twins: d80 (336 ± 10 vs 317 ± 7; P<0.10); d100 (1049 ± 41 vs 952 ± 27; P<0.05); d120 (2826 ± 121 vs 2437 ± 86; P<0.05); d140 (5750 ± 246 vs 5016 ± 108; P<0.05). Greater fetal weights for singles than twins was associated with greater total placentome weights (g)per fetus: d80 (640.1 ± 32.6 vs 486.6 ± 51.7; P<0.05); d100 (516.1 ± 38.2 vs 384.6 ± 62.2; P<0.10); d120 (469.1 ± 45.3 vs 370.8 ± 19.4; P<0.10); d140 (495.7 ± 21.9 vs 380.8 ± 22.8; P<0.01) and placentome number per fetus: d80 (100.7 ± 7.9 vs 55.0 ± 2.7; P<0.01); d100 (99.0 ± 8.9 vs 40.2 ± 10.2; P<0.01); d120 (101.2 ± 7.1 vs 55.0 ± 4.0; P<0.01); d140 (88.5 ± 6.1 vs 56.8 ± 6.3; P<0.05) indicating the presence of maternal constraint on fetal size. Estimated total fibre number differed between ranks at days 80 and 120 of gestation in the *adductor* muscle, while fibre number did not differ between ranks at any other stage of gestation for the other muscles studied. In contrast, the heavier muscle weight observed for singles was associated with greater fibre cross-sectional area in three of the muscles studied at 140 days of gestation: *semitendinosus* (90.60 ± 2.53 vs 75.41 ± 4.39; P<0.05); *adductor* (85.58 ± 4.70 vs 66.32 ± 2.50; P<0.01); *gastrocnemius* (121.26 ± 6.16 vs 94.44 ± 7.34; P<0.05) and a similar trend was observed for the *plantaris* (87.68 ± 10.5 vs 69.36 ± 5.78; P<0.10). These results illustrate that myogenesis in the sheep fetus can be influenced as a result of maternal constraint reflected by placental size. Since fibre number was not consistently affected, but muscle weight and fibre cross-sectional area

Were greater in singles than in twins in later gestation, the impact of maternal constraint on myogenesis is most marked in late gestation, i.e. after fibres are formed.

4.2 INTRODUCTION

Both muscle fibre number and size contribute to the overall mass of a muscle (Buttery *et al.*, 1990). In larger precocial species, the maximum fibre complement of a muscle is reached during gestation (sheep: Ashmore *et al.*, 1972; Swatland and Cassens, 1973; pigs: Staun, 1963; Stickland and Goldspink, 1973; cattle: Russel and Onteruelo, 1981; Robelin *et al.*, 1991), while postnatal muscle growth is accomplished by an increase in muscle fibre size. Factors such as maternal nutritional (Wigmore and Stickland, 1983; Nordby *et al.*, 1987; Ward and Stickland, 1991) and innervation (McLennan, 1983) can affect muscle fibre development in utero. These factors can impose permanent effects on postnatal growth rate (Ezekwe and Martin, 1975; Hegarty and Allen, 1978; Dwyer *et al.*, 1995) and potential size (Stickland, 1995), which emphasizes the importance of the gestational period for the development of muscle.

Although twin and single lambs have similar muscle weights at market weight (Nordby *et al.*, 1987), twins have lower body weight (Donald and Russell, 1970; Nordby *et al.*, 1987) and proportionally smaller muscle s(Sailer *et al.*, 1994; Hunt *et al.*, 1996); Chapter 3) at birth, reflecting their smaller placentas and competition for nutrients. At birth, low-and high-birthweight lambs are reported to have similar numbers of muscle fibres and fibre types, per unit cross-sectional area of muscle (Hunt *et al.*, 1996). However, differential effects on fibre number per unit cross-sectional area of muscle and average fibre cross-sectional (CS) area between muscles, were observed between single and twin fetal lambs as a result of maternal constraint due to placental size (see Chapter 3). These observations suggest that factor acting in utero may have important consequences for the postnatal growth potential of lambs.

Myogenesis in the sheep fetus has been described previously (Ashmore *et al.*, 1972; Maier *et al.*, 1992; Wilson *et al.*, 1992), however, the ontogeny of myogenesis in single and twin fetuses has not been described. Therefore, the aim of this study was to

compare myogenesis between single and twin fetuses during gestation. The development of selected hindlimb muscles from single and twin fetuses was studied at days 80, 100, 120, and 140 of gestation in an attempt to identify morphological characteristics that contribute to the differences in muscle size observed at birth in previous studies.

4.3. MATERIALS AND METHODS

4.3.1. Animals

A 2 x 4 factorial design incorporating two ranks (single vs twin) and four stages of gestation (80, 100, 120 and 140 days) was used. Eighty, three to five year-old Coopworth ewes were mated naturally in March following a two-week period of treatment with progesterone-impregnated CIDRs (Eazi-breed CIDR type G, Carter Holt Harvey Plastic Products, Hamilton, New Zealand) to synchronize oestrus. Coopworth rams fitted with harnesses and crayons were used to identify mated ewes and only those mated in the first two days of the first oestrous cycle were used (n=45). Ewes were managed on perennial ryegrass *(Lolium perenne)*/white clover (*Trifolium repens*) dominant pasture, and were weighed every two weeks. Pregnancy status was determined using ultrasound at day seventy-six of gestation, following which ewes (balanced for age and date of mating) were randomly assigned to one of the four slaughter dates.

4.3.2. Slaughter Procedure

Ewes were slaughtered by captive bolt pistol and exsanguination at 80, 100, 120 and 140 days of gestation. The intact uterus was removed, weighed and the fetus(es) exposed. The umbilical cord was ligated and severed, and fetus(es) weighed. Fetuses were euthanased using sodium pentobarbitone via cardiac puncture, and crown-rump length measured. Eight major hindlimb muscles (*biceps, gastrocnemius, plantaris, adductor, semitendinosus, semimembranosus, quadraceps and gluteus),* were dissected

from the left hindlimb of each fetus and weighed. The femur, tibia and humerus bones were also dissected out, weighed and their length measured using digital callipers. The placenta was dissected, and placentome number and weight recorded.

4.3.3. Histology Samples

From the eight hindlimb muscles dissected, the *plantaris, semitendinosus, adductor* and *gastrocnemius* were chosen for histological characterisation of muscle fibre number, type and cross-sectional (CS) area. The method used for sample collection, storage and processing is the same as described in Chapter 3.

4.3.4. Immunohistochemistry

The methodology used to identify slow-twitch fibres was a modification of that described by Maier *et al.* (1992). The 10 μm cryostat sections, from muscles taken at 100, 120 and 140 days of gestation, were air-dried for at least one hour and fixed in 10% buffered formalin for 5 minutes. Sections were washed in 0.1 M glycine; 0.01 M phosphate buffered saline (PBS) pH 7.4 (4 x 5 min). Sections were incubated in 1% BSA (bovine serum albumin) in PBS for 10 minutes to reduce non-specific binding, and incubated overnight at 4°C in a humid chamber with the mouse monoclonal antibody NOQ.7.1A, abbreviated henceforth as 1A (Harris *et al.*, 1989, a gift from J. Harris and J. McEwan), diluted at 1:50 in diluting buffer (0.5% BSA in PBS + 0.5% Tween 20). Sections were washed twice in washing buffer (PBS; 2% NaCl (w/v); 1% non-fat milk powder (w/v); 5% Tween 20 (w/v), pH 7.4) for 10 minutes, washed 3 x 1 min in PBS to remove milk proteins and incubated in 0.3% hydrogen peroxide in methanol for 10 minutes to stop exogenous peroxidase activity. The second antibody used was biotinylated-anti-mouse IgG (Amersham, Life Science, Buckinghamshire, UK), diluted 1:200 in PBS; 0.5% BSA (w/v); 0.5% Tween (w/v), pH 7.4, and sections were incubated for 30 minutes at room temperature. The immunoreactivity was visualized by incubating the sections in streptavidin biotinylated horseradish-peroxidase complex (Amersham, Buckinghamshire, UK) diluted (1:200) in PBS; 0.5% BSA (w/v); 0.5% Tween (w/v), for 15 minutes, and for 3 minutes in a mixture

containing 5 mg diaminobenzidine (DAB), 8 µl of hydrogen peroxide activator and 10 ml of PBS. Sections were then washed in PBS, dehydrated, mounted using DPX mounting medium, and stored at room temperature until analysis.

These sections were processed simultaneously with two serial sections, one a negative control (methodology as above omitting the primary antibody) and the other, a section incubated with monoclonal antibody MY32 (Sigma Chemical Co.), which detects neonatal IIa and IIb fast myosin heavy chain (MHC) reactivity, at a dilution of 1:50 and using the above methodology. Reactivity of MY32 (Plate 4.1.) and 1A (Plate 4.2.) was compared to ensure correct detection of fast or slow MHC reactivity, respectively.

Plate 4.1. Skeletal muscle tissue from the *adductor* muscle of a sheep fetus at 140 days of gestation. Fast-twitch myofibres have been labelled with an anti-MY32 antibody (brown) and the slow-twitch fibres remain unlabelled. (Mag x500).

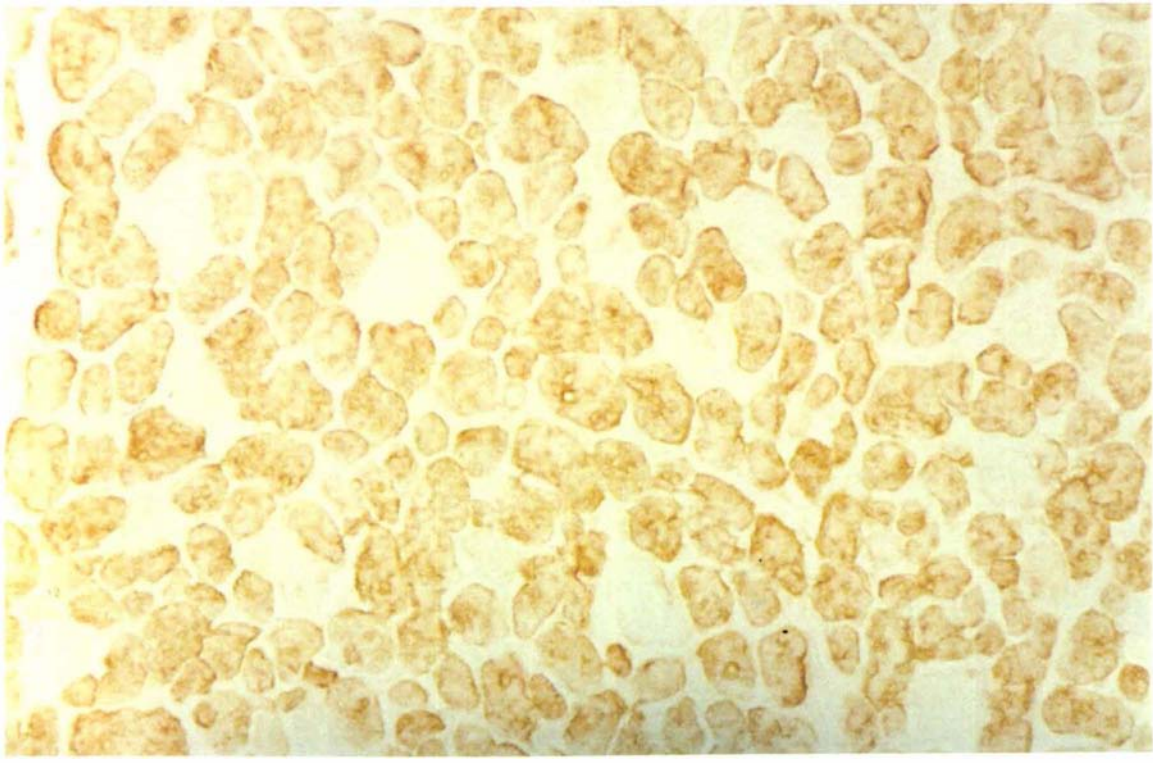
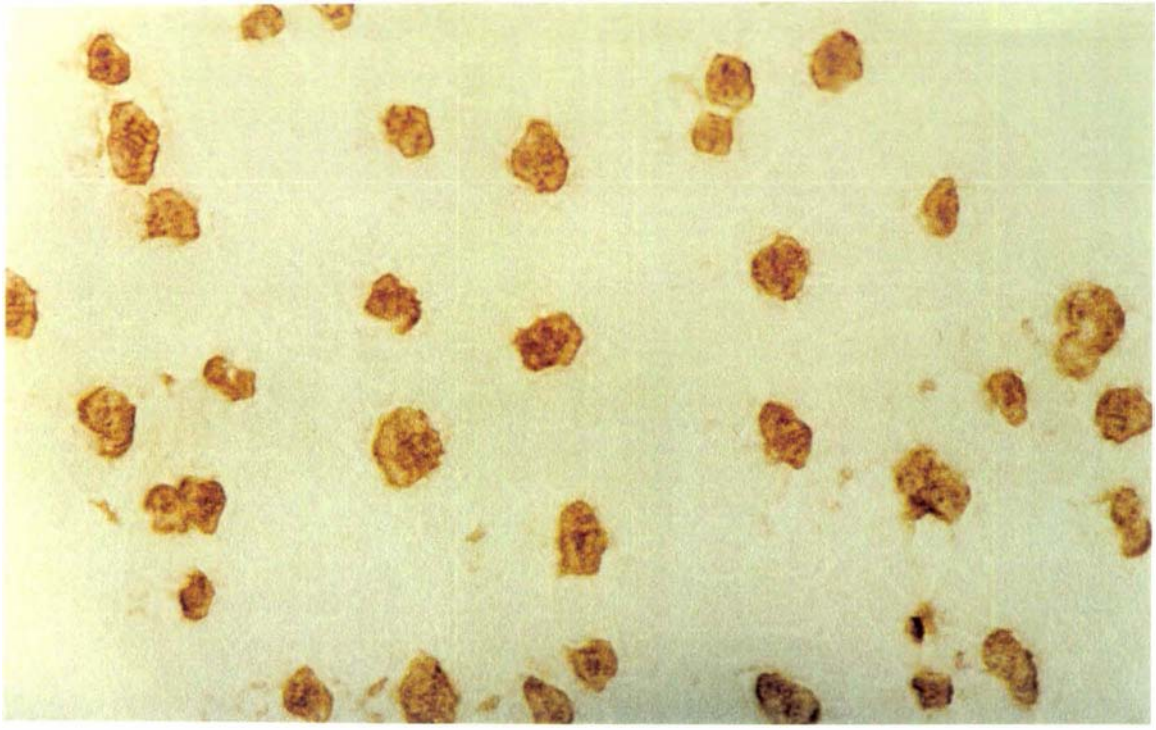


Plate 4.2. Skeletal muscle tissue from the *adductor* muscle of a sheep fetus at 140 days of gestation. Slow-twitch myofibres have been labelled with the 1A antibody (brown) and the fast-twitch fibres remain unlabelled. (Mag x500).



4.3.5. Estimation of Total Fibre Number and Slow-Twitch Fibre Number

The methodology used to estimate total fibre number is described in Chapter 3. The methodology to determine the number of slow-twitch fibres was the same method used to estimate total fibre number. The number of slow-twitch fibres is presented as a percentage of the total number of fibres.

4.3.6. Fibre Cross-Sectional Area

The methodology used to measure fibre CS area is described in Chapter 3.

4.3.7. Statistical Analysis

Data were analysed using a general linear model procedure for Analysis of Variance using Minitab for Windows (Release 10, 1994). Results are expressed as unadjusted

means ± s.e.m. Fetal data were adjusted for sex and this factor remained in the model when significant, and is discussed in the text where relevant.

4.4. RESULTS

The total gravid uterus weight was greater for twin- than single-bearing ewes at each stage of gestation studied (Table 4.1.). Despite a greater total placentome weight in twin-bearing ewes, total placentome weight per fetus was significantly lower for twin- compared to single-bearing ewes at days 80 and 140 of gestation (Table 4.1.). A similar trend of lower total placentome weight per fetus was observed at days 100 and 120 of gestation ($P<0.10$). Although total placentome number was not significantly different between single- and twin-bearing ewes, at each stage when analysed separately, the mean effect of birthrank across all stages was significant, indicating higher numbers of placentomes in twins than in singles. Total placentome number per fetus was considerably lower for twin- than single-bearing ewes (Table 4.1.) at all stages. Placental parameters (weight, number) did not change significantly during the period of study.

Twins had lower fetal weights than singles at all stages of gestation studied in this trial, but twins and singles had similar crown-rump lengths (Table 4.2.). When comparisons were made within data obtained at each stage of gestation studied in this trial, twins were found to have lower fetal weights than singles, but twins and singles had similar crown-rump lengths (Table 4.2.) on each occasion. When data for all stages was simultaneously analysed, a significant rank effect confounded the effect of birth rank on fetal weight. A significant stage effect indicates that foetal weights increased during gestation, and a significant birth rank by stage interaction indicated a gradual divergence in fetal weight between birth ranks in favour of singles. Several fetal organs (liver, heart, spleen, kidneys, thyroid and thymus) were smaller in twins than in singles, particularly at day 140 of gestation, but, after adjusting to a common fetal weight, there were no differences in fetal organ weights, indicating that the fetal organs grew in proportion with body weight (Table 4.2.; data not adjusted for fetal weight).

Table 4.1. Placental characteristics of single- and twin-bearing ewes at 80, 100, 120 and 140 days of gestation (mean ± s.e.m.).

		Day of gestation				Significance		
Parameter	**Rank**	**80**	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Ewe Number	1	7	7	6	7			
	2	5	6	5	4			
TGUW (g)	1	1948 ± 78***	2771 ± 132*	5060 ± 143***	8662 ± 322***	**	***	***
	2	3411 ± 114	4302 ±457	7901 ± 491	14348 ± 523			
Total placentome weight (g)	1	640.1 ± 32.6*	516.1 ± 38.2†	469.1 ± 45.3**	495.7 ± 21.9***	***	NS	NS
	2	973.0 ± 103.0	769.0 ± 124.0	741.5 ± 38.8	761.7 ± 45.6			
Total placentome number	1	100.7 ± 7.9	99.6 ±9.0	101.2 ± 7.1	88.5 ± 6.1†	**	NS	NS
	2	110.0 ± 5.4	80.5 ± 20.3	110.0 ± 8.1	113.5 ± 12.6			
Placentome weight/fetus (g)	1	640.1 ± 32.6*	516.1 ± 38.2†	469.1 ± 45.3†	495.7 ± 21.9**	***	†	NS
	2	486.6 ± 51.7	384.6 ± 62.2	370.8 ± 19.4	380.8 ± 22.8			
Placentome number/fetus	1	100.7 ± 7.9***	99.0 ± 8.9***	101.2 ± 7.1***	88.5 ± 6.1**	***	†	NS
	2	55.0 ±2.7	40.2 ± 10.2	55.0 ± 4.0	56.8 ± 6.3			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.001; TGUW = Total Gravid Uterus Weight; (1) = single-bearing; (2) = twin-bearing.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation stndied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage (RxS) interaction are also shown.

Table 4.2. Fetal weight, crown-rump length (CRL), and fetal organ weights of single and twin fetal sheep at days 80, 100, 120 and 140 of gestation (mean±s.e.m.).

		Day of gestation				Significance		
Parameter	**Rank**	**80**	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Fetal Number	1	7	7	6	7			
	2	10	12	10	8			
Fetal weight (g)	1	336 ± 10†	1049 ± 41*	2826 ± 121*	5750 ± 246*	*	***	*
	2	317 ± 7	952 ± 27	2437 ± 86	5016 ± 108			
CRL (mm)	1	26.14 ± 0.28	35.29 ± 1.34†	45.50 ± 2.19	59.00 ± 1.63	NS	***	NS
	2	25.80 ± 0.32	32.65 ± 0.71	44.50 ± 1.38	56.00 ± 0.66			
Organ weights (g):								
Liver	1	22.58 ±1.54	58.27 ± 3.93	100.38 ± 4.34	140.35 ± 8.85***	***	***	***
	2	20.90 ± 1.06	48.94 ± 1.79	71.85 ± 3.26	107.39 ± 4.13			
Heart	1	2.71 ± 0.16†	7.74 ± 0.21	21.08 ± 0.83	40.15 ± 2.53*	†	***	*
	2	2.33 ± 0.11	6.88 ± 0.31	18.68 ± 1.14	33.99 ± 0.75			
Spleen	1	0.32 ± 0.03	1.43 ±0.08	4.70 ± 0.37	8.24 ± 0.54**	*	***	**
	2	0.33 ± 0.04	1.36 ± 0.09	3.99 ± 0.44	6.45 ± 0.34			

Kidneys	1	3.48 ± 0.13	11.03 ± 0.66†	19.23 ± 0.99	29.69 ± 2.05***	***	***	***
	2	3.20 ± 0.15	9.44 ± 0.59	15.88 ± 0.51	22.53 ± 1.25			
Lungs	1	20.80 ± 4.80	42.75 ± 1.91	86.37 ± 3.34	171.62 ± 14.00	NS	***	NS
	2	15.26 ± 0.59	39.17 ± 1.04	77.63 ± 3.69	147.55 ± 9.53			
Thyroid	1	0.26 ± 0.03*	0.54 ± 0.04†	1.07 ± 0.11	1.55 ± 0.09*	NS	***	NS
	2	0.17 ± 0.02	0.45 ± 0.03	1.04 ± 0.15	1.33 ± 0.06			
Thymus	1	0.94± 0.08	4.41 ± 0.22	14.65 ± 1.03	27.84 ± 2.13***	***	***	***
	2	0.84 ± 0.06	3.59 ± 0.38	10.46 ± 1.07	15.99 ± 2.05			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.001; (1) = single; (2) = twin.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage (RxS) interaction are also shown.

The weights of the femur, tibia and humerus bones were lower in twin fetuses than in singles by day 120 of gestation (Table 4.3.). Both the femur and humerus bones were shorter in the twins than in the singles at 120 days of gestation, while the femur and tibia bones were significantly shorter at day 140 of gestation. The differences in weights and lengths of these bones were not apparent after adjustment to a common fetal weight, implying that the growth of these bones was proportional to body weight.

The weights of all the muscles studied were lower in the twins than in the singles, however, this effect was not seen consistently across all muscles until 140 days of gestation (Table 4.4.). Following adjustment to a common fetal weight, the *semitendinosus, semimembranosus* and *adductor* muscles remained significantly smaller in the twin than the single fetuses implying disproportionately slower growth of these muscles. Birth rank and stage of gestation had significant overall effects on the weights of each muscle studied and the significant rank by stage interaction indicated a gradual divergence in individual muscle weights with advancing gestation in favour of singles.

Table 4.5. shows estimated fibre numbers for the *semitendinosus, plantaris, adductor* and *gastrocnemius* muscles of twin and single fetuses. There was no consistent difference in fibre numbers in these muscles between ranks. The *adductor* muscle of twin fetuses had more fibres at day 80 of gestation than that of single fetuses, an effect that was not apparent at day 100, reversed by day 120, and not significantly different at day 140 of gestation. However, there was no overall effect of birth rank with advancing gestation on estimated total fibre number per CS for any of the muscles studied.

The proportions of slow-twitch fibres present in the muscles studied were similar in both twin and single fetuses throughout gestation (Table 4.6.). Muscle fibre CS areas in the *semitendinosus, adductor* and *gastrocnemius* muscles were all significantly smaller in the twins than in the singles at day 140 of gestation (Table 4.7.), and a similar trend was observed for the *plantaris* muscle.

Table 4.3. Fetal bone weights and lengths of single and twin fetal sheep at days 80, 100, 120 and 140 of gestation (mean±s.e.m.).

		Day of gestation				Significance		
Parameter	**Rank**	**80**	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Fetal Number	1	7	7	6	7			
	2	10	12	10	8			
Femur length (mm)	1	30.32 ± 0.42	47.32 ± 1.25	73.55 ±1.11*	95.62 ± 0.98*	NS	***	NS
	2	29.80 ± 0.45	47.20 ± 0.29	69.68 ± 1.18	93.10 ± 0.74			
Femur weight (g)	1	0.95 ± 0.06	3.69 ± 0.21	15.12 ± 0.54*	37.38 ± 1.58**	NS	***	*
	2	0.83 ± 0.08	3.52 ± 0.09	12.98 ± 0.65	31.55 ± 0.81			
Tibia length (mm)	1	36.21 ± 0.37	56.71 ± 1.01	84.37 ± 2.64	112.00 ± 1.06*	NS	***	NS
	2	35.47 ± 0.57	55.43 ± 0.49	83.16 ± 1.01	108.39 ± 0.95			
Tibia weight (g)	1	0.96 ± 0.04	3.59 ± 0.19	14.60 ± 0.71*	33.29 ± 1.15*	NS	***	NS
	2	0.93 ± 0.03	3.49 ± 0.07	12.27 ± 0.46	29.48 ± 0.95			
Humerus length (mm)	1	29.96 ± 0.54	44.20 ± 0.86	68.69 ± 1.51*	87.30 ± 1.43	NS	***	NS
	2	28.31 ± 0.47	44.11 ± 0.45	65.20 ± 0.71	85.74 ± 0.88			
Humerus weight (g)	1	0.94 ± 0.03*	3.16 ± 0.18†	12.07 ± 0.56*	28.12 ± 1.14**	NS	***	*
	2	0.89 ± 0.04	2.92 ± 0.09	10.69 ± 0.44	23.48 ± 0.59			

*, P<0.05; **, P<0.01; (1) = single; (2) = twin. Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage (RxS) interaction are also shown.

Table 4.4. Fetal hindlimb muscle weights (g) of single and twin fetal sheep at days 80, 100, 120 and 140 of gestation (mean±s.e.m.).

		Day of gestation				Significance		
Muscle	**Rank**	**80**	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Fetal Number	1	7	7	6	7			
	2	10	12	10	8			
Biceps	1	1.68 ± 0.06*	6.25 ± 0.29	13.98 ± 0.80†	27.29 ± 1.18 **	**	***	**
	2	1.42 ± 0.06	6.06 ± 0.25	12.49 ± 0.37	23.22 ± 0.79			
Gastrocnemius	1	0.64 ± 0.03*	2.35 ± 0.13	5.82 ± 0.26*	11.97 ± 0.53**	*	***	*
	2	0.56 ± 0.02	2.18 ± 0.08	5.05 ± 0.17	10.17 ± 0.23			
Plantaris	1	0.22 ± 0.02	0.79 ± 0.04*	2.13 ± 0.11	4.88 ± 0.22**	†	***	*
	2	0.20 ± 0.01	0.69 ± 0.03	1.95 ± 0.09	4.10 ± 0.12			
Semitendinosus	1	0.63 ± 0.04*	2.13 ± 0.10	4.25 ± 0.27	9.53 ± 0.32***	*	***	*
	2	0.54 ± 0.02	1.93 ± 0.09	3.78 ± 0.18	8.05 ± 0.18			
Semimembranosus	1	1.46 ± 0.16	5.36 ± 0.26†	11.12 ± 0.66	21.37 ± 0.73***	***	***	***
	2	1.48 ± 0.04	4.94 ± 0.19	10.23 ± 0.41	16.78 ± 0.59			
Adductor	1	0.68 ± 0.04*	2.54 ± 0.14	5.54 ± 0.47	10.48 ± 0.51***	***	***	***
	2	0.56 ± 0.03	2.42 ± 0.09	4.79 ± 0.18	8.23 ± 0.32			

Gracillus	1	0.33 ± 0.03	1.02 ± 0.69	2.69 ± 0.19*	5.04 ± 0.19**	†	***	*
	2	0.31 ± 0.03	1.52 ± 0.61	2.18 ± 0.11	4.14 ± 1.15			
Gluteus	1	1.28 ± 0.07	3.69 ± 0.22	7.84 ± 0.41†	14.94 ± 0.76**	*	***	***
	2	1.16 ± 0.04	3.47 ± 0.13	6.80 ± 0.37	11.88 ± 0.38			
Quadraceps	1	2.07 ± 0.11	7.88 ± 0.48	18.94 ± 1.02	41.84 ± 2.03**	*	***	**
	2	1.92 ± 0.06	7.69 ± 0.28	17.19 ± 0.74	33.55 ± 1.31			
Lower Mass	1	0.81 ± 0.04*	3.04 ± 0.18	8.04 ± 0.46	17.45 ± 0.67**	NS	***	*
	2	0.71 ± 0.03	2.91 ± 0.10	7.35 ± 0.32	15.11 ± 0.37			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.001; (1) = single; (2) = twin.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction are also shown.

Table 4.5. Estimated muscle fibre numbers (mean±s.e.m., $x10^4$) for *semitendinosus, plantaris, adductor* and *gastrocnemius* muscles of single and twin fetal sheep at days 80, 100, 120 and 140 of gestation.

		Day of gestation				Significance		
Muscle	**Rank**	**80**	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Fetal Number	1	7	7	6	7			
	2	10	12	10	8			
Semitendinosus	1	13.64 ± 1.71	27.27 ± 2.17	33.20 ± 2.73	42.67 ± 2.33	NS	**	NS
	2	10.06 ± 1.58	31.89 ± 2.59	33.18 ± 2.92	40.42 ± 3.09			
Plantaris	1	2.71 ± 0.21	8.02 ±0.96	16.80 ± 0.88	24.68 ± 1.98	NS	**	NS
	2	2.19 ± 0.31	7.99 ± 0.64	16.05 ± 1.13	26.02 ± 2.21			
Adductor	1	11.24 ± 2.05 *	38.01 ± 4.94	50.20 ± 3.54 *	52.94 ± 2.61	NS	***	NS
	2	17.17 ± 1.20	38.37 ± 2.08	42.35 ± 1.39	57.38 ± 5.00			
Gastrocnemius	1	11.57 ± 3.74	7.67 ± 0.94	14.19 ± 1.12	17.30 ± 1.07	NS	***	NS
	2	10.63 ± 1.43	8.58 ± 0.81	14.87 ± 1.05	19.24 ± 1.53			

* $P<0.05$; (1) = single; (2) = twin.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction are also shown.

Table 4.6. Proportion of slow-twitch muscle fibres (%) in the *semitendinosus, plantaris, adductor* and *gastrocnemius* muscles of single and twin fetal sheep at days 100, 120 and 140 of gestation (mean±s.e.m.) relative to total muscle fibre number

		Day of gestation			Significance		
Parameter	**Rank**	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Fetal Number	1	7	6	7			
	2	12	10	8			
Semitendinosus	1	5.86 ± 1.32	8.75 ± 0.94	10.27 ± 1.46	NS	*	NS
	2	5.71 ± 0.88	7.99 ± 1.25	10.17 ± 1.76			
Plantaris	1	13.20 ± 2.15	10.40 ± 2.46	18.26 ± 2.86	NS	**	NS
	2	14.00 ± 2.19	15.93 ±2.14	22.66 ± 1.70			
Adductor	1	12.05 ± 2.11	7.38 ± 0.59	10.68 ± 1.90	NS	NS	NS
	2	12.47 ± 1.12	9.20 ± 0.70	9.05 ± 0.74			
Gastrocnemius	1	9.89 ± 2.56	11.25 ± 1.90	17.01 ± 1.83	NS	*	NS
	2	10.95 ± 1.34	13.90 ± 1.24	14.58 ± 1.71			

(1) = single; (2) = twin.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction are also shown.

Table 4.7. Muscle fibre cross-sectional area (μm^2) from fetal sheep *semitendinosus, plantaris, adductor* and *gastrocnemius* muscles at day 140 of gestation (mean±s.e.m.).

Muscle:	**Singles**	**Twins**
Fetal Number	7	8
Semitendinosus	90.60 ± 2.53	75.41± 4.39*
Plantaris	87.68 ± 10.50	69.36 ± 5.78†
Adductor	85.58 ± 4.70	66.32 ± 2.58**
Gastrocnemius	121.26 ± 6.16	94.44 ± 7.34*

†, P<0.10; *, P<0.05.

4.5. DISCUSSION

Total placentome weight was greater for twin- than single-bearing ewes at all stages of gestation studied. However, placentome weight per fetus was lower for twins at 80 and 140 days of gestation, indicating the effect of maternal constraint on placental size. A similar trend was observed at days 100 and 120 of gestation. Smaller individual placentas for twins were also associated with lower fetal weights at all ages studied. This observation is consistent with the previous study (Chapter 3) and agrees with studies in which small placental size is associated with a small fetus (Alexander, 1964, 1974; Owens *et al.,* 1987 ab; Bassett, 1991) and supports the observation that birth weight is highly correlated with placental weight (Kelly, 1992).

The lower body weight of twins at 140 days of gestation is consistent with previous reports (Wallace, 1948a; Nordby *et al.,* 1987; Hunt *et al.,* 1996). Lower body weight of twins was evident from 100 days of gestation, indicating that the effect of rank on body weight is not just a manifestation of limitations in maternal nutrient supply in late gestation, when most fetal growth occurs. Rather it is an effect that is associated with factors occurring in early gestation, including reduced placental size (per fetus) in twin-bearing ewes (see Chapter 3) as observed in this trial and described in detail by Robinson *et al.* (1977).

Individual muscle weights were lower in the low-weight twin lambs compared to singles. Differential effects on individual muscle weight were observed prior to day 140 of gestation, but the effects were not consistent over time. By day 140 it was evident that all of the hindlimb muscles studied were lighter in twins than in singles. Five of the individual muscles studied (*biceps, gastrocnemius, plantaris, gluteus, quadraceps*) were proportionally smaller in the twin fetuses, while the remaining hindlimb muscles (*semitendinosus, semimembranosus*, and *adductor*) appeared to be disproportionately smaller relative to body weight in the twin than single fetuses in late gestation. These results suggest that morphogens which control or regulate muscle growth *in utero*, and which reflect the consequences of maternal constraint (in this case via birth rank), may impose differential effects between individual hindlimb muscles.

In a previous trial (Chapter 3) disproportionate growth of individual hindlimb muscles between ranks was observed, however, in that study the *gastrocnemius* and *biceps* muscles appeared to be growing disproportionately slower than body weight in the twin compared to single fetuses. In contrast, in the present trial, disproportionately slower muscle growth was observed for the *semitendinosus, semimembranosus* and *adductor*, rather than the *gastrocnemius* and *biceps* muscles. A possible explanation for the differences between these two trials, is that the animals used conceived in different seasons. In the present trial, ewes were mated in their natural breeding season while the ewes from the previous trial were mated outside of their natural breeding season to lamb in autumn. The season of conception can impact on fetal weight, with lambs born out-of-season having considerably lower fetal and birth weights than lambs born in their normal breeding season (Reid *et al.,* 1988; Peterson, 1992; Morris *et al.,* 1993; Jenkinson *et al.,* 1995; see also Chapter 2). Differences in fetal weight (g) at 140 days of gestation were observed between these two trials for both singles (autumn: 5026±194 vs spring: 5750±246, $P<0.05$) and singles and twins pooled (autumn: 4478±141 vs spring: 5016±108, $P<0.01$). Individual hindlimb muscle weights were also significantly different between seasons ($P<0.05$, data not shown). In addition, a previous report (Chapter 3) indicated that factors involved in the regulation of muscle development and growth can act differently between seasons, resulting in differences in the morphological characteristics of muscle fibres. These seasonal differences in fetal weight, muscle weight and muscle fibre morphology suggest that the differences in growth rates between muscles from this and the trial reported in Chapter 3, may be the result of seasonal differences in the factors involved in muscle development and/or growth.

Both fibre number and the sizes of those fibres determine postnatal muscle mass (Buttery *et al.,* 1990). While postnatal muscle growth is characterized by an increase in fibre CS area, the formation of muscle fibres occurs *in utero.* Fibre number has been related to meat content of animals (Luff and Goldspink, 1967; Swatland and Kieffer, 1974), growth rate (e.g., mouse: Ezekwe and Martin, 1975; pig: Dwyer *et al.,* 1995) and postnatal growth potential (Stickland, 1995). Histological analysis of four selected hindlimb muscles (*plantaris, gastrocnemius, semitendinosus* and *adductor*) in this

study indicated that apparent fibre number was not consistently affected by low body weight (as a result of birth rank) at any stage after day 80 of gestation. The increase in the apparent fibre number within a muscle up until 140 days of gestation for both singles and twins in this study, was likely to have been due to the increase in the overlap of intra-fascicularly terminating fibres (Swatland and Cassens, 1973). The lack of effect of low birth weight in twins on apparent muscle fibre number at birth is supported by the observations of Nordby *et al.* (1987) and Hunt *et al.* (1996). In contrast, studies using pigs have indicated an association between low birth weight and low fibre number (Ward and Stickland, 1991; Dwyer and Stickland, 1992) as a result of a reduction in the secondary fibre complement (Wigmore and Stickland, 1983; Handel and Stickland, 1987; Wilson *et al.,* 1988; Ward and Stickland, 1991). In those studies, severe growth-retarded pigs (runts) were compared to normal birth weight littermates. However, when littermates were compared across the normal birth weight range, no significant effect on apparent fibre number was observed (Dwyer *et al.,* 1995). Therefore, it is likely that the growth restriction occurring in twin lambs relative to singles at 80 days of gestation, when fibres are still forming, is not sufficiently severe to cause an effect on apparent fibre number. The impact of maternal constraint on fetal size as reflected by fetal weight, is most marked after 100 days of gestation, once fibre formation is complete. Therefore, the effect of maternal constraint in this situation appears to have little impact on the formation of fibres in both single and twin fetuses.

In agreement with the observations of Hunt *et al.* (1996) no effect of birth rank on the percentage of slow-twitch fibres was observed, suggesting that the phenotypic development of muscle fibres is also not differentially affected by birth rank in sheep.

The difference in muscle weight observed between single and twin fetuses was, however, associated with a difference in the size of the component fibres. Singles had greater fibre CS area than twins at day 140 of gestation. At this stage of gestation, the difference in the size of the muscle fibres was in the order of about 20%, which is similar to the overall difference in muscle weight. From these results it can be concluded that the overall difference in muscle size between singles and twins is not a

consequence of differing fibre number or an effect on fibre type; rather it is associated with smaller fibre CS area in the twin fetuses.

In conclusion, although muscle mass is determined by fibre number and fibre size, the difference in muscle mass between singles and twins was not associated with different fibre numbers. However, average CS area of muscle fibres was substantially smaller in some hindlimb muscles of twin fetuses compared to singles, at 140 days of gestation. If we assume that muscle fibre number is an indicator of the growth potential of animals as measured by body weight or size, which is suggested by previous reports (Luff and Goldspink, 1967; Swatland and Kieffer, 1974; Stickland, 1995), the potential for growth of single and twin lambs would be expected to be similar, as shown by Nordby *et al.* (1987). Despite similar postnatal growth potentials, the smaller fibre CS areas of muscle fibres from twin lambs observed in the present study, suggests that although twins are growing according to a similar growth trajectory as singles, they exhibit a growth lag. This would result in twin lambs taking longer to reach similar slaughter weights with similar fibre CS areas as singles lambs, as observed by Nordby *et al.* (1987). These results emphasise that ultimate muscle size is not only restricted by events during postnatal life such as the sharing of a single milk source between twin lambs, but that differences in muscle fibre morphology can originate during fetal life. This further emphasises that importance of the prenatal period of muscle development and growth for the postnatal growth potential of lambs.

Chapter Five

FIBRE DEVELOPMENT IN THE *ADDUCTOR* MUSCLE OF SINGLE AND TWIN FETAL LAMBS

5.1. ABSTRACT

In Chapters 3 and 4 it was observed that single fetuses have greater body weights, and muscles which are heavier with larger fibre diameters than muscles from lower body weight twins near term. Muscle hypertrophy in late fetal and postnatal stages of development is characterized by increased protein and DNA content. The differences in muscle weights and fibre cross-sectional area between singles and twins observed in Chapters 3 and 4, suggest that the DNA content of the muscles may differ between ranks. Therefore, the first aim of this study was to determine if the DNA content of a selected hindlimb muscle (*adductor*) differs between singles and twins. Three regions (proximal, medial and distal) of the *adductor* muscle from six single and eight twin fetuses at day 140 of gestation, were used in this study. These fetuses were born to ewes mated as part of an independent trial (Chapter 4). Single fetuses had greater body weights 5750 ± 246 vs 5016 ± 108 g; $P<0.01$), *adductor* muscle weights (10.5 ± 0.5 vs 8.2 ± 0.3 g; $P<0.001$) and *adductor* muscle volume (7618 ± 413 vs 6496 ± 210 mm^3; $P<0.05$) compared to twin fetuses. The *adductor* muscle of singles also had greater fibre cross-sectional area than twins in the medial (94.7 ± 4.7 vs 78.9 ± 2.6 μm^2; $P<0.01$) and distal (110.6 ± 3.0 vs 93.8 ± 3.7 μm^2; $P<0.01$) regions but not in the proximal region. Despite differences in fibre cross-sectional area between ranks, the estimated total number of fibres and the number of slow-twitch fibres did not differ between singles and twins at any region of the *adductor* muscle studied. Greater muscle weight and volume and larger fibre cross-sectional area was also associated with greater amount of DNA in the *adductor* muscle of singles compared to twins (6290 ± 617 vs 4939 ± 280 μg; $P<0.05$).

Large muscles such as the *adductor* have intrafascicularly terminating fibres which necessitates accurate sampling of the muscle when investigating possible perturbations in morphological characteristics, e.g., between singles and twins. The second aim of this study was to investigate the impact of the sampling site on the morphological parameters including fibre number and cross-sectional area of the *adductor* muscle. The estimated total fibre number and slow-twitch fibre number differed between the three regions of the *adductor* muscle. Similarly, fibre cross-sectional area differed

between the proximal, medial and distal regions of this muscle. These results indicate that, particularly with large muscles such as the *adductor*, which has intrafascicularly terminating fibres, single site sampling for determination of fibre morphological characterises may generate misleading results and that careful selection of the sampling area may be necessary.

5.2. INTRODUCTION

As a result of maternal constraint, twin fetal lambs are not only significantly lighter than singles at birth, they also have lower hindlimb muscle weights (Chapters 3&4). Histological examination of selected hindlimb muscles (*adductor*, *plantaris*, *semitendinosus* and *gastrocnemius*) from both single and twin fetuses indicated that, although the lower muscle weight observed for the twins was not related to differences in muscle fibre number or type, muscle fibre cross-sectional (CS) area was substantially smaller in the twin than the single fetus.

Early fetal muscle development is characterized by an increase in fibre number (hyperplasia), until about 100 days of gestation in the sheep (Ashmore *et al.,* 1972, Swatland and Cassens, 1973). The subsequent increase in muscle mass, during late fetal and postnatal life until maturity, is achieved by muscle fibre hypertrophy, which is characterized by protein accretion in fibres resulting in both longitudinal growth (Williams and Goldspink, 1971) and an increase in the diameter of the fibres and an increase in DNA content. Total myonuclei number is directly related to body weight, muscle weight (Stickland *et al.,* 1975) and muscle fibre diameter (Allen *et al.,* 1979), suggesting that DNA accretion may be a prerequisite for subsequent muscle growth. This is supported by the observation that DNA accumulation in a muscle, as the result of satellite cell proliferation and fusion with existing fibres (Moss and Leblond, 1970, 1971), parallels protein accretion. In addition, the rapid phase of protein accretion corresponds with the greatest accumulation of DNA or myonuclei (Di Marco *et al.,* 1987).

The differences in body weight, muscle weight and myofibre CS area observed between twin and single fetuses in Chapters 3&4 suggest that there may also be differences in the DNA content of the muscle between birth ranks. Thus, the first aim of this study was to determine if the DNA content of a selected hindlimb muscle (*adductor*) differed between single and twin fetuses at 140 days of gestation. This muscle was selected because it was significantly heavier in single fetuses than in twins, and the longitudinal orientation of the fibres makes it possible to estimate the total number of fibres in a cross-section of the muscle.

In situations in which perturbations in morphological characteristics of a muscle, such as in fibre number or fibre cross-sectional (CS) area, are being investigated, estimation of fibre characteristics from a single region may give misleading results. This may be true of the comparison of fibre number and CS area characteristics of the *adductor* muscle between singles and twins in the present study. A prominent feature of large muscles such as the *adductor*, in which fascicle length is greater than 2-3 cm, is the intrafascicular terminations of their component fibres (reviewed by Gans and Gaunt, 1992). Whereas primary fibres appear to extend the length of the muscle, at least in small animals (e.g., rat, Duxson *et al.,* 1989; guinea pig, Duxson and Sheard, 1995), secondary fibres, which develop after the formation of primary fibres and eventually constitute the main bulk of adult muscle, form exclusively in the regions of innervation of the supporting fibre (Duxson *et al.,* 1989). Large muscles, in large animals such as sheep, are innervated by multiple endplates along the length of the muscle (Coers and Woolf, 1959; Wilson *et al.,* 1992). Since secondary fibres form in the regions of endplates, and since there are multiple endplates along large muscles, the number of secondary fibres formed in each region may be different. Estimates of fibre number from a single mid-belly region of this type of muscle therefore do not account for a large number of intrafascicularly terminating fibres, and greatly underestimate the total number of fibres in the adult muscle. As a result, consistent selection of the portion of muscle from which samples are taken for estimation of fibre number is necessary. In addition, the portion selected must be consistent across all muscles sampled. By selecting a particular portion of a muscle from which to sample, the assumption is made that this portion is representative of the whole muscle. Therefore, in situations in which

perturbations in morphological characteristics such as fibre number or CS area are investigated, determination of fibre characteristics from a single region may give misleading results. Thus, the second aim of this study was to determine whether the site of sampling (i.e., proximal, medial or distal), influenced results for fibre morphology characteristics including fibre number and CS area in the *adductor* muscle of single and twin fetuses.

5.3. MATERIALS AND METHODS

5.3.1. Animals

Muscle samples used in this study were derived from animals used in an experiment described previously (Chapter 4). The *adductor* muscle from eight twin and six single fetuses at 140 days of gestation was selected to compare the effects of birth rank on fibre characteristics (CS area and number) and DNA content of the muscle, and to test whether a single mid-belly cross-section of muscle is representative of the whole muscle.

5.3.2. Sample Collection

The *adductor* muscle was removed from both hindlimbs of each fetus and excess fat and connective tissue removed. Length and weight of the left-hand-side (LHS) muscle were recorded, and the whole muscle was frozen by immersion in melting isopentane chilled over liquid nitrogen, for later histological analysis of fibre number and CS area. The right-hand side (RHS) muscle was snap frozen in liquid nitrogen for later analysis of DNA content. The muscles were wrapped in aluminium foil and stored at -85°C until analysis.

Three regions (proximal, medial and distal) of the LHS muscle were selected for sectioning. In this study, the medial region refers to the middle of the muscle and the proximal and distal regions refer to a site 1cm inward from each end of the muscle.

Two cryostat sections (10μm) were cut from each selected region. One section was air-dried and stained with Haematoxylin Van Geisen (HvG), which differentially stains all fibres, connective tissue and nuclei. Stained sections were mounted using DPX mountant, and stored at room temperature until analysis. The other section was used for immunohistochemical identification of slow-twitch muscle fibres.

5.3.3. Immunohistochemistry

The methodology used to identify slow-twitch fibres is explained in Chapter 4.

5.3.4. Estimation of Total Fibre Number and Slow-Twitch Fibre Number

The sections stained with HvG were used for estimation of total fibre number, and the sections stained with 1A antibody were used to estimate slow-twitch fibre number. The method used to estimate total fibre number and slow-twitch fibre number is explained in Chapter 3.

5.3.5. Fibre Size

The method used to measure fibre size is explained in Chapter 3.

5.3.6. Muscle Volume

Muscle volume was estimated by averaging the cross-sectional areas of the sections at each of the 3 regions and multiplying by the length of the muscle.

5.3.7. DNA Extraction and Quantification

The muscle was divided into three parts: proximal ($^1/_3$), medial (middle $^1/_3$) and distal ($^1/_3$), and subsamples from each region used for estimation of DNA content by a modification of the method of Sambrook *et al.* (1989). A known amount of tissue (75-150 mg) was crushed using a French press chilled with liquid nitrogen, and then

homogenized in DNA extraction buffer (DNAzol, Life Technologies; 1 ml of DNAzol per 25 mg of tissue). The homogenate was centrifuged at 10 000 g for 10 minutes at room temperature, and the supernatant transferred to a fresh tube. The DNA was precipitated by adding 0.5 ml of 100% ethanol for each ml of DNAzol reagent used, and left on ice for 5 minutes before centrifugation at 10 000 g. The DNA pellet was washed twice with 95% ethanol, air dried for 10 minutes, and the DNA resuspended in double-distilled water (0.2-0.3 ml of water per 10-20 mg of animal tissue used), and left overnight at 4°C to dissolve. Protein contamination was minimized by phenol/chloroform/isoamyl alcohol (25:24:1) extraction (twice). The supernatant was transferred to a fresh tube and a half volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol added. The DNA precipitate was recovered by centrifugation at 1700 g for 2 minutes and the pellet washed twice with 70% ethanol. The pellet was air-dried for 10 minutes and resuspended in double-distilled water (0.2-0.3 ml of water per 10-20 mg animal tissue used). Quantification of DNA was achieved by measuring the Absorbance (260nm) value using a Microplate Spectrophotometer (Spectramax 250, Molecular Devices Corporation, Sunnyvale, California). The DNA content was calculated assuming that one Absorbance (260) unit was equivalent to 50 μg of DNA/ml which is the recommended correction factor when using this DNAzol Reagent (Life Technologies, 1995). Given the difficulties in preparing an accurate external standard for DNA, external standardization was not performed.

The total DNA content was determined using the average amount of DNA/mg tissue in each subsample from the three regions of the *adductor* muscle multiplied by the total muscle weight. Results are expressed as the total DNA content (mg) in the whole muscle. Total muscle weight was used as the weight of each of the three regions was not measured.

5.3.8. Statistical Analysis

Data was analysed using a general linear model procedure for Analysis of Variance in Minitab for Windows (Release 10, 1994). Results are expressed as unadjusted means±s.e.m.

5.4. RESULTS

At day 140 of gestation (term is about 147 days), single fetuses had significantly greater body weight, and *adductor* muscle weight and volume compared to twin fetuses (Table 5.1). Although the concentration of DNA did not differ between birth ranks, the total DNA content of the *adductor* muscle was greater in singles than in twins (Table 5.1).

Table 5.1. Fetal weight, and weight, volume and total DNA content of the *adductor* muscle in single and twin fetal sheep at day 140 of gestation (mean ± s.e.m.).

	Singles	Twins
Fetal Number	8	6
Fetal weight (g)	5750 ± 246	5016 ± 108**
Muscle weight (g)	10.5 ± 0.5	8.2 ± 0.3***
Muscle volume (mm^3)	7618 ± 413	6496 ± 210*
Total DNA content (μg)	6290 ± 617	4939 ± 280*
DNA concentration (ng/mg)		
Proximal region	597.7 ± 46.5	652.8 ± 40.3
Medial region	595.2 ± 34.1	572.8 ± 29.5
Distal region	590.1 ± 61.7	567.2 ± 53.5

*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Despite single fetuses having greater *adductor* muscle weights at day 140 of gestation, the total number of fibres and the number of slow-twitch fibres estimated at the proximal, medial and distal regions of the *adductor* muscle did not differ between ranks (Table 5.2). In contrast, although fibre CS area did not differ between ranks in the proximal region, singles had fibres with greater CS areas in the medial and distal regions of the muscle compared to twins (Table 5.2).

Although the estimated total fibre number in a CS did not differ between ranks in any of the three regions of the *adductor* muscle, within each birth rank the estimated total

fibre number per muscle CS was greater in the proximal than medial region of the muscle (P<0.001), and greater in the medial than the distal end of the muscle (P<0.001). Similarly, the estimated slow-twitch fibre number was greater in the proximal than medial region of the muscle (P<0.001), however estimated slow-twitch fibre number did not differ between the medial and distal regions, within each birth rank (Table 5.2.). The muscle fibre CS area within a muscle differed depending on the region of sampling. Fibre CS area was greater in the medial region than the proximal region (P<0.001) but did not differ significantly between the medial and distal regions, within each birth rank.

Table 5.2. Estimates of total fibre number, slow-twitch fibre number and average fibre cross-sectional area in cross-sections from the proximal, medial and distal regions of the *adductor* muscle in single and twin fetal sheep at day 140 of gestation (mean ± s.e.m.).

	Singles	**Twins**
Fetal Number	8	6
Fibre number ($x10^4$)		
Proximal region	86.74 ± 6.91	78.11 ± 5.61
Medial region	52.94 ± 2.61	57.38 ± 5.01
Distal region	25.28 ± 3.58	27.57 ± 3.10
Slow-twitch fibre number ($x10^4$)		
Proximal region	15.88 ± 1.33	16.11 ± 1.26
Medial region	5.51 ± 0.67	5.02 ± 0.36
Distal region	6.09 ± 0.73	5.43 ± 0.74
Fibre CS area (μm^2)		
Proximal region	74.3 ± 1.1	76.5 ± 4.9
Medial region	94.7 ± 4.7	78.9 ± 2.6**
Distal Region	110.6 ± 3.0	93.8 ± 3.7**

*, P<0.05; **, P<0.01.

5.5. DISCUSSION

The difference in body weight between singles and twins is consistent with previous studies (Robinson *et al.,* 1977; Nordby *et al.,* 1987; Sailer *et al.,* 1994; Hunt *et al.,* 1996; see also Chapters 3&4). Single fetuses also had greater *adductor* muscle weights compared to twins, which is consistent with previous studies (Sailer *et al.,* 1994; see also Chapters 3&4). The volume of the *adductor* muscle was also considerably greater in singles than in twins. The difference in muscle mass between singles and twins appears to be primarily due to a difference in fibre CS area, as seen in both the medial and distal regions of this muscle, as the number of muscle fibres at each region of the muscle was similar between birth ranks. The greater muscle fibre CS area observed in singles was also associated with a greater DNA content of the whole muscle. An increase in DNA content of a muscle parallels an increase in protein content during muscle fibre hypertrophy. Therefore, both the greater fibre CS area and the greater DNA content of the *adductor* muscle from singles, indicate that fibre hypertrophy was more advanced in singles than in twins near birth.

It is likely that DNA accretion is a prerequisite for subsequent muscle growth, as total myonuclei number is directly related to body weight, muscle weight (Stickland *et al.,* 1975) and muscle fibre diameter (Allen *et al.,* 1979). The greater DNA content which was associated with greater fibre CS area of the *adductor* muscle of singles in this study, suggests that DNA accretion may allow a greater rate of hypertrophy.

Skeletal muscle consists of multinucleated muscle fibres that gain additional nuclei (DNA) as a result of postmitotic satellite cell fusion (Moss and Leblond, 1971), facilitating muscle hypertrophy (Allen *et al.,* 1979). Since total DNA content is an indicator of myonuclei number (Moss, 1968; Kang *et al.,* 1985), the greater total amount of DNA in the *adductor* muscle of singles suggests that there may be differences in satellite cell proliferation and subsequent fusion to the preformed fibres. This may contribute to the observed difference in the total DNA content between ranks. Whether there are differences in the number of active satellite cells or the proportion

that have fused to become myofibres between muscles from single and twin fetuses is yet to be determined.

Differences in fibre morphological characteristics between singles and twins offered an opportunity to investigate whether taking a single 'representative' sample from a muscle is adequate to illustrate morphological differences which may be characteristic of the whole muscle. Estimation of the total number of muscle fibres in a given cross-section of a large muscle is difficult due to the large number of fibres - in the order of 200,000-800,000 fibres for the *adductor* muscle of fetal sheep in this study. Unless a fully automated system is available, direct counting of all of the fibres is a formidable task. Therefore, when perturbations in the number, type, or CS area of muscle fibres are being investigated, a common method for investigating possible differences in fibre characteristics is to take a cross-section of muscle from a selected region of the muscle in question, often the mid-belly region, and consistently select samples from this region in all the muscles sampled. This minimizes the error due to differences that may be associated with intrafascicular terminations of fibres (reviewed by Gans and Gaunt, 1992) in other regions of the muscle. In doing so, the assumption is made that this region is representative of the whole muscle.

Using the above method for estimating fibre numbers in a CS of muscle, the total number of fibres and the number of slow-twitch fibres in the cross-section did not differ between birth ranks at any of the three sampling points along the muscle. This result suggests that a single mid-belly sample would be representative of the whole muscle when estimating muscle fibre number in a particular CS of the *adductor* muscle. However, the number of fibres decreased from the proximal to the distal end, presumably because of the physical shape of this muscle. The *adductor* muscle is a wedge-shaped muscle, with the proximal region having a much greater CS area than the distal region and, hence, greater space in which fibres can form.

The CS area of the fibres was not significantly different between birthranks in the proximal region, however, the fibres in the muscles from singles had greater fibre CS areas in the medial and distal regions of the muscle compared to twins. In addition,

muscle fibre CS area within each birth rank increased from the proximal to the distal region of the muscle. Although the reasons for these differences are unclear, this result illustrates that a single 'representative' sample would not be adequate to yield accurate information about the CS area of muscle fibres in the *adductor* muscle from both singles and twins.

During growth, an increase in the total muscle DNA content parallels an increase in body and muscle weight (Stickland *et al.,* 1975), and fibre CS area (Allen *et al.,* 1979). Consistent with these observations, the greater total DNA content of the *adductor* muscle of singles than twins was associated with greater muscle weight and fibre CS area, indicating more advanced fibre hypertrophy in singles compared to twins near birth. Since satellite cells are the primary source of new nuclei near birth, it is likely that differences in their proliferation and/or fusion contribute to the observed difference in DNA content between ranks. This hypothesis is investigated in Chapter 6.

The differences in fibre CS area between birth ranks and between regions of the muscle studied indicate that, for some muscles, a mid-belly sample may not be representative of the whole muscle. Therefore, it is important to take samples from several regions along the muscle to accurately identify possible perturbations in fibre characteristics in the muscle under study. If the results are the same in each of the regions studied, such as for fibre number in this study, then taking a representative sample would be valid. However, when differential effects are seen to be dependent on the regions from which the sample was taken, such as for fibre CS areas in this study, sampling along the length of the muscle would be necessary.

Chapter Six

IMMUNOHISTOCHEMICAL DETECTION OF PROLIFERATING CELLS IN MUSCLES OF FETAL AND NEONATAL LAMBS.

6.1. ABSTRACT

The relative role of myogenic precursor nuclei (satellite cells) in myofibre hypertrophy in late fetal and postnatal life is not well understood. This is primarily due to the lack of a reliable marker for these cells. The myogenic regulatory factor MyoD has been used as a marker for active satellite cells in rodents, however, its usefulness in ovine tissue has not been described. In this study, MyoD appeared to be a useful marker for active satellite cells in the sheep fetus/neonate and may also be involved in the maintenance of satellite cells in an active state facilitating myofibre hypertrophy.

The twin fetal/neonatal lamb has lower muscle weights, smaller myofibre cross-sectional areas and reduced muscle DNA contents, but similar myofibre complements, compared to the single. This implies that myonuclei accumulation in a muscle is more important than myofibre number in the determination of muscle size in the fetal/neonatal lamb. Therefore, the aim of this trial was to determine if there were differences in the total nuclei content and myogenic precursor cell number between selected skeletal muscles from single and twin fetuses/neonates. Twenty-two single-bearing and thirteen twin-bearing mature Coopworth ewes were mated naturally. Eighteen single-bearing and nine twin-bearing ewes were randomly assigned to one of three slaughter groups (100, 120 and 140 days of gestation). The remaining ewes were kept on pasture until twenty days postpartum at which time four single and four twin lambs were sacrificed. Following sacrifice, fetal weight, crown-rump length and girth were measured. The weights, lengths and histological characteristics of selected hindlimb muscles were determined.

Twin fetuses/neonates had lower body weights and muscle weights compared to singles. Lower muscle weights in the twins than singles were also associated with smaller myofibre cross-sectional areas and lower total nuclei numbers and myogenic precursor cell numbers per muscle in selected hindlimb muscles. These results suggest that myofibre hypertrophy in late gestation and early postnatal life is related to myogenic precursor cell activity which may have important implications for growth potential of the growth-restricted fetus.

6.2. INTRODUCTION

The classical method for detecting cells engaged in DNA synthesis *in vivo* is by their uptake of [3H]-thymidine, identified using autoradiography (Moss and Leblond, 1970; Denekamp and Kallman, 1973). However, this is very expensive when using large numbers of animals, such as in the present trial. The thymidine analogue 5-bromo-2-deoxyuridine or BrdU, which is incorporated into cellular DNA during the S-phase of cell replication, has also been used and can be localized in histological sections with a monoclonal antibody and an immunoenzyme detection method (horseradish peroxidase-diaminobenzidine) or by immunofluorescence (Gratzner, 1982; Dolbeare *et al.,* 1983; Morstyn *et al.,* 1983; Allison *et al.,* 1985; Gonchoroff *et al.,* 1985; Vanderlaan and Thomas, 1985; Harms *et al.,* 1987; Schutte *et al.,* 1987). This method is not suitable to label replicating cells of fetal sheep following administration to the dam, as this compound does not permeate the feto-placental barrier (see Appendix 1). An alternate marker for detecting actively proliferating cells is MyoD. This member of the basic helix-loop-helix transcription factor family is a nuclear protein that has been used to identify active satellite cells in neonatal mouse tissue (Koishi *et al.,* 1995). However, it may also label some recently incorporated myonuclei in adult mouse tissue (Hughes *et al.,* 1997). It is unknown which nuclei are labelled by MyoD in fetal and neonatal sheep muscle tissue.

In Chapters 3 and 4 it was demonstrated that twin fetuses have lower body and muscle weights than singles as a result of maternal constraint characterized by placental insufficiency. Lower muscle weights in twins compared to singles were also associated with a smaller mean myofibre cross-sectional (CS) area (see Chapter 4) and lower total DNA content (see Chapter 5) of the *adductor* muscle. Since an increase in DNA content accompanies myofibre hypertrophy (Rattray *et al.,* 1975), and satellite cells are the only source of new nuclei during late fetal and early postnatal muscle growth, an effect on satellite cell and subsequent myonuclei number is suggested.

During embryonic and fetal stages of development, presumptive myoblasts are responsible for the development of skeletal muscle (Stockdale, 1992). These cells proliferate, differentiate and fuse end to end to form myotubes and eventually muscle fibres (Swatland, 1984; Franzini-Armstrong and Fischman, 1994; Hauschka, 1994). Fetal muscle development is characterized by an increase in myofibre number as a result of hyperplasia of presumptive myoblasts. Postnatal muscle growth, however, is the result of cellular hypertrophy characterized by increased DNA and protein content (Moss, 1968; Kang *et al.,* 1985) without a significant increase in the number of myofibres (Smith, 1963). The high correlation between total muscle mass and total nuclei number or DNA (Robinson and Bradford, 1969; Ezekwe and Martin, 1975; Powell and Aberle 1975; Aberle and Doolittle, 1976; Harbison *et al.,* 1976), emphasizes the importance of understanding the control of nuclei proliferation in the study of regulation of skeletal muscle growth.

Postnatal myonuclei do not divide and are considered to be postmitotic (Stockdale and Holtzer, 1961). Satellite cells, which are located between the plasmalemma and basal lamina of the muscle fibre (Mauro, 1961), undergo DNA replication and mitosis during muscle growth (Shafiq *et al.,* 1968; Moss and Leblond, 1970) producing daughter nuclei. These nuclei become incorporated into muscle fibres (Moss and Leblond, 1971; Snow, 1978) facilitating postnatal skeletal muscle hypertrophy (Allen *et al.,* 1979). Satellite cells become mitotically quiescent at maturity (Schultz *et al.,* 1978) when muscle mass becomes static. However, given an appropriate stimulus (Allen and Rankin, 1990), they can be induced to proliferate, differentiate and fuse with adjacent fibres (Moss and Leblond, 1971) resulting in increased myonuclei number for fibre repair (Grounds, 1991) and growth, such as that observed following muscle overload (Rosenblatt *et al.,* 1994) and exercise (Alway *et al.*, 1989).

There have been numerous *in vivo* studies of satellite cell content and mitotic activity during postnatal growth (Schultz, 1974; Campion, 1984). It is generally accepted that in growing mammalian muscle the percentage of satellite cell nuclei decreases until mature muscle weight is achieved (Campion, 1984). As a result of maternal constraint operating through limitation of placental size, twin lambs have lower birth weights and

muscle weights (see Chapters 3 & 4). Lower muscle weights in twins than singles are also associated with lower muscle DNA content to accommodate a larger myofibre volume (see Chapter 5). It therefore follows that there may be compensatory changes in the behaviour of satellite cells during the growth period, as a continued high rate of satellite cell proliferation may be required to maintain an appropriate DNA unit size in enlarging muscle fibres. The "DNA unit size" (Cheek, 1985) is a term used to describe the concept that each nucleus has jurisdiction over a certain volume of cytoplasm. This idea is supported by *in vitro* work (Ralston and Hall, 1992) showing that mRNA produced by a myonucleus is confined to the area immediately surrounding that myonucleus. The DNA unit size has been expressed biochemically as the protein to DNA ratio (Cheek, 1985) and histologically as the cytoplasmic volume to nucleus ratio (Landing *et al.*, 1974; Matthew and Moore, 1987).

Since there are no previous reports on the use of MyoD as a marker of satellite cells in fetal and neonatal sheep tissues, the first aim of this study was to determine the usefulness of MyoD as an endogenous marker for identification of active satellite cells in skeletal muscle tissue. The differences in ovine fetal and muscle mass, myofibre CS area and total muscle DNA content observed in previous studies (see Chapters 3,4 & 5) suggest that the slower rate of myofibre hypertrophy in the growth-restricted twin is related to differences in myonuclei number. Since satellite cells are the only source of new nuclei during late fetal and postnatal muscle growth, an effect on satellite cell activity is suggested. Therefore, the second aim of this experiment was to identify possible differences between birth ranks in the total nuclei content, and proliferation and subsequent fusion of satellite cells into existing muscle fibres, using MyoD as a marker for active satellite cells.

6.3. MATERIALS AND METHODS

6.3.1. Animals

Two-hundred, five to seven year-old Coopworth ewes were mated in late March. Oestrus was synchronized with progesterone-impregnated controlled internal drug releasers (Eazi-Breed CIDR type G; Carter Holt Harvey Plastic Products, Hamilton, New Zealand) and ewes were mated naturally using rams fitted with mating harnesses and crayons to enable identification of mated ewes. Only those mated in the first three days of the first oestrous cycle were used. Pregnancy status was determined by ultrasound at day fifty-five of gestation, and twenty-two single-bearing and twelve twin-bearing ewes (balanced for age and date of mating) were randomly selected. Ewes were grazed on pasture as a single group following mating.

6.3.2. Sample Collection

Six single-bearing and three twin-bearing ewes were sacrificed at each of three gestational ages (100, 120 and 140 days) and four single lambs and four twin lambs were sacrificed at postnatal day twenty (PN20). Ewes and lambs were slaughtered by captive bolt pistol and exsanguination. The intact uterus was removed, weighed and the fetuses exposed. The umbilical cord was ligated and severed, and the fetuses weighed and euthanased with sodium pentobarbitone via cardiac puncture. The crown-rump length (CRL) and chest girth were measured and the sex recorded. Eight major hindlimb muscles (*biceps, gastrocnemius, plantaris, adductor, semitendinosus, semimembranosus, quadraceps, gluteus*) were dissected from the left hindlimb of each fetus, and their weight and length (using digital calipers) measured. The femur, tibia and humerus bones were also dissected, and weights and lengths recorded. A portion of the *semitendinosus* and *plantaris* muscles from one dam was also taken for immunohistochemical determination of MyoD-positive cells. The placenta was dissected out and the numbers and weights of caruncles and cotyledons determined.

6.3.3. Histology Samples

From the eight hindlimb muscles dissected, the *plantaris*, *semitendinosus*, *adductor* and *gastrocnemius* muscles were selected for histological characterisation of muscle fibre number and CS area, total muscle nuclei number (myonuclei + connective tissue nuclei) and satellite cell nuclei. These muscles were selected on the basis of their size and ease of sectioning. The *gastrocnemius* muscle was divided into two as it is too large to cryostat section whole. Both portions were sectioned and data were pooled.

Dissected muscles were frozen rapidly (whole) in isopentane chilled over liquid nitrogen, wrapped in aluminium foil and stored initially in liquid nitrogen (up to 4 hours), then long term at -80°C, until sectioning.

Three regions (proximal, medial and distal, see Chapter 5) of each muscle were selected for sectioning. Four cryostat sections (10 μm) were cut from each region selected. Two sections were air-dried and stained with Haematoxylin and Eosin (H&E), which differentially stains all fibres pink and nuclei blue/black. Stained sections were mounted using DPX mountant, and stored at room temperature until analysis. The other sections were used for immunohistochemical identification of MyoD-positive nuclei. Cryostat sections (10 μm) were also made from the portions of the *semitendinosus* and *plantaris* muscles taken from the pregnant dam. These sections were used for immunohistochemical identification of MyoD-positive nuclei.

6.3.4. Immunohistochemistry

Cryostat sections (10 μm) were air-dried for at least 1 hour, then fixed in 10% buffered formalin for 5 minutes. Sections were washed in 0.1 M phosphate buffered saline (PBS) pH 7.4 (2x5 min) to stop fixation. A glue ring was applied and sections were incubated in 1% BSA (bovine serum albumin) in PBS for 10 minutes to reduce non-specific binding, and incubated overnight at 4°C in a humid chamber with an affinity-

purified rabbit antiserum raised against TrpE fusion of amino acids 3-318 of mouse MyoD (Koishi *et al.*, 1995) (a gift from J. Harris, University of Otago Medical School, Dunedin, New Zealand), diluted at 1:50 in diluting buffer (PBS; 0.5% BSA (w/v)). Sections were washed twice in PBS (2x5 min) and incubated for 30 minutes at room temperature with biotinylated-anti-rabbit IgG (Amersham, Life Science, Buckinghamshire, UK), diluted (1:200) in PBS; 0.5% BSA (w/v). The immunoreactivity was visualised by incubating the sections in streptavidin biotinylated horse-radish-peroxidase complex (Amersham, Buckinghamshire, UK) diluted (1:200) in PBS; 0.5% BSA (w/v) for 15 minutes, and for 3 minutes in a mixture containing 5 mg diaminobenzidine (DAB), 8 µl of hydrogen peroxide activator and 10 ml of PBS. Sections were then washed in PBS, dehydrated, and mounted using DPX mounting medium and stored at room temperature until analysis.

For analyses two sections were mounted on each slide. One section was incubated with the primary antibody (anti-MyoD; Plates 6.1., 6.3. & 6.5.) while the other section was used as a negative control (method as above omitting the primary antibody; Plates 6.2., 6.4. & 6.6.) .

Unfortunately, the samples from day 140 of gestation for immunohistochemistry were irreversibly damaged following collection, and therefore there are no data for myogenic precursor nuclei number at this age.

6.3.5. Double-Labelling

In order to determine whether the MyoD-positive nuclei were located in the satellite cell position or whether they were in fact myonuclei, double-labelling with anti-dystrophin, which labels the plasmalemma of the myofibre, was necessary. The same methodology as above was employed, but replacing anti-MyoD with the mouse monoclonal antibody anti-dystrophin (Sigma Chemical Company) diluted (1:10) in PBS; 0.5% BSA (w/v). The

secondary antibody, biotinylated-anti-mouse IgG (Amersham, Buckinghamshire, UK), was also diluted 1:200 in PBS; 0.5% BSA (w/v).

Plate 6.1. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 100 days of gestation. The nuclei have been labelled with a MyoD antibody (brown) and the myofibres counterstained pink with Eosin (Mag. x500).

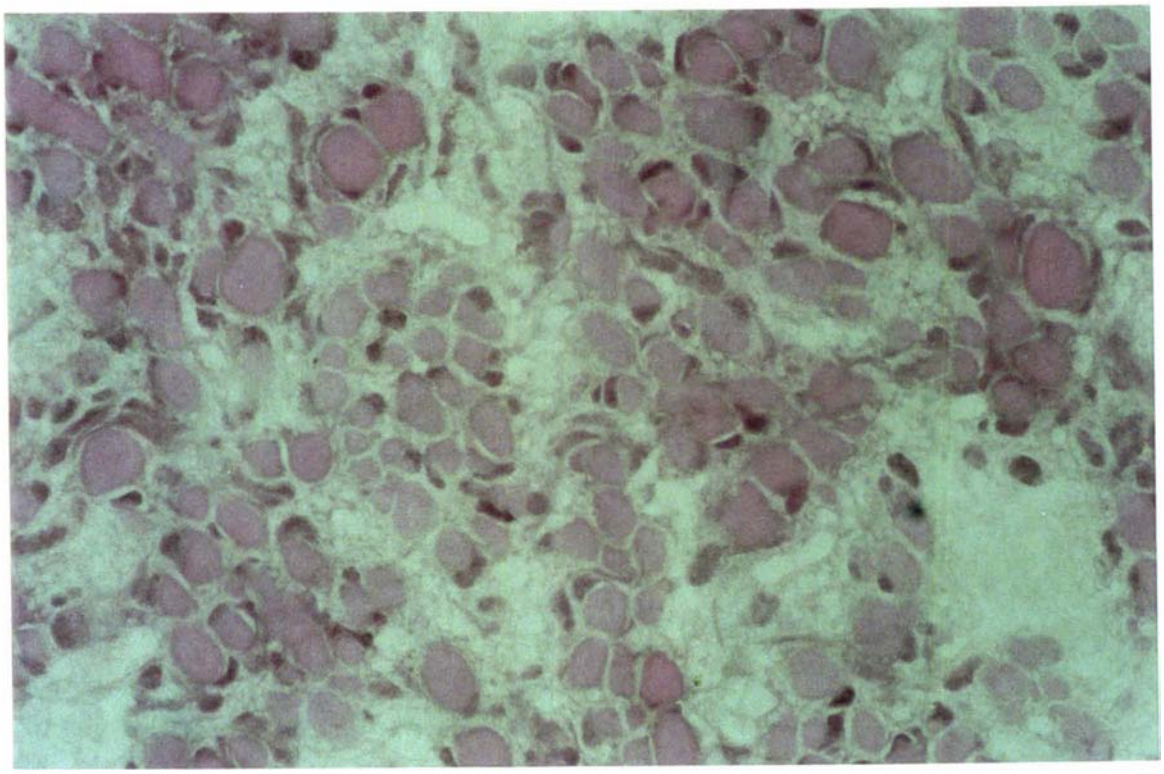


Plate 6.2. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 100 days of gestation. This section has been treated the same as the section in plate 6.1. omitting the primary antibody (negative control). Myofibres are counterstained pink with Eosin (Mag. x500).

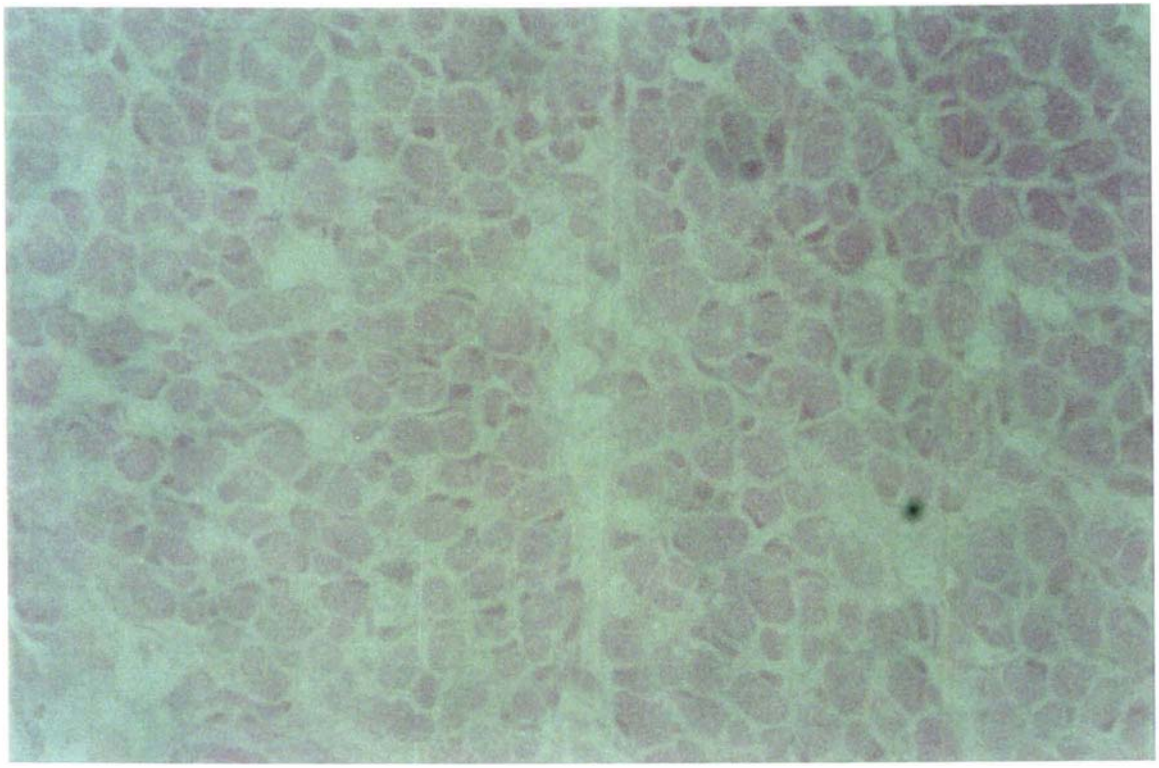


Plate 6.3. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 120 days of gestation. The nuclei have been labelled with a MyoD antibody (brown) and the myofibres counterstained pink with Eosin (Mag. x500).

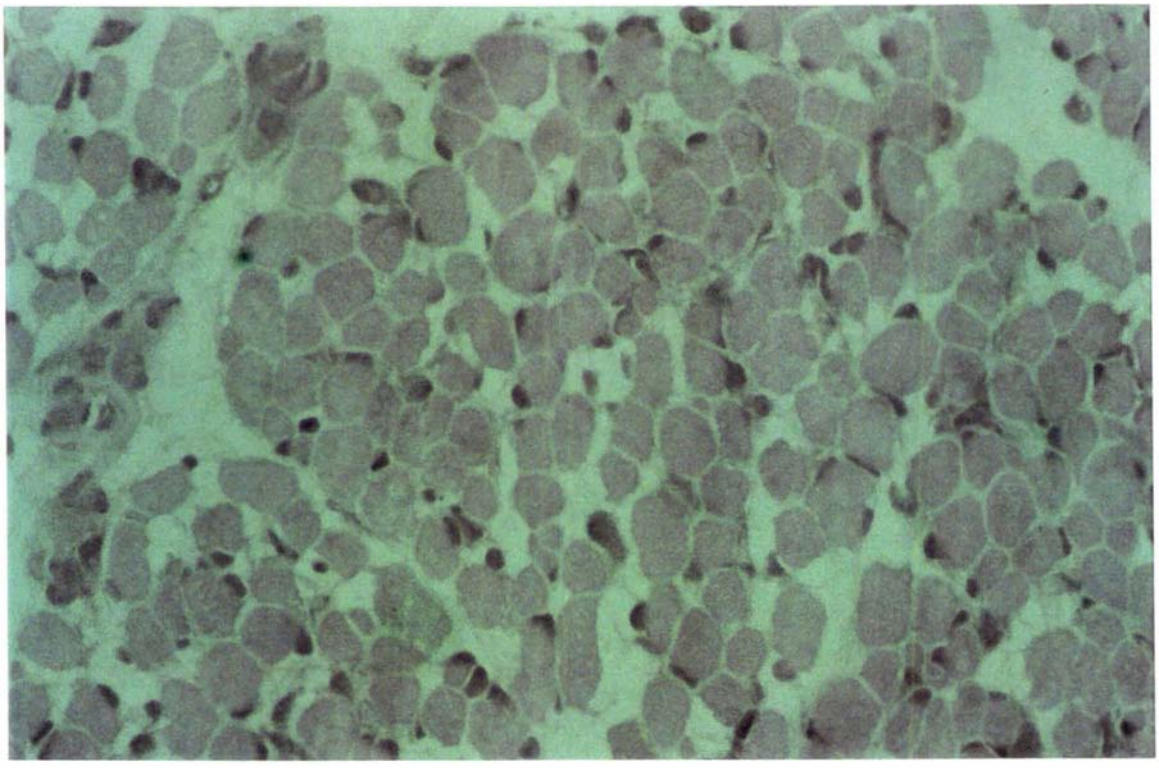


Plate 6.4. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 120 days of gestation. This section has been treated the same as the section in plate 6.3. omitting the primary antibody (negative control). Myofibres are counterstained pink with Eosin (Mag. x500).

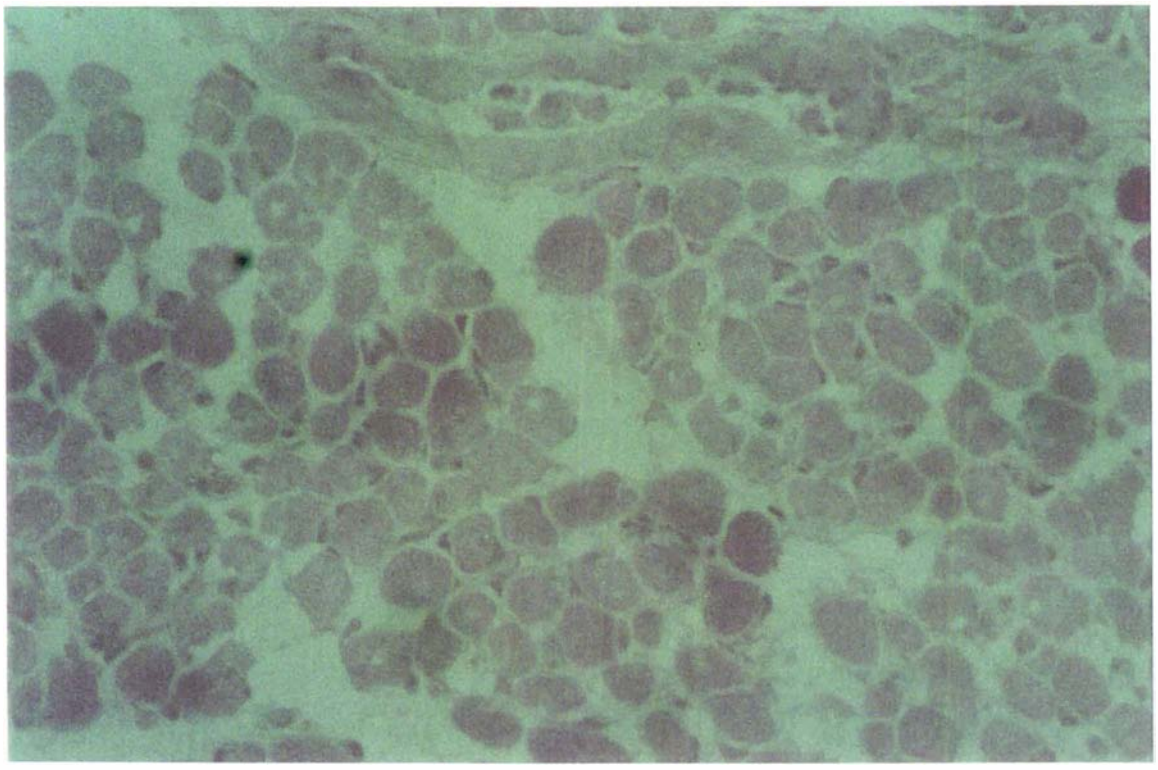


Plate 6.5. Photomicrograph of a portion of the *plantaris* muscle from a lamb at postnatal day 20. The nuclei have been labelled with a MyoD antibody (brown) and the myofibres counterstained pink with Eosin (Mag. x500).

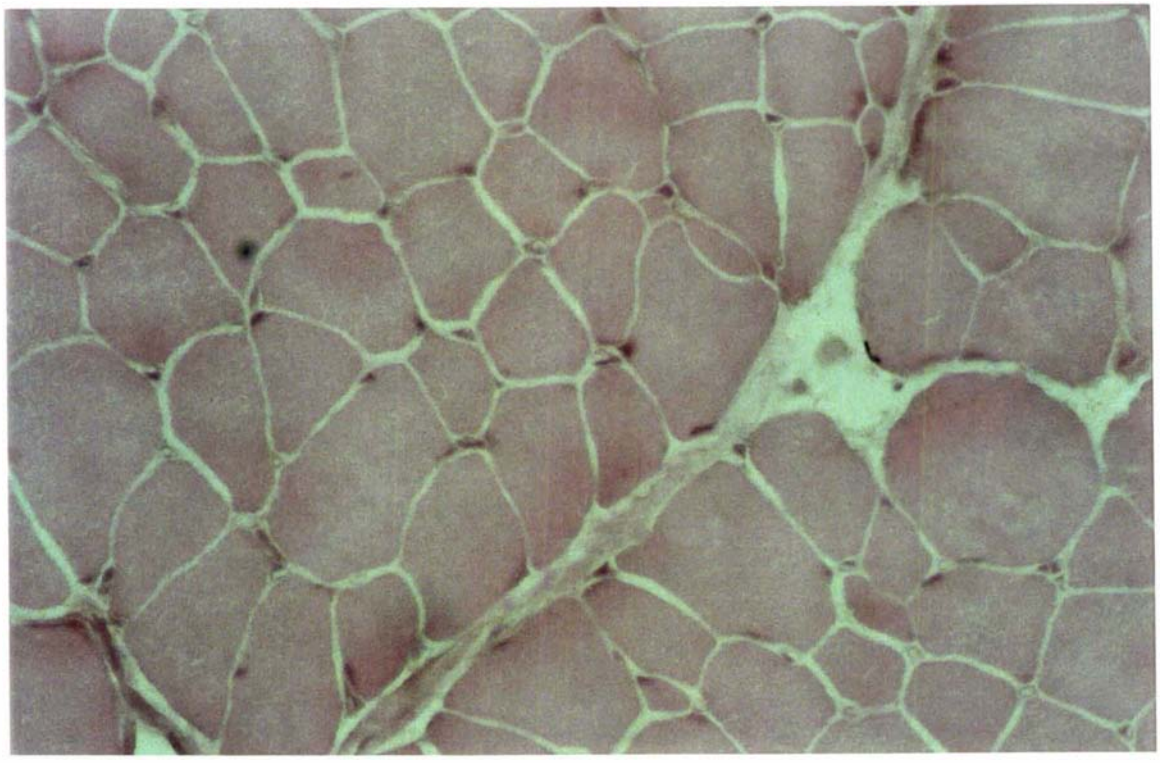
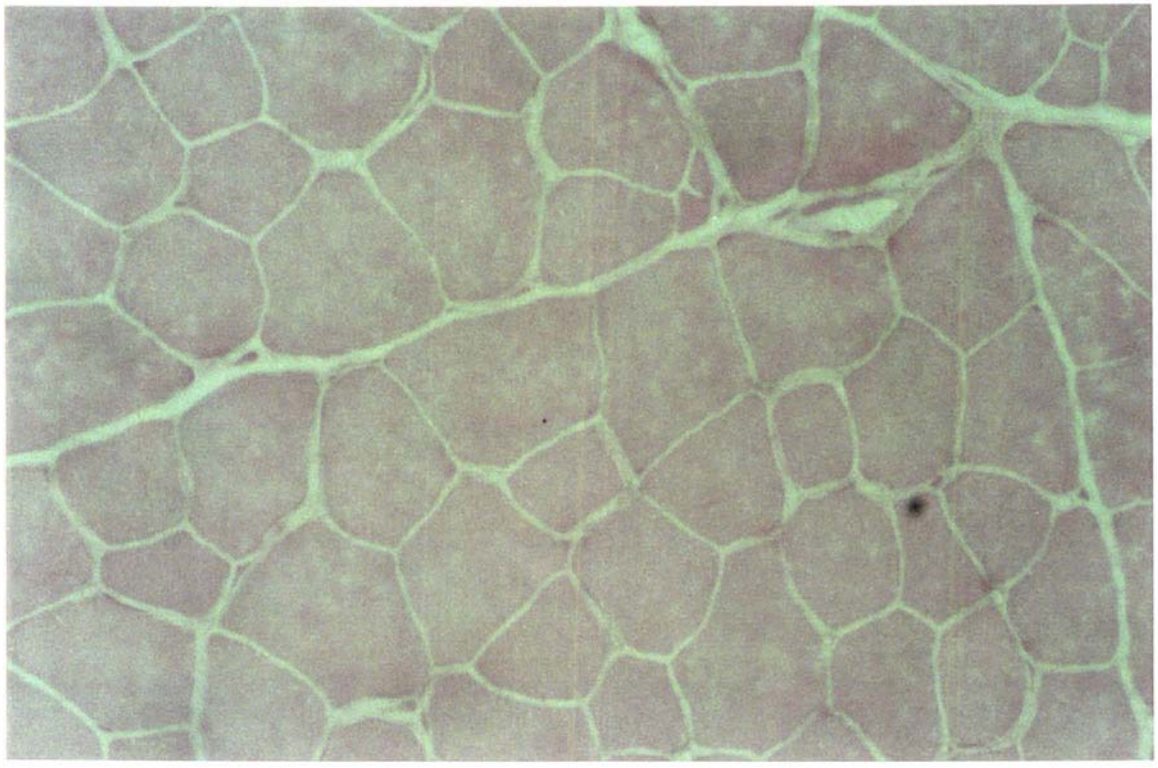


Plate 6.6. Photomicrograph of a portion of the *plantaris* muscle from a lamb at postnatal day 20. This section has been treated the same as the section in plate 6.5. omitting the primary antibody (negative control). Myofibres are counterstained pink with Eosin (Mag. x500).



6.3.6. Estimation of Total Fibre Number and Fibre Cross-Sectional Area

The sections stained with H&E were used for estimation of total fibre number (day 140 animals only) and fibre CS area (day 140 and PN20 animals only). The methodology used is explained in Chapter 5.

6.3.7. Estimation of Total Nuclei Number and MyoD-Positive Nuclei Number

The sections stained with H&E were used for estimation of total nuclei number per muscle at each stage of gestation (Plates 6.7.-6.10.). Using a digitiser pad attached to a computer using the Sigma Scan Scientific Measurement Programme (Jandel Scientific, 1988 - Version 3.90), the total number of nuclei in a 200 μm^2 area was determined. A total of five

areas, selected using a random stratified sampling method (Gundersen *et al.,* 1988) were counted, resulting in direct counting of 500-1500 nuclei per cross-section of muscle. Estimates were made from all sections, i.e., sections from the proximal, medial and distal regions of each muscle from each animal. The total cross-sectional areas of the muscle sections were determined by projecting the section image onto a screen, tracing its outline, and determining its area using the Sigma Scan Scientific Measurement Programme. An estimate of the total number of nuclei in each cross-section of muscle was determined from the following equation: estimated total nuclei number per muscle CS = (muscle CS area/200 μm^2) x average nuclei number per photo. Muscle volume was estimated by averaging the cross-sectional areas of the sections at each of the three regions and multiplying by the length of the muscle. An estimate of the total number of nuclei in each muscle was calculated using the following equation: total number of nuclei in muscle = total number of nuclei per muscle CS x (muscle length/section thickness). It is the product of this equation that is referred to in the Results section.

The number of MyoD-positive nuclei per muscle was calculated using the same method described above. Only the medial sections from muscles of PN20 lambs were analysed. In order to determine the proportion of MyoD-positive nuclei that lie in the satellite cell position, sections double labelled with MyoD/anti-dystrophin were analysed. A total of up to 400 nuclei from each of fifteen regions were classified as either inside the plasmalemma (myonuclei) or outside the plasmalemma (in the satellite cell position). Due to the tedious and time-consuming nature of this analysis, detailed analysis of only one section from each muscle from each PN20 animal was carried out. PN20 animals were chosen because the nuclei in the satellite cell position are likely to be true satellite cell nuclei rather than fetal/late myoblasts which may be present in the fetal muscle tissues.

Plate 6.7. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 100 days of gestation. The skeletal muscle had been stained with Haematoxylin and Eosin with differentially stains nuclei blue/black and myofibres pink. (Mag. x500).

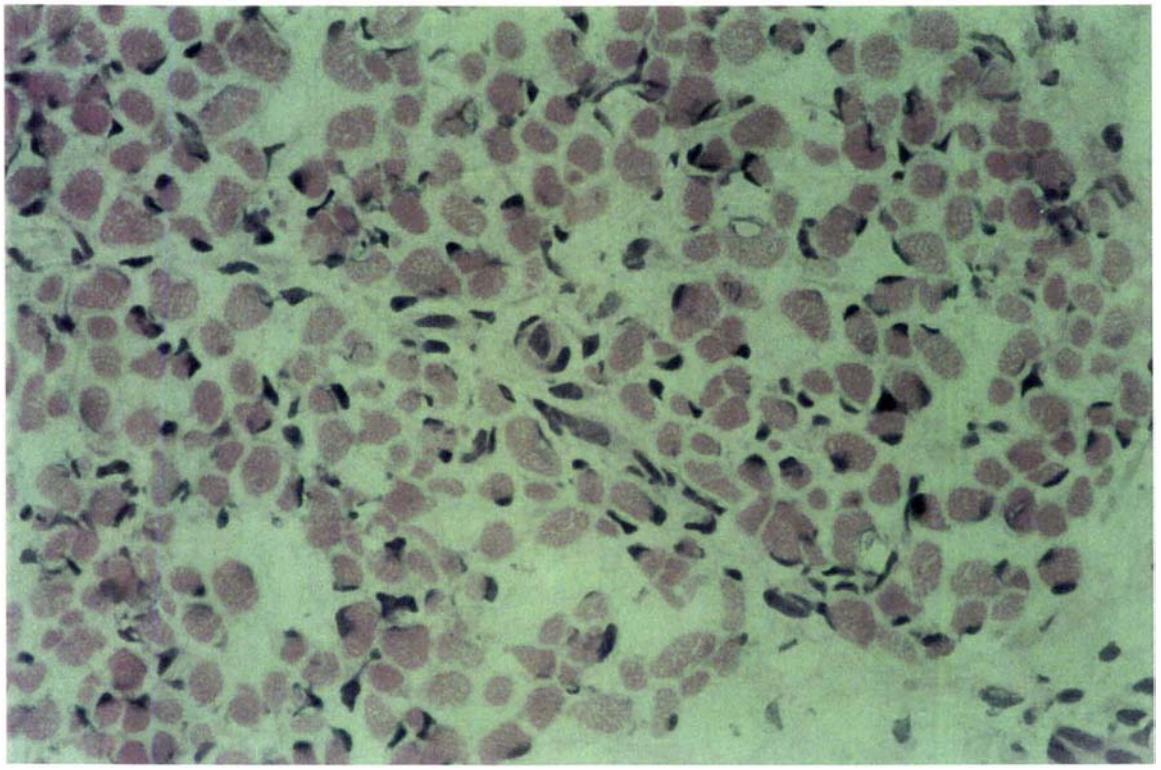


Plate 6.8. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 120 days of gestation. The skeletal muscle has been stained with Haematoxylin and Eosin with differentially stains nuclei blue/black and myofibres pink. (Mag. x500).

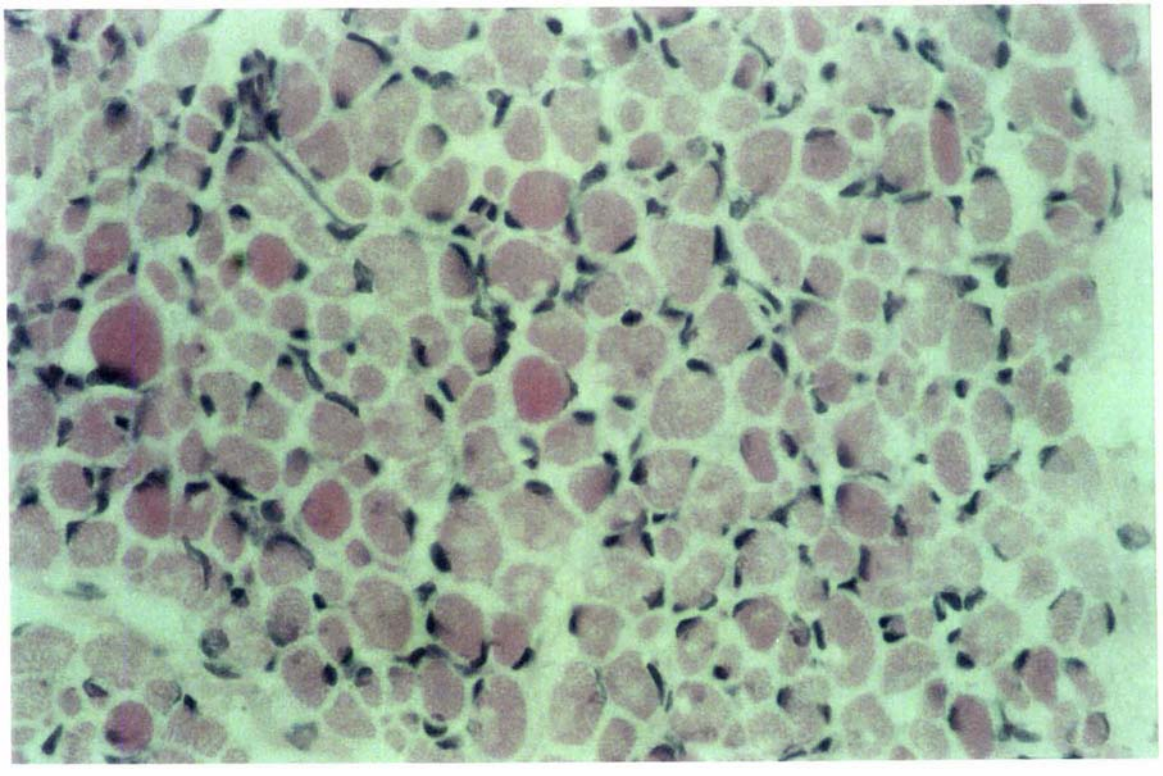


Plate 6.9. Photomicrograph of a portion of the *plantaris* muscle from a sheep fetus at 140 days of gestation. The skeletal muscle has been stained with Haematoxylin and Eosin with differentially stains nuclei blue/black and myofibres pink. (Mag. x500).

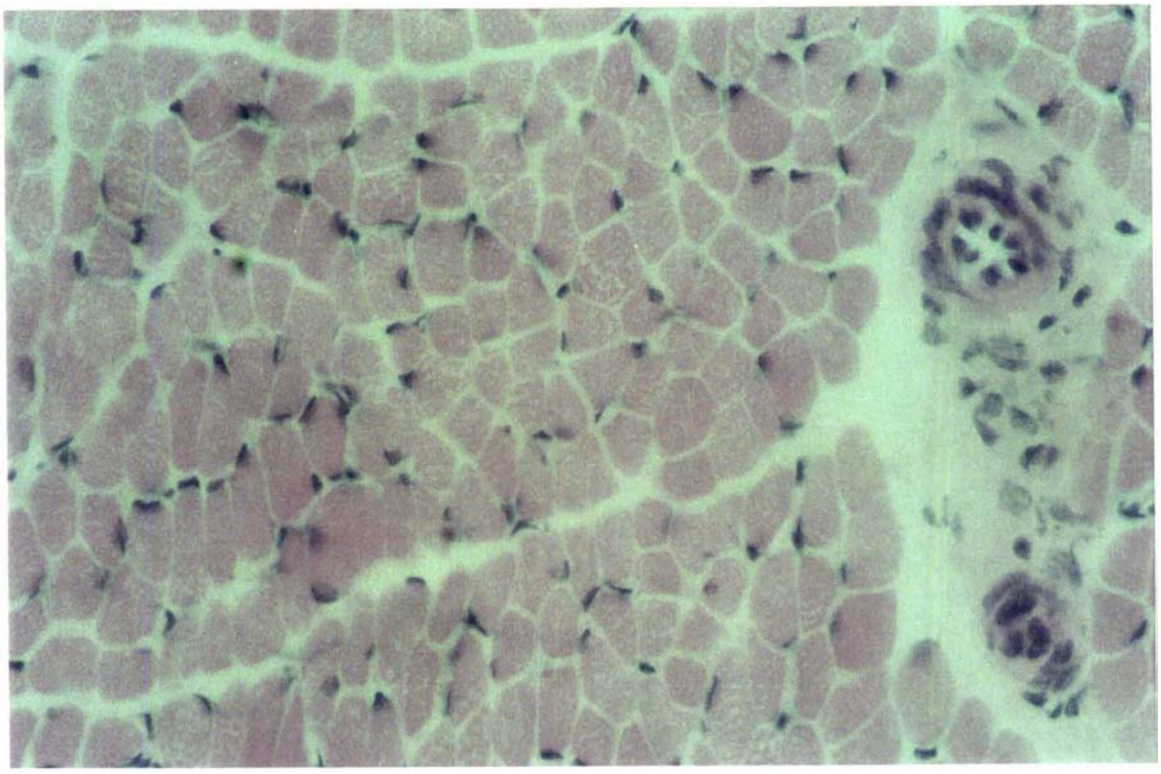
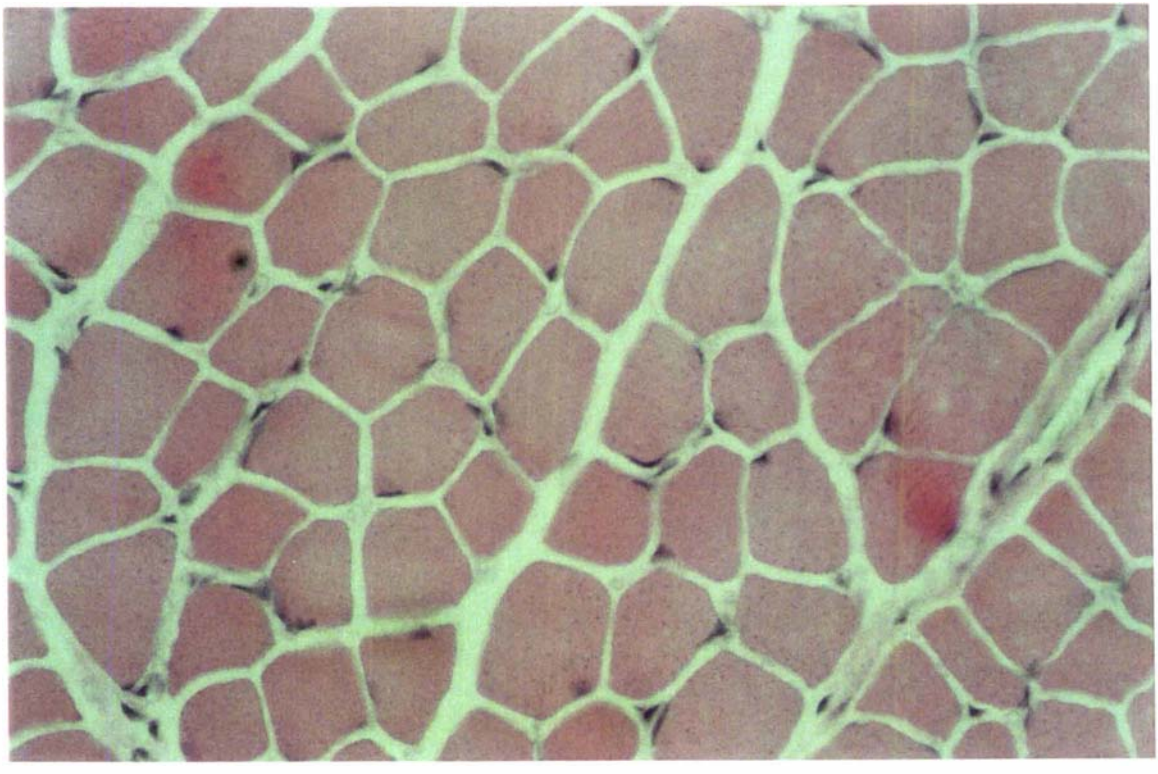


Plate 6.10. Photomicrograph of a portion of the *plantaris* muscle from a lamb at postnatal day 20. The skeletal muscle has been stained with Haematoxylin and Eosin with differentially stains nuclei blue/black and myofibres pink. (Mag. x500).



6.3.8. Statistical Analysis

Data were analysed using a general linear model procedure for Analysis of Variance in Minitab for Windows (Release 10, 1994) to determine the individual effects of birth rank and stage of gestation, and the overall effects of birth rank, stage of gestation and the birth rank by stage interaction. Results are expressed as unadjusted means±s.e.m.. Fetal data were adjusted for sex and common maternal environment (for twins). These effects remained in the model when significant and are discussed in the text where relevant.

6.4. RESULTS

Twin-bearing ewes had a greater TGUW than single-bearing ewes at each stage of gestation studied (Table 6.1.). Total placentome weight was significantly greater for twin- compared to single-bearing ewes. However, total placentome weight per fetus was lower in twin- compared to single-bearing ewes at day 100 and 140 of gestation (Table 6.1.). A similar trend for total placentome weight per fetus was observed at day 120 of gestation. Although total placentome number did not differ between twin- and single-bearing ewes at any stage of gestation studied, total placentome number per fetus was considerably lower for twin- than for single-bearing ewes at both day 100 and 140 of gestation (Table 6.1.).

Although singles and twins had similar body weights at day 100 of gestation, twins had considerably lower body weights than singles at day 140 of gestation and PN20 (Table 6.2.). A similar trend for body weight was observed at day 120 of gestation. Overall, there were significant birth rank and stage of gestation effects on body weight ($P<0.001$), and a birth rank by stage of gestation interaction ($P<0.001$). At day 140 of gestation, twins had a smaller CRL than singles, but this effect was not apparent at PN20 (Table 6.2.). Twins had a smaller girth compared to singles at day 120 of gestation, an effect that was also apparent at day 140 of gestation and PN20 (Table 6.2.). Overall, both birth rank and the stage of gestation had significant effects on girth

($P<0.001$) and there was a birth rank by stage interaction ($P<0.001$), reflecting a divergence between the birth ranks with increasing age in favour of singles.

All the muscles studied were lighter in the twins than in the singles at day 120 and 140 of gestation, and at PN20 (Table 6.3.). At day 120 of gestation (Table 6.4.) the *plantaris* and *gastrocnemius* muscles were shorter in twins than in singles, but this effect was not observed at any other stage studied. Birth rank and the stage of gestation ($P<0.001$) had overall effects on the weights of each of the muscle studied. A significant interaction between birth rank and stage ($P<0.001$) was also observed.

The estimated total nuclei number per muscle was lower for twins than for singles at PN20 in the *plantaris* and *gastrocnemius* muscle, an effect that was also observed at day 120 of gestation in the *gastrocnemius* muscle (Table 6.5.). A similar trend was also observed at day 140 of gestation and PN20 in the *adductor* muscle, at day 120 of gestation in the *semitendinosus* muscle, and at day 140 in the *gastrocnemius* muscle (Table 6.5.). Overall, birth rank had a significant ($P<0.05$) impact on estimated total nuclei number per muscle for the *adductor*, *plantaris* and *gastrocnemius* muscles, with a similar trend observed for the *semitendinosus* muscle. The stage of gestation also had a large impact on estimated total nuclei number for all muscles studied. There was also a significant ($P<0.001$) birth rank by stage interaction for each muscle.

Table 6.1. Placental characteristics (mean ± s.e.m.) of single- and twin-bearing ewes at 100, 120 and 140 days of gestation.

		Day of gestation			Significance		
	Rank	**100**	**120**	**140**	**Rank**	**Stage**	**RxS**
Ewe Number	1	6	6	6			
	2	3	3	3			
TGUW (g)	1	2824 ± 129***	5226 ± 329***	9071 ± 365***	***	***	***
	2	5398 ± 73	9211 ± 130	15516 ± 611			
Placentome wt (g)	1	594.8 ± 23.7***	519.6 ± 42.6**	649.5 ± 67.2*	***	NS	NS
	2	925.0 ± 57.4	835.5 ± 84.1	905.0 ± 47.6			
Placentome no.	1	94.2 ± 2.3	78.7 ± 14.6	107.4 ± 9.5	***	***	NS
	2	100.0 ± 5.2	104.7 ± 12.7	115.3 ± 8.9			
Placentome wt/fetus	1	594.8 ± 23.7**	519.6 ± 42.6†	649.5 ± 67.2*	***	NS	NS
	2	462.5 ± 28.7	417.8 ± 42.1	452.5 ± 23.8			
Placentome no./fetus	1	94.2 ± 2.3***	78.7 ± 14.6	107.4 ± 8.5***	†	***	***
	2	50.0 ± 2.6	52.3 ± 6.3	57.6 ± 4.5			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.005; TGUW = total gravid uterus weight; (1) = single-bearing; (2) = twin-bearing.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction (RxS) are also shown.

Table 6.2. Body weight (g), Crown-rump Length (CRL, cm) and Girth (cm) measurements (mean ± s.e.m.) of single and twin fetuses at 100, 120 and 140 days of gestation and in 20-day-old lambs (PN20).

		Day of Gestational/Postnatal Age				Significance		
Muscle	**Rank**	**d100**	**d120**	**d140**	**PN20**	**Rank**	**Stage**	**RxS**
N	1	6	6	6	4			
	2	6	6	6	4			
Body weight	1	1004 ± 60	3069 ± 211 †	5883 ± 208*	11619 ± 102***	***	***	***
	2	1007 ± 46	2707 ± 111	5062 ± 187	7744 ± 607			
CRL	1	36.1 ± 0.7 †	48.2 ± 1.9	63.5 ± 1.2***	75.5 ± 6.9	NS	***	NS
	2	37.8 ± 0.6	48.8 ± 0.5	59.1 ± 0.7	75.9 ± 0.9			
Girth	1	22.3 ± 0.5	31.8 ± 0.7*	39.6 ± 0.7***	58.6 ± 4.6*	***	***	***
	2	22.8 ± 0.5	29.8 ± 0.3	36.6 ± 0.5	47.5 ± 0.9			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.005; (1) = single; (2) = twin; *N* = Number of Fetuses/Lambs.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction (RxS) are also shown.

Table 6.3. Selected hindlimb muscle weights (g) from single and twin fetuses (mean ± s.e.m.) at 100, 120 and 140 days of gestation and in 20-day-old lambs (PN20).

		Day of Gestational/Postnatal Age				Significance		
Muscle	**Rank**	**d100**	**d120**	**d140**	**PN20**	**Rank**	**Stage**	**RxS**
N	1	6	6	6	4			
	2	6	6	6	4			
Biceps	1	6.35 ± 0.25	15.16 ± 1.31*	28.08 ± 2.49***	95.63 ± 1.01***	***	***	**
	2	6.83 ± 0.32	12.26 ± 0.41	22.97 ± 1.06	60.62 ± 3.75			
Semitendinosus	1	2.12 ± 0.66	4.97 ± 0.25***	9.32 ± 0.56***	31.83 ± 4.99***	*	***	**
	2	2.00 ± 0.67	3.85 ± 0.11	7.58 ± 0.42	21.02 ± 1.68			
Semimembranosus	1	5.49 ± 0.40	13.01 ± 1.15**	22.70 ± 1.37***	90.33 ± 3.79* **	***	***	***
	2	5.43 ± 0.29	9.47 ± 0.49	17.65 ± 0.66	48.50 ± 7.02			
Plantaris	1	0.79 ± 0.04	2.43 ± 1.49***	4.37 ± 0.23*	13.30 ± 0.71***	***	***	***
	2	0.77 ± 0.04	1.85 ± 0.07	3.79 ± 0.21	8.43 ± 0.53			

Gastrocnemius	1	2.48 ± 0.13	6.25 ± 0.46**	11.85 ± 0.74**	36.80 ± 0.44***	***	***	***
	2	2.47 ± 0.10	4.98 ± 0.09	9.84 ± 0.50	23.83 ± 1.41			
Adductor	1	2.48 ± 0.16	6.49 ± 0.77*	11.23 ± 1.14**	36.00 ± 0.84***	**	**	***
	2	2.48 ± 0.15	6.12 ± 0.19	8.91 ± 0.53	24.15 ± 1.43			
Gluteus	1	4.04 ± 0.25	8.53 ± 1.37*	14.90 ± 1.36***	66.35 ± 1.91***	**	***	***
	2	3.85 ± 0.22	6.79 ± 0.15	11.90 ± 0.70	39.05 ± 3.07			
Quadraceps	1	5.87 ± 1.20	20.79 ± 1.51**	40.41 ± 2.19***	117.32 ± 3.57***	***	***	***
	2	8.02 ± 0.27	16.25 ± 0.46	34.18 ± 1.47	73.38 ± 3.92			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.005; (1) = single; (2) = twin; *N* = Number of Fetuses/Lambs.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction (RxS) are also shown.

Table 6.4. Selected hindlimb muscle lengths (cm) from single and twin fetuses (mean ± s.e.m.) at 100, 120 and 140 days of gestation and in 20-day-old lambs (PN20)).

		Day of Gestational/Postnatal Age				Significance		
Muscle	**Rank**	**d100**	**d120**	**d140**	**PN20**	**Rank**	**Stage**	**RxS**
N	1	6	6	6	4			
	2	6	6	6	4			
Semitendinosus	1	28.52 ± 1.94	49.64 ± 1.51†	59.75 ± 0.60	85.0 ± 0.35	***	***	NS
	2	25.81 ± 1.43	44.95 ± 1.82	56.46 ± 2.32	76.7 ± 0.17			
Plantaris	1	29.94 ± 2.11	52.51 ± 1.58	69.82 ± 0.75*	102.500 ± 0.43	*	***	NS
	2	31.53 ± 1.21	50.35 ± 2.07	63.08 ± 2.17	97.30 ± 0.27			
Gastrocnemius	1	31.01 ± 2.34	52.35 ± 0.64	68.60 ± 1.73**	90.00 ± 0.35	NS	NS	NS
	2	33.17 ± 0.55	50.50 ± 1.04	62.54 ± 1.37	85.70 ± 0.47			
Adductor	1	23.93 ± 1.53	43.85 ± 2.14	55.60 ± 1.63†	74.00 ± 0.25	NS	***	NS
	2	23.43 ± 0.83	42.35 ± 1.56	53.38 ± 1.47	70.70 ± 0.07			

†, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.005; (1) = single; (2) = twin; *N* = Number of Fetuses/Lambs.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction (RxS) are also shown.

Table 6.5. Estimated total nuclei number per muscle ($x10^7$) of single and twin fetuses (mean ± s.e.m.) at 100, 120 and 140 days of gestation and in 20-day-old lambs (PN20).

		Day of Gestational/Postnatal age				Significance		
Muscle	**Rank**	**d100**	**d120**	**d140**	**PN20**	**Rank**	**Stage**	**RxS**
N	1	6	6	6	4			
	2	6	6	6	4			
Adductor	1	38.7 ± 3.3	69.9 ± 5.7	82.9 ± 4.3 †	159.1 ± 1.6 †	***	***	NS
	2	34.1 ± 3.0	62.0 ± 1.9	69.7 ± 5.8	119.3 ± 1.0			
Plantaris	1	10.8 ± 1.3	27.9 ± 2.6	37.8 ± 1.5	91.4 ± 4.9	***	***	***
	2	9.7 ± 0.2	24.3 ± 1.2	33.9 ± 2.4	84.5 ± 12.6			
Semitendinosus	1	29.2 ± 2.2	51.7 ± 3.8 †	62.2 ± 2.8	105.2 ± 1.1	†	***	NS
	2	32.3 ± 1.6	43.7 ± 2.5	55.5 ± 4.1	90.7 ± 1.3			
Gastrocnemius	1	24.5 ± 2.4	42.3 ± 5.4*	50.5 ± 4.5 †	169.2 ± 0.9**	***	***	***
	2	23.9 ± 1.5	32.6 ± 3.2	42.3 ± 2.9	130.0 ± 0.8			

†,P<0.10; *, P<0.05; **, P<0.01; ***, P<0.001; (1) = single; (2) = twin; *N* = Number of Fetuses/Lambs.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction (RxS) are also shown.

At day 100 of gestation, the *semitendinosus* muscle of twin lambs had more MyoD-positive nuclei per muscle compared to singles. However, this effect was reversed at day 120 and PN20 for the *gastrocnemius* muscle (Table 6.6.). There was a similar trend at day 120 in the *adductor* and *semitendinosus* muscle and at PN20 in the *semitendinosus* muscle (Table 6.6.). Birth rank had an overall effect on estimated total MyoD nuclei number per muscle in the *adductor* ($P<0.05$), *semitendinosus* ($P<0.05$) and *gastrocnemius* ($P<0.001$) muscles. The estimated total number of MyoD-positive nuclei per muscle was also significantly influenced by the stage of gestation ($P<0.001$). An interaction between birth rank and stage of gestation ($P<0.001$) was observed only for the *gastrocnemius* muscle reflecting a divergence between the birth ranks with increasing age in favour of singles. The percentage of MyoD-positive nuclei that were located outside, but in close association with the plasmalemma, and thus in the satellite cell position at PN20, were: *adductor* (95%), *plantaris* (87%), *semitendinosus* (75%) and *gastrocnemius* (93%). There were no MyoD-positive nuclei present in the adult skeletal muscle samples.

Average myofibre CS area was smaller in the *adductor* and *semitendinosus* muscles of the twins than the singles at day 140 of gestation (Table 6.7.). At PN20 the average myofibre CS area was also smaller for twins than for singles for all muscles studied.

Table 6.6. Estimated total MyoD nuclei number per muscle ($x10^7$) of single and twin fetuses (mean ± s.e.m.) at 100, 120 and 140 days of gestation and in 20-day-old lambs (PN20).

		Day of Gestational/Postnatal Age			Significance		
Muscle	**Rank**	**d100**	**d120**	**PN20**	**Rank**	**Stage**	**RxS**
N	1	6	6	4			
	2	6	6	4			
Adductor	1	27.90 ± 2.15	59.20 ± 5.37 †	99.00 ± 8.08	*	***	NS
	2	26.30 ± 2.07	48.90 ± 2.43	81.30 ± 12.10			
Plantaris	1	7.55 ± 1.29	23.80 ± 2.54	80.80 ± 0.9*	NS	***	NS
	2	7.03 ± 0.38	20.00 ± 1.13	54.4 ± 0.3			
Semitendinosus	1	21.40 ± 0.96*	48.00 ± 7.81 †	43.00 ± 4.33 †	*	***	NS
	2	24.10 ± 0.54	34.70 ± 2.12	30.10 ± 4.01			
Gastrocnemius	1	18.30 ± 1.91	35.60 ± 6.29*	154.00 ± 15.50*	***	***	***
	2	16.30 ± 1.66	24.70 ± 2.49	96.30 ± 12.34			

†, P<0.10; *, P<0.05; ***, P<0.001; (1) = single; (2) = twin; *N* = Number of Fetuses/Lambs.

Note: Significance symbols at each time point refer to comparisons made between mean parameters for singles and twins at each day of gestation studied. Significance of effects on the overall mean for each birth rank, each stage and the rank by stage interaction (RxS) are also shown.

Table 6.7. Average cross-sectional area (μm^2) of muscle fibres from the medial region of the *adductor* and *semitendinosus* muscle of single and twin fetuses (mean ± s.e.m.) at 140 days of gestation, and from the medial region of the *adductor*, *plantaris, semitendinosus* and *gastrocnemius* muscles from PN20 lambs.

		Day of Gestational/Postnatal Age	
Muscle	**Rank**	**d140**	**PN20**
N	1	6	4
	2	6	4
Adductor	1	64.5 ± 5.5***	187.1 ± 10.4*
	2	51.0 ± 3.7	139.3 ± 19.9
Plantaris	1	ND	205.8 ± 19.7**
	2	ND	118.5 ± 13.5
Semitendinosus	1	75.5 ± 6.3***	209.4 ± 9.3**
	2	60.7 ± 4.2	139.5 ± 14.9
Gastrocnemius	1	ND	210.5 ± 14.9*
	2	ND	162.7 ± 5.9

ND, not determined; *, P<0.05; **, P<0.01; ***, P<0.005.
(1) = single; (2) = twin; *N* = Number of Fetuses/Lambs.

6.5. DISCUSSION

The first aim of this study was to determine the effectiveness of the MyoD protein as a marker for active satellite cells in sheep skeletal muscle. MyoD, a nuclear protein belonging to the muscle-specific regulatory factor family (for reviews see Sassoon, 1993; Weintraub, 1993; Buckingham, 1994; Olson and Klein, 1994), has previously been shown to be a relatively reliable marker for active satellite cells in fetal and neonatal rat muscle (Koishi *et al.,* 1995). However, it has been shown to label some recently incorporated myonuclei (Hughes *et al.,* 1997). Until now, the effectiveness of the MyoD protein as a marker for active satellite cells in developing ovine skeletal muscle has not been described.

Differentiating between a satellite cell nucleus and a myonucleus can be difficult since satellite cells are often indented into the side of fibres. The plasmamembrane of the fibre and the nuclear membrane of the satellite cell are in such close proximity that they appear to be a single membrane. Therefore, satellite cells can often be mistaken for myonuclei or vice versa. Zhang and McLennan (1994) described a double-labelling technique using anti-dystrophin to label the plasmalemma and anti-laminin to label the basal lamina, which enables accurate determination of the location of MyoD-labelled satellite cells. In the present study, in order to determine whether the MyoD-labelled nuclei were in the satellite cell position, the sections were double-labelled with anti-dystrophin to label the plasmalemma. By definition, satellite cells lie outside the plasmalemma and beneath the basal lamina. Double-labelling with anti-laminin to label the basal lamina, as described by Zhang and McLennan (1994), was attempted but was unsuccessful. Nuclei were classified as either outside or inside the labelled plasmalemma as described by Zhang and McLennan (1994). As discussed by these authors, the plasmalemma immunoreactivity was not always continuous, but by focusing up and down through a section, accurate location of the MyoD labelled nuclei, i.e., either inside or outside the plasmalemma, was always possible. The percentages of labelled cells that were in the satellite cell position were: *adductor* (95%), *plantaris* (87%), *semitendinosus* (75%) and *gastrocnemius* (93%). These nuclei were always closely associated with the plasmalemma of the myofibre and, therefore, were likely to

be satellite cells and were classified accordingly. Very few of the nuclei were inside the plasmalemma, and they were likely to be recently incorporated myonuclei as reported by Koishi *et al.* (1995) and Hughes *et al.* (1997).

Double-labelling was carried out on sections from PN20 animals only, to ensure that late myoblasts did not confound the results. In addition, since the distribution of satellite cells is heterogeneous within a muscle (Kelly, 1978; Snow, 1981; Gibson and Schultz, 1982), unbiased sampling of each muscle was necessary and was achieved using random systematic sampling (Gundersen *et al.,* 1988). Using this approach, estimates of nuclei number were made from the superficial and deep regions of each section, from the proximal, medial and distal regions of a muscle. In order to determine whether MyoD labels all active satellite cells in a muscle, an alternative marker for satellite cells is required. However, there is no reliable alternative marker for active satellite cells. In the present study, MyoD appeared to be a reliable marker for active satellite cells for three main reasons. First, MyoD-positive cells were abundant during the rapid stages of growth, i.e., during late gestation and early postnatal life, which agrees with studies in rodents (Koishi *et al.,* 1995). Second, the majority of MyoD-positive cells were located outside, but in close proximity to, the plasmalemma, and thus in the satellite cell position. Finally, no MyoD-positive cells were observed in skeletal muscle from adult sheep, in which muscle mass is static and satellite cells are quiescent. The results from the present study further support the usefulness of MyoD as a marker for active satellite cells as reported in rodents (Zhang and McLennan, 1994; Koishi *et al.,* 1995; Hughes *et al.,* 1997), but also illustrates the usefulness of this endogenous protein as a marker for active satellite cells in sheep skeletal muscle tissue.

As a result of maternal constraint operating through limitation of placental size, twin lambs have a lower body weight and a lower mass of muscle than singles (Chapters 3&4), implying growth-restriction in twins compared to singles. A lower muscle mass in the twin fetuses compared to the singles is also associated with smaller average myofibre CS area and a lower muscle DNA content (see Chapter 5) implying retarded myofibre hypertrophy in the muscles of twin lambs. Since an increase in DNA content accompanies myofibre hypertrophy, and satellite cells are the only source of new nuclei

during late fetal and postnatal muscle growth, an effect on satellite cell and subsequent nuclei number is suggested. Therefore, the second aim of this study was to determine whether the differences in myofibre hypertrophy between singles and growth-restricted twins is associated with differences in total nuclei number and myogenic cell cycle activity, using MyoD as a marker for active satellite cells.

The growth-restriction of twins compared to singles in this study was associated with lower placentome weight and number per fetus in twin- compared to single-bearing ewes, indicating the presence of maternal constraint. Placental insufficiency in this situation resulted in a gradual divergence in body weight between birth ranks as gestation advanced, as illustrated by the birth rank by stage interaction ($P<0.001$), and was particularly marked at PN20.

In addition to lower body weights observed between singles and twins in this trial, by 120 days of gestation all of the hindlimb muscles studied were heavier in the single than in the twin fetuses, an effect which persisted through gestation and was still evident at PN20. As observed with body weight, the interaction between birth rank and stage of gestation ($P<0.001$) demonstrated a gradual divergence in muscle weights with the most marked differences evident at PN20 in favour of singles compared to twins. A greater muscle mass in singles compared to twins was also associated with greater mean muscle fibre CS area. At day 140 of gestation myofibre CS area differed by about 20% between birth ranks in the *adductor* and *semitendinosus* muscles. However, by PN20 the difference in myofibre CS area increased to about 25% and 35% in the *adductor* and *semitendinosus* muscles respectively. The difference in myofibre CS area was similar to the difference in the weight of these muscles, illustrating continued enhanced growth of singles compared to twins following parturition.

The greater DNA content of the *adductor* muscle from singles compared to twins observed in Chapter 5 implies differences in myonuclei number between birth ranks. Since an increase in DNA content accompanies myofibre hypertrophy, and satellite cells are the only source of new nuclei during late fetal and postnatal muscle growth, an effect on satellite cell number is suggested. At 100 days of gestation there were no

differences between birth ranks in the total number of nuclei per muscle, but twins had more myogenic precursor cells in the *semitendinosus* muscle compared to singles. However, by 120 days this effect was reversed, as shown by a lower total nuclei number and myogenic precursor cell number per muscle in the *gastrocnemius* muscle from twins than from singles. There was also a trend for the total nuclei number and myogenic precursor cell number in the *semitendinosus* muscle to be lower in twins than in singles. There were no differences between birth ranks in the total nuclei number or the total myogenic precursor cell number in the *adductor* and *plantaris* muscles at 120 days of gestation. Although there was a tendency for the *adductor* and *gastrocnemius* muscles from twins to have fewer nuclei per muscle compared to singles at 140 days of gestation, there were no data for myogenic precursor cell number due to irreversible damage to the samples collected for immunohistochemistry. By PN20, the *plantaris* muscle and the *gastrocnemius* muscle from twins had fewer nuclei per muscle than singles, and the *gastrocnemius* muscle from twins had fewer myogenic precursor nuclei than singles. There was a trend for lower total nuclei number in the *adductor* muscle of twins but there was no difference in myogenic precursor nuclei number between birth ranks for this muscle. Although there was no difference in the total nuclei number in the *semitendinosus* muscle of twins at PN20, there tended to be fewer myogenic precursor nuclei in this muscle in twins than in singles.

Overall, birth rank had a significant impact on estimated total nuclei number in the *adductor*, *plantaris* and *gastrocnemius* muscles in favour of the singles compared to twins. A similar trend was also observed for the *semitendinosus* muscle. The overall effect of the stage of gestation on total myonuclei number in all of the muscles studied illustrates that myonuclei number increases with advancing gestation. It was interesting to note that there was a significant birth rank by stage interaction for total nuclei number in the *gastrocnemius* and *plantaris* muscles, demonstrating a gradual divergence in total nuclei number in these muscles in favour of singles compared to twins. Overall, there was a greater total estimated MyoD-positive nuclei number in the *adductor*, *semitendinosus* and *gastrocnemius* muscles of singles than twins, while MyoD-positive nuclei number did not differ between birth ranks in the *plantaris* muscle. The overall effect of the stage of gestation for all of the muscles illustrates that

the myogenic precursor cell population increases with age. The significant "birth rank by stage of gestation" interaction for MyoD-positive cells in the *gastrocnemius* muscle illustrates a gradual divergence in myogenic precursor cell numbers as gestation advances, in favour of singles compared to twins, which parallels the result for total nuclei number. It should be noted that although the myogenic precursor cells at PN20 are likely to be mostly satellite cells, the nuclei observed at earlier ages may be a mixture of satellite cells and late myoblasts (Stockdale, 1992).

The smaller total nuclei content of muscles in twins compared to singles observed at various stages during late gestation and early postnatal life in this study is consistent with previous studies in pigs (Ezekwe and Martin, 1975; Powell and Aberle, 1975; Buhlinger *et al.,* 1978). Termination, or a decrease in the rate, of nuclear proliferation has been reported to be associated with either a cessation of muscle growth or reduced growth rate (Winick and Noble, 1966; Robinson, 1969; Beermann *et al.,* 1983; Purchas *et al.,* 1985). The results from the present study are consistent with these observations as the reduction in total nuclei number and the number of myogenic precursor nuclei in some muscles at various stages of development, and the overall effects of birth rank and stage of gestation particularly in the *gastrocnemius* muscle, relate to the restricted growth of twins compared to singles. Further evidence to support the relationship between myonuclei number and muscle hypertrophy is provided by the difference in myofibre CS area between birth ranks.

DNA proliferation in muscle of postnatal animals is highly sensitive to total food intake (Howarth and Baldwin, 1971; Glore and Layman, 1983b), however, cessation or restriction of muscle growth is usually observed only when the level of food restriction is greater than 60% (Glore and Layman 1983, ab). Myogenic nuclei are especially active during late gestation and early postnatal life, coinciding with rapid growth rates. Therefore, it is not surprising that, at this time, nuclear proliferation is particularly sensitive to nutritional restriction (Winick and Noble, 1967). The limitation in nutrient supply to twin fetuses during late gestation, as a result of smaller placental size per fetus, coupled with the competition for a single nutrient source postpartum, may explain some of the differences in total nuclei and myogenic nuclei content of muscles

between birth ranks in this study. The fact that twins and singles can reach similar market weights (Nordby *et al.,* 1987), suggests that the restriction of muscle hypertrophy in early life, as measured by DNA content and muscle fibre CS area, is not permanent and that there is some potential for recovery following reduced nutrient intake. This is supported by studies investigating the potential for recovery after prolonged growth retardation (Glore and Layman, 1987; Sika and Layman, 1995).

Several hormones and growth factors have been attributed roles in the control of proliferation and differentiation of myogenic cells (Allen and Boxhorn, 1989; review by Florini and Magri, 1989). Hathaway *et al.* (1994) reported that myogenic cells isolated from fetal lambs during mid-gestation exhibited a concentration-dependent decrease in proliferation in response to TGF-β1. Similar, but less marked responses were seen in ovine satellite cells isolated from 5-day-old lambs, while TGF-β1 failed to suppress serum-stimulated proliferation of satellite cells isolated from 30- or 50-day-old lambs. Another member of the TGF-β superfamily, growth differentiation factor-8 (GDF-8), is also reported to negatively regulate skeletal muscle growth (McPherron *et al.,* 1997).

Members of the TGF-β family, and other growth factors also play an intimate role in the regulation of the myogenic regulatory factor family, of which MyoD is a member. MyoD, in addition to being a useful marker of myogenic cells in rodents (Zhang and McLennan, 1994; Koishi *et al.,* 1995; Hughes *et al.,* 1997), and in sheep skeletal muscle tissue as shown in the present study, has been implicated in the formation and differentiation of myogenic cells, at least in rodents (Eftimie *et al.,* 1991; Witzeman and Sakmann, 1991). The use of null mutant mice has also led to the suggestion that a MyoD-like function is necessary for muscle formation (Rudnicki *et al.,* 1992, 1993). The importance of MyoD in myoblast differentiation during early development implies that it is likely to be involved in the regulation of satellite cells. The fact that MyoD is present in active satellite cells in the rodent led Koishi *et al.* (1995) to suggest that MyoD may be required to maintain satellite cells in an active state. In the present study, the MyoD-positive cells observed in muscle tissue of fetal and neonatal lambs, and the absence of MyoD-positive cells in adult sheep skeletal muscle (in which satellite cells are quiescent), further suggests a role for MyoD in the maintenance of

satellite cells in an active state. In addition, since MyoD-positive cells are observed in satellite cells following muscle damage (Koishi *et al.*, 1995), MyoD may enable satellite cells to remain within the myogenic lineage to facilitate repair following damage.

In conclusion, the present study illustrates not only that the use of MyoD to identify myogenic precursor nuclei is a reliable endogenous marker for active satellite cells in the lamb, but also that MyoD may have an important role in maintaining satellite cells in a active state, facilitating myofibre hypertrophy and the attainment of ultimate muscle size. In addition, this study illustrates that growth restriction during late fetal development has important implications for birth size and skeletal muscle growth. The differences in myonuclei number and myogenic precursor cell numbers which relate to differences in muscle weight and myofibre CS area imply that myogenic precursor cell activity is related to skeletal muscle hypertrophy in late fetal and early postnatal life, and may have important consequences for postnatal growth potential.

Chapter Seven

DISCUSSION AND CONCLUSIONS

Myogenesis is a complex process integrating several developmental events resulting in functional muscle tissue. These developmental processes are initiated during the embryonic period and continue throughout gestation. At birth, precocial species such as sheep are able to stand and walk within minutes of parturition, indicating considerable functional development of the muscle tissue during the prenatal period. Therefore, the prenatal developmental period must be crucial for myogenesis.

A common method to study developmental processes is to compare normal versus growth-perturbed models. The resulting difference in body size has obvious implications for animal survival and competitiveness, but is also useful for studying developmental processes such as myogenesis. Therefore, the first objective of these studies was to identify and characterize a model that could be used to study myogenesis in the prenatal lamb.

Apart from the contribution of connective tissue, muscle mass is determined by both the number of myofibres and the size of those fibres. Thus, in order to identify the factors regulating ultimate muscle size, the relative impacts of myofibre hyperplasia and hypertrophy within muscles of growth-restricted versus normal animals must be determined. Therefore, the second objective of these studies was to identify possible differences in fibre hyperplasia and/or hypertrophy between normal and growth-restricted lambs, and the stages of gestation at which possible differences in fibre number and/or size were evident. The final objective was to identify factors or processes that may be involved in the regulation of these developmental events, and that may contribute to the morphological differences between muscles from normal versus growth-restricted lambs during prenatal and early postnatal life.

Severe manipulation of the ewe (e.g., undernutrition) or the placenta (e.g., uterine ligation or carunclectomy) can result in fetal growth retardation, and models such as these have been commonly used to study fetal development. Other models which do not involve severe manipulation of the ewe, placenta or fetus but result in fetal growth restriction (e.g., low vs high birth weight lambs (Hunt *et al.*, 1996), normal vs runted piglets (Dwyer and Stickland, 1991) or enhanced growth (e.g., double-muscled animals

(Swatland and Kieffer,. 1974; Shahin, 1995)) have also been described. Models which result in growth restriction without manipulation of the fetus are desirable as they are simple and economical to produce, and provide an experimental environment which closely mimics or represents the natural environment of the developing fetus.

Two models were investigated in this programme. Both were expected to result in restriction of body size as a result of maternal constraint, and illustrate the importance of the intra-uterine period for the development and growth of the fetus. The first model involved lambs born to ewes which conceived outside of their natural breeding season. Such lambs generally have lower birth weights than their spring-born counterparts (Reid *et al.,* 1988; Peterson *et al.,* 1992; Morris *et al.,* 1993; Jenkinson *et al.,* 1995). The difference in body weight of the fetuses between seasons was associated with impaired placental development in the autumn- compared to spring-lambing ewes, however placental insufficiency did not consistently impair fetal growth (Chapter 3). In this situation, placental efficiency must have improved to compensate for the reduction in placentome weight and number. Despite the lack of differences in body and muscle weights between autumn and spring fetuses, it was interesting to note that maternal constraint in this situation can have differential effects on myofibre morphology between muscles, without influencing overall body or muscle size, an effect which warrants further investigation. Since the effect of season was not consistent between Chapters 2 and 3, it was concluded that this approach for restricting fetal growth in order to study myogenesis *in utero*, was unreliable. Perhaps manipulation of maternal progesterone levels (Rhind *et al.,* 1980) could result in more consistent effects on fetal size between seasons, however, there is no experimental evidence to support this theory. Thus the seasonal model was not pursued further in this programme.

The second model involved the comparison between singles and twins which provided a natural, non-invasive growth-restriction model that was easy to produce and reliable. When ewes are mated in their natural breeding season and maintained under normal on-farm conditions throughout gestation, competition between littermates, such as between twins, consistently results in twins having lower body and muscle weights compared to singles, as observed in this and other studies (Donald and Russell, 1970;

Mellor and Murray, 1981). Growth-restriction in this situation was primarily a result of maternal constraint reflected by there being fewer placentomes, each of lower mean weight per twin fetus than per single fetus. The observation that placentome number per fetus declines as fetal number increases generally agrees with earlier reports (Alexander, 1964, 1974; Stegeman, 1974; Rhind *et al.,* 1980; see also Chapters 3 & 4) and illustrates the importance of the placenta for fetal growth. Placental insufficiency in this situation resulted in a gradual divergence in fetal weight between birth ranks as gestation advanced. The gradual divergence in body weight between singles and twins, as illustrated by the birth rank by stage interaction, and which was particularly marked at PN20 (see Chapter 6), illustrates that birth weight and postpartum rearing conditions have important consequences for postnatal growth. Although artificial rearing conditions may (Greenwood, 1997) or may not (Schnickel and Short, 1961; Taplin and Everitt, 1964; Penning *et al.,* 1980; Villette and Theriez, 1981) compensate for effects of differences in birth size on postnatal growth potential, birth weight has important implications for neonatal survival (Dalton *et al.,* 1980; McCutcheon *et al.,* 1981) and subsequent growth rates (Nordby *et al.,* 1987; Bell, 1992). Coupled with lower body weights, twin fetuses also have smaller muscles than singles suggesting that the comparison between single and twin fetuses is a more reliable method of imposing fetal growth-restriction, in order to study factors controlling or regulating myogenesis *in utero,* than the "season of birth" model. Therefore, this model was used to study myogenesis *in utero* in the remainder of these studies.

Several important methodological considerations were raised in this programme. First, differences exist in myofibre number and myofibre CS area between the proximal, medial and distal regions of a muscle, as a result of intrafascicularly terminating fibres (for review see Gans and Gaunt, 1992) and multiple generative centres for fibres along large muscles (Duxson and Sheard, 1995). This illustrates that careful selection of samples for histological analysis of myofibre characteristics is necessary, in order to yield accurate results. Second, and in contrast to rodents (see Appendix 1), while direct administration of BrdU to the fetus, such as via intra-peritoneal injection with the aid of ultrasound (Greenwood, 1997), resulted in labelling of replicating cells in the sheep fetus, administration via the dam was not efficacious for fetal labelling. Third, MyoD

appears to be a useful marker for active satellite cells in fetal and neonatal sheep skeletal muscle, when it is used in conjunction with a plasmalemma marker such as anti-dystrophin. Finally, due to the large variation in fibre number between animals, there was quite substantial error associated with the estimation of mean fibre number in each birth rank. By using stereological analysis (Gundersen *et al.,* 1988) and counting relatively small numbers of fibres in randomly selected areas, as described by other authors (Dwyer and Stickland, 1991; Hunt *et al.,* 1996), the samples taken in these studies are sufficient to make accurate estimates of fibre number in a cross-section of muscle tissue. Therefore, it is unlikely that the error associated with the counting procedure and the between-animal variation masked true differences in fibre number between single and twin lambs.

Although twin fetuses/neonates had lower body weights and muscle weights than singles, myofibre hyperplasia was not consistently affected in any of the muscles studied, a result which supports work by others (Nordby *et al.,* 1987; Hunt *et al.,* 1996). Differential effects on fibre number between birth ranks were observed in the *gastrocnemius* muscle at 140 days of gestation (see Chapter 3), and the *adductor* muscle at 80 and 120 days of gestation (see Chapter 4). These effects were not observed at every stage of gestation (see Chapter 4) and were not consistently observed between studies. The extent and timing of growth-restriction in the present study may be a reason for the lack of effect on fibre number. In the present studies, twin lambs were approximately 20% lighter at birth than singles, but had similar muscle fibre complements which is consistent with previous observations (Nordby *et al.,* 1987; Hunt *et al.,* 1996). Although differences in body and muscle weights were observed by birth, there was no difference in fetal or muscle weights at 80 days of gestation, when fibres are still forming, and fibre complements were similar. This observation suggests that fibre hyperplasia in the sheep fetus is completed prior to any major nutritional impact which results in growth-restriction of the twin fetus compared to singles in later gestation.

Since fibre hyperplasia is complete by 100 days of gestation in the sheep (Ashmore *et al.,* 1972), a model which restricts development early in gestation would be necessary

in order to perturb this process. Such models may include prolonged exposure of ovine embryos during embryo transfer procedures to culture conditions which result in enhanced body size (Walker *et al.,* 1992; Bishonga *et al.,* 1994; Farin *et al.,* 1994; Thompson *et al.,* 1995; Farin and Farin, 1995; Holm *et al.,* 1996; Young *et al.,* 1996; Sinclair *et al.,* 1997; Thompson, 1997). Severe growth retardation, such as observed in piglet runts, can retard fibre hyperplasia (Dwyer and Stickland, 1991) and similar differences have been observed in lambs following severe maternal undernutrition from early in gestation (Everitt, 1964). Although models such as this can result in severe growth-retardation prior to 100 days of gestation, the resulting growth restriction does not mimic that observed under natural conditions. One model which does mimic natural growth restriction, in addition to those used in the present studies, involves the use of rapidly-growing adolescent ewes (Wallace *et al.,* 1996), but the possible effects on fibre hyperplasia have not been investigated under experimental conditions.

Although birth rank did not influence myofibre number, the difference in muscle weights between single and twin fetuses near birth was associated with parallel differential effects on myofibre hypertrophy. Myofibre CS area was considerably smaller in muscles from twins as compared to singles at 140 days and PN20 in all muscles studied except for the *adductor* muscle at 140 days of gestation (in Chapter 3). Restricted myofibre hypertrophy in the twins was also related to lower total nuclei number and myogenic precursor nuclei numbers. This indicates that the activity of myogenic precursor nuclei in late fetal and early postnatal life has a significant impact on myofibre hypertrophy and thus, muscle mass, and is more important than myofibre number for determining postnatal growth potential. This finding is consistent with previous studies (Beermann *et al.,* 1983; Greenwood, 1997).

Fetal growth is described as following an exponential growth curve which illustrates that, during the early stages of gestation, fetal growth rate is relatively slow in comparison to that in late gestation. Despite slow growth-rates, as measured by body weight, these earlier stages of development are crucial for laying down the body plan and the development of the body systems and tissues. Although placental insufficiency was evident in the twin-bearing ewes compared to single-bearing ewes by mid-

gestation, fetal and muscle weights and myofibre numbers were not affected between birth ranks by 100 days of gestation. This indicates that when ewes are well-fed during early pregnancy, nutrition via the placenta has little impact on fetal and muscle mass and myofibre hyperplasia. During the later stages of gestation, fetal weight increases rapidly in an exponential fashion. It is during this time that the fetus gains most of its birth weight and is sensitive to limitations in nutrient supply. The growth restriction evident in twin lambs compared to singles illustrates the importance of fetal nutrition in late gestation. This series of studies demonstrates that not only is fetal size compromised by inadequate nutrition in the twin fetus as a result of placental insufficiency, but that myofibre hypertrophy is also affected.

Despite differences in body weights, muscle weights, and myofibre morphology at birth, singles and twins are able to reach similar market weights (Nordby *et al.,* 1987). This suggests that the differences seen in body size and muscle characteristics between birth ranks at birth are not permanent, and implies compensatory growth in the twin lambs following birth. Compensatory growth during the first 3 weeks of postnatal life was not evident in these studies as differences in body and muscle weights and myofibre CS area were still apparent at PN20. The observation of less DNA in muscles of twins than singles at this time also suggests longer-term consequences for muscle growth. Nutrition during the early stages of postnatal life is likely to be a limiting factor to muscle growth as nutrition of the twin lamb is compromised by the need to share a single milk supply. The importance of nutrition during this time is demonstrated by the ability of low birth weight newborns to grow at similar rates to high birth weight newborns when fed *ad libitum* following birth (Greenwood, 1997). However, by weaning, the primary source of nutrients has changed from milk to a pasture-based diet, at which time twin lambs are no longer in direct competition with their siblings. It is likely that most of the compensatory growth occurs from weaning until market weight. However, despite similar growth potential, the growth restriction imposed on twin lambs during gestation and early postnatal life is still evident in late postnatal life as twins can take considerably longer to reach market weight than singles (Nordby *et al.,* 1987).

This study also shows that nutrition has an impact on myogenic cell cycle activity and that myogenic precursor nuclei play an important role in determining muscle mass. However, this programme does not provide extensive information about the regulation of cell cycle activity in muscle. The fact that MyoD-positive cells were evident in rapidly growing muscle of fetuses and neonates, but not in mature muscle tissue, coupled with the role of MyoD in early fetal myogenesis, illustrates that the presence of this transcription factor may be required to maintain satellite cells in an active state in order to facilitate growth. However, further investigation is required to determine the exact role of this transcription factor, and possibly other myogenic regulatory factors and/or growth factors such as the insulin-like growth factors and the transforming-growth factor-beta family, in the control of cell cycle activity in fetal sheep skeletal muscle. This will be necessary to gain a better understanding of the process of myogenesis during this growth phase, and for devising strategies to manipulate myogenesis in order to meet consumer and production demands.

Appendix 1

IMMUNOHISTOCHEMICAL DETECTION OF PROLIFERATING CELLS USING 5-BROMO-2-DEOXYURIDINE (BRDU)

A.1. INTRODUCTION

The classical method for *in vivo* and *in vitro* detection of cells synthesizing DNA (S-phase) is by their uptake of tritiated thymidine, identified using autoradiography. This method has generated a valuable database for toxicological and carcinogenic studies. However, there are several negative aspects associated with this method, including radioactive contamination of personnel and waste disposal, long autoradiographic exposure times and personnel safety. The thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which is also incorporated into cellular DNA during the S-phase of cell replication, has proven to be a useful non-radioactive alternative to study cell cycle kinetics *in vitro* and *in vivo* (Dolbeare *et al.,* 1983; Wynford-Thomas and Williams, 1986). BrdU can be localised in histological sections with a monoclonal antibody and immunoenzyme detection method (horseradish peroxidase-diaminobenzidine) or by immunofluorescence, which recognizes BrdU in single-stranded DNA.

Although there is a good correlation between BrdU and tritiated thymidine labelling in the S-phase (Thornton *et al.,* 1988), and it is an accurate sensitive measure of DNA synthesis (Dolbeare *et al.,* 1983; Miller *et al.,* 1986), the procedure involving BrdU incorporation may result in false negatives due to either insufficient doses to label all dividing cells, or because there may be inadequate uncoiling of the DNA (McClure *et al.,* 1988). BrdU is also mutagenic *in vitro* (Davidson *et al.,* 1988; Kaufman, 1988) and carcinogenic in rats (Napalkov *et al.,* 1989) following administration of high doses of the compound. Further, it has been shown to inhibit DNA synthesis and cell replication *in vitro* (Morris *et al.,* 1989) and *in vivo* (Chwalinski *et al.,* 1988; Weghorst *et al.,* 1991), and can inhibit cell differentiation and thymidine uptake *in vitro* (Tapscott *et al.,* 1989). Since BrdU is not an endogenous marker for DNA synthesis, the length of time that the tissue is exposed to BrdU can also have a significant impact on the number of labelled cells. Evaluation of a small window of time can be achieved by using single- or multiple-pulse injections of BrdU hours before sacrifice (Weghorst *et al.,* 1991). However, this method can fail to detect cells that are not induced to synthesize DNA during that window. The development of slow-release pellets and osmotic minipumps has allowed DNA precursors such as BrdU to be administered continuously for days or

weeks at a time, enabling long-term studies, by avoiding the complication of rapid dehalogenation in the liver (Kriss and Revesz, 1962). Although the method used for chronic administration of BrdU to animals has an important role in presenting the true proliferative scenario in cell kinetic studies (Weghorst *et al.,* 1991), further investigation is required to determine the suitability of BrdU replacement for tritiated thymidine in chronic exposure experiments. Despite these negative aspects, BrdU appears to be a suitable replacement for tritiated thymidine in experiments utilizing pulse- or multiple-injections of BrdU hours prior to sacrifice (Ward *et al.,* 1988; Lanier *et al.,* 1989).

The first experiment of this study was designed to confirm the mode of administration and dose of BrdU required to adequately label cells in the S-phase of the cell cycle in gut, skin and skeletal muscle tissue in the rat. There are many studies describing the use of BrdU to label proliferating cells in rodent fetuses. In contrast, there are no studies describing use of the method in pregnant sheep in order to label the proliferating cells of the fetus without fetal catheterization or intra-peritoneal injection of BrdU with the aid of ultrasound. Experiment 2 was therefore designed to confirm the dose of BrdU required to label proliferating cells in gut, skin and skeletal muscle tissue in ovine tissue. These samples were also used to develop the immunohistochemical method to detect BrdU labelled nuclei. Once the necessary dose of BrdU was identified, experiment 3 enabled determination of whether BrdU crosses the ovine feto-placental barrier to adequately label DNA synthesizing cells in the fetus following administration of BrdU to the dam.

A.2. MATERIALS AND METHODS

A.2.1. Animals

A.2.1.1. Experiment 1

Two neonatal (d10) Sprague Dawley rats were randomly selected and given an intraperitoneal (i.p.) injection of BrdU at a dose of 0.5mg/kg body weight. An additional two neonates were given an i.p. injection of saline (controls). One treatment and one control neonate were sacrificed by exposure to an atmosphere of 100% carbon dioxide one hour post-injection and samples collected. The remaining two neonates were tagged and returned to their mother until sacrifice and sample collection 72 hours post-injection. In addition, one pregnant female Sprague Dawley rat was given an i.p. injection of BrdU at a dose of 3mg/kg body weight at 18 days of gestation and sacrificed by exposure to carbon dioxide one hour later. Samples of gut, skin and skeletal muscle were taken from both the dam and fetuses.

A.2.1.2. Experiment 2

Two pairs of twin lambs were obtained from an independent study, 3 days following birth. One lamb from each pair was given an intravenous injection of BrdU at a dose of 5mg/kg of body weight. The remaining two lambs were given an injection of saline (controls). One pair of twins (i.e., one treatment and one control) were euthanased with sodium pentobarbitone (i.v.) one hour post-injection and samples of gut, skin and skeletal muscle collected. The remaining pair of lambs were bottle fed with ewes milk obtained from an independent sheep lactational study, and euthanased at 72 hours post-injection, and samples taken.

A.2.1.3. Experiment 3.

The pregnant ewe used for this experiment was obtained from the group of animals mated for the trial described in Chapter 5. The ewe was randomly selected and given

an intravenous injection of BrdU (10mg/kg body weight) at 98 days of gestation. One hour post-injection, the ewe was euthanased by captive bolt gun and exsanguination. The fetus was exposed, the umbilical cord ligated and severed and the fetus euthanased with sodium pentobarbitone via cardiac puncture. Samples of gut, skin and skeletal muscle were taken from both the dam and the fetus.

A.2.2. Immunohistochemistry

Immediately following sacrifice, a sample of gut, skin and hindlimb skeletal muscle tissue was obtained from each of the animals in the 3 experiments outlined above. The skeletal muscle tissue was pinned out on cork boards at resting length. All samples were fixed in Bouins fluid for up to 12 hours (smaller samples for about 6 hours) and then stored in 70% ethanol until paraffin embedding. Following embedding, 10μm sections were cut using a microtome, mounted on slides, and air-dried at 37°C for 24 hours.

Sections were incubated for 10 minutes in 1% BSA (Bovine Serum Albumin) in 0.01 M phosphate buffered saline (PBS) pH 7.4, to reduce non-specific binding, then incubated overnight at 4°C in a humid chamber with the mouse anti-BrdU antibody (Amersham International) diluted at 1:10 in diluting buffer (PBS; 1% BSA (w/v)). Sections were washed twice in PBS for 5 minutes and then incubated with biotinylated anti-mouse IgG (Amersham International) diluted at 1:200 in diluting buffer (PBS; 0.5% BSA (w/v)) for 30 minutes at room temperature. The immunoreactivity was visualized by incubating the sections in streptavidin biotinylated horse-radish-peroxidase complex (Amersham, Buckinghamshire, UK) diluted 1:20 in PBS; 0.5% BSA (w/v) for 15 minutes, and for 3 minutes in a mixture containing 5mg diaminobenzidine (DAB), 8μl of hydrogen peroxide activator and 10ml of PBS. Sections were then washed in PBS, dehydrated and mounted using DPX mounting medium and stored at room temperature until analysis.

For analysis, two sections were mounted on each slide. One section was incubated with the anti-BrdU antibody (treatment) and the other was incubated with 1% BSA in PBS omitting the mouse anti-BrdU antibody, to act as a negative control. In addition, with each batch of slides, a section of sheep skin known to contain BrdU was incubated with the mouse anti-BrdU antibody, to act as a positive control. Cells that had incorporated BrdU in their DNA were easily identified under a light microscope, by the brown colour of their nuclei.

A.3. RESULTS

For each experiment, the mode of administration of BrdU (i.v. for the sheep and i.p. for the rats) and the dose rate (pregnant ewe: 10mg//kg/b.w.; lambs: 5mg/kg/b.w.; adult rat: 3 mg/kg/b.w.; neonatal rats: 0.5 mg/kg/b.w.) was sufficient to label cells in the S-phase of the cell cycle from gut, skin and skeletal muscle tissues (Table A.1.).

In Experiment 1, BrdU-positive cells, i.e., those cells in the DNA synthetic phase of the cell cycle, were observed in gut, skin (Plate A.1.) and skeletal muscle (Plate A.3.) of neonatal rats injected with BrdU, both one hour and 36 hours prior to slaughter (Table A.1.). The neonatal rats in the control group were negative for BrdU in all tissues sampled (Plates A.2. & A.4.). Similarly, BrdU-positive cells were evident in the tissues from the pregnant adult rat (Table A.1.), and in gut, skin (Plate A.5.) and skeletal muscle tissue (Plate A.7.) collected from its fetus (Table A.1.), but absent from the controls (Plates A.6. & A.8.).

In Experiment 2, labelled cells were evident in gut, skin and skeletal muscle tissue taken from the neonatal lambs one hour and 48 hours after injection of BrdU. Similarly, gut, skin (Plate A.9.) and skeletal muscle tissue collected from the adult pregnant ewe in Experiment 3, showed BrdU-positive cells (Table A.1.). BrdU-positive cells were absent from tissue taken from control animals in both cases (Plate A.10.). In contrast to the study with the pregnant rat, BrdU-positive cells were not present in the skin (Plate A.11.) or skeletal muscle tissues collected from the E98 fetal lamb (Table A.1.).

Table A.1. BrdU labelling of replicating cells from gut, skin and skeletal muscle tissues of adult, neonatal and fetal rats and sheep.

		Tissue		
	Experiment	Gut	Skin	Skeletal Muscle
d10 neonatal rat	1	✓	✓	✓
Adult rat	1	✓	✓	✓
E18 fetal rat	1	✓	✓	✓
d3 neonatal lamb	2	✓	✓	✓
d6 neonatal lamb	2	✓	✓	✓
Adult sheep	3	✓	✓	✓
E98 fetal lamb	3	X	X	X

Positive=✓ ; Negative=X

d = postnatal day

E = embryonic day

Plate A.1. Photomicrograph of a portion of 10-day-old neonatal rat skin. The nuclei have been labelled with a BrdU antibody (brown) and the hair follicles and connective tissue are counterstained pink with Eosin (Mag. x500).

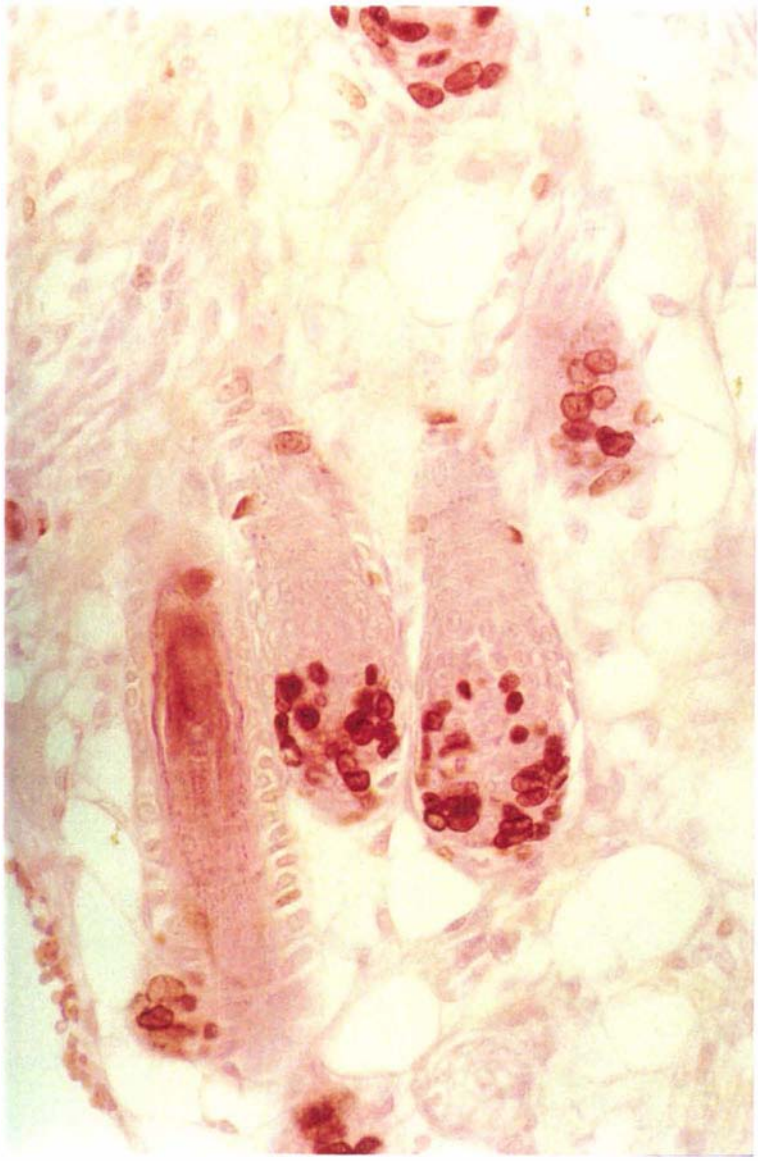


Plate A.2. Photomicrograph of a portion 10-day-old neonatal rat skin. This section has been treated the same as the section in plate 1 omitting the primary antibody (negative control). Hair follicles and connective tissue are counterstained pink with Eosin (Mag. x500).

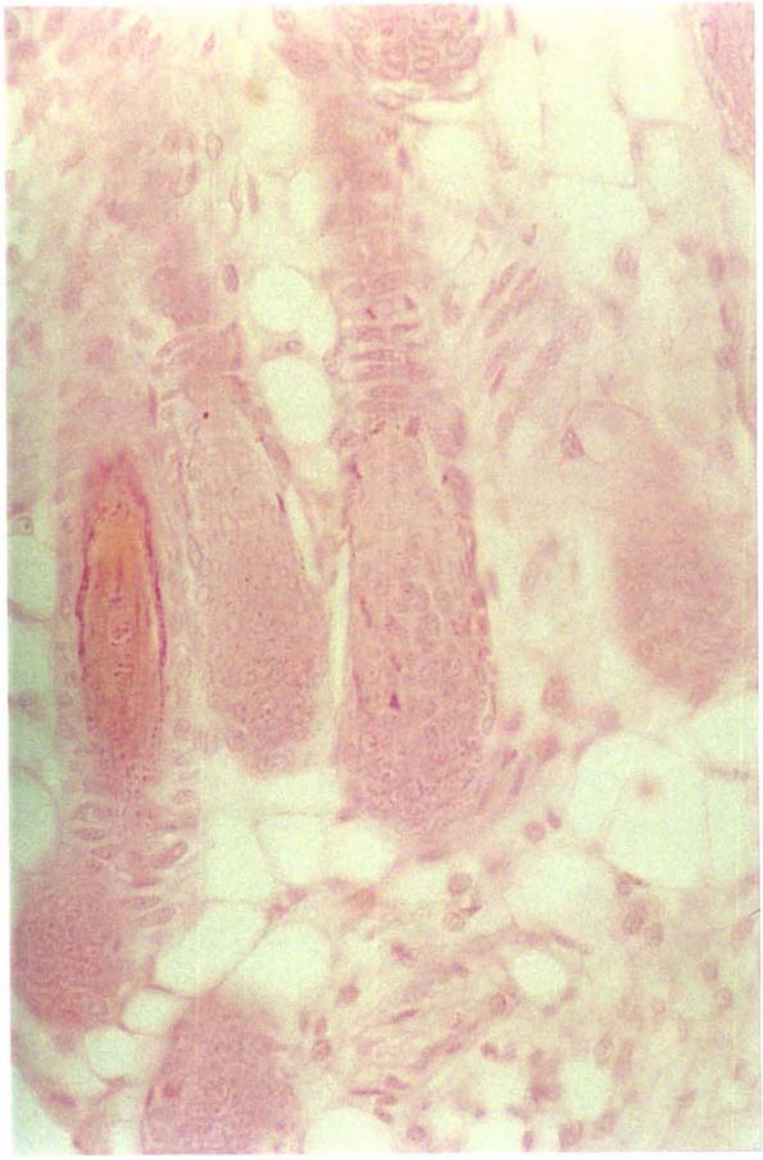


Plate A.3. Photomicrograph of a portion 10-day-old neonatal rat skeletal muscle. The nuclei have been labelled with a BrdU antibody (brown) and the myofibres and connective tissue are counterstained pink with Eosin (Mag. x250).

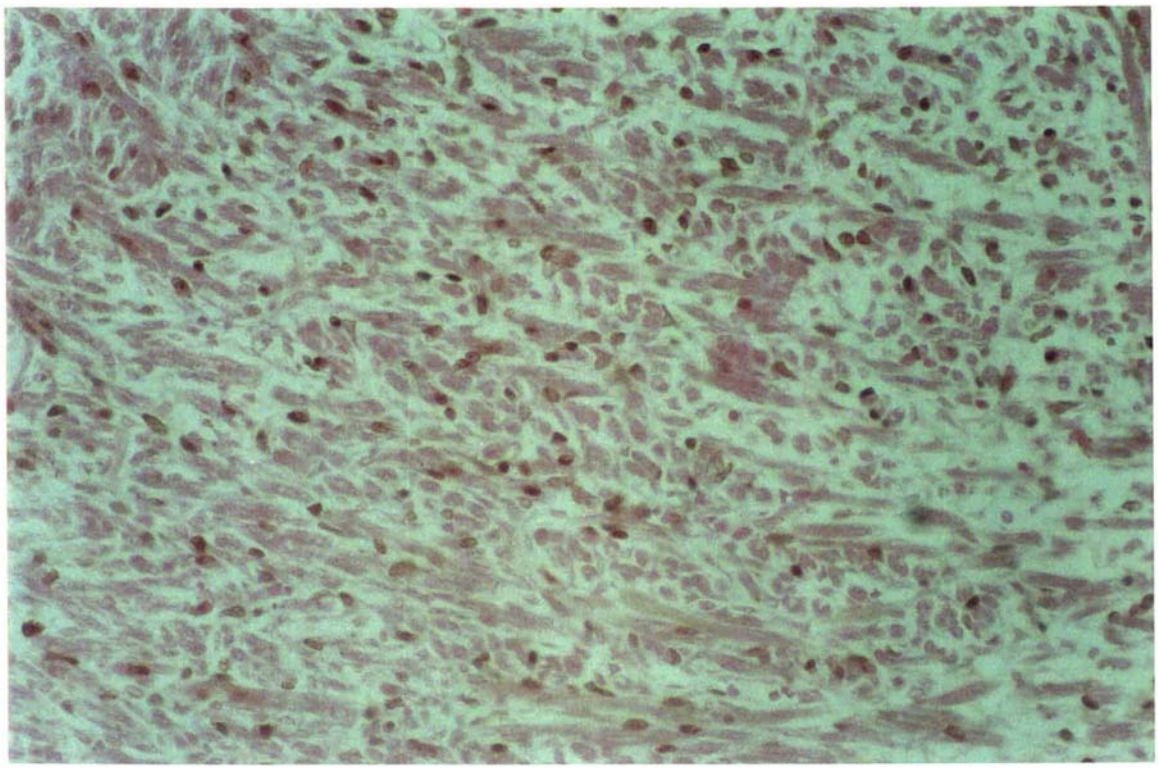


Plate A.4. Photomicrograph of a portion 10-day-old neonatal rat skeletal muscle. This section has been treated the same as the section in plate 3 omitting the primary antibody (negative control). Myofibres and connective tissue are counterstained pink with Eosin (Mag. x250).

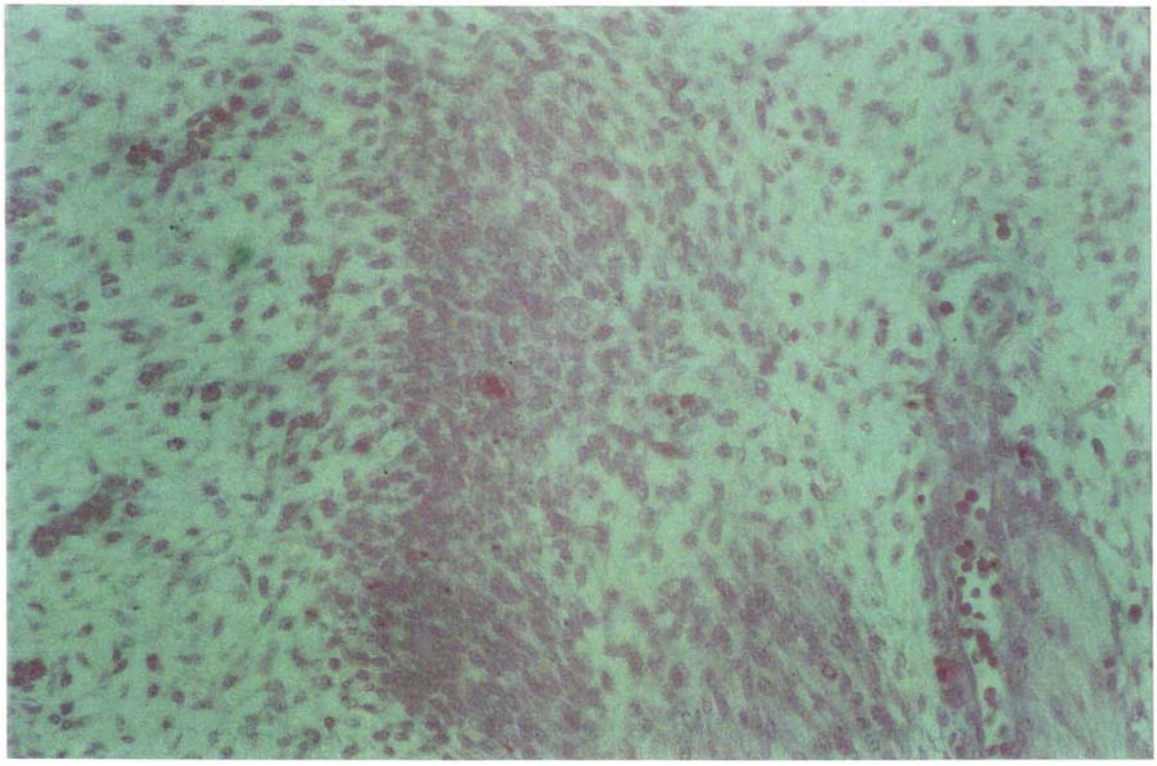


Plate A.5. Photomicrograph of a portion of embryonic day 18 rat skin. The nuclei have been labelled with a BrdU antibody (brown) and the hair follicles and connective tissue counterstained pink with Eosin (Mag. x500).

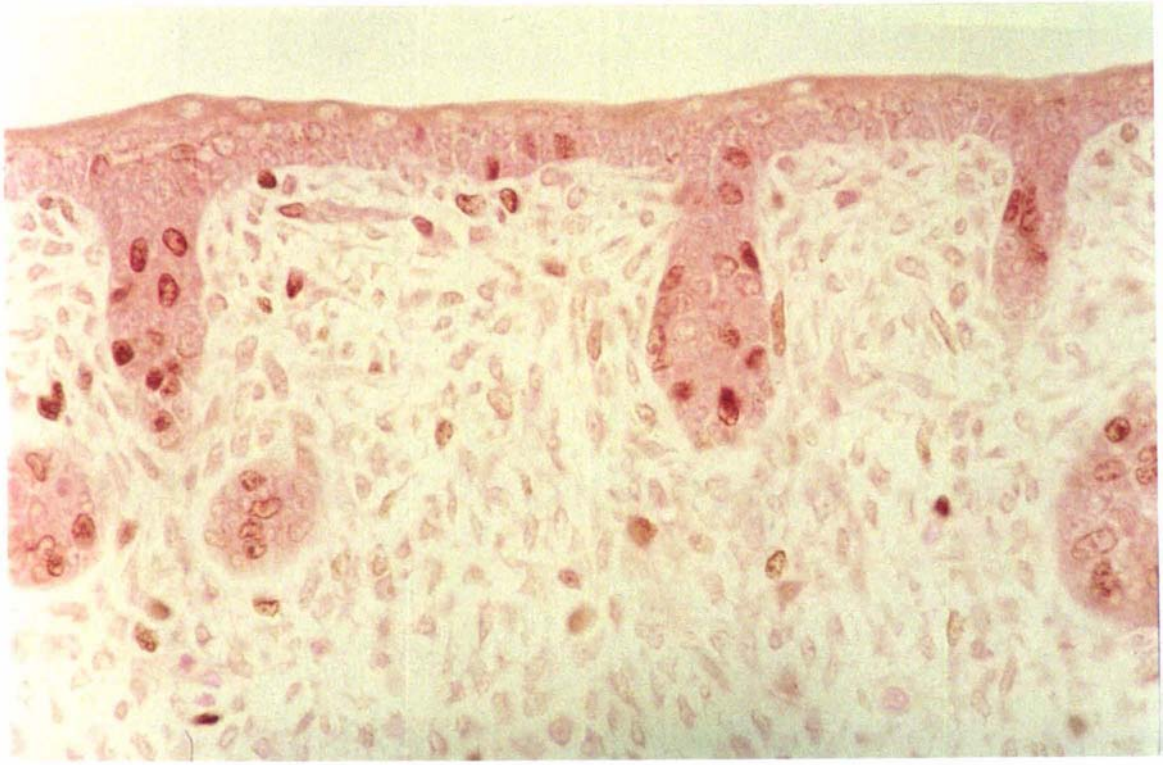


Plate A.6. Photomicrograph of a portion embryonic day 18 rat skin. This section has been treated the same as the section in plate 5 omitting the primary antibody (negative control). Hair follicles and connective tissue are counterstained pink with Eosin (Mag. x500).

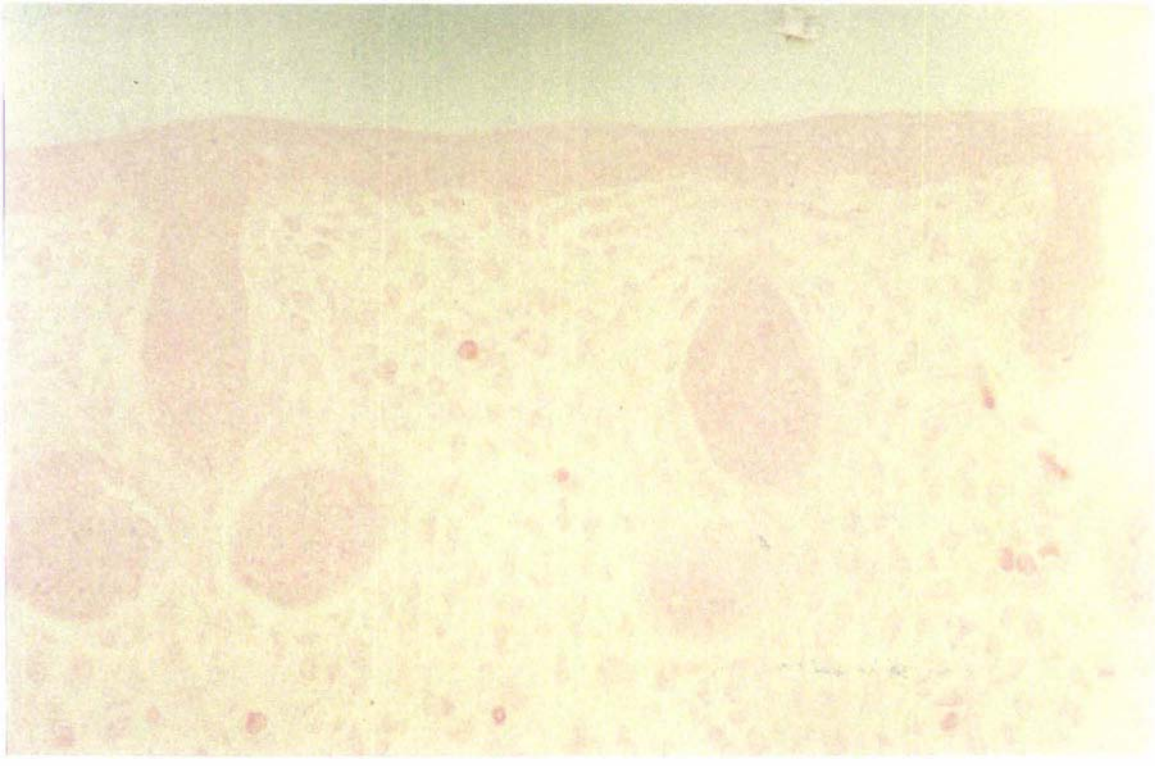


Plate A.7. Photomicrograph of a portion of embryonic day 18 fetal rat skeletal muscle. The nuclei have been labelled with a BrdU antibody (brown) and the myofibres and connective tissue are counterstained with Eosin. (Mag. x500).

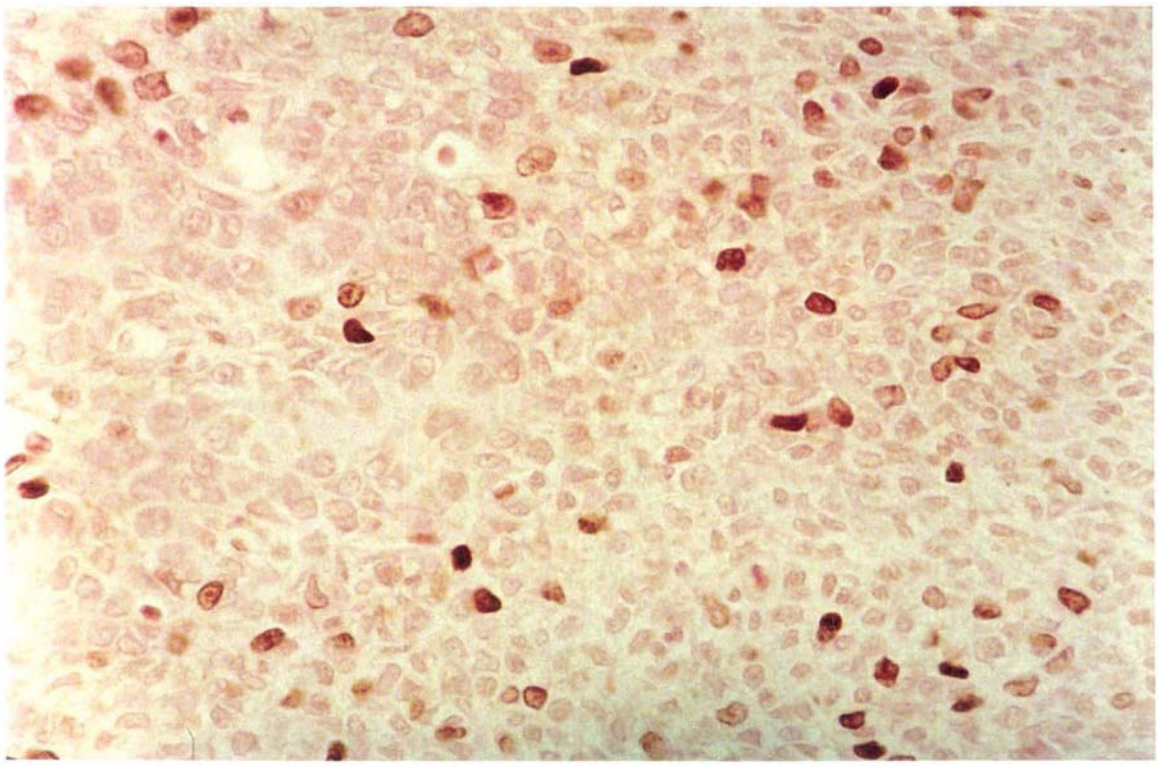


Plate A.8. Photomicrograph of a portion embryonic day 18 rat skeletal muscle. This section has been treated the same as the section in plate 7 omitting the primary antibody (negative control). Myofibres and connective tissue are counterstained pink with Eosin (Mag. x500).

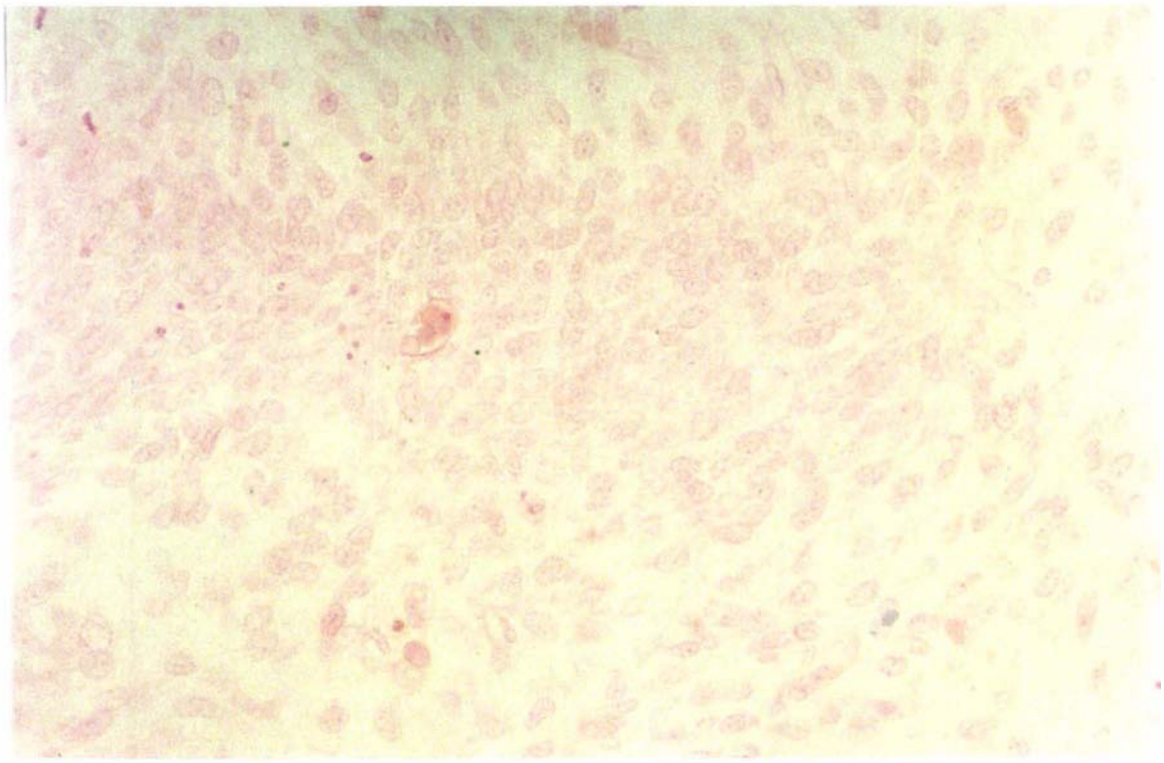


Plate A.9. Photomicrograph of a portion of adult sheep skin. The nuclei have been labelled with a BrdU antibody (brown) and the hair follicles and connective tissue are counterstained pink with Eosin. (Mag. x500).

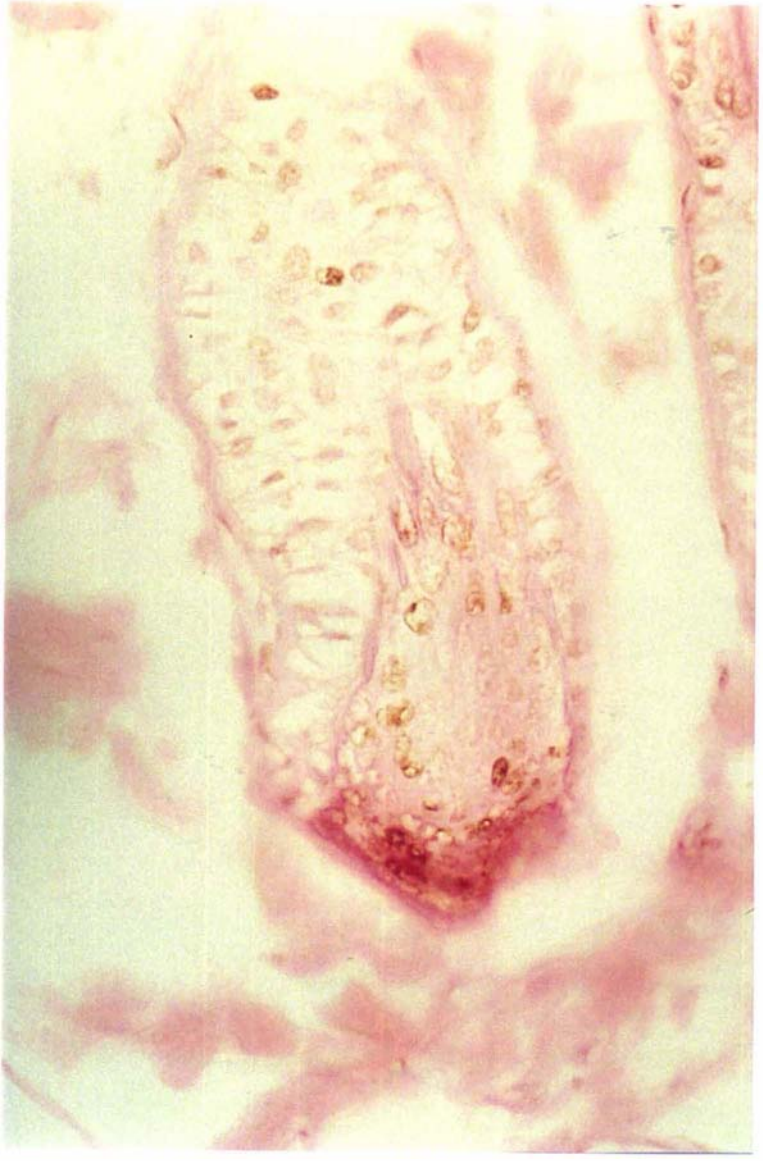


Plate A.10. Photomicrograph of a portion adult sheep skin. This section has been treated the same as the section in plate 9 omitting the primary antibody (negative control). Hair follicles and connective tissue are counterstained pink with Eosin (Mag. x500).

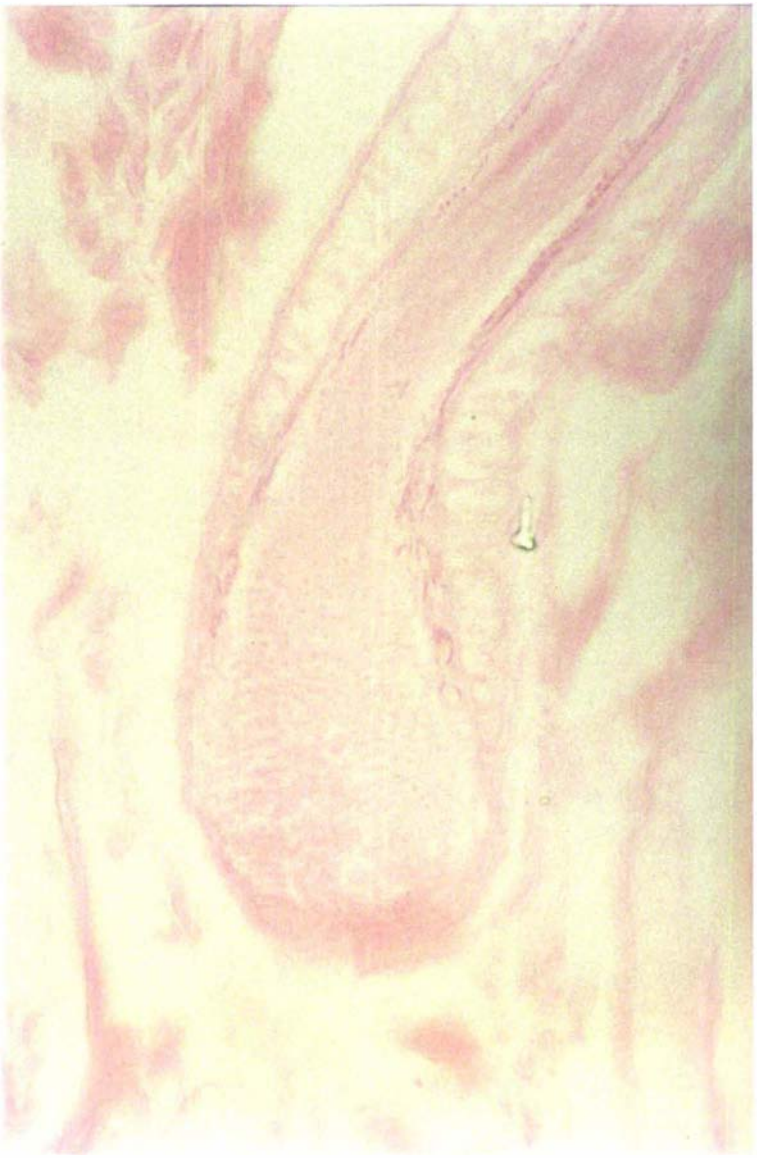
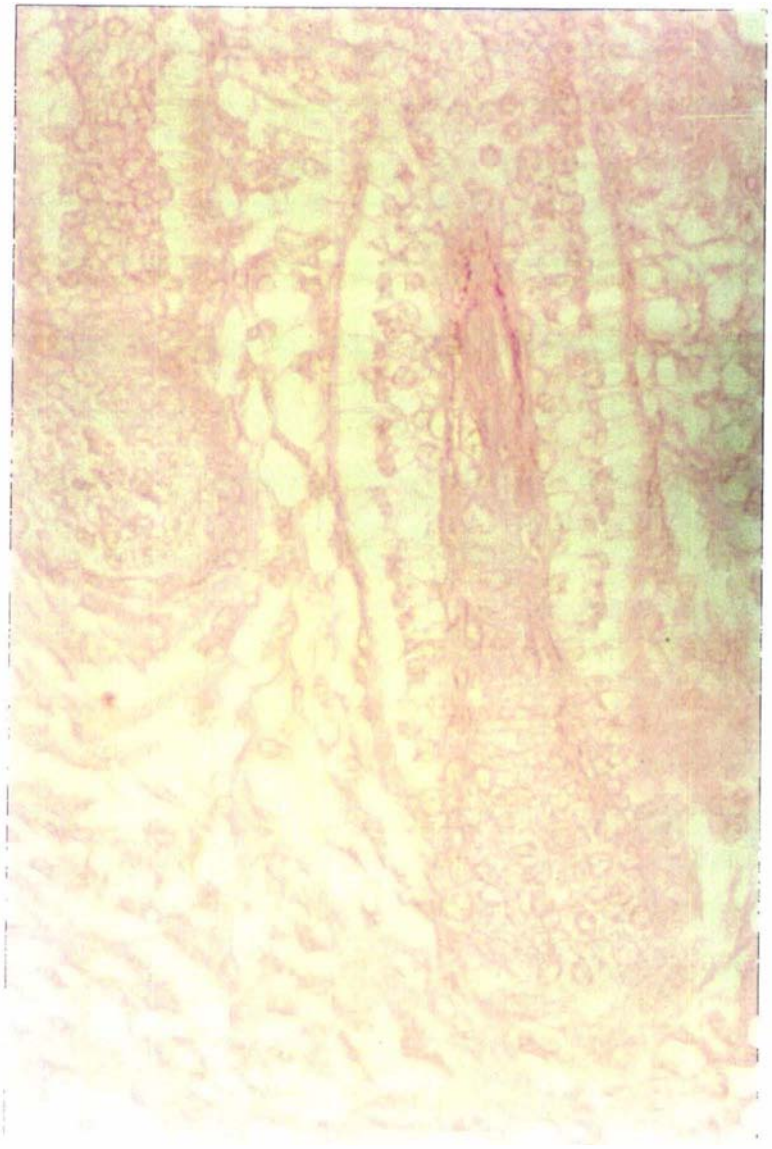


Plate A.11. Photomicrograph of a portion of fetal sheep skin at 98 days of gestation. The nuclei are unlabelled with a BrdU antibody (no staining) and the hair follicles and connective tissue are counterstained with Eosin. (Mag. x500).



A.4. DISCUSSION

A single injection of BrdU hours before sacrifice followed by immunohistochemical analyses resulted in discrete staining of some nuclei believed to be in the DNA synthetic phase of the cell cycle. The dose used in each experiment resulted in labelled nuclei without any noticeable adverse effects on the animals. However, the effect of BrdU on cell proliferation and differentiation within various tissues was not measured in this study. Therefore, the dose rate and the mode of administration appeared to be sufficient to adequately label at least some of the cells which were in the S-phase of the cell cycle between the time of injection and sacrifice.

Intraperitoneal injection of BrdU into neonatal and adult rats resulted in dark staining of nuclei in gut, skeletal muscle, and hair follicle bulbs in the skin. The darkly-stained BrdU-positive nuclei in all the fetal rat tissues studied (Plates A.5. & A.7.) indicate that the dose administered to the dam was sufficient to label replicating cells in her tissues as well as the tissues of her fetuses. In addition, this result illustrates that BrdU can successfully permeate the feto-placental barrier of the pregnant rat, enabling labelling of proliferating cells in the fetus following administration of the chemical to the dam.

In contrast, although BrdU-positive cells were evident in neonatal and adult sheep gut, skin and skeletal muscle tissues (Table A.1.), i.v. injection of BrdU into the dam, did not result in labelling of replicating cells in the tissues of the fetus. Thus, although BrdU is able to permeate the rat feto-placental barrier, it is unable to cross the feto-placental barrier in sheep, at least at the dose used here. To check whether this animal was representative of all pregnant ewes, a further 6 single-bearing and 3 twin-bearing ewes from an independent trial (Chapter 6), were given a similar i.v. dose of BrdU at 100 days of gestation. Muscle, gut and skin samples from the dams and their fetus(es) were processed in the same manner as described in this study. Although maternal tissues were labelled with BrdU, cells in the S-phase of the cell cycle in the fetus were not labelled (data not shown). As a result, in order to label replicating cells of the sheep fetus using BrdU, administration must be direct, such as via an i.p. injection with the aid of ultrasound, or by direct injection into the bloodstream of the fetus following fetal

catheterization. Both methods have obvious financial and time disadvantages if using large numbers of animals.

An alternative approach is to use an endogenous marker of replicating cells such as proliferating cell nuclear antigen or PCNA (McCormick and Hall, 1992) or MyoD (Koishi *et al.,* 1995; Hughes *et al.,* 1997; see also Chapter 6) antibodies detected by an immunoenzyme detection method or immunofluorescence. PCNA is a nuclear protein closely associated with DNA synthesis. Although PCNA has the advantage of being an endogenous protein, thus avoiding the requirement for *in vivo* labelling, there are some important technical considerations which must be recognized. The duration of fixation and type of fixative (Hall *et al.,* 1990) can influence the pattern of staining, and low levels of PCNA may be present throughout the cell cycle (Cells and Cells, 1985; Bravo and Macdonald-Bravo, 1987; Morris and Matthews, 1989) which, depending on the sensitivity of the immunohistochemical detection system, may result in either an over- or under-estimation of the number of PCNA immunoreactive cells. In addition, some cells which have recently exited the cell cycle may express PCNA, such as in normal tissue adjacent to tumours (Hall *et al.,* 1990). Therefore, although PCNA has the advantage of being an endogenous protein that enables the detection of replicating cells *in vivo*, allowances must be made for these technical problems.

MyoD, a muscle-specific nuclear protein and a member of the basic helix-loop-helix transcription factor family, is readily detectable in many nuclei in all muscles, but not in a variety of non-muscle tissues (Hughes *et al.,* 1997). Many of these nuclei are located in the myoblast/satellite cell position (Koishi *et al.,* 1995) based on their physical location and their lack of reactivity with a marker for myoblast differentiation (sarcomeric MyHC). However, some nuclei appear to be located within nascent muscle fibres (Koishi *et al.,* 1995; Hughes *et al.,* 1997). MyoD-labelled nuclei are also located in the "satellite cell" position in fetal and neonatal sheep skeletal muscle (see Chapter 6), indicating that it may also be a useful alternative to BrdU for detection of proliferating cells in skeletal muscle tissue of fetal sheep.

In conclusion, although BrdU appears to be a useful method to detect cells in the S-phase of the cell cycle in many tissues, it has limitations when investigating proliferating tissues of fetal sheep, due to the failure of this compound to permeate the feto-placental barrier. The financial and time limitations of large-scale experiments often do not permit the direct administration of BrdU to the fetus(es), therefore an alternative approach is necessary, such as the use of endogenous proteins (e.g., MyoD) for muscle tissue as described in Chapter 6.

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