EFFECTS OF THE INTRAUTERINE GROWTH RETARDATION (IUGR) CONDITION ON CENTRAL HOMEOSTATIC SYSTEMS REGULATING ENERGY METABOLISM IN IMMEDIATE POSTNATAL LIFE IN THE SHEEP.

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Jose Manuel Ramos-Nieves

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Obesity has detrimental effects beyond wellbeing, increasing susceptibility to metabolic and cardiovascular diseases. Multiple factors like, genetics, calorie-dense foods, physical activity and social environment contribute to its development. In the last decades, epidemiological and clinical research has revealed associations between perinatal events and propensity to develop obesity in adult life. In particular intrauterine growth retardation (IUGR) has been associated with young and adult obesity and metabolic diseases. The IUGR condition induces accelerated growth, hyperphagia and obesity in rodents offered high caloric diets after birth. It has been discovered that hypothalamic regulation of energy homeostasis is defective in these animals due to an abnormal leptin profile during the first 2 postnatal weeks. During this period, leptin stimulates the development of axonal projections between various hypothalamic centers. However, the ontogeny of this phenomenon is different between altricial rodents and precocial species such as humans and sheep. Therefore, we used sheep to study the effects of the IUGR condition and high fat diets on immediate postnatal energy metabolism. We confirmed that IUGR and normal lambs display similar intake relative to their metabolic size, however, they accrete excess fat when compared on similar body weight. Our observations indicate that interactions between birth size and diet alter energy expenditure and retention through changes in thyroid hormones. Second, we

evaluated the functionality of the melanocortin system, the best known mechanism of central control of energy intake and expenditure, and its effects on IUGR lambs. Using a melanocortin receptor agonist, we confirmed that this system is functional as early as postnatal day 4 in IUGR and its stimulation reduces adiposity by decreasing appetite and, presumably, increasing energy expenditure. Finally, we examined the role of leptin signaling during early life in the control of energy homeostasis and programming of future metabolic alterations and obesity. We discovered that treatment with a long-lasting leptin antagonist during the first two weeks of life acutely increases appetite and growth but has no long-term effects on appetite, growth or body composition.

BIOGRAPHICAL SKETCH

Jose Manuel Ramos Nieves was born in Mexico City on May 31st of 1978 to Jose Manuel Ramos Villegas and Maria Blanca Nieves Silva. He grew up with his parents and his younger brother and sister, Pablo and Paulina. Although he was raised in Mexico City, he spent most of his weekends and summers at the countryside in the state of Queretaro, where he developed his interest in agriculture and animal production. After graduating with honors from high school, he moved to the city of Queretaro to enroll in the local campus of the Tecnologico de Monterrey and pursue professional studies in agronomy and animal science. Throughout this time, he collaborated in numerous experiments and product tests in commercial dairy farms. During the last year of his professional studies, he had his first international experience in a one-semester academic exchange at Cornell University. After returning home, he graduated Magna Cum Laude with University Honors in 2001. During more than 2 years, he worked for the Tecnologico de Monterrey evaluating the economic and technological impact of several state and federal agricultural subsidies. In 2004, he joined Elanco Animal Health© as sales representative in the dairy area of La Laguna, in northern Mexico. In 2005, he returned to Cornell University as a CONACYT scholar to conduct his Master of Science studies with Dr. Thomas Overton in nutrition of the transition cow. He continued his doctoral studies under the mentorship of Dr. Yves Boisclair using the sheep as biomedical model to study the effects of intra-uterine growth retardation and diet in the growth and composition of the neonate as well as the functionality of the central control of energy homeostasis.

A mis padres.

Porque ni mis días ni mis sueños serían posibles sin su vida y sin su amor…

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LIST OF ABBREVIATIONS

α-MSH	alpha-melanocyte-stimulating hormone
ADG	average daily gain
AGRP	agouti-related peptide
ALS	acid-labile subunit
ANOVA	analysis of variance
ARC	arcuate nucleus
BAT	brown adipose tissue
BMI	body mass index
BS	body size
BSA	bovine serum albumin
BW	body weight
CART	cocaine-amphetamine regulated peptide
CP	crude protein
CRH	corticotropin-releasing hormone
CRL	crown-rump length
DM	dry matter
DMN	dorsomedial nucleus
DMV	dorsal motor nucleus of the vagus
DVC	dorsal vagal complex
EBW	empty body weight
FAS	fatty acid synthase
FWG	fractional weight gain
G	gestation day
GH	growth hormone

GI	gastrointestinal tract
HF	high fat
HPT	hypothalamic-pituitary-thyroid
ICV	intracerebroventricular
IGF-1	insulin-like growth factor 1
IUGR	intrauterine growth retardation or intrauterine growth retarded
IV	intravenous
KIU	kilo international unit
LF	low fat
LHA	lateral hypothalamic area
LW	live weight
MBW	metabolic body weight
MC3R	melanocortin receptor 3
MC4R	melanocortin receptor 4
Mcal	megacalorie
MCH	melanin-concentrating hormone
MCs	melanocortins
N	nitrogen
NEFA	non-esterified fatty acids
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
Ob-Rb	leptin receptor b
P	postnatal day
pO2	oxygen partial pressure
POMC	pro-opiomelanocortin protein
PVN	paraventricular nucleus

SD	standard deviation
SE	standard error
SOLA	super ovine leptin antagonist
T3	triidothyronine
T4	thyroxine
TCA	tricarboxylic acid
TRH	thyrotropin releasing hormone
UCP(-1, -2, -3),	uncoupling protein (-1, -2, -3)
VMH	ventromedial hypothalamus

CHAPTER 1: INTRODUCTION

Through evolution, animals have regulated their energy reserves to successfully cope with the uncertainty of food availability, and simultaneously preserve body fitness to thrive within their ecological niche. Consequently, the control of fat reserves is tightly regulated and involves biological mechanisms that regulate the two determinants of overall energy balance, namely energy intake and expenditure (1). With more than 500 million individuals suffering from obesity in the world, it is evident that these mechanisms frequently fail in humans (2). In the U.S.A. alone more than 35% of the adult population is obese and an additional 33% presents some degree of overweight (3). Beyond the evident detrimental effects on human wellbeing, obesity increases susceptibility to non-communicable diseases like dyslipidemia, hypertension, type 2 diabetes and hepatic damage and is also associated with higher mortality rates (4).

Scientists have shown that factors promoting obesity include genetics, greater availability of calorie-dense foods, lower physical activity and even the social environment (1, 5, 6). In the last 4 decades however, evidence has accumulated for an additional factor contributing to the development of adult obesity and metabolic diseases. Specifically, Dorner, in Germany, and Barker, in England, suggested that nutrition during pregnancy has a major role in increasing the propensity of the offspring to develop diabetes or obesity during adult life (7, 8). Since then, multiple epidemiological studies and clinical studies have supported their hypothesis that perinatal events can have long lasting effects leading to the promotion of excessive fatness (9–13). This phenomenon is commonly referred as fetal or perinatal programming (14).

Among the different obesity programming paradigms, the intra-uterine growth retardation (IUGR) condition has generated interest due to its relevance to human populations (15). Experiments with rodents have shown that the IUGR condition is associated with higher growth rates, higher appetite, lower locomotor activity, higher fatness and the incapacity to regulate caloric intake when offered calorie-dense foods (16–19).

In parallel, remarkable advances have occurred in our understanding of the central control of energy intake and its interactions with the environment. It is now well accepted that the central system regulates intake and energy expenditure through the integration of peripheral cues and signals of nutritional and environmental origin (20, 21). Interestingly, rodent models have shown that the IUGR condition is linked to a defective development of hypothalamic circuits responsible for the control of energy homeostasis (22, 23). One current limitation in this field is near total reliance on rodent models. Rodents are altricial animals and appear to undergo key developmental events in a different window of life than the precocial human infant (24). Most importantly, final development of the hypothalamus, the brain structure most involved in energy homeostasis, occurs during the first two postnatal weeks in rodents whereas it is mostly completed at birth in humans and other precocial animals (25–28). Therefore, rodent models may not be ideal to study the programming of central regulatory mechanisms by the IUGR condition.

The pregnant sheep has long been viewed as an adequate model to study the progression and physiology of pregnancy (29). Some of the advantages of this large animal model include relatively short gestation period, mother:fetus weight ratio similar to humans and the possibility of surgical intervention in both, mother and fetus (29). Because of these reasons and their precocial nature, they offer the possibility of more

accurately duplicating the events occurring in humans. In this dissertation a naturally occurring sheep IUGR model was used. This model relies on inadequate placental mass during the last third of gestation to induce low birth weight. Previous work has already described the propensity of these IUGR lambs to develop accelerated growth and higher adiposity by presumably increasing intake and reducing energy expenditure during early life (30). However, multiple questions remain unanswered in this animal model.

High-fat diets during the post-weaning period exacerbate the hyperphagia of IUGR rodents (18). Would IUGR lambs be affected similarly if offered a high caloric diet during the accelerated growth period in early life? What effects would this diet have on energy homeostasis and body composition?

Also, it is known that both, energy intake and expenditure are regulated by the central system in response mainly to a peripheral hormonal cue called leptin that reflects the status of energetic reserves in the body (31). This central modulation is mediated through a well-known system that involves peptides known as melanocortins and their receptors (20). Given the immature state of IUGR lambs, is this system defective at birth in these animals? Does the melanocortin system effectively regulate energy homeostasis and body composition during early life?

Finally, as mentioned before, the development of hypothalamic centers responsible for energy homeostasis takes place during the first two weeks of life in rodents and leptin seems to be essential to this process (32). Does leptin has the same neurotrophic role during early life in precocial species like sheep? Would a defective leptin signaling induce a permanent alteration in energy homeostasis of these animals?

The overall objective of this body of research is to explore the role of IUGR and dietary fatness in the ontogeny of obesity in a species that more closely resembles the developmental stages of humans. We focused on the functionality of the leptin-melanocortin system responsible for the control of voluntary intake and energy expenditure.

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CHAPTER 2: LITERATURE REVIEW

The obesity epidemic in the human population

Extent of problem. Excessive adiposity or obesity is a growing concern in the world. It is a considered a disease with clear etiology, signs and symptoms that affect not only the health but also the social and economic wellbeing of affected individuals (1, 2). Obesity has also been associated with increased morbidity of non-communicable diseases like dyslipidemia, hypertension, type-2 diabetes and hepatic damage (3).

The need for a consistent way to assess adiposity resulted in the adoption of the body mass index (BMI). BMI is defined as body weight divided by the square of height (4). The World Health Organization has accepted BMI as an indirect measure of fatness in the general population. In most world regions, the normal BMI range is 18 to 25 kg/m^2 (5). A BMI falling between 25 and 29.9 kg/m^2 indicates overweight whereas a BMI in excess of 30 kg/m^2 indicates obesity (5). Supporting the association between obesity and non-communicable diseases, any increment in BMI above 21 kg/m^2 raises the risk to develop ischemic heart disease, stroke, hypertensive heart disease, diabetes, osteoarthritis, breathlessness, back pain, dermatitis, menstrual disorders and infertility, gallstones, as well as different types of cancer (6). It is important to emphasize that although BMI has been an effective tool to describe the prevalence of obesity in a population, it is regarded as a poor predictor of overall fatness at the individual level. Piers et al. (7) demonstrated that the BMI is a poor predictor of the actual body fat content and has poor sensitivity. Some researchers have suggested that other indexes such as waist circumference provide a better estimation of adiposity, especially for the visceral fat depot (8).

Many developed and developing societies face a serious threat to future wellbeing, health and productivity due to increased obesity rates (5). In the US, 33% of the adult population suffer some degree of overweight (BMI between 25 and 30 kg/m^2) and an additional 35% is obese (BMI>30 kg/m^2) (3). Worldwide the situation is not better: in 2005, 23% of the world's population was overweight and 9.8% was obese (9). Moreover, the BMI is still increasing in many parts of the world: these increments average 0.4 units per decade for men and 0.5 units for women (calculated from 1980 to 2008) (5).

Causes of obesity. In the most basic sense, excessive adiposity is the result of a positive imbalance between energy intake and energy expenditure. Greater availability of calorie-dense foods, reduced physical activity and the social environment promote and facilitate this imbalance, increasing fat storage and obesity (10). Evidence for a major environmental component for obesity is provided by significant increases in BMI for population as diverse as Pima Indians, Africans or Asians when living in developed countries such as the U.S. and U.K. (11, 12). In addition, studies of monozygotic and heterozygotic twins have provided evidence of the remarkable genetic component of obesity. Researchers have estimated that the heritability of BMI ranges between 0.50 and 0.70 (13, 14), and is second only to the heritability of height among physical traits (15).

An additional factor that has received attention in recent years is the intrauterine environment (16, 17). Over the last four decades, epidemiological studies have found significant correlations between various fetal events and the susceptibility to develop non-communicable diseases in postnatal life, including obesity and insulin resistance (18). David Barker's group (19) elaborated on these associations to develop the 'thrifty phenotype' hypothesis. This hypothesis posits the existence of developmental plasticity

during prenatal life whereby the fetus engages metabolic adaptations to face adverse *in utero* conditions. If irreversible, however, these adaptations become detrimental when postnatal conditions are normal. For example, fetal undernutrition has been associated with insulin resistance in 8-year old infants and 50-year old adults (20, 21). Phillips (22) suggested that this insulin resistance was the consequence of fetal adaptations that insured adequate glucose supply to the brain in uterine life but became unfitting after birth.

Perinatal insults linked to excessive fatness in postnatal life

Programming of future obesity and metabolic disorders has been associated with both, maternal over- or under-nutrition (23). Several epidemiological studies have supported the association between maternal high BMI during gestation to higher birth weight and to the later development of obesity and metabolic syndrome in the offspring (24, 25). The postnatal environment contributes to the higher adiposity in offspring but does not explain all of it. For example, Dorner et al. (26) studied Germans born during and soon after World War II: diabetes was higher in individuals whose mother was well-fed during pregnancy than those born from underfed mothers. Also Dabelea et al. (27) analyzed pairs of siblings born before and after their mother developed diabetes mellitus. They found that children born after the onset of maternal diabetes were 3.7 times more likely to develop diabetes than siblings born before.

At the other side of the spectrum, low birth weight has been linked to obesity and metabolic abnormalities in adulthood as well. Barker and colleagues (28) showed that women born to mothers exposed to the Dutch famine of 1944-1945 during the first third of gestation had higher BMI at age 50 than those born from non-exposed mothers. Also,

a retrospective study of 64-72 year old white men indicated that low birth weight was associated with higher fat mass and fat percent as well as lower fat-free soft tissue and muscle mass than individuals of high birth weight (29). Another study comparing elderly adults (55-73 years of age) monozygotic and dizygotic twin pairs discovered negative associations between birth weight on one hand, and fasting plasma glucose, fasting plasma insulin and insulin resistance index on the other, indicating a defective glucose metabolism in IUGR individuals (30).

Gluckman et al. (31) made an important observation with regard to associations between the IUGR condition and non-communicable diseases. They remarked that many studies that associate birth size with chronic diseases offer no indication of the effect that fetal adaptations may have in early post-natal life. Their observation is relevant in the sense that most of the detrimental consequences of pre-natal growth retardation are known to relate to accelerated growth soon after birth (also known as catch-up growth) (32–34). Studies conducted in different populations indicate a strong association between catch-up growth during the first months of life and higher BMI and adiposity of pre-adolescent children (35–37). In addition, some researchers have made observations about the possibility of a metabolic programming window during the early postnatal period. For example, Stettler et al. (38) determined that high milk replacer consumption during the first 8 days of life is positively associated with BMI of young adults (20 to 32 years old). Also, Plagemann et al. (39) indicated that consumption of breast-milk from diabetic mothers during the first 7 days of life is positively correlated with body weight at 2 years of age.

The present dissertation uses a multifoetal sheep model to explore the consequences of the IUGR condition on appetite and energy metabolism. Accordingly

the review will focus on the biology of IUGR models in both rodents and sheep and their long-term effects on systems responsible for postnatal energy homeostasis.

IUGR models and postnatal consequences on energy metabolism

Results from research in rodent and large animal models support associations between the IUGR condition, and aberrations in postnatal energy metabolism and increased susceptibility to metabolic diseases. In the early 70's, Widdowson and McCance (40, 41) recognized that undernutrition during prenatal life or during early postnatal life can permanently alter growth trajectories and decrease mature size. The following section will describe commonly used rodent and sheep IUGR models.

Rodent models of IUGR

One simple method to induce prenatal growth retardation is to restrict maternal feed intake during gestation. Desai et al. (42) restricted pregnant rats to 50% of ad libitum fed controls from gestation day 10 (G10) to term, inducing a mild reduction of 15% in birth weight. These IUGR pups had higher growth rates and even surpassed the body weight of control animals at weaning on postnatal day 21 (P21) when crossfostered by ad libitum fed dams. This difference in body weight persisted and was amplified by 36 weeks of age. This model may not be completely repeatable because a similar experimental design failed to generate differences in adult weight between IUGR and Control rats at P160 (43). A greater degree of maternal nutritional limitations can induce permanent stunting in the offspring. For example, Vickers et al. (44) imposed a 70% nutritional restriction to pregnant dams throughout pregnancy and observed a 39%

reduction in birth weight. These offspring were never able to recover from the severe IUGR and were 85% of normal weight at P125.

Both, the mild and severe prenatal undernutrition models described above have detrimental effects on postnatal energy metabolism. In the milder model of feed restriction, IUGR offspring developed a higher absolute dry matter intake during the post-weaning period, but only if cross-fostered to mothers that were unrestricted during pregnancy and lactation (42). However, this difference between IUGR rats and controls disappeared when intake was normalized to body weight (42). In the extreme model of Vickers et al. (44), male IUGR rats developed higher relative caloric consumption than control animals before and after puberty, as well as during adulthood. This difference was exacerbated when IUGR rats were fed a hypercaloric diet containing 55% of calories as fat (44). In both models, hyperphagia of IUGR animals was associated with increases in adiposity: the mild model induced an increase of 83% in total body fatness at 9 months of age (42) whereas the more severe prenatal model induced a 10% increase in retroperitoneal fatness at P125 when rats were offered a normal diet (44). A very important observation is that IUGR pups did not develop an adverse phenotype when their mothers were restricted during the lactation period (42). Thus, a mismatch between fetal and postnatal nutrition exacerbates the effects of the IUGR condition. IUGR can even affect energy expenditure by reducing physical activity. Vickers et al. (45) demonstrated that IUGR rats had lower locomotor activity at P35, P145 and P420, independently of the post-weaning dietary conditions.

Another popular method to induce IUGR in rats involve the use of low protein diets (8-10% instead of 20%) throughout gestation (46–49). Berends et al. (49) found that offspring of pregnant rats fed a low-protein diet were ~17% lighter at P3 and remain lighter until P7. However, when cross-fostered onto dams fed a normal diet, male pups

became numerically heavier than normal pups at P110. Surprisingly, these animals were not fatter than control rats at this age. It is important to point out that other laboratories have failed to see higher body weight in this IUGR model at 4 months or 10 months of age (50, 51). This difference is probably the consequence of slightly different post-natal diets. Low protein diets are also effective in inducing the IUGR condition in mice (52, 53). Similarly to rats, IUGR mice developed higher rates of gain and adiposity when cross-fostered onto normally fed dams and offered highly palatable diets (52, 53). IUGR male mice and rats arising from this model have a 25% shorter lifespan when they are allowed to express accelerated growth before weaning (46, 48).

It is not surprising that IUGR rodents that develop obesity also show defects in glucose metabolism and insulin action (54–56). IUGR arising from sever maternal undernutrition had elevated circulating insulin concentration and hepatic glycogen content at P250 (54). Also, leptin levels in these animals are disproportionately higher than expected from their fatness at 9 months of age (55). At the same age, IUGR arising from the milder model of maternal restriction and offered unrestricted postnatal nutrition suffered increased plasma glucose and insulin (56). IUGR offspring from dams fed a low-protein diet during gestation and lactation show a better glucose tolerance at 3 months of age with normal insulin levels (49). However, by 15-mo of age, IUGR rats become even more glucose intolerant relative to control animals, presumably due to lower insulin sensitivity in males and lower insulin secretion in females (46).

Sheep models of IUGR

Sheep models have been extensively used in the study of maternal-fetal interactions (57). Sheep models offer many advantages including the feasibility of

catheterization and frequent sampling of both, fetus and dam, under non-anesthetized and stress-free conditions (58, 59). Additionally, it is one of the few precocial models used in biomedical sciences. The advanced degree of development of the neonatal lamb makes this species more comparable to infants than rodent models. These characteristics and the renewed interest in the prenatal environment and its post-natal consequences have prompted the use of sheep models to study the IUGR condition.

Several experimental manipulations have been used to induce IUGR in the sheep. They include maternal heat stress, carunclectomy, large litters, overnutrition of adolescent ewes and nutrient restriction (60–63). A common feature of all these models is the significant reduction in placental mass or its nutrient transfer capacity. The small placenta becomes unable to provide adequate nutrition to the fetus. This limitation is most obvious over the last third of gestation when most of fetal growth occurs (64, 65).

Exposure of pregnant ewes to a high temperature regime (40°C 9h/d, 30°C 15 h/d, 40% rel. hum.) during the middle and/or final third of gestation can induce a 24-50% reduction in the weight of the offspring (66, 67). For example, Alexander and Williams (66) demonstrated that placental weight is reduced ~67% by chronic heat stress of pregnant ewes during the last two thirds of gestation. This placental weight reduction was associated with a 50% reduction in the birth weight of lambs. They also demonstrated that placental size is restricted by 34% or 44% when heat stress is applied exclusively during the second or last third of gestation, respectively. In both of these cases, size at birth is reduced by 30% (66). In another study, Bell et al. (67) induced heat stress in pregnant ewes from day 64 to 136-141 of pregnancy, causing a 43% reduction in placentome weight, and a 17% reduction in fetal weight at day 141. Regnault et al. (68) initiated heat stress at day 37 of gestation and measured a 27% stunting of fetal weight by P93.

Another method consists on the surgical removal of the maternal site of placental attachment in the uterus prior to mating (69). These structures are known as caruncles. Carunclectomy can induce a ~35% reduction in fetal weight, but is also frequently ineffective due to compensatory placental adaptations (69–71). Alexander (69) performed a series of experiments in which he removed up to 84 caruncles. Birth size was negatively correlated with the number of caruncles removed, and even more closely related to the number and weight of the fetal portion of the placentome (also known as cotyledons). Moreover, the weight of individual cotyledons was inversely related to the number of caruncles removed. These observations suggested the existence of compensatory mechanisms to preserve placental capacity.

Multifoetal pregnancies give rise to naturally occurring IUGR animals. In the 1980's, two studies described the effects that ovulation rates, fetal number and uterine distribution of fetuses have on fetal growth of sheep (72, 73). IUGR occurs frequently during multifoetal pregnancies because the fixed number of caruncles in the female uterus (80 to 100) and the uneven distribution of fetuses in the uterine horns impose a limit on placental development. For example, the usual number of placentomes ranges from 27 to 73 for a singleton but is reduced to 8 to 32 for each fetus of a litter of 5 (72). The birth weight of individual lambs from multifoetal pregnancies is not only lower than that of singletons, but also more variable (73).

Finally, maternal nutrient restriction can be an effective method to induce IUGR in the sheep (74, 75). A 20-40% nutrient restriction throughout gestation induces a 40% reduction in fetal growth rate during the last third of gestation (74). A 50% restriction in ME intake during the last third of gestation induced a 22% reduction in birth size (76). However, when the same restriction is limited to days 30 to 80 of a 148-day gestation, it does not alter birth weight (77). Paradoxically, maternal overnutrition also leads to the

IUGR condition naturally if applied to adolescent ewes (78). In this model, adolescent ewes are fed twice the normal maintenance energy requirement during the entire gestation period. Overnutrition induces maternal growth and obesity, a 45% reduction in placental mass and a 28-38% reduction in the weight of the neonate (78). The mechanistic basis for this effects appears to be a re-direction of nutrients towards maternal growth at the expense of placental and fetal tissues (62). Overnutrition limited to early gestation does not induce IUGR because it does not limit placental growth during the second third of gestation. This suggests that, in adolescent ewes, placental and fetal tissues are most sensitive to overnutrition during the last two thirds of gestation.

Effect on postnatal appetite. A unique advantage of ovine models is the feasibility of measuring voluntary feed intake before weaning (63). Lambs can be separated from the mother at birth and transferred to a controlled-environment facility where they are fed a milk replacer. Using this system, Greenwood et al. (63) evaluated growth, intake, development and metabolism of Normal and IUGR lambs arising from the naturally occurring multifoetal model. Lambs were either fed *ad libitum* or restricted to grow at a rate of 150 g/day. In their experiment, IUGR lambs were 52% smaller at birth (2.3 vs. 4.8 kg) and presented signs of altered appetite during early life. Relative intake was higher in IUGR for around 20 days after birth, with a maximum difference of around 20% reached during the first or second week of life, depending on the plane of nutrition. Interestingly, when relative intake was compared at equal weight, IUGR and Normal lambs did not differ. In other words, IUGR had a higher relative feed intake only until they reached the birth weight of Normal lambs.

De Blasio et al. (79) studied IUGR neonates arising from the carunclectomy model. These lambs suffered a 25% reduction in birth weight (4.15 vs. 5.53 kg). Lambs were reared naturally and no measurements of intake were obtained in these animals.

However, at day 15 of life, the number of suckling events, total suckling time and average suckling time were recorded for 1.5 h after a 1-h fast. None of the parameters differed between IUGR and Normal lambs. Nevertheless, the authors suggested that IUGR exhibited an altered pattern of suckling that correlates with higher growth rates and adiposity.

Effect on postnatal growth and adiposity. Given a lower weight at birth, IUGR lambs typically have lower absolute rates of gain than normal-birthweight lambs (63). On the other hand, IUGR lambs consistently achieve higher fractional weight gain (i.e. gain normalized to body weight) than Normal lambs for the first 4 to 8 weeks of life (61, 63, 80). Interestingly, just as observed with relative feed intake, the fractional weight gain of IUGR lambs does not differ from Normal lambs when compared at the same body weight.

Just as observed in humans, the IUGR condition in sheep is associated with higher total adiposity later in life (61, 63, 80, 81). For example, Greenwood et al. (63) observed higher levels of total body fatness in IUGR than Normal lambs at any body weight up to 20 kg. This was true for lambs offered unlimited amounts of milk replacer or restricted to grow at the fixed rate of 150 g/day. Loey et al. studied female IUGR that were 38% lighter than controls at birth as a result of placental embolization (80). When studied at 2-3 years of age, they had 27% higher total fat mass and 39% higher abdominal adiposity. Finally, when naturally reared, IUGR lambs resulting from carunclectomy had higher relative mass of perirenal (0.8 vs. 0.5% of BW), omental (1.0 vs. 0.8% of BW) and total visceral fat depots (2.3 vs. 1.5% of BW) than normal weight lambs at day 43 of age (61, 81).

Effect on glucose metabolism. As mentioned above the sheep offers the feasibility of repeated blood sampling in both fetus and dam. Data on glucose and insulin in fetal sheep illustrate adaptations induced by the IUGR condition. Placental insufficiency induces a reduction in uterine and umbilical blood flow along with lower glucose and oxygen delivery to the fetus (78, 82). Surprisingly, despite having lower relative glucose uptake, IUGR fetuses have similar weight-specific glucose utilization rates as Normal fetuses, indicating an increase in endogenous glucose production (82). In line with this observation, the expression of phosphoenolpyruvate carboxykinase and glucose 6 phosphatase mRNA is increased in liver of IUGR lambs (83). Under basal conditions or during a glucose clamp, glucose utilization in IUGR fetuses is almost identical to controls, despite a significant ~70% reduction in plasma insulin concentration (82). Using the mid-gestation heat-stress model, Limesand et al. (84) reported that pancreatic islets of the IUGR fetus have lower insulin biosynthesis than control fetuses. They also found that β-cell mass is decreased in severe cases of IUGR as a result of longer cell cycle and lower mitosis rate (85). These data are consistent with major metabolic adaptations in IUGR lambs during fetal life.

Hypoglycemia and hypoinsulinemia are often observed at birth in the different IUGR models (62, 83, 86, 87) and are seen as an extension of the prenatal condition. De Blasio et al. (81) studied glucose metabolism in IUGR lambs arising from the carunclectomy model at 1 month of age. By the hyperinsulinemic-euglycemic clamp, they determined that insulin sensitivity with respect to glucose metabolism was not different between control and IUGR lambs when studied at 1 month of life (81). However, they determined that the ability of insulin to decrease circulating free fatty acids was greater in IUGR lambs, and argued that this adaptation contributed to the increased visceral adiposity of these lambs. On the other hand, Husted et al. (88)

performed glucose tolerance tests at 10 and 19 weeks of age on control and IUGR lambs born to feed-restricted ewes. Glucose tolerance was not altered at any time in IUGR animals but they had higher glucose-stimulated insulin secretion at 19 weeks of age. It is important to mention that in both studies, IUGR lambs were at least 10% smaller than Normal lambs when these studies were performed.

Effect on metabolic hormones. It is known that the growth hormone/insulin-like growth factor-1 (GH/IGF-1 system) partially mediates the effect of nutrition on growth in the young ruminant (89). This important growth system is also sensitive to nutrition during prenatal life. Circulating GH concentration is elevated in IUGR fetuses of ewes restricted during the last third of pregnancy or subjected to a 48 h starvation period between day 120-130 of gestation (90, 91). In response to a low maternal plane of nutrition, IUGR fetuses present low circulating IGF-1 during the last third of gestation (92). During this period, plasma IGF-1 is positively associated with fetal weight, fetal liver weight, blood pO2 and circulating glucose. In fact, although fetal IGF-1 plasma concentration is relatively low during gestation, it can rapidly respond to starvation and fluctuations in circulating glucose during the last 10 days of gestation (93). Along the same line, Rhoads et al. (94) reported that hepatic expression of IGF-1 and the acid-labile subunit (ALS) is reduced in IUGR fetuses as early as day 130 day of gestation.

After birth, IUGR lambs continue to have a higher plasma concentrations of GH than Normal lambs (49.1 vs. 10.8 µg/l) for the first two weeks of life (95). Circulating IGF-1 remained significantly lower at birth (36 vs. 158 µg/l) but becomes responsive to postnatal plane of nutrition during the first week of life, independently of birth weight (95). IGF-1 concentration rose and reached a maximum before 13 days of life in IUGR and normal lambs fed *ad libitum* but concentrations remained lower in IUGR animals (95).

When neonatal lambs were feed restricted to gain only 150 g/d, however, plasma IGF-1 concentration was consistently low in both, IUGR than Normal lambs (95).

IUGR lambs are in a hypothyroid state at birth (96), similar to their human counterparts (97, 98). Cabello and Levieux (96) analyzed lambs ranging from 1.22 to 3.55 kg born to prolific Romanov ewes and found positive correlations (>0.55) between birth weight and T3 and T4 status during the immediate postnatal life. Furthermore, Cabello (99) found that IUGR neonatal lambs had a reduced ability to increase T4 production during cold challenges, resulting in hypothermia and high mortality rates. Later Dwyer and Morgan (100) confirmed that the low T3 and T4 status of IUGR lambs is not only associated with poor thermogenic capacity, but also with poor behavioral progress to stand and suckle. However, thyroid status of naturally reared IUGR and normal lambs is not related to their fractional growth rate (79).

Using the multifoetal ovine model, Ehrhardt et al. (101) demonstrated that plasma leptin concentration in early life is not affected by birth weight and is better associated with lipid accretion rates than adiposity itself. On the other hand, De Blasio et al. (102) found that leptin plasma concentration of IUGR lambs from carunclectomized ewes was higher than control lambs on day 5 of postnatal life but not different afterwards. Additionally, they suggested a negative relationship between leptin concentration and feeding activity (suckling events) on postnatal day 15 in Normal lambs and that the IUGR condition reversed this relationship. They reached this conclusion even though suckling events did not differ between IUGR and Normal lambs (102).

Central control of energy homeostasis.

Early evidence for involvement of the hypothalamus. The role of hypothalamic centers in the control of energy homeostasis was first suggested by studies conducted during the 1940's in which electrolytic lesions of specific regions of the rat hypothalamus induced hyperphagia and fatness (103). Years later, a parabiosis experiment between rats with hypothalamic lesions and intact animals offered the first evidence of peripheral cues indicative of energy reserves acting on hypothalamic feeding center (104): hyperphagia and marked obesity occurred in the lesioned rat whereas the intact animal ate less and lost weight. This suggested the presence of a circulating satiety factor emanating from the obese rat and acting in the intact rat to reduce intake and body weight. During the 1950-1970 period, the identification of the *ob* and *db* mouse strains as well as parabiosis experiments with these mutant mice offered further evidence of a circulating factor acting through its cognate receptor to control appetite and metabolism (105, 106). This model was finally confirmed in the 1990's by the discovery that the *ob* gene encodes the protein leptin and that the *db* gene encodes its receptor (107, 108). These studies also showed that leptin is expressed in proportion to fat mass and regulates appetite and energy expenditure by acting predominantly on the central nervous system (107). The hypothalamus receives and integrates many other peripheral cues produced by adipose tissue and the gastrointestinal tract. This integration allows for the coordinated control of energy and glucose homeostasis in the organism (109).

Hypothalamic organization. The hypothalamus consists of nuclei or groups of neurons sharing common functions (110). A key hypothalamic nucleus is the arcuate nucleus (ARC). It is located near the medium eminence where the blood-brain barrier is more permeable (111). Most of its residing neurons express receptors for metabolites

and hormones (112). Because of these properties, the ARC is believed to be an ideal center to read peripheral cues indicative of energy balance and adiposity such as leptin.

The ARC contains 2 neuron-populations that are important for regulation of energy intake and energy expenditure (110). The first population consists of NPY/AGRP neurons, based on their co-expression of two neuropeptides called neuropeptide Y (NPY) and agouti-related peptide (AGRP). The NPY/AGRP neurons are considered orexigenic because intracerebroventricular (ICV) injection of these two neuropeptides induces significant increases in food intake and weight gain (113, 114). The second group is known as POMC/CART neurons based on co-expression of pro-opiomelanocortin protein (POMC) and cocaine and amphetamine-related transcript (CART). The POMC/CART neurons have anorexigenic properties because ICV injection of CART or α-melanocortin stimulating hormone (α-MSH), the proteolytic product of POMC, inhibit voluntary intake (115, 116).

Genetically modified mouse models have provided further evidence about the reciprocal role of the NPY/AGRP and CART/POMC neurons. Hypothalamic overexpression of NPY or AGRP in mice causes hyperphagia and obesity (117, 118). On the other hand, acute ablation of AGRP/NPY neurons in adult mice cause severe anorexia but has no effect if done in neonatal animals, perhaps due to developmental compensation by other pathways (119). Interestingly, NPY null mice did not show alterations in appetite or body weight (120). Null AGRP mice have a normal intake and energy expenditure when young but develop lower body weight and elevated metabolic rate by adulthood (121). Null POMC mice, on the other hand, show higher voluntary intake, higher fat accretion, lower circulating T4 and depressed metabolic rate (122).

ARC neurons are considered first-order or sensory neurons because they have an abundance of receptor for leptin, insulin and other peripheral cues (112). In general, activation of these receptors has reciprocal effects on AGRP/NPY and CART/POMC neurons. For example, leptin inhibits production and release of AGRP and NPY by orexigenic neurons whereas it increases production of POMC and CART by the anorexigenic neurons (123, 124). Thus, when energy reserves are high, plasma leptin is elevated leading to increased POMC/CART and reduced AGRP and NPY production, inducing a reduction in voluntary feed (123, 124).

However, ARC neurons are not directly responsible for functional outputs such as changes in feed intake and energy expenditure (125). These functions are carried out by neurons located in other hypothalamic nuclei and brain centers. These 'second-order' nuclei include the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial nucleus (DMN), and perifornical and lateral hypothalamic areas (LHA) (126). ARC neurons are linked to these second order neurons via an extensive network of axonal projections. They themselves project to other central nervous structures such as the dorsal vagal complex (DVC), which includes the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV).

The central leptin-melanocortin system

Leptin is obviously not the only peripheral cue read by hypothalamic neurons (110). Similarly, the melanocortins (MC) AGRP and POMC are not the only important neuropeptides regulating energy homeostasis (127). Nevertheless, the following section will focus on leptin and the role of the central MC system in mediating its effect. This will

be done because it is by far the better understood system involved in the energy homeostatic function of the central nervous system.

Both, insulin and leptin circulate in proportion to body fat content and are sensed by receptors in the hypothalamus (110). They are regarded as playing important roles in short- and long-term regulation of energy homeostasis. In fact, ICV infusion of either hormone reduces voluntary intake in non-human primates and mice (128, 129). Although both, insulin and leptin circulate in proportion to adiposity, only a leptin deficiency causes hyperphagia and obesity, indicating the predominant importance of this hormone in the control of energy intake and expenditure (110). Leptin actions are mediated via the functional long form of the leptin receptor (Ob-Rb) located on both, NPY/AGRP and POMC/CART neurons (130).

As reviewed above, leptin increases POMC expression and production of its proteolytic product α-MSH and has the opposite effect on AGRP production. α-MSH and AGRP are known as melanocortins. There are 5 known melanocortin receptors, but only melanocortin receptor 3 (MC3R) and 4 (MC4R) are found in the brain and prominently expressed in many second order neurons (131). In terms of their actions on MC3R and MC4R, α-MSH and AGRP are functional opposites with α-MSH acting as an agonist and AGRP as an antagonist (132). As a result, activity of central MC receptors is regulated by the ratio of α-MSH and AGRP (α-MSH/AGRP ratio) produced in the ARC.

The importance of MC action via central melanocortin receptors is illustrated by the hyperphagia, hyperinsulinemia, hyperglycemia and obesity seen after total ablation of MC4R in the hypothalamus (133).

The PVN and LHA are second order nuclei abundantly supplied with axonal projections from both, NPY/AGRP and POMC/CART neurons. This has been shown in

both, rodents and non-rodent species, like sheep (134, 135). Injections of AGRP directly into the PVN induce a significant increase in voluntary intake (136). Also, re-expression of the MC4R only in the PVN was sufficient to correct the hyperphagia of MC4R knockout mice, but did not normalized their reduced energy expenditure (137). Together, these experiments indicate that the MC α-MSH acts in the PVN to modulate feed intake but its effect on metabolic rate likely involves another nuclei. Importantly, neurons within the PVN express corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), oxytocin and vasopressin, integrating energy homeostasis with systems regulating glucocorticoid production, metabolic activity and fluid balance (126). The LHA is another important second-order nucleus that also receives significant efferent axonal projections from the ARC. This nucleus contains groups of neurons that express the additional orexigenic neuropeptides, orexins and melanin concentrating hormone (MCH) (126).

Central melanocortin and regulation of energy expenditure. In addition to effects on energy intake, the hypothalamus regulates energy expenditure via endocrine and autonomic pathways (138). MC are partly responsible for the regulation of synthesis and secretion of TRH in neurons located in the PVN and, therefore, for the activity of the hypothalamic-pituitary-thyroid (HPT) axis (139). Thyroid hormones are central modulators of both basal and induced thermogenesis. In fact, both basal and cold-induced thermogenesis can be severely reduced in the absence of thyroid hormones (140). The thermogenic effect of thyroid hormones involves uncoupling proteins not only in brown adipose tissue (BAT) (141) but also in muscle (142), where substrate flux to the TCA cycle is increased without increases in ATP production (143). When circulating leptin is low during fasting, the levels of thyroid hormones are reduced (144–146). In contrast the systemic or central administration of leptin during fasting is

sufficient to normalize TRH expression in the PVN (139). Legradi et al. (147) found that pharmacological ablation of the ARC eliminates not only the drop in TRH and TSH during fasting, but also the stimulatory effects of exogenous leptin treatment. TRH neurons in the PVN have been shown to express MC3R and MC4R (148). ICV administration of α-MSH in fasted animals restored the expression of TRH in the PVN while infusions of AGRP induced a state of hypothyroidism in *ad libitum* fed rats, similar to that observed in fasting animals (139). These experiments support an important role for the melanocortin system in mediating effects of leptin and ARC neurons on TRH synthesis and therefore, thyroid hormone-dependent energy expenditure.

The central melanocortin axis also regulates metabolic rate by stimulating the autonomic nervous system. Experiments with rats conducted by Haynes et al. (149) demonstrated that leptin is capable of increasing significantly the activity of sympathetic nerves serving all tissues and organs (e.g. BAT, muscle, kidney and adrenal glands). In order to determine if leptin effects on sympathetic activity were mediated by melanocortin receptors, Haynes et al. (149) used both, MC4R agonist and antagonist to evaluate regulation of sympathetic activity in brown adipose tissue. They discovered that ICV infusion of a melanocortin receptor agonist induced a dose-dependent increase in sympatho-excitation of brown adipose tissue, kidney and hind limb (149). Moreover, Brito et al. (150) demonstrated that central melanocortin administration induced lipolysis and increased temperature not only in BAT but also in some white fat depots. In support for a role of central MC in the control of energy expenditure, MC4R knockouts have lower energy expenditure, lower oxygen consumption as well as an elevated respiratory exchange ratio indicative of reduced lipid oxidation (151).

There are few data in sheep regarding regulation of energy expenditure by the leptin-central MC system. A significant finding in this context was the observation that

central leptin administration increases thermogenesis in sheep (152). This increase was associated with increased expression of UCP2 and UCP3 genes in muscle as well with increased uncoupled respiration in mitochondria isolated from muscle (152).

Central melanocortin and modulation of metabolism. In addition to the overall regulation of energy balance by modulating intake and metabolic rate, hypothalamic centers also contribute to glucose and lipid partitioning and utilization in peripheral tissues. For example, chronic peripheral leptin therapy of *ob*/*ob* mice for five days reduces circulating insulin and glucose concentrations before obesity and hyperphagia are resolved (153). Furthermore, these reductions are larger than those observed in pair-fed *ob*/*ob* controls, indicating that leptin has intake independent effects on glucose metabolism. Later, Rossetti et al. (154) used hyperinsulinemic clamps to demonstrate that leptin enhances the ability of insulin to reduce hepatic glucose output. They also showed that leptin actions included intra-hepatic suppression of glycogenolysis and a stimulation of gluconeogenesis. In support of the previous experiments, ICV or peripheral leptin infusions alone reduced hyperglycemia induced by chemical or autoimmune β-cell destruction (155, 156).

The actions of leptin in the central control of glucose metabolism has been found to take place within the ARC (157). Effects of leptin on hepatic glucose metabolism are seen even if leptin is administered only in the brain (158). Despite the modest effects on intake and body weight, molecular restoration of leptin receptor expression exclusively in the ARC of *db*/*db* mice greatly improves hyperglycemia and hyperinsulinemia in these animals (157). This indicates that a lack of leptin signaling in the ARC of *db*/*db* mouse plays an important, adiposity independent, role in glucose metabolism. Furthermore, rescue of leptin receptors only in POMC/CART neurons solved the hyperglycemia and ameliorated hepatic insulin resistance, hyperglucagonemia, and dyslipidemia in leptin

receptor deficient mice (159). Acute peripheral leptin infusion also induces a reduction in lipogenesis in liver and white adipose tissue in mice (160). ICV leptin infusion in rats represses the expression of key lipogenic genes such as acetyl-coenzyme A-carboxylase, fatty acid synthase and stearoyl-coenzyme A desaturase-1 (161).

A significant portion of leptin effects on glucose and lipid metabolism is mediated by the MC receptor. Fan et al. (162) demonstrated that a melancortin agonist inhibited basal insulin release by *ob*/*ob* mice in a dose dependent manner via the sympathetic nervous system. Interestingly, glucose tolerance was also impaired during melanocortin treatment. In order to dissect the effect of obesity and melanocortin receptor stimulation on glucose metabolism, the same group evaluated concentrations of circulating insulin as well as glucose tolerance in MC4R null mice. At 4 weeks of age hyperglycemia and glucose intolerance were evident even before these mice became fatter than wild-type mice (162).

Nogueiras et al. (163) explored the role of hypothalamic melanocortin signaling on lipid metabolism. They demonstrated that pharmacological inhibition of MC4R induced greater body weight, feed efficiency, greater fat accretion and lower fat oxidation than pair-fed controls. In line with this phenotype, expression of genes linked to lipid uptake and lipogenesis was increased in white adipose tissue of these animals. Reciprocally, they also showed that melancortin receptor activation reduced weight, feed efficiency and fat accretion to a greater extent than pair fed controls. This was associated with higher adipose tissue expression of lipolytic genes such as hormone-sensitive lipase and adipose-triglyceride lipase. Their experiment also demonstrated the relevance of the sympathetic nervous system in mediating these effects. During ICV treatment with a melanocortin agonist, sympathetic nervous activity in white adipose tissue increased in a dose dependent manner. This increase was prevented by co-

infusion of melanocortin agonist and antagonist. Although these results only showed the direct autonomic connection between MC4R-neurons and white adipose tissue, an experiment in mice lacking all β-adrenergic receptors offered further insight: chronic ICV infusion of a melanocortin receptor antagonist did not increase body weight and failed to induce FAS expression (163). These results confirmed the importance of β-adrenergic signaling in mediating the effects of central melanocortin on lipid metabolism. Overall, this work demonstrates that the central nervous system modulates lipogenesis and lipid accretion in white adipose tissue via the sympathetic nervous system.

The role of perinatal nutrition and leptin in regulating hypothalamic development

As mentioned previously in this review, different hypothalamic nuclei are involved in the regulation of appetite (110). The neurons populating these nuclei are interconnected via axonal and dendritic projections. This network allows reception and integration of signals by the hypothalamus so that appropriate responses are mounted. Remarkably this network of axon connections develops at different stages of life in altricial and precocial animals (e.g. rats and mice vs. humans, non-human primates and sheep). For example, ARC, DMH, VMH and LHA neurons can be detected in mice embryos as early as day 11 of fetal life (164). However, ARC axonal projections to second-order neurons located in the PVN, VMH and LHA are virtually absent at birth and are not fully developed until the end of the third week of age (165). Because of this, leptin regulation of energy homeostasis is not present in early postnatal rodents. For example, leptin treatment for 7 days did not induce changes in body weight in 10-day old mouse pups (165), whereas the same treatment is highly effective in adult mice. In

humans, non-human primates and sheep, ARC neurons can be identified during the 2nd trimester of fetal life (166–168). In stark contrast with rodents most axonal development between the ARC and the other hypothalamic nuclei occurs during the last third of gestation in these animals (167, 169).

Several research groups have described a 5 to 10-fold increase in plasma leptin during the second week of life in rodents (170, 171). Ahima et al. (170) observed that this increase in circulating leptin was not associated with adiposity, could not be prevented by feed restriction and did not induce a reduction in body weight. Other groups demonstrated that exogenous leptin was able to induce changes in POMC and NPY expression in the ARC of pre-weaned rodents but did not alter food intake or body weight (165, 172). In pre-weaned rodents, leptin-dependent activation of second-order neurons was proportional to the density of efferent axonal projections received from the ARC (165). Based on the timing of activation of hormonal systems regulated by the hypothalamus, Ahima et al. (170) suggested that leptin could have a neurotrophic role that allowed for adequate development of hypothalamic-pituitary-adrenal, gonadal and thyroid axis. In agreement with this hypothesis, Bouret et al. (173) later demonstrated that leptin was essential for the proper development of the axonal network between the ARC and secondary hypothalamic nuclei involved in energy homeostasis. They demonstrated that chronic leptin treatment from day 4 to 12 of postnatal life could rescue the poor density of such axonal projections in leptin deficient (*ob/ob*) mice. Moreover, intake was somewhat normalized in these *ob/ob* mice on postnatal day 32 in absence of concurrent leptin administration. Remarkably, leptin therapy was ineffective in restoring this neural network when it was administered to adult *ob/ob* mice. This suggested that the developmental role of leptin is largely restricted to the early neonatal period, which coincides with a natural surge of circulating leptin (170).

As mentioned earlier in this review, the IUGR condition induces a hyperphagic and obese phenotype in postnatal rodents, particularly when they are offered high fat diets during the post-weaning period (56, 174). Delahaye et al. (175) showed that the IUGR condition induced by maternal undernutrition ablates the normally occurring surge of leptin in neonatal rats. Also, Vickers et al. (174) tested whether exogenous leptin treatment during early life (P3 to P13) could correct the abnormal energy metabolism of IUGR rat pups. This neonatal therapy completely normalized adult body weight, body weight gain, caloric intake, locomotor activity, fat mass, as well as fasting plasma insulin, glucose and leptin in IUGR rats. Overall, these findings lead to a model whereby the IUGR condition leads to a neonatal leptin deficiency, followed by inadequate axonal development in the hypothalamus. Failure to complete this developmental event would then account for inappropriate energy metabolism later. The applicability of such a model to precocial species that complete hypothalamic axonal development in late fetal life is unknown. There is no evidence relative to the potential neurotrophic effect that leptin might have in the proper development of the leptin-MC system in precocial species or the time window when this could take place. Also, it is unknown if the hyperphagic and fat phenotype observed in IUGR lambs during early life is the result of a defective central leptin-MC system as it is the case in rodents.

Summary

Epidemiological evidence indicate that the IUGR condition is associated with higher risk for obesity and metabolic disorders (28–30). These observations are supported by clinical experiments with rodents in which the IUGR condition can induce hyperphagia and excess fatness, especially if these animals experience over nutrition or are fed high-caloric diets (42, 44). This adverse phenotype is associated with poor development of hypothalamic networks involved in the control of energy intake and

expenditure. It has been discovered that hypothalamic development of energy homeostasis is defective in these animals due to abnormal leptin profile during the first 2 postnatal weeks. Leptin has neurotrophic capacities exclusive to this period and is essential for the development of proper connectivity between hypothalamic centers. However, ontogenesis is different in altricial rodents compared to precocial humans, non-human primates and sheep. This leaves unanswered questions. 1) Does higher energy diet exacerbate the negative effect of the IUGR condition when offered immediately after birth? 2) Is the central melanocortin system functional immediately after birth? 3) What is the role of leptin in the regulation of energy metabolism in the immediate postnatal period? and, 4) Does a lack of leptin signaling in early postnatal life have long-term consequences?

Our multifoetal-model of IUGR and our artificial rearing system offers the opportunity to test the capacity of precocial animals to regulate intake of diets with different caloric density during early life. Furthermore, we studied the impact that these diets have on body composition in interaction with the IUGR condition. Also, the functionality of the melanocortin receptor has never been tested during early life in precocial species. We were able to do this using a melanocortin agonist capable of crossing the blood-brain barrier. Finally, to explore the role of leptin during early life in the short- and long-term control of energy homeostasis and body composition, we used a long-lasting leptin antagonist during the first weeks of life in sheep. This novel series of experiments will offer important insight in the mechanisms regulating energy intake and expenditure in a relevant precocial animal model.

References chapter 2

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CHAPTER 3: EFFECT OF BIRTH SIZE AND DIETARY FAT ON INTAKE, GROWTH AND BODY COMPOSITION OF NEONATAL LAMBS

Introduction

A chronic positive imbalance between energy consumption and energy expenditure results in excessive adiposity. Several factors like genetics, greater availability of caloric-dense foods, reduced physical activity and social environment have been implicated as promoters of this imbalance (1–3). Excessive adiposity has developed into a medical and economic problem over the last decades. Obesity is not only associated with an increased susceptibility to non-communicable diseases like dyslipidemia, hypertension, type 2 diabetes and hepatic damage but also with higher mortality rates (4, 5). Based on the body mass index (BMI), over 65% of the population in the US is either overweight (BMI between 25 and 30 kg/m^2) or obese (BMI>30 kg/m^2) (5). The situation is not better worldwide: an extensive analysis of BMI indicates that it has increased globally since 1980 at the rate of 0.4 and 0.5 kg/m^2 per decade in men and women, respectively (6).

Obesity is also becoming an alarming condition of childhood and adolescence. Over 170 million children and adolescents are estimated to be overweight worldwide (7). In the US alone, obesity affects 12.5 million children and adolescents (8). There is growing evidence in recent years that various fetal insults, including both under- and over-nutrition, contribute to the increased adult obesity and metabolic disorders of this cohort (9–12).

Various rodent models have been used to explore how fetal insults lead to greater adiposity in adult life (13–15). A common feature of many of these models is the

imposition of caloric or protein restriction to the dam, leading to fetal undernutrition and the birth of pups suffering from intrauterine growth retardation (IUGR). Remarkably, these IUGR pups have an increased predisposition to develop obesity after weaning, particularly when offered a high fat (HF) diet (13–15).

Rodent models, however, have two limitations. First, it is technically difficult to manipulate the diet before weaning. This relates to the absolute dependence of the newborn rodent on maternal milk for the first 2-3 weeks of life and the difficulty to alter milk composition. Accordingly, the consequences of varying diet composition in early life on the abnormal energy metabolism of IUGR animals have not been well studied. Second, the central system responsible for appetite regulation is less developed in rodents than in human at birth (16–20). Accordingly, it is unclear whether metabolic defects identified in pre-weaned IUGR rodents apply to their human counterpart. The newborn sheep is an excellent model with respect to these two limitations. Neonatal lambs can be fed artificially from birth and milk replacer composition can be easily manipulated (21). Moreover, hypothalamic development at birth is comparable in sheep and primates (18, 19). Greenwood and colleagues (21–24) described an IUGR sheep model characterized by increased appetite and reduced energy expenditure in early postnatal life. Accordingly, our objective was to assess whether these and other features of the IUGR condition were exacerbated in this sheep model by increasing the energy density of the diet in the immediate postnatal period.

Materials and Methods

Animals and study design. All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Lambs were males from Finn x Dorset genotype. They were born from ewes selected for aseasonal breeding and prolificacy at the Cornell Teaching and Research Farm.

Forty-six lambs were classified at birth on the basis of their body weight as IUGR (<3.0 kg, n = 23) or Normal weight (>4.0 kg, n = 23). The actual birth weight of IUGR and Normal lambs were 2.6 kg and 4.1 kg (P<0.001). IUGR lambs also had shorter crown-rump length (CRL) than Normal lambs at birth (44.5 vs. 50 cm; P<0.001). Lambs were towel-dried, weighed and bottle-fed artificial colostrum (LandO'Lakes®) reconstituted in warm water at the rate of 60 g/kg body weight (BW). Animals were transported immediately to the Cornell University Large Animal Research and Teaching Unit and housed in individual cages (75 cm width x 80 cm length x 80 cm height) at constant temperature (25° to 27 °C) and photoperiod (light on between 0700 and 1900 h).

Four Normal and four IUGR lambs were killed within 4 hours of birth and served as reference groups for body composition at birth. Remaining IUGR and Normal lambs were randomly assigned to receive unlimited amounts of a Low Fat (LF; 22% of dry matter (DM)) or a High Fat (HF; 38% of DM) milk replacer (Milk Specialties Global, Carpentersville, IL Table 3.1). Two lambs receiving the LF diet were removed from the experiment, one IUGR lamb that developed an intestinal infection and one Normal lamb that developed a lung infection. A first group of Normal (n = 15) and IUGR (n = 15) lambs was killed at 14 days of age. An additional group of 3 IUGR-LF and 3 IUGR-HF lambs was killed at the live weight (LW) reached by Normal lambs at 14 days of life (8.5 kg).

Table 3.1 Nutrient composition of milk replacers.

	Milk replacer[a]	
Nutrient	LF	HF
	--- per kg dry matter ---	
Gross energy, Mcal	5.54	6.54
Crude protein, g	301	276
Fat, g	218	380
Nitrogen free extract, g	411	287
Ash, g	70	56
Vitamin A, KIU	50.67	50.71
Vitamin D3, KIU	12.21	12.21
Vitamin E, IU	124	124

[a]Low fat (LF) and high fat (HF) milk replacers.

Procedures during the experiment were as follows: both replacers were reconstituted with water at 18.8% DM and served at 0900 h. They were replenished if needed at 1600 and 2000 h. Offered volume was incrementally adjusted on a daily basis. CRL was measured at birth and on postnatal day 5, 10 and 14. BW was recorded daily at 1600 h. Blood samples were obtained by jugular venipuncture within 2 h of birth and at 1600 h on postnatal days 1, 3, 5, 7, 9, 11, 13 and 14. Blood samples were collected in 6-ml tubes containing 90 USP lithium heparin. Plasma was prepared by centrifugation and stored at -20°C until analyzed for glucose and hormones.

Tissue collection and body composition. Lambs were killed by lethal jugular injection of sodium pentobarbital (88 mg/kg LW; Virbac AH, Inc. TX). The head was severed immediately between the cranium and the first cervical vertebrae and processed for isolation of the hypothalamus (25). Blood was collected and weighed. The gastrointestinal (GI) tract was dissected and weighed before and after contents were removed. Omental, perirenal, retroperitoneal and pericardial fat depots were dissected and weighed separately. The viscera fraction was obtained by combining GI tract, visceral fat depots, visceral organs (respiratory tract, heart, liver and spleen) and blood whereas the carcass fraction consisted of the carcass (without the head), skin and hoofs.

Each fraction was ground separately by extrusion through grinding plates (Viscera: 2x through a 13-mm plate and 3x through a 4-mm plate; Carcass: 1x through a 50-mm kidney plate, 3x through a 13-mm plate and 3x through a 4-mm plate). A 250 g subsample of each fraction was freeze-dried for determination of dry matter. The freeze-dried samples were then pulverized with dry ice using a Waring blender and stored at -20°C until analyzed for nitrogen, ether extract and ash content. Nitrogen content was measured by macro-Kjeldahl digestion (26) with steam distillation into boric acid using a

Kjeltec 2300 (FOSS Analytical AB, Sweden). Crude protein (CP) of body fractions was calculated as nitrogen (N) x 6.25. Fat content of body fractions was determined by petroleum-ether extraction (27). Ash content was determined by incinerating samples in a furnace at 506 °C for 16 hours. All samples were analyzed in duplicate. Milk replacers were analyzed similarly except that a factor of 6.38 was applied to N content to calculate CP due to its amino-acid profile (28). Fat content of milk replacer was measured by an hexane/isopropanol extraction (29).

Glucose and hormones analyses. Plasma glucose was measured by the glucose oxidase method (510A, Sigma Chemical, St. Louis, MO) (23). Total plasma thyroxine (T4) was measured using a commercial solid-phase radioimmunoassay (RIA) (Coat-a-Count, Siemens, Germany) previously validated in the sheep (30). Internal RIA previously validated with ovine plasma were used to measure the plasma concentration of insulin, leptin and IGF-1 (23, 31). These RIA use bovine proteins for iodination and standards. The insulin RIA was performed with a guinea pig anti-bovine insulin primary antibody (Sigma I-6136) and a goat anti-guinea pig secondary antibody (Equitech-Bio Inc., Kerrville, TX) (31). The leptin RIA was described previously (31). For IGF-1, IGF-binding proteins were first removed by ethanol-acetic acid-acetone extraction. The IGF-1 concentration of supernatant was then analyzed using an assay based on a rabbit anti-human IGF-1 primary antibody (lot AFP4892898; National Hormone and Peptide Program) and a caprine anti-rabbit γ-antibody (lot 12515, Biotech Source Inc.) (23). Inter-assay and Intra-assay coefficients of variation were less than 7% for all assays.

Calculations. Feed intake was calculated by subtracting unconsumed milk replacer from the previous feed allowance. Average daily gain (ADG) was calculated for each individual as the slope of the regression of body weight over time. Relative intake and fractional weight gain (FWG) were calculated by dividing daily intake or BW gain by

the BW of the previous day, respectively. Metabolic body weight (MBW) was calculated by scaling live body weight to the 0.58 power. According to indirect calorimetry work conducted by Graham et al. (32), this exponent relates BW more closely to fasting metabolic rate in neonatal lambs than the commonly used 0.75. In this experiment, empty body weight (EBW) is the sum of wet viscera and beheaded carcass weights. Indices of fatness (mass of fat depots, visceral fat or carcass fat) were expressed as a % of EBW. Composition of the EBW was calculated from the composition of viscera and carcass fraction.

Statistical analyses. Averages were calculated over the 14-day period for intake and growth data (e.g. intake, FWG, etc.). Averages and cumulative end-point data (e.g. BW gain, ADG and body composition data) were analyzed by a model accounting for Body size (Normal vs. IUGR), Diet (LF vs. HF) and their interaction using the ANOVA procedure.

A MIXED procedure of SAS was used to analyze repeated measures data (feed intake, body weight, metabolic data). Our model accounted for BS, Diet, Day and their interactions as fixed effects and lamb as the random effect. In presence of Diet x Day interactions, linear and quadratic regressions were performed within Diet category. Statistical significance was set at $P<0.05$ for main effects and $P<0.10$ for interactions. Statistical tendency was set at $P<0.10$ for main effects and $P<0.15$ for interactions.

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Results

Intake. DM and energy intake data were averaged over the 14-day experimental period and reported in table 3.2. IUGR lambs had lower voluntary DM intake than Normal lambs (Diet, $P<0.001$) but the opposite effect was observed when intake was expressed relative to LW (Diet, $P<0.001$). Dietary fat content had no effect on either variable.

BS had similar effects on energy intake: IUGR lambs consumed less energy than Normal lambs when analyzed in absolute terms but more when energy intake was normalized to LW (Diet, $P<0.001$ for both variables). Effects of BS on energy intake were eliminated by correcting for MBW. Dietary fat content increased absolute energy intake as well as energy intake corrected for LW or MBW ($P<0.01$ or less for all). BS x Diet interactions were not detected for any dry matter or energy intake values.

Finally, we asked whether BS and Diet effects varied over time by analyzing intake responses as repeated measures over the period of treatment. This analysis revealed the presence of the same Diet x Day interaction for all intake responses ($P<0.15$ or less). The nature of this interaction is illustrated by the profiles of absolute DM intake (Fig. 3.1) and energy intake corrected for MBW (Fig. 3.2). In brief, dry matter intake and energy intake corrected for MBW increased linearly for the LF diet (linear time effect, $P<0.001$) whereas they plateau by day 7 to 9 for the HF diet (quadratic time effect, $P<0.001$).

Growth. Growth data averaged over the 14 d treatment period are given in Table 3 and the cumulative growth curves are shown in Fig. 3.3. IUGR and Normal lambs grew at steady rates throughout the entire treatment period (Fig. 3.3). IUGR lambs had a 20% lower ADG than Normal lambs (Table 3.3; BS, $P<0.001$), resulting

Table 3.2 Effect of birth size and dietary fat content on dry matter and energy intake between birth and slaughter on day 14 of postnatal life.

	Normal[b]		IUGR[b]			Probability level[c]		
Variable[a]	LF	HF	LF	HF	SD	BS	Diet	BS x Diet
Dry matter intake								
Absolute, g/d	276	280	228	209	31	<0.001	NS	NS
Relative to LW, g/kg • d	42	43	50	50	4	<0.001	NS	NS
Energy intake								
Absolute, kcal/d	1,529.8	1,831.6	1,264.4	1,369.7	186.7	<0.001	<0.01	NS
Relative to LW, kcal/kg • d	232.0	283.6	278.8	328.0	26.3	<0.001	<0.001	NS
Relative to MBW, kcal/$kg^{0.58}$•d	504.1	614.5	519.1	585.9	56.6	NS	<0.001	NS

[a]LW, live weight; MBW, metabolic body weight ($kg^{0.58}$).
[b]Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment).
[c]Type I error probability for birth size (BS), diet (Diet) and their interaction (BS x Diet). NS = $P>0.05$ for main effects and $P>0.15$ for the interaction.

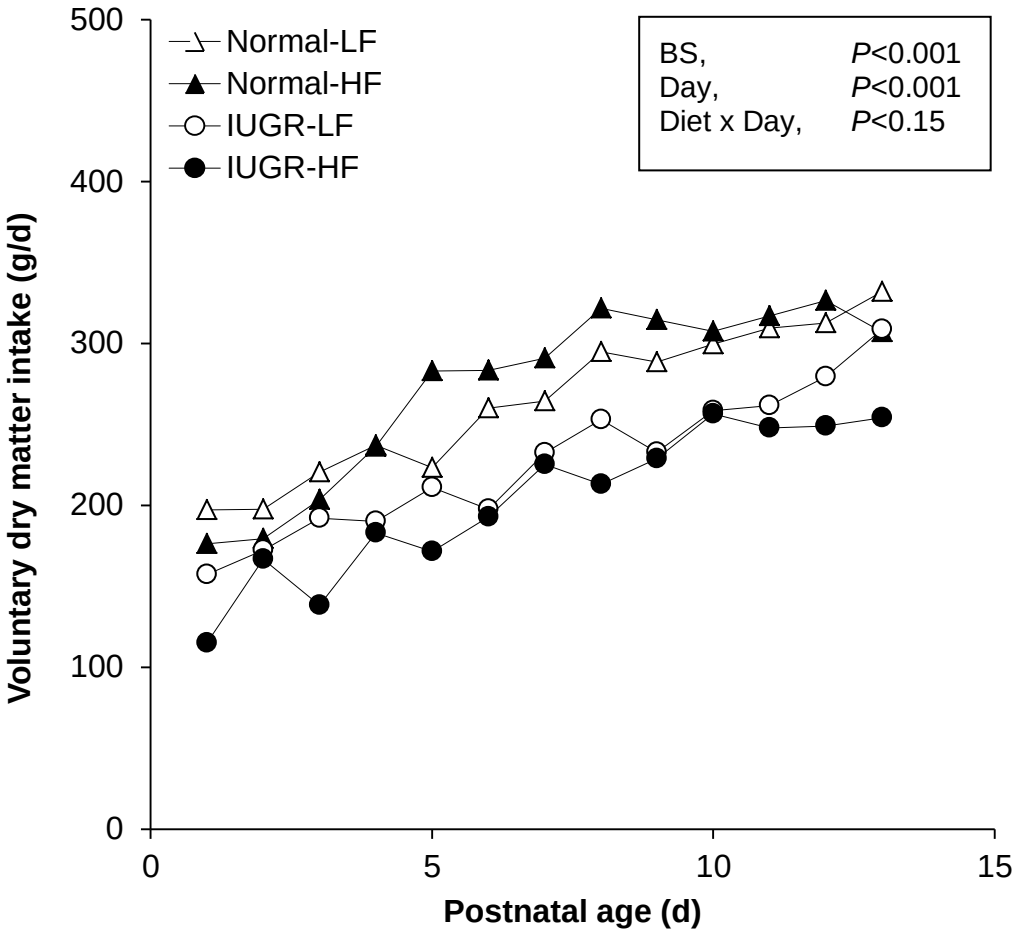

Figure 3.1 Effect of birth size and dietary fat content on voluntary dry matter intake between birth and slaughter on day 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded (IUGR) lambs were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Voluntary dry matter intake was recorded daily. The pooled SE was 16.4 for n = 8 and 18.5 for n = 7. Significant effects of birth size (BS), and day (Day) and Diet x Day are reported. All other effects were non-significant.

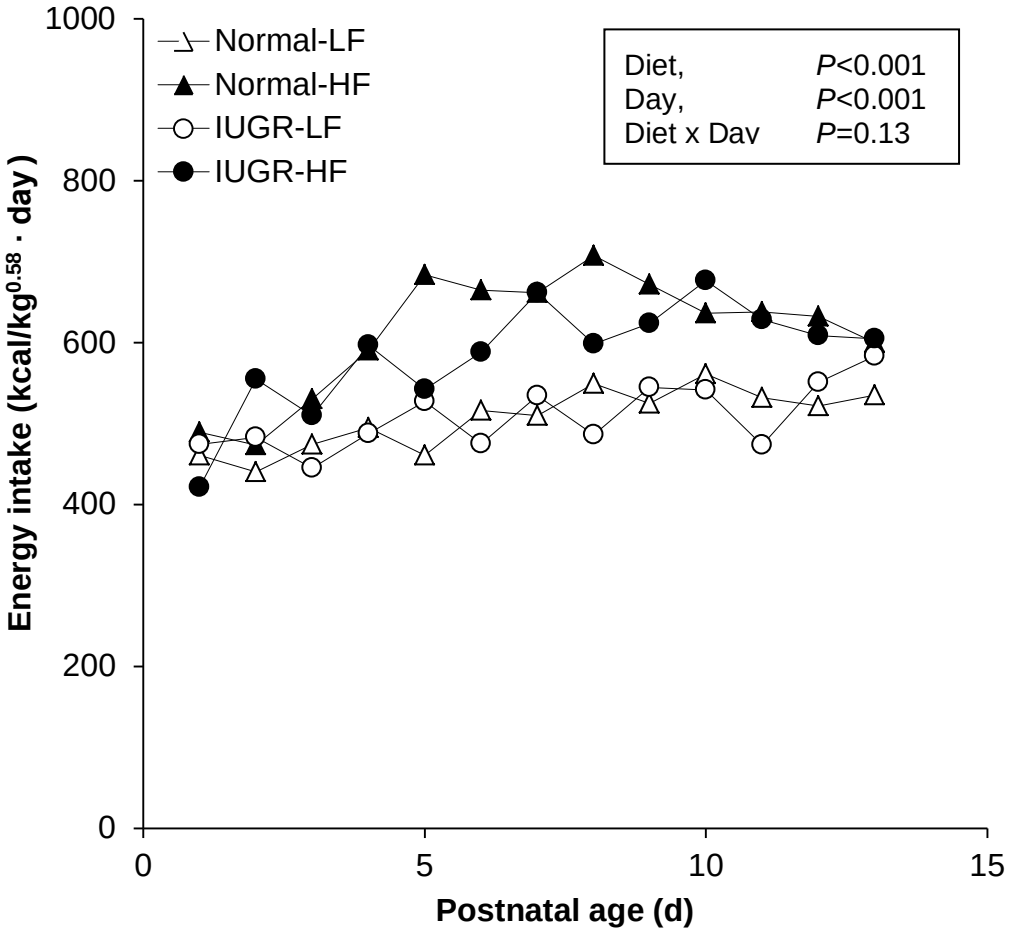

Figure 3.2 Effect of birth size and dietary fat content on caloric intake adjusted for metabolic body weight between birth and slaughter on day 14 of postnatal.

Normal size (Normal) or intrauterine growth-retarded (IUGR) lambs were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Caloric intake was measured daily and expressed as ratio of metabolic body weight ($kg^{0.58}$). The pooled SE was 38.9 for n = 8 and 41.5 for n = 7. Significant effects of dietary treatment (Diet), day (Day) and Diet x Day interaction are reported. All other effects were non-significant.

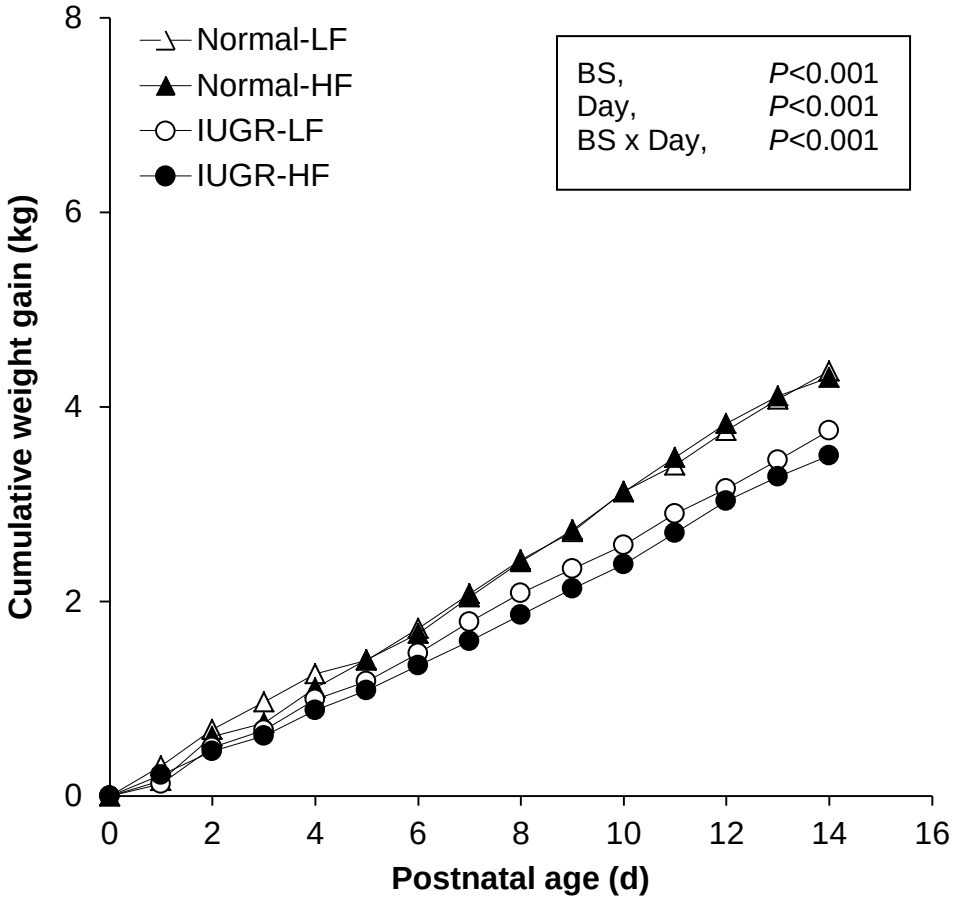

Figure 3.3 Effect of birth size and dietary fat content on cumulative weight gain between birth and slaughter on day 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded (IUGR) lambs were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Body weights were recorded daily and cumulative weight gain calculated by difference from body weight at birth. The pooled SE was 0.11 for n = 8 and 0.12 for n = 7. Significant effects of birth size (BS), day (Day) and BS x Day interaction are reported. All other effects were non-significant.

Table 3.3 Effect of birth size and dietary fat content on lamb growth between birth and slaughter on day 14 of postnatal life.

	Normal[b]		IUGR[b]			Probability level[c]		
Variable[a]	LF	HF	LF	HF	SD	BS	Diet	BS x Diet
Cumulative weight gain, kg	4.37	4.30	3.76	3.50	0.46	<0.001	NS	NS
Final weight, kg	8.49	8.46	6.52	5.92	0.59	<0.001	NS	NS
ADG, kg/day	0.31	0.32	0.25	0.27	0.03	<0.001	NS	NS
FWG, % day	5.7	5.9	6.9	6.8	0.6	<0.001	NS	NS
CRL gain, cm	8.6	10.8	9.1	10.9	3.2	NS	NS	NS
Final CRL, cm	58.1	60.8	54.2	54.4	2.9	<0.001	NS	NS

[a]ADG, average daily gain; FWG, fractional weight gain; CRL, crown rump length.
[b]Normal size (Normal) or intrauterine growth-retarded (IUGR) lambs were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment).
[b]Type I error probability for birth size (BS), diet (Diet) and their interaction (BS x Diet). NS = $P>0.05$ for main effects and $P>0.15$ for the interaction.

in progressively diverging cumulative growth curves (Fig. 3.3; BS x Day, $P<0.001$). Accordingly, IUGR lambs also had lower final cumulative gain and remained lighter at slaughter (BS, $P<0.001$). The average weight difference between Normal and IUGR lambs actually grew from 1.6 kg at birth to 2.0 kg by day 14. Nevertheless, the fractional growth rate (FGR) of IUGR lambs was 18% higher than that of Normal lambs (BS, $P<0.001$). Finally, both groups had similar crown-rump growth during the treatment period and therefore IUGR remained ~9% shorter at slaughter (BS, $P<0.001$). Diet did not have any effects on any growth parameters.

Body composition. At birth, IUGR and Normal lambs had similar fat (1.6 vs. 1.6%) and ash content (15 vs. 17%) in the EBW, but protein content was higher in Normal than IUGR lambs (18.3 vs. 17.0%, $P<0.02$). Effects of BS and Diet on EBW characteristics at day 14 of postnatal life are given in Table 3.4. IUGR lambs had a 27% lower EBW mass than Normal lambs (BS, $P<0.001$), and a 5% higher visceral fraction in the EBW (BS, $P<0.03$). Diet did not alter these variables.

Composition analysis was performed separately on the visceral and carcass fractions of EBW. In the viscera fraction, both the IUGR condition and the HF diet increased fat content (Fig. 3.4; BS and Diet, $P<0.03$ or less). For example, the visceral fat content was 42% higher in IUGR than Normal lambs on the LF diet, whereas it was 58% higher when Normal lamb were fed the HF rather than the LF diet (Fig. 3.4). These data are supported by dissection of the individual visceral fat depots at slaughter (Fig. 3.5). With the single exception of the pericardial fat depot, both BS and Diet increased the fraction of the EBW occupied by each depot (BS and Diet, $P<0.04$ or lower).

In the carcass, effects of BS and Diet were less obvious, reflecting in part a blunted increase in fat content in IUGR lambs receiving the HF diet (Fig. 3.4; BS x Diet,

Table 3.4 Effect of birth size and dietary fat content on body composition on day 14 of postnatal life.

	Normal[b]		IUGR[b]			Probability level[c]		
Variable[a]	**LF**	**HF**	**LF**	**HF**	**SD**	**BS**	**Diet**	**BS x Diet**
EBW, kg	7.10	7.17	5.42	4.95	0.51	<0.001	NS	NS
Carcass (% EBW)	77.4	77.9	76.8	76.4	1.1	<0.03	NS	NS
Viscera (% EBW)	22.6	22.1	23.2	23.6	1.1	<0.03	NS	NS
Composition of EBW								
Water %	72.6	68.9	72.3	70.3	1.6	NS	<0.001	NS
Fat %	6.8	10.6	8.1	10.1	1.6	NS	<0.001	NS
Protein %	17.5	17.3	16.6	16.4	0.7	<0.01	NS	NS
Carbohydrate %	0.4	0.4	0.5	0.7	0.3	0.05	NS	NS
Ash %	2.8	2.7	2.6	2.5	0.3	0.02	NS	NS

[a]EBW, empty body weight.
[b]Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment).
[c]Type I error probability for body weight category at birth (BWC), diet (Diet) and their interaction (BW x Diet). NS = $P>0.05$ for main effects and $P>0.15$ for the interaction.

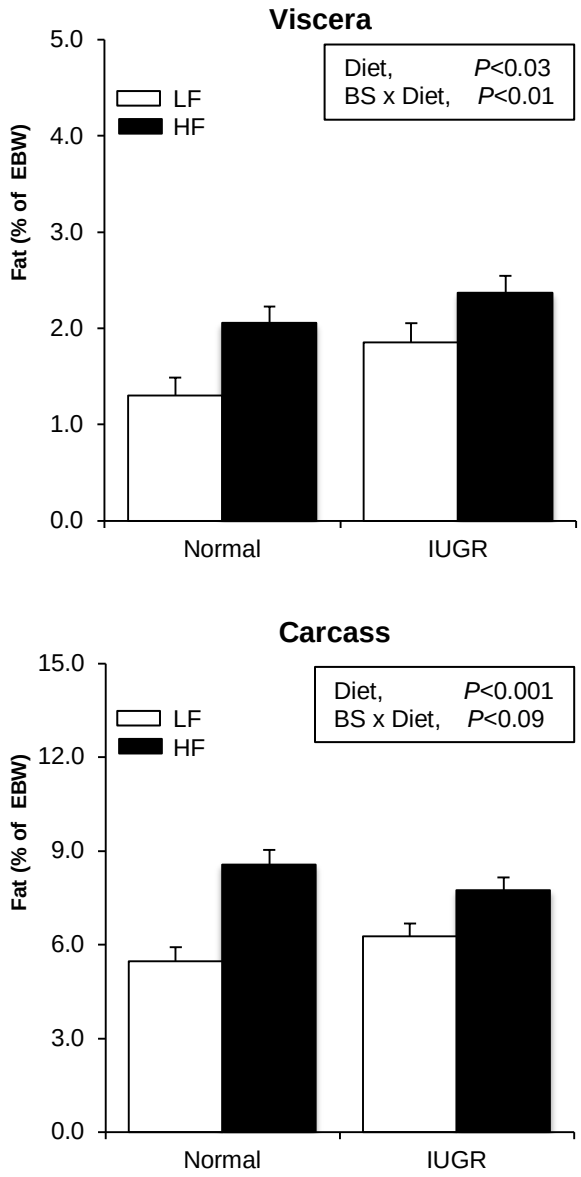

Figure 3.4 Effect of birth size and dietary fat content on viscera and carcass.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a Low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Fat was measured separately in carcass and viscera and expressed as a % of empty body weight (EBW). Each bar represents the mean ± SE of 7-8 lambs. The significant effects of birth size (BS), diet (Diet) and their interaction (BS x Diet) are reported.

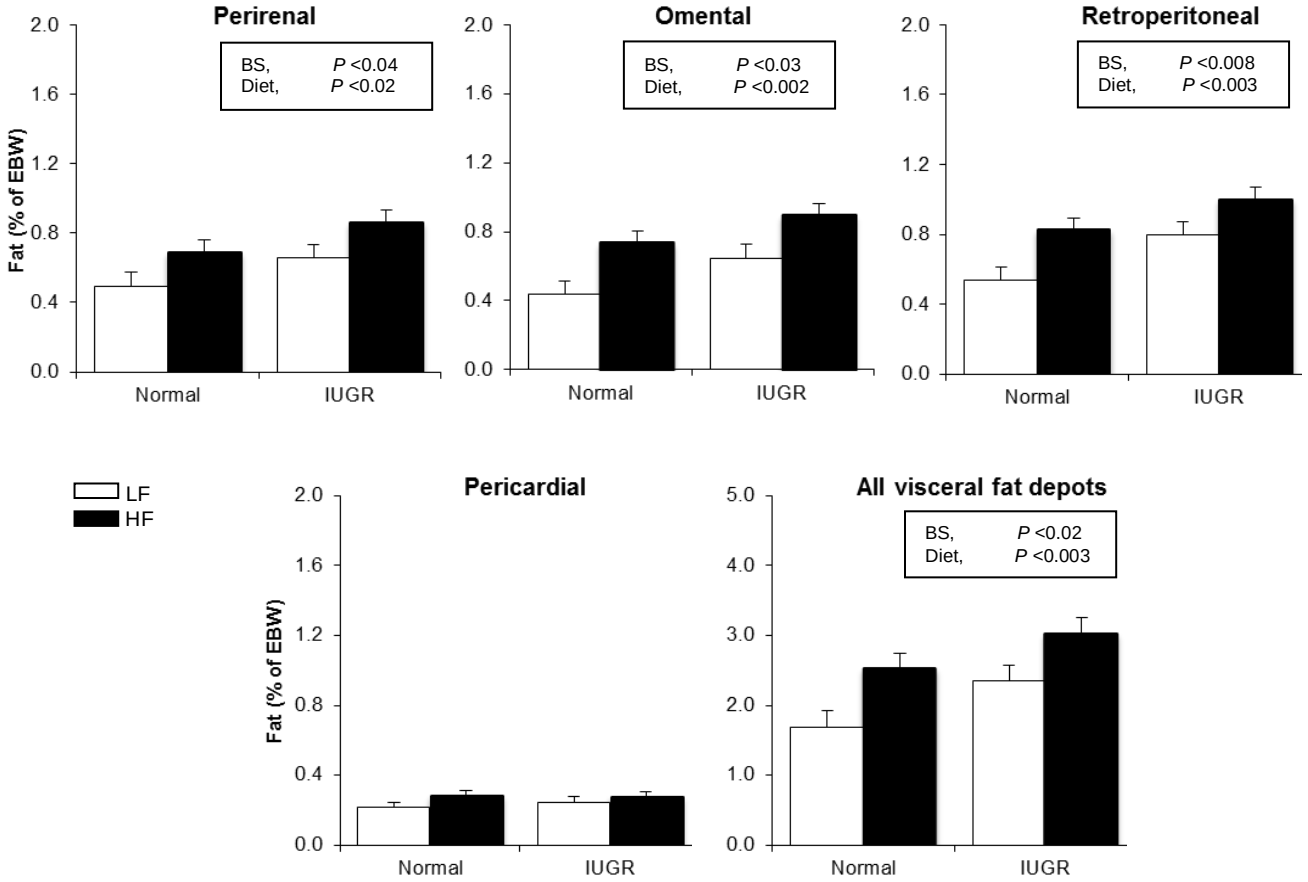

Figure 3.5 Effect of birth size and dietary fat content on visceral depots.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Fat depots were dissected, weighed, and expressed as % of empty body weight (EBW). Each bar represents the mean ± SE of 7-8 lambs. The significant effects of birth size (BS) and diet (Diet) are reported.

P<0.09). As a consequence, the stimulatory effects of the IUGR condition on fat content were not significant in the EBW (Table 3.4). This lack of BS effect in the EBW was associated with attenuation in the retention of dietary fat and energy in IUGR lambs fed the HF diet (Fig. 3.6; BS x Diet, *P*<0.04). Effects of BS and Diet on the EBW content of the other chemically defined components are also reported in Table 3.4. IUGR lambs had slightly lower protein and ash content and slightly higher carbohydrate content (BS, *P*<0.05 or less). The only effects of the HF diet were to increase the fat content of the EBW and to cause the opposite for water content (Diet, *P*<0.001).

Metabolic indices. Blood samples were obtained at birth before ingestion of colostrum and analyzed for the plasma concentration of glucose and metabolic hormones (Table 3.5). Consistent with a state of fetal undernutrition, IUGR lambs had lower plasma concentration of glucose and IGF-1 than Normal lambs (*P*<0.02 or less) but had similar plasma leptin. Moreover, plasma insulin was only detectable in Normal lambs. IUGR lambs also had lower plasma T4 (*P*<0.001) and plasma insulin levels that were below the detection level of the assay. Similar analyses were performed on plasma samples obtained between day 1 and 14 of treatment. The LF diet caused a reduction in plasma glucose but only on day 1 (Fig. 3.7; Diet x Day, *P*<0.006). Normal lambs experienced an elevation in plasma insulin concentration by the end of treatment versus lower and steady concentration in IUGR lambs (Fig. 3.8; BS x Day, *P*<0.04). In the case of IGF-1 the plasma concentration rose until day 9 in Normal lambs whereas it was still rising by day 14 in IUGR lambs (Fig. 3.9; BS x Diet, *P*<0.04). Plasma leptin remained relatively steady over time irrespective of BS and tended to be higher in lambs fed the HF diet (Fig. 3.10; Diet, *P*=0.14). The most prominent effects of treatments were on plasma T4 (Fig. 3.11), with effects of both BS and Diet varying over time. IUGR lambs had lower plasma T4 than Normal lambs for the first 3 days irrespective of Diet,

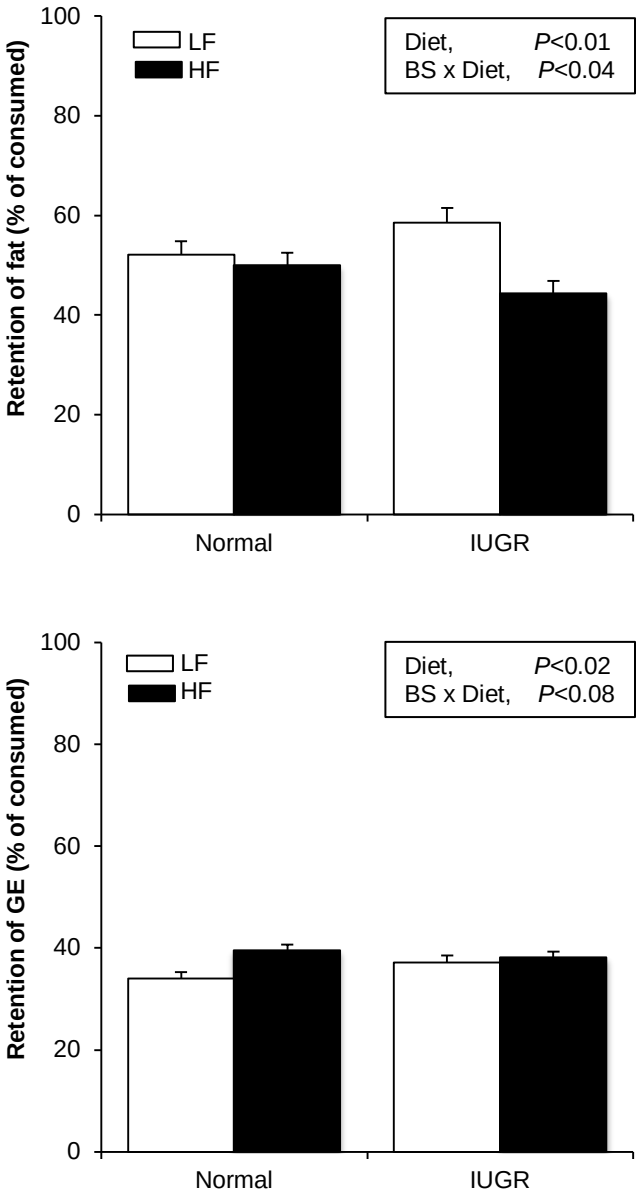

Figure 3.6 Effect of birth size and dietary fat content on fat and energy retention.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a Low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Fat was measured by chemical method and energy by bomb calorimetry. Each bar represents the mean ± SE of 7-8 lambs. The significant effects of birth size (BS), diet (Diet) and their interaction (BS x Diet) are reported.

Table 3.5 Effect of birth size on glucose and the concentration of selected metabolic hormones at birth.

	Birth Size			
Variable	**Normal[a]**	**IUGR[a]**	**SD**	**Probability level[b]**
Glucose, mg/dl	58	35	25	<0.02
Insulin, ng/ml	0.11	<0.10	0.2	NS
IGF-1, ng/ml	147	93	43	<0.01
Leptin, ng/ml	4.0	3.7	1.0	NS
T4, nmol/l	163	109	28	<0.001

[a]Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were sampled within 2 hours of birth before consuming colostrum (n = 15 lambs per group).
[b]Type I probability error. NS = $P>0.05$.

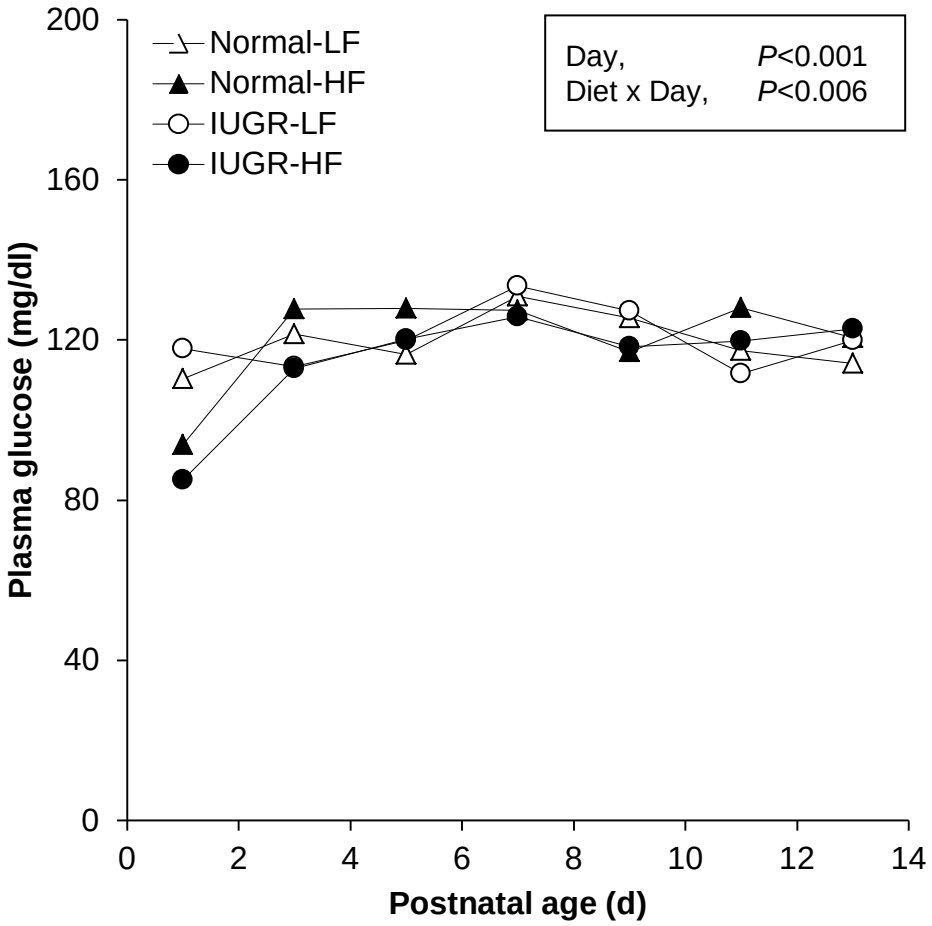

Figure 3.7 Effect of birth size and dietary fat content on plasma glucose concentration between day 1 adn 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Plasma samples were obtained between 1600 and 1700 h. Pooled SE was 6.6 for n = 8 and 7.1 for n = 7. Significant effects of day (Day) and the interaction between Diet and Day are reported. All other effects were non-significant.

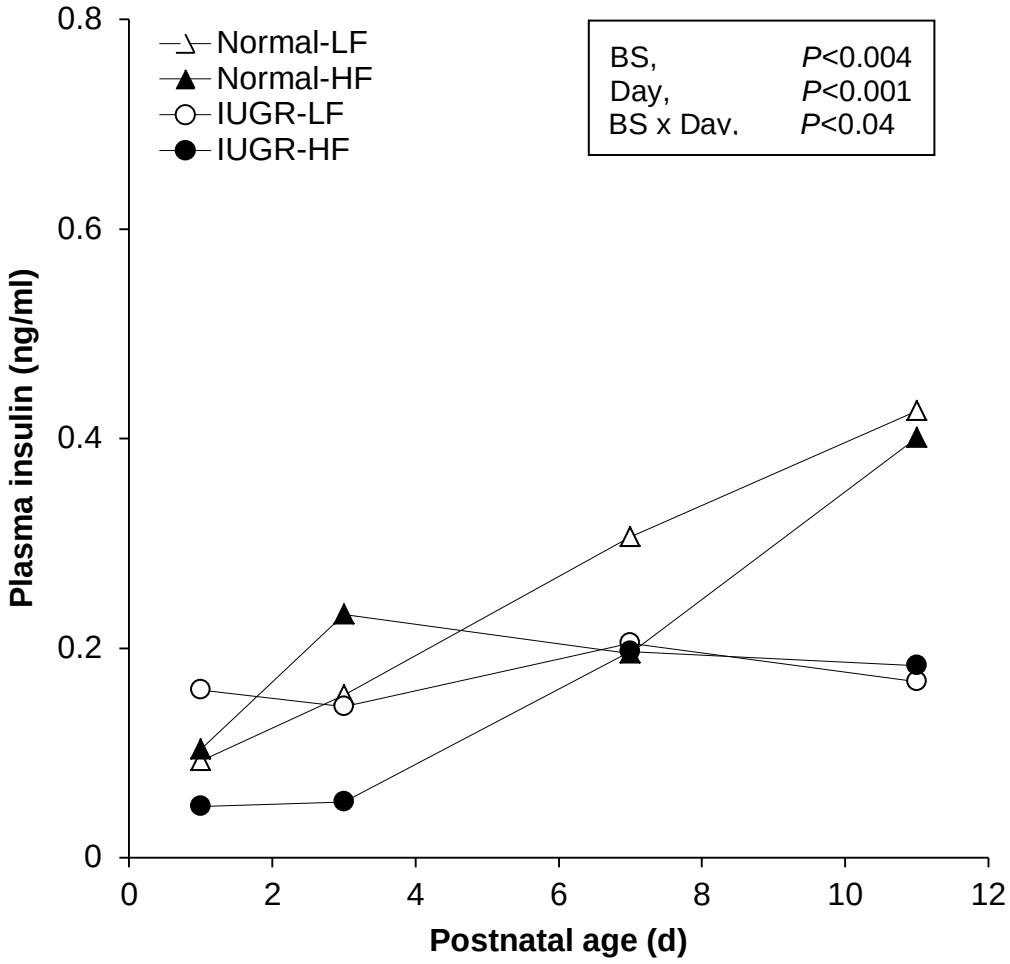

Figure 3.8 Effect of birth size and dietary fat content on plasma insulin concentration between day 1 and day 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Plasma samples were obtained between 1600 and 1700 h. Pooled SE was 0.06 for n = 8 and 0.07 for n = 7. Significant effects of birth size (BS), day of treatment (Day) and their interaction are reported. All other effects were non-significant.

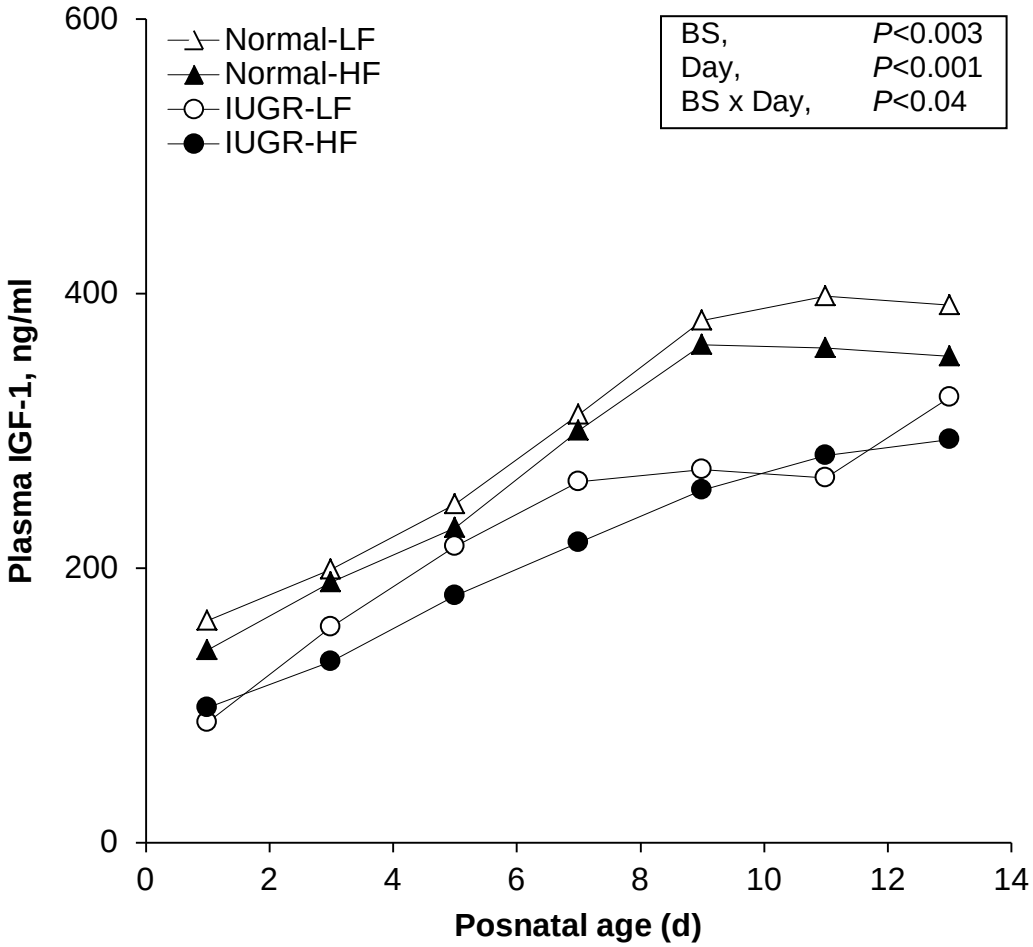

Figure 3.9 Effect of birth size and dietary fat content on plasma IGF-1 concentration between day 1 and 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded (IUGR) lambs were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Plasma samples were taken between 1600 and 1700 h. Pooled SE was 27 for n = 8 and 29 for n = 7. Significant effect of birth size (BS), day (Day) and their interaction are reported. All other effects were non-significant.

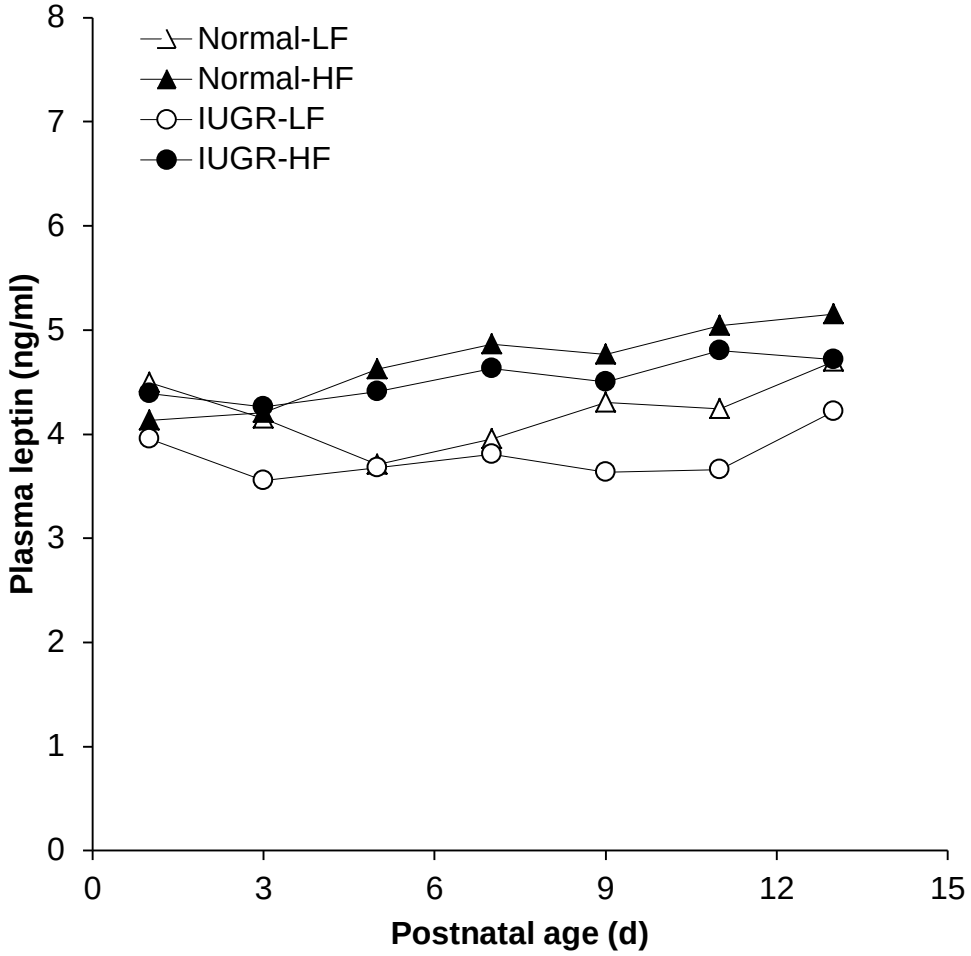

Figure 3.10 Effect of birth size and dietary fat content on plasma leptin concentration between day 1 and 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Plasma samples were taken between 1600 and 1700 h. Pooled SE was 0.4. No effects were significant.

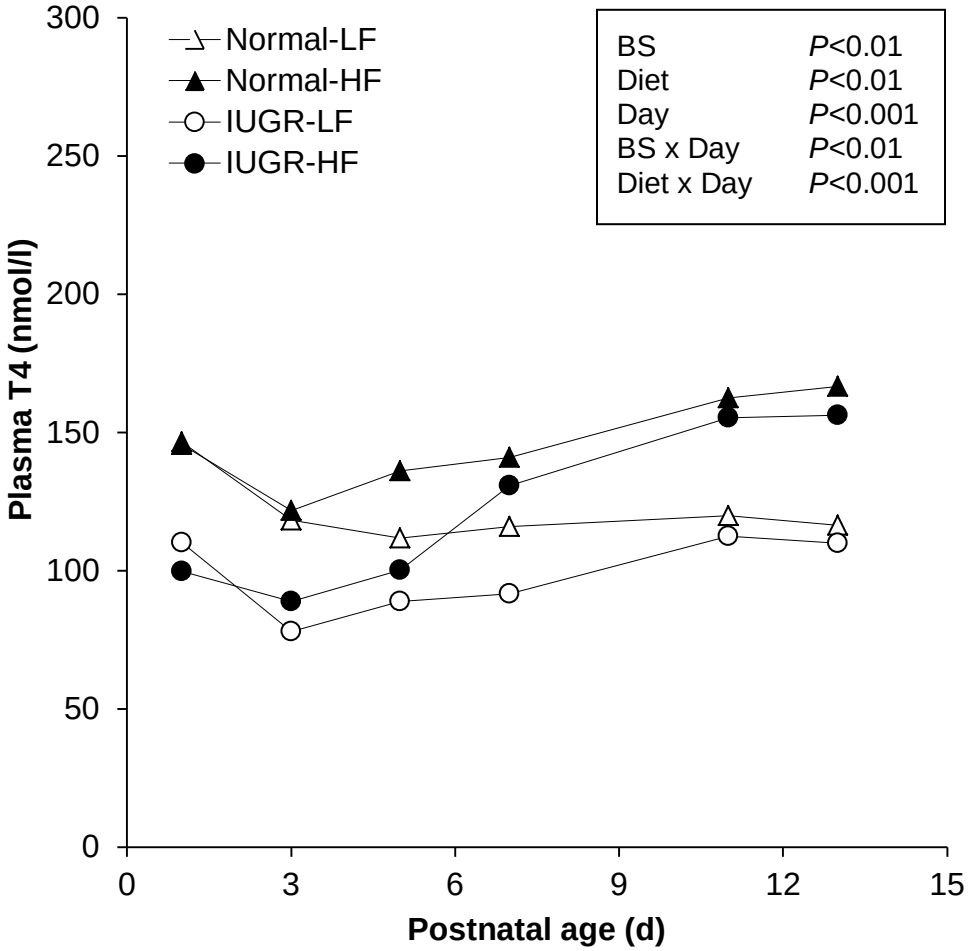

Figure 3.11 Effect of birth size and dietary fat content on plasma T4 concentration between day 1 and 14 of postnatal life.

Normal size (Normal) or intrauterine growth-retarded lambs (IUGR) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter on day 14 of life (n = 7-8 lambs per treatment). Plasma samples were taken between 1600 and 1700 h. Pooled SE was 9.1 for n=8 and 9.9 for n = 7. Significant effects of birth size (BS), diet (Diet), day (Day) and their interactions are reported. All other effects were non-significant.

followed by disappearance of the BS effect by day 14 (BS x Day, $P<0.01$). Dietary fat on the other hand had no effect over the first 3 days but caused increased plasma T4 irrespective of BS by day 14 (Diet x Day, $P<0.001$).

Body composition at fixed live weight. IUGR lambs were 27% lighter than Normal lambs by the end of treatment on day 14 of life (6.2 vs. 8.5 kg), and therefore BS effects on fatness may be underestimated. To assess whether this is the case, a second group of IUGR lambs were slaughtered when they reached 8.5 kg LW. IUGR fed the LF diet tended to reach this target weight sooner than their HF counterparts (18.7 vs. 21.3 days, $P=0.07$). Body composition analysis was performed exactly as described earlier and compared to Normal lambs killed on day 14. When compared in this manner, neither BS nor Diet affected EBW (Fig. 3.12A). The fraction of EBW occupied by viscera was increased in IUGR lambs fed the HF diet, whereas the reciprocal effect was seen for the carcass fraction (Fig. 3.12B and results not shown, BS x Diet, $P<0.02$). As seen at 14 days of age, IUGR lambs had higher fat content in the viscera than Normal lambs irrespective of Diet (Fig. 3.12C, BS, $P<0.001$). This fat content difference was now significantly larger than seen at day 14 of age: when fed the LF diet, IUGR had a greater than 2 fold difference (Fig. 3.12C) relative to Normal lambs at 8.5 kg vs. 1.3 fold difference at day 14 (Fig. 3.4). IUGR lambs also had heavier visceral fat depots than Normal lambs on both diets, except for the pericardial fat depot (Fig. 3.12D and results not shown). Finally, IUGR also had more fat in the carcass at 8.5 kg (Fig. 3.12C) whereas this effect was not significant at 14 days of age (Fig. 3.4).

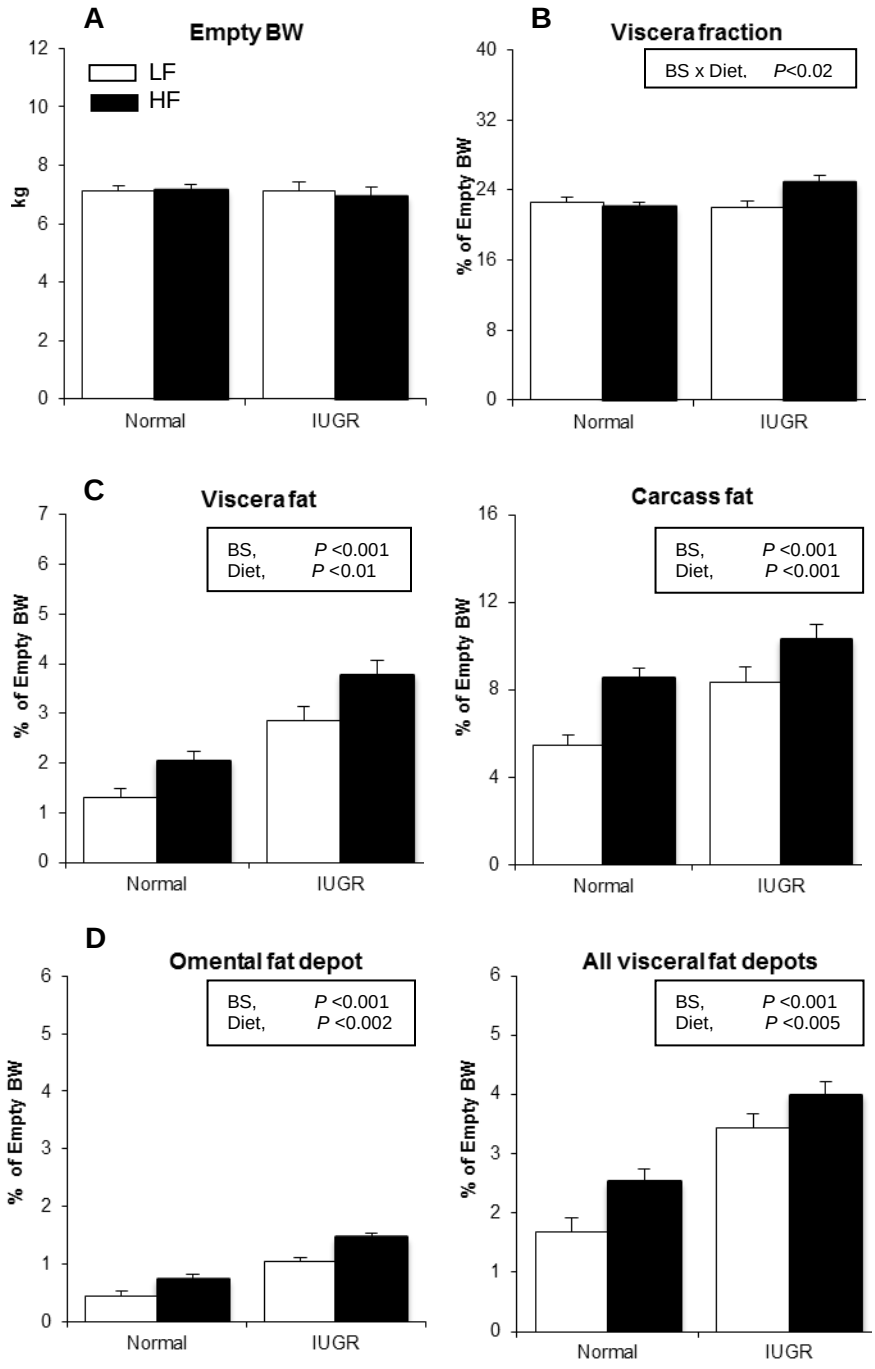

Figure 3.12 Effect of birth size and dietary fat content on carcass and visceral parameters at 8.5 kg live weight.

Normal size (Normal; n = 7-8 per group) or intrauterine growth-retarded lambs (IUGR; n = 3 per treatment) were fed a low fat (LF) or high fat (HF) milk replacer between birth and slaughter at the fixed weight of 8.5 kg. **A**. Empty body weight (EBW). **B**. Viscera fraction expressed as % of EBW. **C**. Fat content of viscera and carcass fractions. Fat was measured separately in carcass and viscera and expressed as % of EBW. **D**. Mass of omental and all visceral fat depots. Fat depots were dissected, weighed, and expressed as a % empty body weight (EBW). Each bar represents the mean ± SE of 3, 7 or 8 lambs. The significant effects of birth size (BS), diet (Diet) and their interaction (BS x Diet) are reported.

Discussion.

In this study we used an IUGR model arising naturally in prolific ewes (21). The cause of the IUGR condition in this model is fetal undernutrition, which results from the combination of 2 factors: first, ewes have a fixed number of uterine sites where fetal and maternal tissues meet to form the placentome, the functional nutrient exchange unit of the placenta. It follows that the number of placentomes serving each fetus decreases with increasing litter size, inevitably leading to smaller placentas for one or more fetuses in litters of 3 or 4 lambs (33). This placental insufficiency induces a state of fetal undernutrition and growth retardation mainly during the last third of gestation when 80% of fetal growth occurs (34). Consistent with fetal undernutrition, IUGR lambs had reduced concentration of nutritionally-regulated plasma variables at birth (i.e. glucose, IGF-1 and insulin). The 37% reduction in birth size in our study agrees with the 20-50% reduction obtained in other sheep models limiting placental size or function, such as artificial placental embolization (35), endometrial carunclectomy (36), overnutrition of pubertal ewes or hyperthermia (37).

Studies performed mostly in rodents have shown that the IUGR condition has long-term effects on postnatal energy metabolism such as increased appetite, lower energy expenditure and overall adiposity (38–40). These studies have also shown that HF diets exacerbate these detrimental effects (38, 41). All of this work however, has been performed during the post-weaning period and the interaction between IUGR and caloric density during early postnatal life remains unknown. We sought to investigate this question by rearing Normal and IUGR lambs with artificial milk varying in fat content.

Similar to the results obtained with this model by Greenwood et al (21), IUGR lambs had higher relative dry matter and caloric intake during the first 14 days of life. When adjusted for MBW, however, differences in relative energy intake between IUGR

and Normal lambs disappeared, implying that intake is not energetically disproportionate in IUGR lambs. This adjustment is used to normalize the fasting or basal energy expenditure per unit of body weight (kg) across animals differing substantially in size. We chose the 0.58 exponent because Graham et al. (32) showed that it offers a closer association to basal metabolic rate in milk-fed lambs than the commonly used 0.75 exponent. It would be interesting to determine whether the application of this normalizing procedure would eliminate differences in intake associated with the IUGR condition in other species as well.

A second important set of observations relate to the effects of dietary fat content on intake. The absence of caloric density effects on the average feed intake suggests that homeostatic regulation of energy consumption does not fully operate during this early stage of postnatal life in sheep. Other investigators have made the same observation in small ruminants of normal birth size that were offered milk replacers of differing in fat content (42, 43). Furthermore, the same phenomenon was observed in studies conducted with newborn infants (44, 45). In contrast, Corl et al. (46) reported a reduction in intake when newborn piglets were offered a 25% fat diet compared to a 3% diet. It is possible that such extreme dietary treatment would also trigger intake regulation in neonatal sheep. Finally, our data suggest that the profile of intake over time follows a quadratic relation in HF-lambs versus a linear relation in LF-animals. These data are consistent with emergence of a calorie-based appetite control after the first week of age. We did not observe, however, any differences between Normal and IUGR lambs with respect to this phenomenon.

The significant 18% increase in fractional weight gain observed in IUGR lambs indicates that these animals developed accelerated weight gain or ‘catch-up’ growth during the first 14 days of life. It is well established that human infants experiencing

catch-up growth, have higher risk of developing obesity and non-communicable diseases later in life (47, 48). Furthermore, catch up growth in mice not only increased adiposity but also reduced their lifespan dramatically (49, 50). De Blasio et al. (36, 51) observed that IUGR lambs reared naturally catch-up with the weight of their normal controls at approximately day 35 of age. At 43 days of life, these IUGR animals had 50% heavier visceral fat depots than control animals but the timing of the effect was not established. On the other hand, Greenwood et al. (21) demonstrated that total body fatness of *ad libitum*-fed IUGR lambs was 33% higher than normal lambs as early as postnatal day 11. Our data shows that visceral fat content is increased by the IUGR condition as early as day 14 of life, and effects are independent of the fat content of the diet. Negative effects of the IUGR condition on adiposity were most evident in lambs fed the LF diet. These lambs showed an increase of 42% in visceral fatness. This was supported by the significantly higher relative weight of all visceral fat depots dissected at slaughter. Remarkably, these effects of the IUGR condition on fat deposition increased dramatically when comparisons were performed at the similar BW of 8.5 kg. For instance, IUGR lambs offered the LF diet had a greater than two fold increase in visceral fat and in the mass of the visceral fat depots relative to their normal counterparts. Moreover, IUGR lambs also had significantly more fat in the carcass. Finally, these differences in fatness were achieved after only 3 weeks of life. It is likely that the obesity penalty of the IUGR condition is significantly higher at maturity.

Finally, we profiled plasma glucose and selected metabolic hormones as a first step to understand the predisposition for fat accretion in IUGR. The plasma concentrations of glucose and insulin were not very instructive, perhaps because plasma samples were obtained without a standardized fasting period. In contrast, plasma

concentrations of T4 and leptin are not acutely affected by feeding and both provided information relevant to the IUGR condition.

As observed previously in sheep and infants (52–54), IUGR lambs had lower plasma T4 plasma levels at birth. Hypothyroxinemia continued for the length of the experiment in IUGR lambs fed the LF diet and those are the IUGR lambs with the highest fat deposition relative to their matched counterparts. It is well known that sheep, as well as humans, rely on adaptive thermogenesis by brown adipose tissue (BAT) to maintain an adequate temperature at birth (55). A normal thyroid function is essential to develop such thermogenesis in the newborn lamb (56, 57). In the past, researchers have demonstrated the association between the evident hypothyroidism observed in IUGR lambs and their incapacity to initiate this process to maintain adequate body temperature at birth (57, 58). Moreover, key evidence indicates that prenatally thyroidectomized lambs show inadequate thermogenesis, reduced UCP-1 content and increased lipid deposition in perirenal depots (59). Importantly, the rate of lipid deposition increases as soon as thermogenic capacity of BAT decreases in early life (60). IUGR animals also mobilize less NEFA upon adrenergic stimulation (61), presumably due to reduced expression of β_2 adrenergic receptor in white adipose tissue (62).

Finally, concentration of thyroid hormones is positively associated to energy expenditure in adult animals (63, 64). Higher concentration of thyroid hormones is associated with greater energy expenditure by uncoupled oxidation of lipids in muscle, while hypothyroid states promote lipid retention in adult animals (64, 65). We speculate that neonatal hypothyroidism contributes to the increased visceral adiposity observed in IUGR lambs. In fact, the expression of key genes regulating β-oxidation in muscle is reduced in IUGR lambs at birth (Ehrhardt and Boisclair, unpublished data). Moreover,

persistently lower T4 concentration found in IUGR-LF lambs could exacerbate this effect, leading to a significant increase in perirenal and retroperitoneal adiposity observed in these animals. On the other hand, it was clear that the fatness-promoting effect of the HF was blunted in IUGR relative to that of Normal lambs. This effect was associated with a significantly lower apparent retention of dietary fat in this group (44% vs. 50% in Normal lambs; results not shown). The significant effect of dietary fat on the dynamics of T4 could be a factor to consider in understanding these differences. Although we can only speculate, the explanation for this observation could range from a lower absorption of dietary fat to a relative hypersensitivity to rising T4 levels in the IUGR lambs offered the HF diet.

A second important finding in this experiment is the absence of any notable effect of BS on either the average plasma leptin concentration or its profile over time. Rodents experience a 5- to 10-fold increase in plasma leptin concentration during the first two weeks of life that is not related to either adiposity or changes in voluntary intake (66). It was later determined that this leptin surge had neurotrophic effects and is essential to the proper development of axonal projections between hypothalamic centers involved in the regulation of energy homeostasis (67). Interestingly, this neonatal surge of leptin is blunted in IUGR offspring born of rat dams subjected to a 50% diet restriction during the last third of gestation (68). This endocrine defect is associated with hyperphagia, obesity, hyperinsulinemia and hyperleptinemia during adulthood when IUGR offspring are fed a high fat diet (38). Interestingly, leptin treatment of rats during early life (P3 to P13) reverses this adverse phenotype (13). Long et al. (69) recently suggested that sheep, like rats and mice, also experience a leptin surge that could be altered by fetal nutritional insults and consequently, affect the adult phenotype of the offspring. Close examination of these data show a rise from ~1.8 ng to 2.5 ng/ml between day 5 and 9 of postnatal

age. This is no different than normal daily variation observed previously by others (70, 71), and more importantly nothing to what is seen in rats (6 to 45 ng/ml) or mice (1 to 10 ng/ml) (66, 72). Additionally, this and other studies did not detect any surge in plasma leptin in neonatal lambs (31, 73). Due to the significant differences in developmental maturity at birth between precocial and altricial species, any potential neurotrophic effect of leptin in the sheep is more likely to occur during intrauterine rather than early life.

In summary, our study confirmed that the IUGR condition in lambs is associated to higher relative intake, accelerated growth and excess adiposity in the immediate postnatal life. On a MBW basis, however, both Normal and IUGR lambs were on the same plane of energy consumption. Moreover, a higher fat diet did not exacerbated appetite in IUGR lambs. Rather, our data suggest that lower energy expenditure could be a determinant factor behind the obesity observed in IUGR lambs within the first weeks of life.

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CHAPTER 4: EFFECT OF MELANOCORTIN AGONIST TREATMENT ON INTAKE, GROWTH AND BODY COMPOSITION OF INTRAUTERINE GROWTH RETARDED LAMBS

Introduction

Greenwood and colleagues (1) characterized the early life phenotype of intrauterine growth retarded (IUGR) lambs arising from multifoetal sheep pregnancies and reared artificially with milk replacer. These IUGR lambs were born at 60-70% of normal weight as a result of undernutrition during the last third of gestation. Immediately after birth, these animals had higher relative intake and dietary fat retention as well as lower basal energy expenditure (1). As a consequence, they were fatter than their normal counterparts, particularly when assessed at similar body weight. We have recently extended this work by showing that the IUGR condition promotes visceral lipid deposition and that this effect is associated with decreased thyroid hormone levels (Chapter 3). Additionally, IUGR offspring from carunclectomized ewes also developed heavier visceral fat depots than control lambs after only 43 days of natural rearing (2, 3). Promotion of lipid deposition by the IUGR condition has also been observed in humans (4) and is associated with the development of metabolic disorders (5–9).

Over the last few decades, the central nervous system has been shown to be the primary regulator of energy-dependent variables, including all of those found to be altered in IUGR offspring (10, 11). One of the most important circuits involved in central regulation of energy homeostasis is controlled by peptides known as melanocortins (MC). MC are produced predominantly by neurons located in the arcuate (ARC) region of the hypothalamus and include the proteolytic product of the POMC gene α-MSH, and Agouti

related peptide (AGRP) (12, 13). α-MSH and AGRP are subject to reciprocal regulation by peripheral cues indicative of energy status such as leptin (14). Both, peptides bind to melanocortin receptor -3 and -4 (MC4R), located in distal hypothalamic nuclei such as the PVN and LHA (15) with α-MSH acting as an agonist and AGRP as an antagonist. Energy dependent variables such as appetite, energy expenditure and thyroid hormone production are regulated by these reciprocal actions of α-MSH and AGRP (16).

The IUGR condition, however, has been found to alter the ability of leptin and other cues to regulate the activity of the MC system leading to hyperphagia and obesity in rodents (17–19). This has prompted efforts to directly activate this system via exogenously administrated MC agonist in an effort to normalize energy metabolism. MC agonists have been successfully used in various adult models of disrupted energy expenditure and obesity (20, 21). It is unknown, however, whether the MC system is functional in early postnatal life in the sheep and other precocial mammals and therefore whether MC agonists could be used to normalize the energy metabolism of these IUGR animals in the immediate postnatal life. The objectives of this experiment were to explore the functionality of the central MC system in the immediate postnatal life and to evaluate MC4R's potential use to correct some of the detrimental phenotypes observed in IUGR lambs.

Materials and Methods

Animals and study design. All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Lambs were males from Finn x Dorset genotype. They were born from ewes selected for aseasonal breeding and prolificacy at the Cornell Teaching and Research Farm.

Seventeen lambs were selected at birth prior to suckling on the basis of their body weight as IUGR (<3.0 kg) or Normal weight (>4.0 kg). The average birth weight of selected lambs was 2.46 kg for IUGR lambs (n = 12) and 4.27 kg for Normal lambs (n = 5) (P<0.001). Lambs were towel-dried, weighed and bottle-fed colostrum replacer (LandO'Lakes®) reconstituted in warm water at the rate of 60 g/kg BW. Animals were transported immediately to the Cornell University Large Animal Research and Teaching Unit and housed in individual cages (75 cm width x 80 cm length x 80 cm height) at constant temperature (25° to 27 °C) and photoperiod (light on between 0700 and 1900). Lambs were offered unlimited amounts of a milk replacer (Milk Specialties Global, Carpentersville, IL; Table 4.1) throughout the experiment.

The period between birth and day 3 was used to collect basal data on feed intake, growth and plasma concentration of glucose and selected metabolic hormones. Treatments were administered between day 4 and 15 of postnatal life. IUGR lambs were treated for 12 days with 450 nmol/kg LW • day of the MC4R agonist BIM-22493 (IUGR-MC4R, n = 6) or excipient solution (IUGR, n = 6). BIM-22493 was solubilized in excipient solution (0.9% saline, 0.1% BSA and 5% N,N-dimethylacetamide) at the concentration of 2.3 µmol/ml as recommended by the supplier (Biomeasure Incorporated, IPSEN, Milford, MA). The daily dose was administered as 3 equal subcutaneous injections at 0 h, 0800 h and 1600 h. A group of Normal lambs was injected with

Table 4.1 Nutrient composition of milk replacer.

Nutrient	-- per kg dry matter --
Gross energy, Mcal	5.54
Crude protein, g	301
Fat, g	218
Nitrogen free extract, g	411
Ash, g	70
Vitamin A, KIU	50.67
Vitamin D3, KIU	12.21
Vitamin E, IU	124

excipient solution (Normal, n = 5) and served as an additional control. The last injection was administered at 0800 h on day 15.

Experimental procedures during the basal and treatment periods were as follows. The milk replacer was reconstituted with water at 18.8% dry matter (DM) and served at 0900 h with replenishment at 1600 and 2000 h if needed. Offered volume was adjusted on daily basis as needed. Crown-rump length was measured on day 5, 10 and 15. Body weight was recorded daily at 1600 h. Blood samples were obtained by jugular venipuncture within 2 h of birth and at 1600 h of postnatal days 1, 3, 5, 7, 9, 11 and 13. Blood samples were collected in 6-ml tubes containing 90 USP lithium heparin and plasma was prepared by centrifugation. Plasma samples were stored at -20°C until analyzed for glucose and hormones.

Tissue collection and analysis of body composition. Lambs were killed on day 15 of life by lethal intra-jugular injection of sodium pentobarbital (88 mg/kg LW; Virbac AH, Inc. TX). The head was severed immediately between the cranium and the first cervical vertebrae and processed for isolation of the hypothalamus. Blood was collected and weighed. The gastrointestinal (GI) tract was dissected and weighed before and after contents were removed. Omental, perirenal, retroperitoneal and pericardial fat depots were dissected and weighed separately. The viscera fraction was obtained by combining the empty GI tract, visceral fat depots, visceral organs (respiratory tract, heart, liver and spleen) and blood. The carcass fraction consisted of the carcass (without the head), skin and hoofs.

Each fraction was grounded separately by extrusion through grinding plates (Viscera: 2x through a 13-mm plate and 3x through a 4-mm plate; Carcass: 1x through a 50-mm kidney plate, 3x through a 13-mm plate and 3x through a 4-mm plate). A 250 g

subsample of each fraction was freeze-dried for determination of dry matter. The freeze-dried samples were then pulverized with dry ice using a Waring blender and stored at -20°C until analyzed for nitrogen, ether extract and ash content. Nitrogen content was measured by macro-Kjeldahl digestion (22) with steam distillation into boric acid using a Kjeltec 2300 (FOSS Analytical AB, Sweden). Crude protein (CP) was calculated as nitrogen (N) x 6.25. Fat content of body fractions was determined by petroleum-ether extraction (23). Ash content was determined by incinerating samples in a furnace at 506 °C for 16 hours. All samples were analyzed in duplicate. Milk replacers were analyzed similarly except that a factor of 6.38 was applied to N content to calculate CP due to its amino-acid profile (24). Fat content of milk replacer was measured by a hexane/isopropanol extraction (25).

Analyses of plasma glucose and hormones. Plasma glucose was measured using a commercial glucose oxidase enzymatic assay (510A; Sigma Chemical, St. Louis, MO) (26). Total plasma thyroxine (T4) was measured using a commercial solid-phase RIA (Coat-a-Count, Siemens, Germany) previously validated in sheep (27). Internal RIA previously validated with ovine plasma were used to measure the plasma concentration of insulin, leptin and IGF-1 (26, 28). These RIA use bovine proteins for iodination and standards. The insulin RIA was performed with a guinea pig anti-bovine insulin primary antibody (Sigma I-6136) and a goat anti-guinea pig secondary antibody (Equitech-Bio Inc., Kerrville, TX). The leptin RIA is based on a primary rabbit antibody raised against bovine leptin and a secondary goat antibody raised against rabbit gamma-globulin. For IGF-1, IGF-binding proteins were first removed by acid-ethanol extraction. Supernatant was then analyzed using an IGF-1 assay based on a rabbit anti-human IGF-1 primary antibody (lot AFP4892898; National Hormone and Peptide Program) and a caprine anti-

rabbit gamma-antibody (lot 12515, Biotech Source Inc.) (26). Inter-assay and Intra-assay coefficients of variation were less than 7% for all assays.

Calculations. Feed intake was calculated by subtracting unconsumed milk from the previous feed allowance. Average daily gain (ADG) was calculated for each lamb as the slope of the regression of body weight over time. Intake and weight gain relative to live weight (LW) were calculated by dividing daily intake and BW gain by the BW of the previous day. Metabolic body weight (MBW) was calculated by scaling live body weight to the 0.58 power as suggested by Graham et al. (29) for neonatal lambs. Empty body weight (EBW) is the sum of wet viscera and beheaded carcass weights. Indices of fatness (mass of fat depots, visceral fat or carcass fat) were expressed as % of EBW. Composition of the EBW was calculated from the composition of viscera and carcass fraction.

Data analysis. All data were analyzed by ANOVA using SAS (SAS Institute, Raleigh, NC). Data collected during the basal period were analyzed by a model accounting for body size at birth (BS, Normal vs. IUGR). Averages were calculated for data collected over the treatment period (intake data, FWG, etc.). Averages and end-point data were analyzed by a model accounting for treatment (Normal, IUGR or IUGR-MC4R). When *P*-value was less than 0.15, the treatment effect was partitioned into contrasts accounting for the effect of BS (Normal vs. IUGR) and the effect of MC4R (IUGR vs. IUGR-MC4R). With the exception of plasma variables, repeated measures data were analyzed by ANOVA using the MIXED procedure of SAS. The model accounted for TRT, day and TRT by day as fixed effects and lamb as the random effect. In the case of plasma data, they were analyzed for each individual day by a model accounting for treatment as above.

To assess the possibility of intake-independent effects by the MC4R agonist, we conducted multiple regression analysis with data from treated and untreated IUGR lambs. Effects of lipid intake and MC4R treatment on total lipid deposition, carcass lipid deposition and visceral lipid deposition were analyzed by ANOVA. Statistical significance and tendency were set at $P<0.05$ and $P<0.1$ for main effects and $P<0.1$ and $P<0.15$ for interactions.

Results

Growth and Intake. Performance data collected between birth and initiation of treatment on day 4 are provided in Table 4.2. IUGR lambs had lower absolute dry matter intake but tended to have higher relative dry matter intake ($P<0.06$). Energy intake relative to live body weight was higher in IUGR than Normal lambs ($P<0.04$), but this difference disappeared when energy intake was expressed relative to MBW. Total weight gain during the pretreatment period was not significantly different between IUGR and Normal lambs but ADG tended to be greater in Normal lambs ($P<0.09$). IUGR lambs, however, had significantly higher FWG before treatment ($P<0.02$). Normal lambs continued to be heavier than IUGR lambs on day 3, immediately before treatment ($P<0.001$).

Intake and growth data for the treatment period are reported in Table 4.3. Normal lambs continued to outperform IUGR lambs in terms of dry matter and energy intake, average daily gain and total weight gain (Table 4.3; BS<0.04 or less). The reciprocal was observed when dry matter intake, energy intake and growth rate were normalized to LW, with higher values seen in IUGR than Normal lambs (BS, $P<0.05$ or less). Finally, IUGR and Normal lambs had similar energy intake normalized to MBW and identical CRL growth rates. Overall, these data are in close agreement with the performance data we observed previously in Normal and IUGR lambs in early postnatal life (Chapter 3).

MC4R agonist administration to IUGR lambs caused a 25% reduction in dry matter and energy intake relative to their matched control when averaged over the entire period (MC4R, $P<0.001$). As a consequence, relative dry matter and energy intake were less in IUGR-MC4R than in IUGR lambs (Table 4.3; MC4R, $P<0.03$), but numerically identical to those of Normal lambs. These anorexic effects of the MC4R agonist were

Table 4.2 Effect of birth size on dry matter, nutrient intake and growth between birth and day 3 of postnatal life.

Variable[a]	Birth size[b]		SD	Probability level[c]
	Normal	IUGR		
Dry matter intake				
Absolute, g/d	218	156	32	<0.003
Relative to LW, g/kg • d	45	52	7	0.06
Energy intake				
Absolute, kcal/d	1180.6	864.1	168.9	<0.004
Relative to LW, kcal/kg • d	241.3	290.2	40.0	<0.04
Relative to MBW, kcal/$kg^{0.58}$• d	469.3	457.4	65.9	NS
Growth				
Weight gain, kg	0.96	0.78	0.24	NS
Weight on day 3, kg	5.2	3.2	0.5	<0.001
ADG, kg/day	0.34	0.26	0.08	0.09
FWG, %/day	7.0	9.7	1.8	<0.02

[a]LW, live weight; MBW, metabolic body weight ($kg^{0.58}$); ADG, average daily gain; FWG, fractional weight gain.
[b]Lambs were normal size (Normal, n = 5) or intrauterine growth-retarded (IUGR, n = 12).
[c]Type I error probability. NS = *P*>0.10.

Table 4.3 Effect of birth size and MC4R agonist on dry matter intake between day 4 and 15 of postnatal life.

	Treatment[b]				Probability level[c]		
Variable[a]	Normal	IUGR	IUGR-MC4R	SD	TRT	BS	MC4R
Dry matter intake							
Absolute, g/d	340	275	207	24	<0.001	<0.001	<0.001
Relative to LW, g/kg • d	46	53	46	5	<0.05	<0.04	<0.03
Energy intake							
Absolute, kcal/d	1877.4	1513.6	1150.7	126.7	<0.001	<0.001	<0.001
Relative to LW, kcal/kg • d	253.2	292.2	253.7	28.6	0.05	<0.05	<0.03
Relative to MBW, kcal/$kg^{0.58}$• d	595.8	584.2	479.8	46.7	<0.002	NS	<0.002
Growth							
Weight gain, kg	4.01	3.45	2.72	0.45	<0.001	<0.04	<0.01
Final BW, kg	9.71	6.95	6.00	0.46	<0.001	<0.001	<0.003
ADG, kg/day	0.37	0.31	0.25	0.03	<0.001	<0.01	<0.005
FWG, %/day	5.1	6.4	5.5	0.8	0.05	<0.03	0.08
CRL gain, cm/day	1.0	1.1	1.2	0.36	NS	NS	NS

[a]LW, live weight; MBW, metabolic body weight ($kg^{0.58}$); ADG, average daily gain; FWG, fractional weight gain; CRL, crown-rump length.
[b]Lambs were normal size (Normal) or intrauterine growth retarded (IUGR). They were treated between day 4 and 15 of postnatal life with either excipient (Normal n = 5 and IUGR n = 6) or MC4R agonist (IUGR-MC4R, n = 6).
[c]Type I error probability for the effect of Treatment (TRT) and preplanned comparisons of birth size (BS, Normal vs. IUGR) and MC4R agonist therapy (MC4R, IUGR vs. IUGR-MC4R). NS = $P>0.10$.

seen on the very first day of treatment and sustained over the entire period of treatment (Fig. 4.1; MC4R, *P*<0.005).

MC4R agonist treatment reduced energy intake corrected for MBW by ~18% (Table 4.3; MC4R, P<0.002). As expected from the feed intake response, MC4R stimulation reduced average daily gain and weight gain by ~20% (MC4R, *P*<0.01 or less) and tended to reduce the FWG to that seen in Normal lambs. The profile of BW suggests that effects of the MC4R agonist on growth were also constant over time (Fig. 4.2). MC4R agonist treatment had no effect on daily CRL gain.

Body composition. Body composition was analyzed after 12 days of treatment on day 15 of postnatal life. The EBW was less for the IUGR than Normal lambs (Table 4.4; BS, *P*<0.01). BS had no effect on visceral and carcass fat (Fig. 4.3), the mass of the various visceral fat depots (Fig. 4.4) or percent fat in the EBW (Table 4.4) even though all indices were numerically higher in IUGR than Normal lambs. In contrast, MC4R agonist treatment caused a 28% reduction in the fat content of the EBW (Table 4.4; MC4R, *P*<0.002). This reduction was apparent in the carcass (~27%) fraction of the EBW (Fig. 4.4; MC4R, *P*<0.001) and in all visceral fat depots with the exception of the perirenal depot (Fig. 4.4; MC4R, *P*<0.03 or less). For the viscera fraction of the EBW, the overall TRT effect tended to be significant (Fig. 4.3, *P* = 0.10) and the MC4R effect was significant.

De novo lipogenesis is almost non-existent in neonatal ruminants. This is due to near total absence of the major source of carbon for fatty acid synthesis in milk-fed ruminants (i.e. acetate) and low ability to use glucose as an alternate substrate (30, 31). It follows that lipid intake is the major determinant of lipid accretion in neonatal ruminants (32). To determine whether the anorexic effects of the MC4R agonist on fatness were

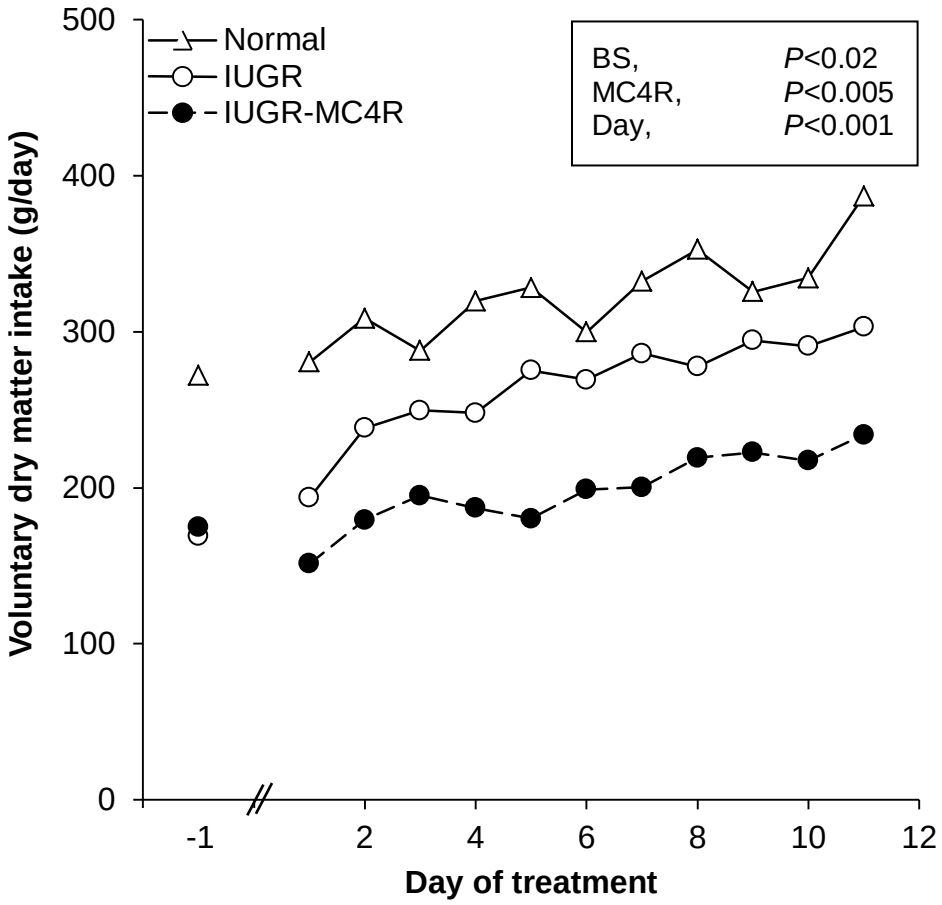

Figure 4.1 Effect of body size and MC4R agonist on voluntary dry matter intake.

Lambs were Normal size (Normal) or intrauterine growth retarded (IUGR) at birth. Treatments started on day 4 of life and consisted of thrice daily injection of excipient (Normal, n = 5; IUGR, n = 6) or MC4R agonist (IUGR-MC4R, n = 6). Voluntary intake was recorded daily. The effect of Treatment (TRT) was significant at $P<0.001$ and was further analyzed by preplanned contrast accounting for the effect of body size (BS = Normal vs. IUGR) and MC4R agonist (MC4R = IUGR vs. IUGR-MC4R). The *P*-values of these contrasts are given when significant. The pooled SE was 17.7 for n = 6 and 19.4 for n = 5.

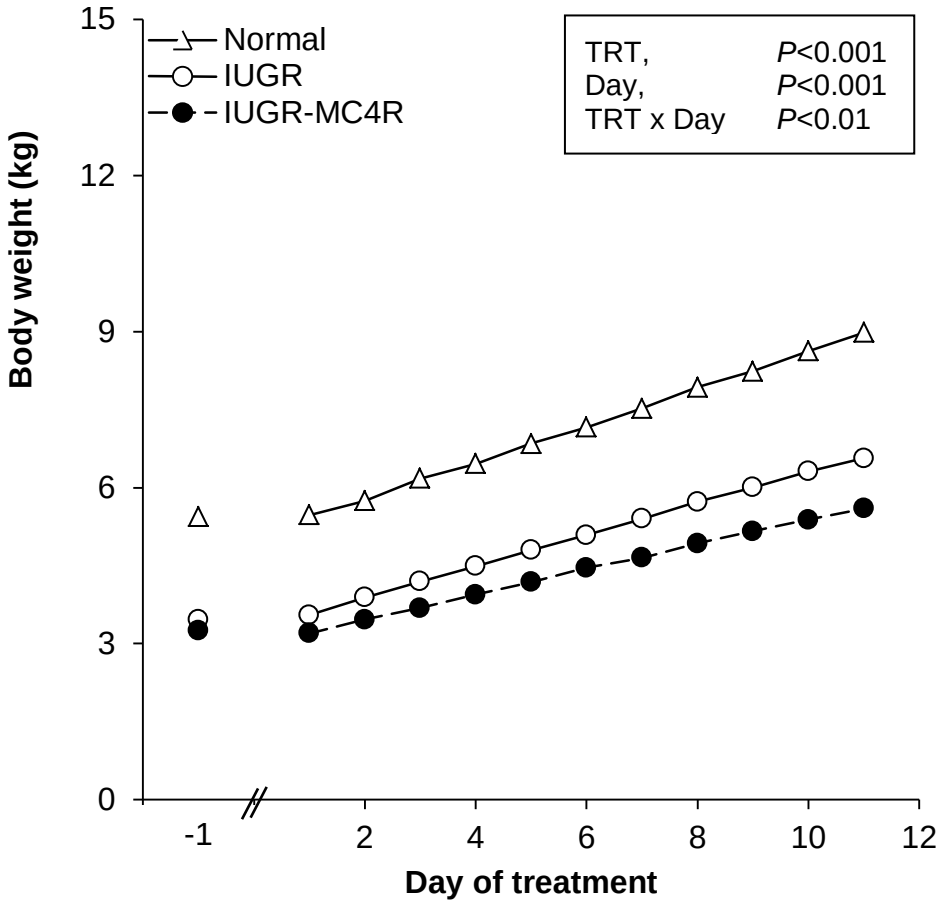

Figure 4.2 Effect of birth size and MC4R agonist or excipient treatment on body weight.

Lambs were Normal size (Normal) or intrauterine growth retarded (IUGR) at birth. Treatments started on day 4 of life and consisted of thrice daily injection of excipient (Normal, n = 5; IUGR, n = 6) or MC4R agonist (IUGR-MC4R, n = 6). Body weights were recorded daily. The significant effects of Treatment (TRT), Day and TRT x Day are reported. The TRT x Day interaction was analyzed further by a first contrast accounting for the interaction between BS (BS = Normal vs. IUGR) and Day and a second contrast accounting for the interaction between MC4R agonist (IUGR vs. IUGR-MC4R) and Day. Neither of these contrasts was significant. The pooled SE was 0.22 for n = 6 and 0.24 for n = 5.

Table 4.4 Effect of birth size and MC4R agonist on body composition on day 15 of postnatal life.

	Treatment[b]				Probability level[c]		
Variable[a]	**Normal**	**IUGR**	**IUGR-MC4R**	**SD**	**TRT**	**BS**	**MC4R**
EBW, kg	8.07	5.90	4.91	0.36	<0.001	<0.001	<0.001
Carcass (% EBW)	78.1	77.3	78.2	1.2	NS	NS	NS
Viscera (% EBW)	21.9	22.7	21.8	1.2	NS	NS	NS
Composition of EBW							
Water %	71.1	71.1	73.2	0.8	<0.001	NS	<0.001
Protein %	17.6	17.1	17.6	0.4	0.08	0.06	0.04
Fat %	8.3	9.0	6.5	1.0	<0.003	NS	<0.002
Ash %	2.7	2.7	2.8	0.3	NS	NS	NS

[a]EBW, empty body weight.
[b]Lambs were normal size (Normal) or intrauterine growth retarded (IUGR). They were treated between day 4 and 15 of postnatal life with either excipient (Normal n = 5 and IUGR n = 6) or MC4R agonist (IUGR-MC4R, n = 6).
[c]Type I error probability for the effect of Treatment (TRT) and preplanned comparisons of birth size (BS, Normal vs. IUGR) and MC4R agonist therapy (MC4R, IUGR vs. IUGR-MC4R). NS = $P>0.10$.

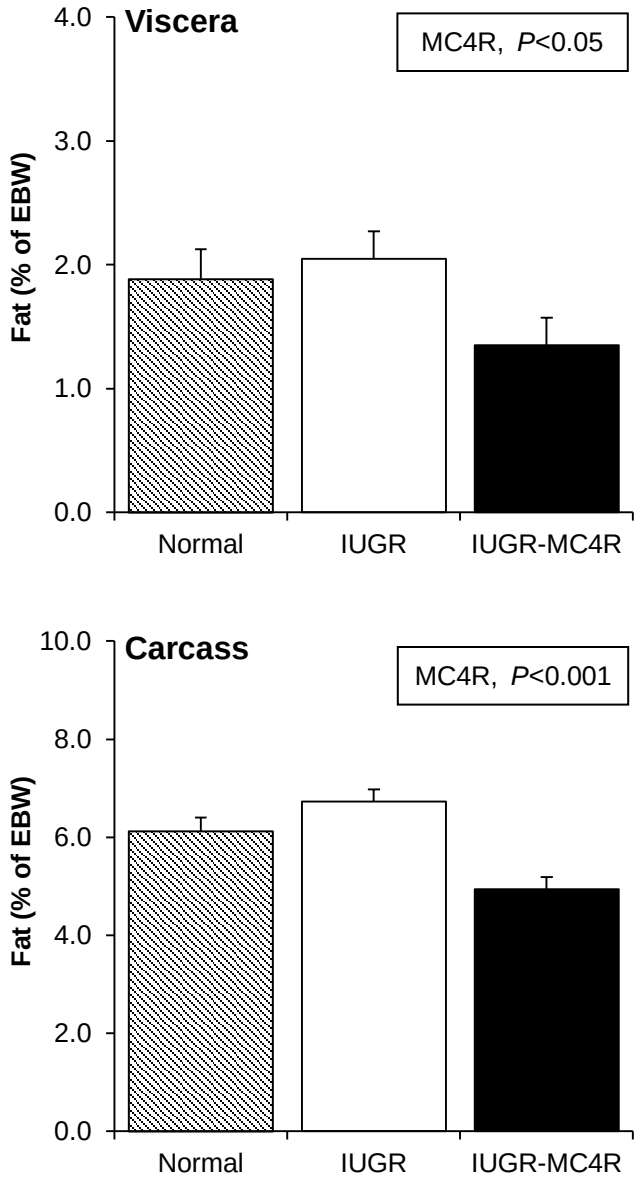

Figure 4.3 Effect of birth size and MC4R agonist on the fat content of the carcass and viscera.

Lambs were Normal size (Normal) or intrauterine growth retarded (IUGR) at birth. Treatments started on day 4 of life and consisted of thrice daily injection of excipient (Normal, n = 5; IUGR, n = 6) or MC4R agonist (IUGR-MC4R, n = 6). Each bar represents the mean ± SE of 5-6 lambs. The treatment effect tended towards significance for the viscera fraction ($P = 0.10$) and was significant for carcass ($P<0.001$). These effects were further analyzed by contrasts accounting for the effect of body size (BS, Normal vs. IUGR) and MC4R agonist (MC4R, IUGR vs. IUGR-MC4R). The *P*-values of these contrasts are given when significant.

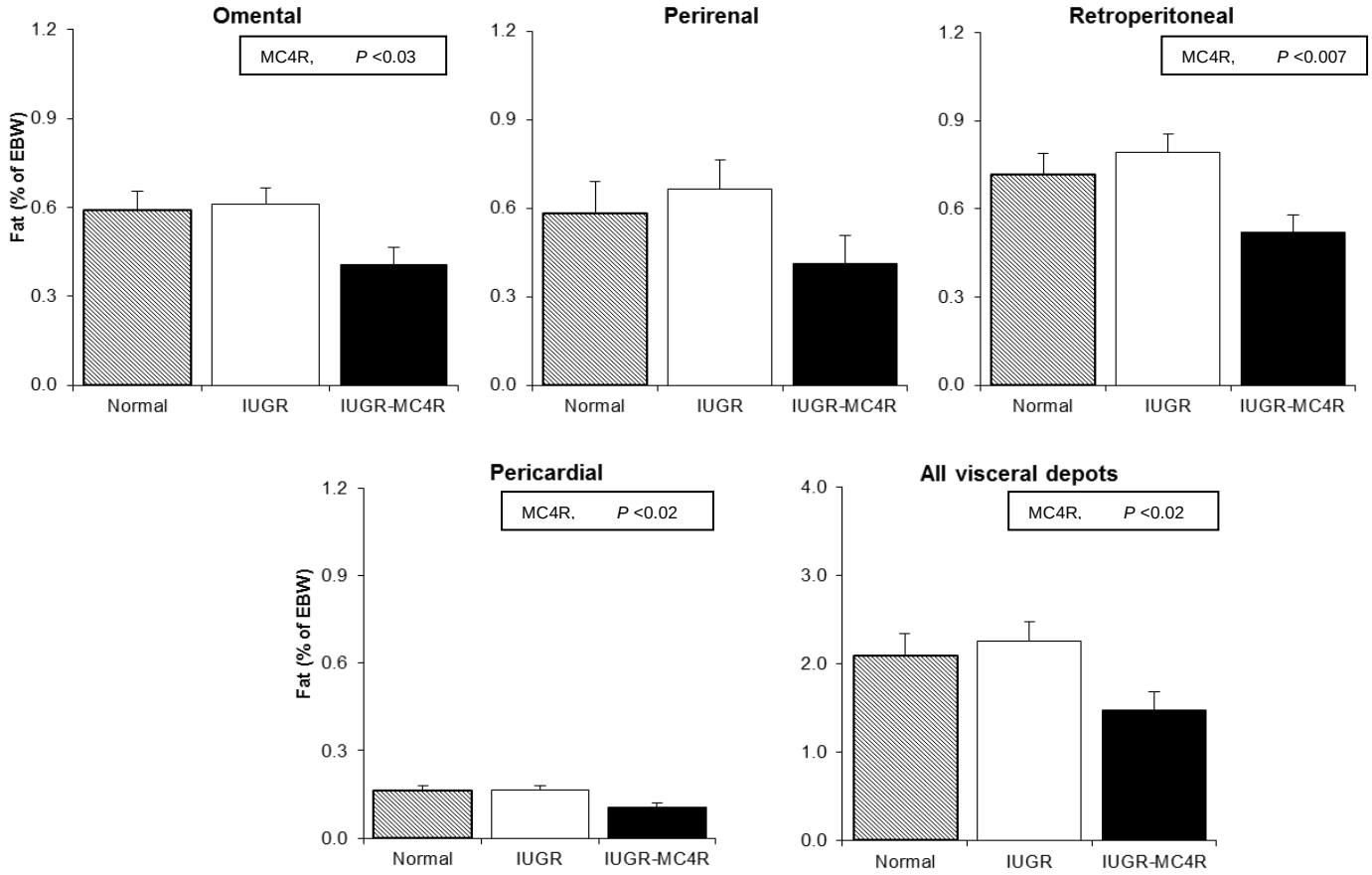

Figure 4.4 Effect of birth size and MC4R agonist on the mass of visceral fat depots.

Lambs were Normal size (Normal) or intrauterine growth retarded (IUGR) at birth. Treatments started on day 4 of life and consisted of thrice daily injection of excipient (Normal, n = 5; IUGR, n = 6) or MC4R agonist (IUGR-MC4R, n = 6). Fat depots were dissected, weighed, and expressed as a % of empty body weight (EBW). When *P*-value for treatment was less than 0.15, it was further analyzed by preplanned contrasts of body size (BS, Normal vs. IUGR) and MC4R agonist (MC4R, IUGR vs. IUGR-MC4R). The *P*-values of these of the contrasts are given when significant.

accounted entirely by its anorectic effect, the relation between lipid deposition in the EBW and lipid intake was determined by regression analysis. Lipid intake accounted for 90% of the variation in lipid deposition (P<0.001). However, an additional 4% was accounted by a model including MC4R treatment (P<0.04). When the same analysis was performed separately for lipid deposition in the carcass and in the viscera, the MC4R effects was present only in the carcass, explaining an additional 7% of the observed variation (r^2= 0.88 vs. 0.95; P<0.007). This suggests that MC4R agonist reduces lipid accretion by both intake and intake-independent mechanisms, with the latter occurring predominantly in the carcass.

Metabolic indices. Blood samples were obtained the day before treatment on day 3 of postnatal life and analyzed for the plasma concentration of glucose and selected metabolic hormones (Table 4.5). BS did not affect plasma variables with the exception that IUGR lambs had significantly lower plasma T4 (P<0.003) and tended to have lower plasma IGF-1.

These analyses were repeated on day 2, 4 and 10 of treatment except for plasma insulin (Fig. 4.5). Results are reported as absolute values for variables that did not differ before treatment (glucose and leptin) and as changes from pretreatment values for those that differed on day 3 (IGF-1 and T4). BS had no effect on plasma glucose, leptin or on the increase in plasma IGF-1 and T4 at any time point during the treatment period. Similarly, MC4R agonist treatment did not have a significant effect on plasma leptin or on the increase in plasma IGF-1. MC4R agonist treatment significantly reduced plasma glucose on day 2 of treatment, but this effect disappeared thereafter. The increase in T4 was significantly greater in MC4R agonist treated lambs on day 4 and tended to be greater on day 10 of treatment (Fig. 4.5).

Table 4.5 Effect of birth size on glucose and hormones concentration on day 3 of postnatal life.

Variable	Birth size[a]		SD	Probability level[b]
	Normal	IUGR		
Glucose, mg/dl	131	124	17	NS
Insulin, ng/ml	2.2	1.5	1.4	NS
IGF-1, ng/ml	239	187	48	<0.07
Leptin, ng/ml	3.9	3.8	0.8	NS
Total T4, nmol/l	105	73	16	<0.003

[a]Lambs were normal size (Normal, n = 5) or intrauterine growth-retarded (IUGR, n = 12).
[b]Type I error probability. NS = *P*>0.10.

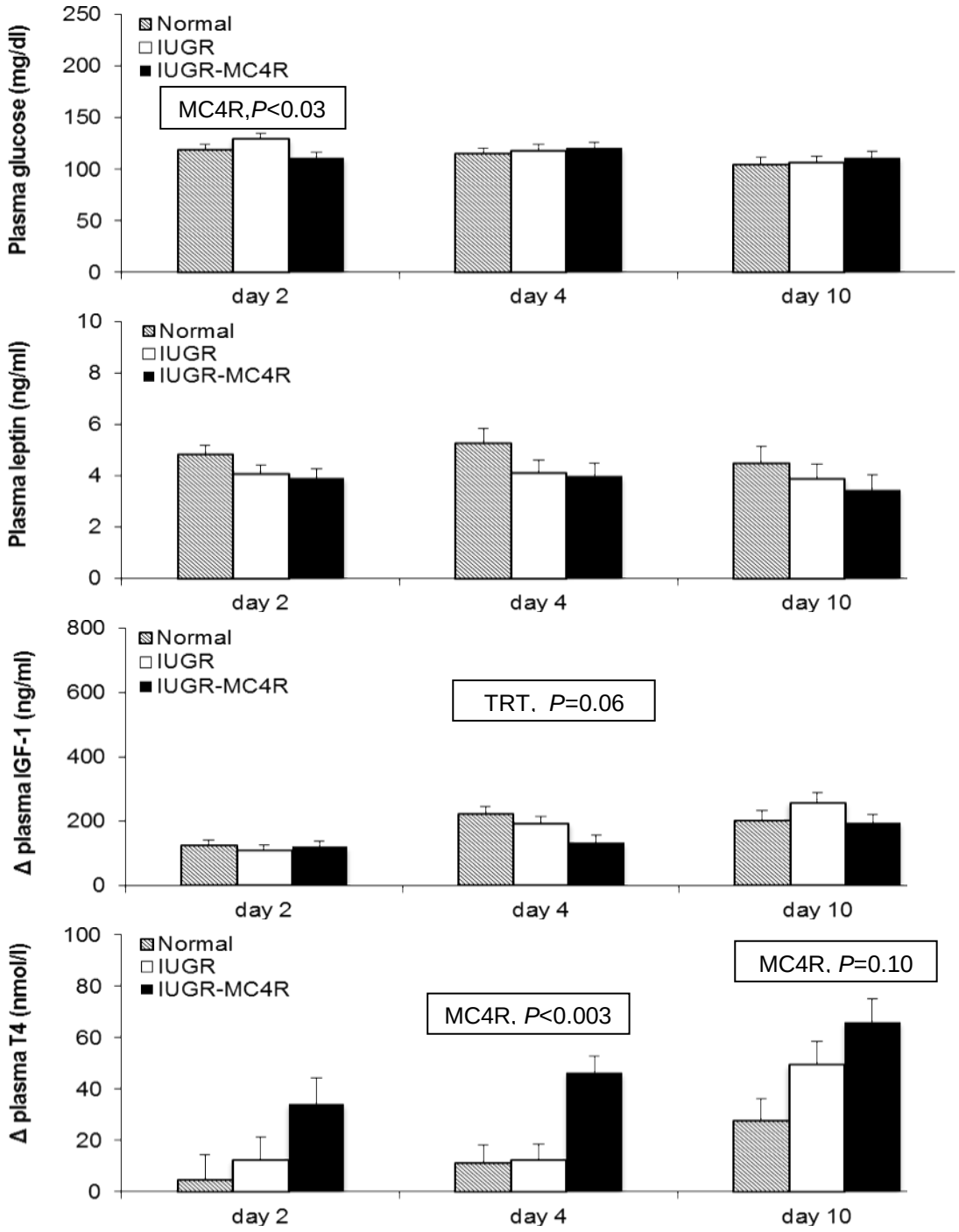

Figure 4.5 Effect of birth size and MC4R agonist on the plasma concentration of glucose and selected hormones.

Lambs were Normal size (Normal) or intrauterine growth retarded (IUGR) at birth. Treatments started on day 4 of life and consisted of thrice daily injection of excipient (Normal, n = 5; IUGR, n = 6) or MC4R agonist (IUGR-MC4R, n = 6). Blood was obtained at 1600 h on day 2, 4 or 10 of treatment. When *P*-value of treatment (TRT) was less than 0.15 effects were further analyzed by contrast accounting for the effect of body size (BS = Normal vs. IUGR) and MC4R agonist (MC4R = IUGR vs. IUGR-MC4R).

Discussion

The IUGR condition is frequently associated with accelerated growth or 'catch-up' growth soon after birth (33–35). Epidemiological studies have also revealed positive associations between early catch-up growth and higher indices of adiposity in pre-adolescent children (36–38). Similar results have been obtained in both rodent and ovine IUGR models. In rodents, numerous experiments show that the IUGR condition has long-term consequences on energy metabolism, such as increased appetite, lower energy expenditure and overall adiposity (18, 39, 40). Our own work with the multifoetal IUGR sheep model confirmed the positive association between accelerated growth and fatness as early as day 14 of postnatal life (chapter 3). Considering that obesity is linked to insulin and leptin resistance, early obesity could be a self-amplifying phenomenon that progressively increases the risk of metabolic syndrome during postnatal life. Therefore, it is crucial to understand which energy homeostatic systems operate in early postnatal life so that strategies can be developed to address increased lipid deposition of IUGR individuals in early life.

The central nervous is regarded as the most important regulator of energy homeostasis in postnatal life. However, the ability of this system to respond to leptin and other peripheral cues is limited in neonatal rodents (41, 42). Moreover, the therapeutic value of leptin appears limited in normoleptinemic humans (43, 44). In this context, targeting signals operating downstream of leptin, such as the MC system is attractive. The crucial role of this system is supported by the decreased energy expenditure and increased appetite and fatness of mice (45–47) and humans (48–50) harboring null mutations in the genes associated this system.

With this in mind, MC analogues have been designed to incorporate two major features (51, 52). First, they must preferably target the MC4R. While endogenous MC

can activate any of the 5 MC receptors, the MC4R is found almost exclusively in the central nervous system and is by far the most important MC receptor regulating energy metabolism (53). Second, any peripherally administered MC4R analogue must cross the blood-brain barrier to act. BIM-22493 is a MC agonist that incorporates both of these features. For instance, it has a ~5 fold higher affinity and 20 times lower EC_{50} for MC4R over MC3R, the other major MC receptor present in the brain (54). Moreover, BIM-22493 has no actions in MC4R knockout mice but retains efficacy in MC3R knockout mice (54). So far, natural MC and chemical agonists, such as BIM-22493, have been tested only in adult animals (54, 55). In the case of BIM-22493, these experiments demonstrated that a dose of 300 and 447 nmol/kg/day reduced voluntary intake and weight in mice and rhesus macaques (54, 56) . Using a dose of 450 nmol/kg/day, we were able to induce similar effects as early as 4 days of life in neonatal sheep. The reduction in intake observed in rodents and rhesus macaques initially reached 20 to 35% but dissipated completely after 12 days in mice and 4 weeks in monkeys (54, 56). Tachyphylaxis has been a recurrent observation with the use of artificial or natural MCR agonists in rodents and non-human primates (56–59). This did not seem to be the case in our experiment as feed intake was reduced by 25% in IUGR neonatal lambs throughout the 12-day experimental period. As we previously reported IUGR lambs have elevated relative feed intake in early life. Accordingly, it is interesting to see that MC4R agonist treatment completely corrected this elevation.

Hypothalamic stimulation with either BIM-22493 or other MC4R agonists is associated with consistent weight loss in adult animals (54, 56–58, 60). These weight reductions were explained by the loss fat and lean in rodents and non-human primates (56–58). In growing animals, it is known that a reduction in feed intake causes reduced fat content in the EBW. For example, Greenwood et al. (1) compared body composition

in neonatal IUGR lambs fed to grow at 150 g/d or fed unlimited amounts and growing at 239 g/d. At the same EBW of 4.9 kg, restricted lambs had 23% lower fat content but similar rate of retention for protein and ash. Our data shows that the 25% feed intake reduction seen with BIM-22493 was associated with reductions of 21% for BW growth, 28% for fat content in the EBW and 25% for the mass of visceral fat depots. Previous data indicate that reduction in body weight and fatness induced by melanocortin stimulation is mostly explained by lower voluntary intake (58). However, pair-fed studies have revealed that a portion of the MC agonist effect is intake-independent and likely accounted by stimulation of basal metabolic rate (56–58, 60). Unfortunately, we were not able to include a pair-fed group to MC4R treated animals due to a lack of IUGR lambs.

As an alternative step to ask if MC has intake-independent effects on fat deposition, we examined the relation between lipid intake and lipid deposition in treated and untreated IUGR animals given that accreted lipids in milk-fed ruminants originate nearly exclusively from dietary lipids (30, 31). This analysis suggests that MC evoked an additional mechanism to reduce lipid accretion operating predominantly in the carcass fraction. In agreement with these data, mice and rats treated with MC agonist had lower subcutaneous fat but similar visceral fat than pair-fed controls (57). Previous experiments in MC-treated rodents and humans, indicate that MCR agonists increase lipolysis (deduced from glycerol release and gene expression data) and fatty acid oxidation (deduced from changes in respiratory quotient) (20, 54, 60, 61). A second related mechanism for an intake-independent effect could be increased T4 production. In our experiment, MC4R agonist caused an increase in plasma T4 that was sustained for most of the experimental period. It is now known that ARC-derived α-MSH activates MC4R in TRH-neurons located in the PVN, ultimately stimulating T4 production (62).

Thyroid hormones increase energy expenditure by increasing uncoupling proteins in brown adipose tissue and muscle (63, 64), leading to substrate flux in the TCA cycle without increasing ATP production (65). Interestingly, in a previous experiment we showed that neonatal IUGR lambs have lower circulating T4 concentration than normal lambs and that this reduction is associated with higher body fatness (Chapter 3). It has been recently demonstrated that T3 can suppress the expression of MC4R in the PVN, providing a negative feedback mechanism to protect from inappropriate melanocortin stimulation (66). This may explain the tachyphylaxis observed previously in adult rodents and rhesus macaques treated chronically with a MC4R agonist (54, 56). We did not observe any loss of MC effectiveness in our experiment, raising the possibility that this mechanism does not operate in early life or in this species.

MC4R deficiency in humans is associated with increased linear growth that starts in early childhood (67, 68). Martinelli et al. (67) offered a possible explanation for the phenomenon. They observed that mean growth hormone concentration is greater in MC4R deficient patients than in controls matched for the same level of fatness. Surprisingly, the concentration of IGF hormones and their binding proteins was not affected by MC4R deficiency. Work with rats supported this observations by demonstrating that direct stimulation of hypothalamic melanocortin receptors in rats did not decrease circulating IGF-1 concentration beyond the level observed in pair-fed controls (69). In our experiment, BIM-22493 appeared to halt the normal increase in IGF-1 observed in the untreated IUGR lambs on day 4 but changes were not significant. Likely, as observed in rodents, this is a reflection of the lower plane of nutrition experienced by MC4R-treated animals. Finally, MC4R did not impact linear growth during our 11-day experimental period.

In mice, MC treatment causes acute increases in glucose uptake, skeletal and cardiac muscle and in BAT (70, 71). Chronic MC therapy of rodents and rhesus macaques improved various indices of insulin action (improved glucose clearance combined with lower insulin secretion), particularly in animals exposed to high fat diets (56). These favorable effects on insulin action likely relate to reduced fatness in these treated adult animals (54, 56). In the present work, blood was collected under free-feeding conditions, making it difficult to assess MC4R agonist effect on insulin action. Finally, we were not able to detect differences in circulating leptin with BIM-22593 treatment. This is not completely surprising as we have previously shown that plasma leptin does not dynamically respond to plane of nutrition in early life (28).

In summary, chronic treatment with MC4R agonist reduced voluntary intake, normalized fractional growth rates and reduced carcass and visceral adiposity. These reductions in fatness induced by MC4R agonist are likely explained by lower nutrient intake as well as by other effects such as increased metabolic rate. Finally our data shows that MC4R signaling operates immediately after birth and could be harnessed to combat the excessive fatness observed in IUGR individuals.

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CHAPTER 5: ACUTE AND LONG-TERM EFFECTS OF EARLY LEPTIN ANTAGONIST TREATMENT ON INTAKE, GROWTH AND BODY COMPOSITION OF NEONATAL LAMBS.

Introduction

Based on epidemiological studies, David Barker and Osmond (1) suggested in the mid 1980's that the health of the adult could be influenced by pre-natal events. This group observed that women born to mothers that suffered the Dutch famine of 1944-45 during early pregnancy were lighter at birth but had higher BMI at age 50 than those born to non-exposed women (2). Additionally, retrospective epidemiological studies of twins uncovered negative non-genetic effects of low birth weight on fatness and glucose metabolism of young and elderly individuals (3, 4). Since then, researchers have continued to explore the consequences of intra-uterine growth retardation (IUGR) using animal models. For instance, several experiments with rodents have shown that IUGR pups develop higher intake, body weight gain and adiposity either when nursed by mothers fed ad libitum or are offered high fat diets during the post-weaning period (5–9).

The hypothalamus plays a central role in the regulation of energy metabolism (10). A simplified model of hypothalamic function proposes that first order neurons located in the arcuate nucleus play a predominant role in assessing the overall energy condition via peripheral cues such as leptin, insulin and glucose (11). These sensory neurons are connected to second order neurons located in other hypothalamic nuclei via a network of axonal projections. Secondary neurons are responsible for initiating appropriate responses, including regulation of intake, energy expenditure and metabolism. In rodents, fetal insults including the IUGR condition impacts the

development of this hypothalamic network (12). Bouret et al. (13) demonstrated that this network was nearly inexistent in *ob*/*ob* pups. They demonstrated that leptin therapy between day 4 and 12 of postnatal life normalizes the axonal density of this system in *ob*/*ob* mice and partially rescues the hyperphagia observed in these mice. These observations led to the hypothesis that defective development of this network could mediate the detrimental effects of inadequate nutrition in fetal and early postnatal life (14). In fact, Vickers et al. (5) demonstrated that leptin therapy between day 3 and 13 of postnatal life eliminated this propensity to develop obesity and hyperphagia in later life.

Hypothalamic development in rodents however, is temporally distinct from precocial animals such as primates, humans and sheep (15–19). This hypothalamic network system is believed to be substantially more developed at birth in these precocial species, even though some additional maturation occurs after birth (18). However, it is not known if leptin is capable of regulating appetite during early life or if its early signaling has long-term consequences on metabolism, body composition or appetite in precocial animals. To address this question we treated newborn lambs with a leptin receptor antagonist (20) during the first 14 days of life and evaluated effects of this treatment on appetite and body composition until day 40 of life. We also evaluated effects of this antagonist in 1-month old lambs.

Materials and Methods

Animals and study design. All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Male lambs from Finn X Dorset genotype parents were obtained from the Cornell Teaching and Research.

Twenty lambs were obtained at birth, towel-dried, weighed and bottle-fed a single dose of 10% of body weight of a common pool of bovine colostrum. Animals were transported immediately to the Cornell University Large Animal Research and Teaching Unit and housed in individual cages (75 cm width x 80 cm length x 80 cm height) at constant temperature (25° to 27 °C) and photoperiod (light on between 0700 and 1900 h). Immediately after birth, animals were randomly allocated to 3 treatment groups. 1) Control: lambs received twice daily intravenous (IV) injection of saline from birth until day 14 of postnatal life, and again between day 30 and 35 of life (n = 6); 2) Early super ovine leptin antagonist (SOLA): lambs received twice daily IV injection of SOLA from birth to day 14 of life (total daily dose = 0.46 mg/$kg^{0.75}$) followed by twice daily IV injection of saline between day 30 and 35 of life (n = 6); 3) Late SOLA: lambs received twice daily IV injection of saline between birth and day 14 of life followed by twice daily IV injection of SOLA from day 30 to 35 of life (total daily dose = 0.91 mg/ $kg^{0.75}$) (n = 8). For all treatments, the twice daily IV injections were performed at 0800 and 2000 h.

SOLA was obtained from PLR Ltd. (Rehovot, Israel). SOLA is a mutant ovine leptin in which amino acid 23 (aspartic acid) is replaced by leucine and amino acids 39 to 41 (leucine-aspartic acid-phenylalanine) have been replaced by alanine (21). This mutant leptin has higher affinity for leptin receptors than wild type ovine leptin, but it is completely unable to activate signaling. In addition, a polyethylene glycol molecule was attached to SOLA to prolong its half-life (20, 22).

Experimental procedures were as follows. Lambs received unlimited amounts of a commercial milk replacer (Milk Specialties Global, Carpentersville, IL; Table 5.1). Milk replacer was reconstituted with water at 18.8% dry matter and served at 0900 h. The milk replacer was replenished if needed at 1600 and 2000 h. Offered volume was incrementally adjusted on daily basis. Body weight (BW) was recorded daily at 1600 h. Blood samples were obtained by jugular venipuncture within 2 h of birth. Thereafter, blood samples were obtained at 1600 h on postnatal day 1, every other day between day 2 to 20 and daily between day 28 to 35. Blood samples were collected in 6-ml tubes containing 90 USP lithium heparin. Plasma was prepared by centrifugation and stored at -20°C until analyzed for glucose and selected hormones.

Tissue collection and body composition. All lambs were killed on day 40 of postnatal life by IM injection of Xylazine (55 µl/kg LW, AnaSed, Lloyd Inc. IA) followed by IV injection of Propofol (4 mg/kg LW, PropoFlo, Abbott Laboratories, IL). The head was severed immediately between the cranium and the first cervical vertebrae and processed for isolation of the hypothalamus. Blood was collected and weighed. The gastrointestinal (GI) tract was dissected and weighed before and after contents were removed. Omental, perirenal, retroperitoneal and pericardial fat depots were dissected and weighed separately. The viscera fraction was obtained by combining GI tract, blood, visceral fat depots and visceral organs (respiratory tract, heart, liver and spleen) whereas the carcass fraction consisted of the carcass (without the head), skin and hoofs.

Each fraction was grounded separately by extrusion through grinding plates (Viscera: 2x through a 13-mm plate and 3x through a 4-mm plate; Carcass: 1x through a 50-mm kidney plate, 3x through a 13-mm plate and 3x through a 4-mm plate). A 250 g subsample of each fraction was freeze-dried for determination of dry matter. The freeze-dried samples were then pulverized with dry ice using a Waring blender and stored at

Table 5.1 Nutrient composition of milk replacer.

Nutrient	-- per kg dry matter --
Gross energy, Mcal	5.87
Crude protein, g	253
Fat, g	302
Nitrogen free extract, g	363
Ash, g	81
Vitamin A, KIU	44.0
Vitamin D3, KIU	11.0
Vitamin E, IU	330

-20°C until analyzed for nitrogen, ether extract and ash content. Nitrogen (N) content was measured by macro-Kjeldahl digestion (23) with steam distillation into boric acid using a Kjeltec 2300 (FOSS Analytical AB, Sweden). Crude protein was calculated as N x 6.25. Fat content of body fractions was determined by petroleum-ether extraction (24). Ash content was determined by incinerating samples in a furnace at 506 °C for 16 h. All samples were analyzed in duplicate. Milk replacers were analyzed similarly except that a factor of 6.38 was applied to N content to calculate CP and fat content was measured by an hexane/isopropanol extraction (25).

Glucose and hormones analyses. Plasma glucose was measured by the glucose oxidase method (510A; Sigma Chemical, St. Louis, MO) (26). Total plasma thyroxine (T4) was measured using a commercial solid-phase RIA (Coat-a-Count, Siemens, Germany) previously validated in the sheep (27). Internal RIA previously validated with ovine plasma were used to measure the plasma concentration of insulin, IGF-1 and leptin. These RIA use bovine proteins for iodination and standards. The insulin RIA was performed with a guinea pig anti-bovine insulin primary antibody (Sigma I-6136) and a goat anti-guinea pig secondary antibody (Equitech-Bio Inc., Kerrville, TX) (28). For IGF-1, IGF-binding proteins were first removed by acid-ethanol extraction. Supernatant was then analyzed using an IGF-1 assay based on a rabbit anti-human IGF-1 primary antibody (lot AFP4892898; National Hormone and Peptide Program) and a caprine anti-rabbit γ-antibody (lot 12515; Biotech Source Inc.) (26). Leptin was analyzed with a bovine leptin RIA previously validated in sheep (28). The same assay was used for samples obtained during SOLA treatment, except that SOLA was used to generate the standard curve. Inter- and intra-assay coefficients of variation were less than 12% for the SOLA leptin assay and less than 7% for all other assays.

Calculations. Daily feed intake was calculated by subtracting unconsumed milk replacer from the previous feed allowance. Average daily gain (ADG) was calculated for each individual as the slope of the regression of body weight over time. Relative intake and fractional weight gain were calculated by dividing daily intake or BW gain by the BW of the previous day. Metabolic body weight (MBW) was calculated by scaling live body weight to the 0.58 power based on studies showing that this exponent relates more closely to basal energy expenditure in neonatal lambs than the commonly used 0.75 (29). Empty body weight (EBW) is the sum of wet viscera and beheaded carcass weights. Indices of fatness (mass of fat depots, visceral fat or carcass fat) were expressed as % of EBW. Composition of the EBW was calculated from the composition of viscera and carcass fraction.

Statistical analyses. All data were analyzed by ANOVA.

Effects of Early and Late SOLA treatment were analyzed separately. For Early SOLA, averages were calculated for intake and growth data (e.g. intake, FWG, etc.) for both the treatment period (day 1 to 14) and post-treatment period (day 15 to 39). These data were analyzed separately with a model accounting for SOLA effects (saline vs. SOLA). Repeated measures data (e.g. intake, body weight, plasma variables) were analyzed with the MIXED procedure of SAS using a model accounting for SOLA treatment, day and their interaction as fixed effects and animal as the random effect.

For the Late SOLA, the treatment period was day 30 to 35 and the post-treatment period was day 36 to 39. Procedures and models used were the same as for the Early SOLA analysis except for the following: pre-treatment body weight (average of day 25 to 29) was included as a covariate for any intake or growth variables not already

normalized by body weight. Statistical significance and tendencies were set at $P<0.05$ and $P<0.15$ for main effects and $P<0.10$ and $P<0.15$ for interactions.

Results

Effect of leptin antagonist therapy from birth to day 14 of post-natal life.

Intake and Growth. Treatments were initiated before any colostrum or milk was provided. Both groups weighed exactly the same at birth (Table 5.2). Leptin antagonist therapy tended to cause a 26% increase in absolute dry matter and energy intake when averaged over the entire experimental period ($P = 0.12$). The same effects were observed even when intake of dry matter and energy were expressed relative to body weight or MBW ($P = 0.10$). Examination of the daily profile shows that tendency of SOLA treatment to increase intake variables was seen throughout the treatment period (Fig. 5.1 and 5.2, treatment period, SOLA, $P = 0.13$ or less).

Consistent with intake data, SOLA treated lambs showed greater absolute average daily gain and fractional weight gain (Table 5.2, $P<0.04$ or less). In agreement with this effect, SOLA treatment increased total body weight gain (Table 5.2, $P<0.07$) and the profile of body weight over time (Fig. 5.3, treatment period, SOLA x Day, $P<0.03$). SOLA treatment had no effect on the rate of linear growth expressed as CRL gain (Table 5.2).

Next, carry-over effects of early SOLA therapy were assessed between day 15 to 39 of postnatal life. When averaged over this period, all intake and growth parameters were identical between Control and SOLA groups, including body weight on day 39 (Table 5.3). In agreement with these data, Control and SOLA treated lambs had an overlapping profile of absolute intake and relative dry matter intake over this period of time (post-treatment period, Fig. 5.1 and 5.2). Moreover, profiles of body weight over time became parallel (post-treatment period, Fig. 5.3).

Table 5.2 Effect of early leptin antagonist therapy on intake and growth during the treatment period (birth to day 14 of postnatal life).

	Treatment[b]			
Variable[a]	**Control**	**SOLA**	**SE**	**Probability level[c]**
Dry matter intake				
Absolute, g/d	193	244	22	0.12
Relative to LW, g/kg • d	37	43	2	0.10
Energy intake				
Absolute, kcal/d	1131.3	1433.6	127.2	0.12
Relative to LW, kcal/kg • d	218.9	254.0	13.5	0.10
Relative to MBW, kcal/$kg^{0.58}$• d	433.8	521.6	34.1	0.10
Growth				
Birth weight, kg	3.61	3.64	0.21	NS
Weight gain, kg	3.05	3.95	0.32	0.07
Weight on day 15, kg	6.66	7.59	1.08	NS
ADG, kg/day	0.19	0.26	0.02	<0.03
FWG, %/day	4.2	5.2	0.3	<0.04
CRL gain, cm/day	0.6	0.6	0.1	NS

[a]LW, live weight; MBW, metabolic body weight ($kg^{0.58}$); ADG, average daily gain; FWG, fractional weight gain; CRL, crown-rump length.
[b]Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6).
[c]Type I error probability for the effect of leptin antagonist . NS = $P>0.15$.

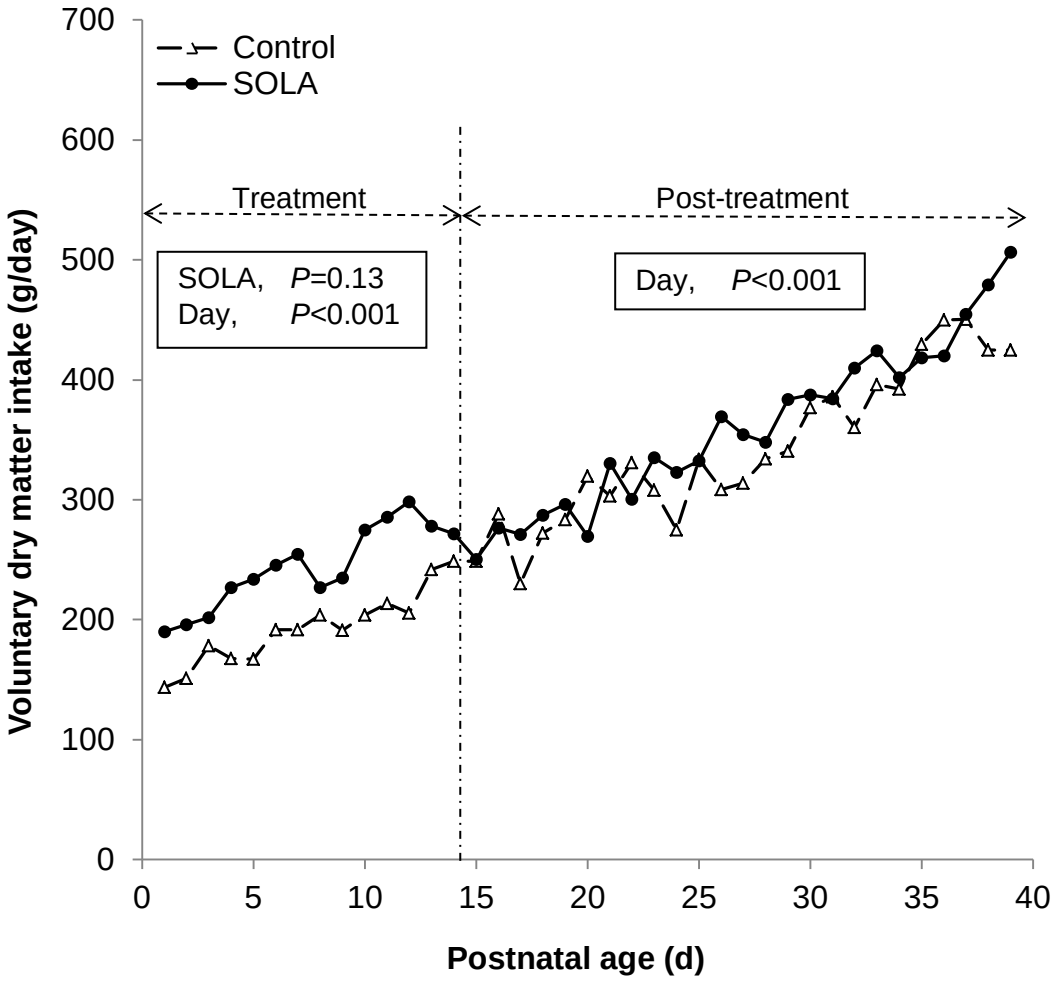

Figure 5.1 Effect of leptin antagonist therapy on voluntary dry matter intake in early postnatal life.

Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6). Treatment and post-treatment periods were analyzed separately. The P-values are given only for effects that were significant (P<0.05) or tended to be significant (P<0.15). The pooled SE during the treatment period was 26 and 34 during the post-treatment period.

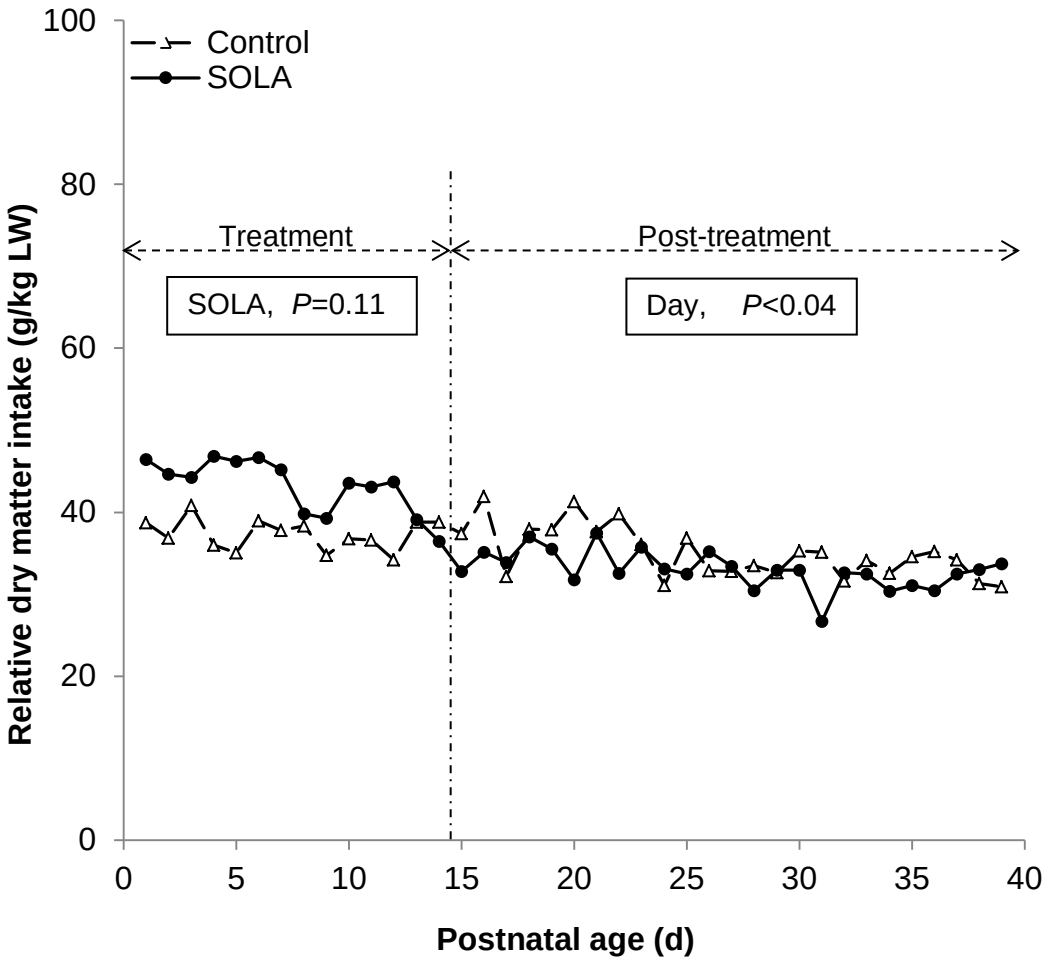

Figure 5.2 Effect of leptin antagonist therapy on relative dry matter intake in early postnatal life.

Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6). Treatment and post-treatment periods were analyzed separately. The P-values are given only for effects that were significant (P<0.05) or tended to be significant (P<0.15). The pooled SE during the treatment period was 3.8 and 2.6 during the post-treatment period.

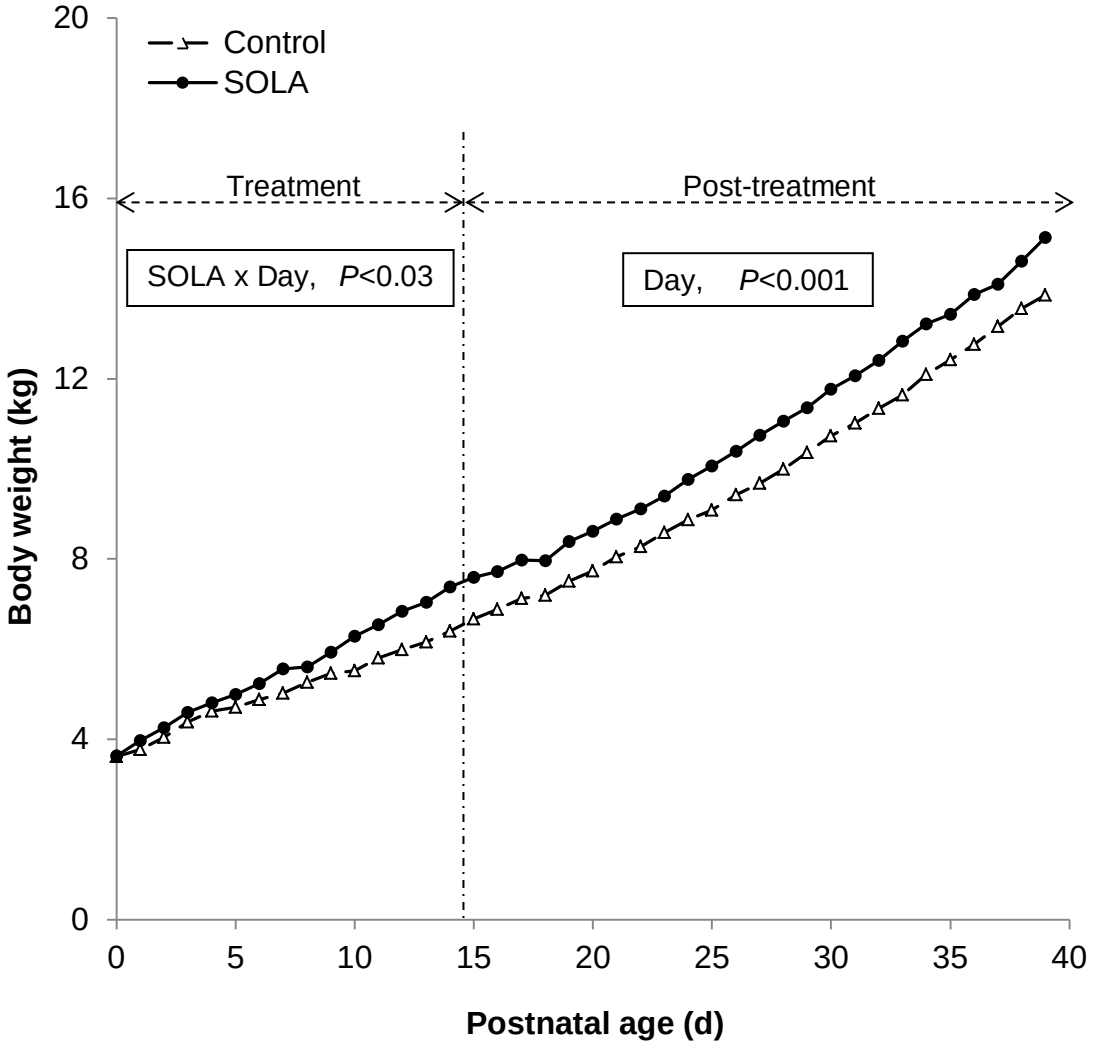

Figure 5.3 Effect of leptin antagonist therapy on body weight in early postnatal life.

Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6). Treatment and post-treatment periods were analyzed separately. The P-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE during the treatment period was 0.35 and 0.70 during the post-treatment period.

Table 5.3 Effect of early leptin antagonist therapy on intake and growth during the post-treatment period (day 15 to 39 of postnatal life).

	Treatment[b]			
Variable[a]	**Control**	**SOLA**	**SE**	**Probability level[c]**
Dry matter intake				
Absolute, g/d	343	357	24	NS
Relative to LW, g/kg • d	35	33	1	NS
Energy intake				
Absolute, kcal/d	2012.4	2097.6	143.3	NS
Relative to LW, kcal/kg • d	205.6	192.9	6.3	NS
Relative to MBW, kcal/$kg^{0.58}$• d	531.6	520.9	21.9	NS
Growth				
Weight gain, kg	7.19	7.55	0.46	NS
Weight on day 39, kg	13.85	15.14	0.85	NS
ADG, kg/day	0.30	0.32	0.02	NS
FWG, %/day	3.2	3.0	0.1	0.14

[a]LW, live weight; MBW, metabolic body weight ($kg^{0.58}$); ADG, average daily gain; FWG, fractional weight gain.
[b]Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6).
[c]Type I error probability for the effect of leptin antagonist. NS = P>0.15.

Finally, body composition was analyzed on day 40 of postnatal life, 26 days after the end of treatment (Table 5.4). Leptin antagonist therapy during the first 14 days of post-natal life did not have any effect on empty body weight, its composition or distribution in viscera and carcass fractions at day 40. Also, fatness assessed in carcass, viscera or EBW did not differ between treatments. SOLA treatment had no effect on the mass of visceral fat depots considered individually or as a group.

Plasma variables. The concentration of plasma SOLA was measured every 2 days during treatment and over the first 6 days post-treatment. All samples were obtained 8-9 hours after the first IV injection. Plasma SOLA rose rapidly to reach a near steady concentration of 970 ng/ml by day 4 of treatment (Fig. 5.4). Over the same period, plasma leptin in Control animals averaged 4.2 ng/ml and was nearly constant (Fig. 5.4). Accordingly, plasma SOLA excess over the endogenous plasma leptin concentration during the treatment period was 237-fold. As expected, plasma SOLA dropped after treatment, reaching 175 ng/ml 56 h after last injection, and 20 ng/ml after another 56 h. SOLA treatment had no effect on the concentration of plasma glucose, insulin or T4 when averaged over the treatment period (day 1 to 14, Table 5.5). SOLA treatment was also devoid of effect over time on these variables with the single exception of glucose: SOLA caused on elevation in plasma glucose over the first 2 day of treatment (Fig. 5.5; SOLA x Day, $P<0.03$) but failed to have the same effect on plasma insulin (Fig. 5.6).

Table 5.4 Effect of early leptin antagonist therapy on body composition on day 40.

Variable[a]	Treatment[b] Control	Treatment[b] SOLA	SE	Probability level
EBW, kg	11.5	12.7	0.8	NS
Carcass				
Fraction of EBW (%)	80.9	80.1	0.8	NS
Fat (% EBW)	13.8	14.2	1.0	NS
Viscera				
Fraction of EBW (%)	19.1	19.9	0.8	NS
Fat (% EBW)	4.1	4.3	0.5	NS
Visceral fat depot (% of EBW)				
Perirenal	1.0	1.1	0.2	NS
Omental	1.7	2.0	0.2	NS
Retroperitoneal	1.4	1.4	0.2	NS
Pericardial	0.3	0.3	0.1	NS
All depots	4.4	4.8	0.6	NS
Composition (% of EBW)				
Water	62.9	62.6	1.4	NS
Protein	15.4	15.0	0.2	NS
Fat	17.9	18.5	1.4	NS
Ash	2.9	2.8	0.1	NS

[a]EBW, empty body weight.
[b]Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6).
[c]Type I error probability for the effect of leptin super antagonist. NS = *P*>0.15.

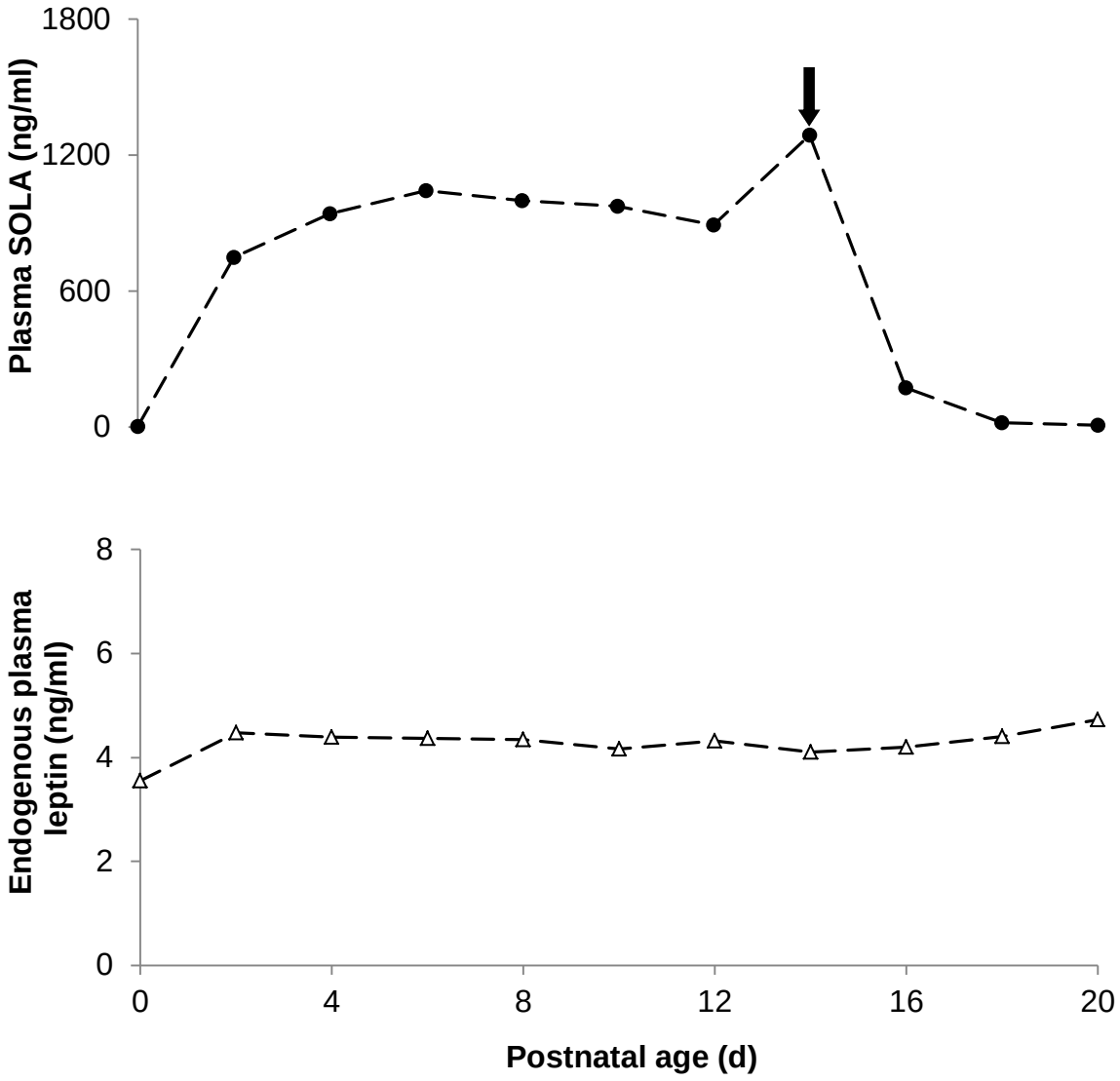

Figure 5.4 Concentration of endogenous plasma leptin or leptin antagonist during treatment.

Lambs were treated from birth (before first feeding) until day 14 of postnatal life with twice daily injection of either excipient (Control, n = 6) or leptin antagonist (SOLA, n=6). Blood samples were obtained in the indicated days at 1600 h, 8 hours after the first daily IV injection. Arrow indicates the last day of treatment. Plasma SOLA was measured only in SOLA-treated lambs (top panel), and endogenous plasma leptin was measured only in control animals (bottom panel). The pooled SE was 241 for SOLA and 0.4 for leptin.

Table 5.5 Effect of leptin antagonist therapy on the plasma concentration of glucose and selected hormones during treatment period (birth to day 14 of postnatal life).

Variable	Treatment[a]		SD	Probability level[b]
	Control	SOLA		
Glucose, mg/dl	101	105	8	NS
Insulin, ng/ml	1.9	2.2	1.8	NS
Total T4, nmol/l	94	105	17	NS
Leptin, ng/ml	4.2	----	0.3	----
SOLA, ng/ml	----	985	165	----

[a]Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n=6) or leptin antagonist (PEGSOLA, n=6).
[b]Type I error probability. NS = $P>0.15$.

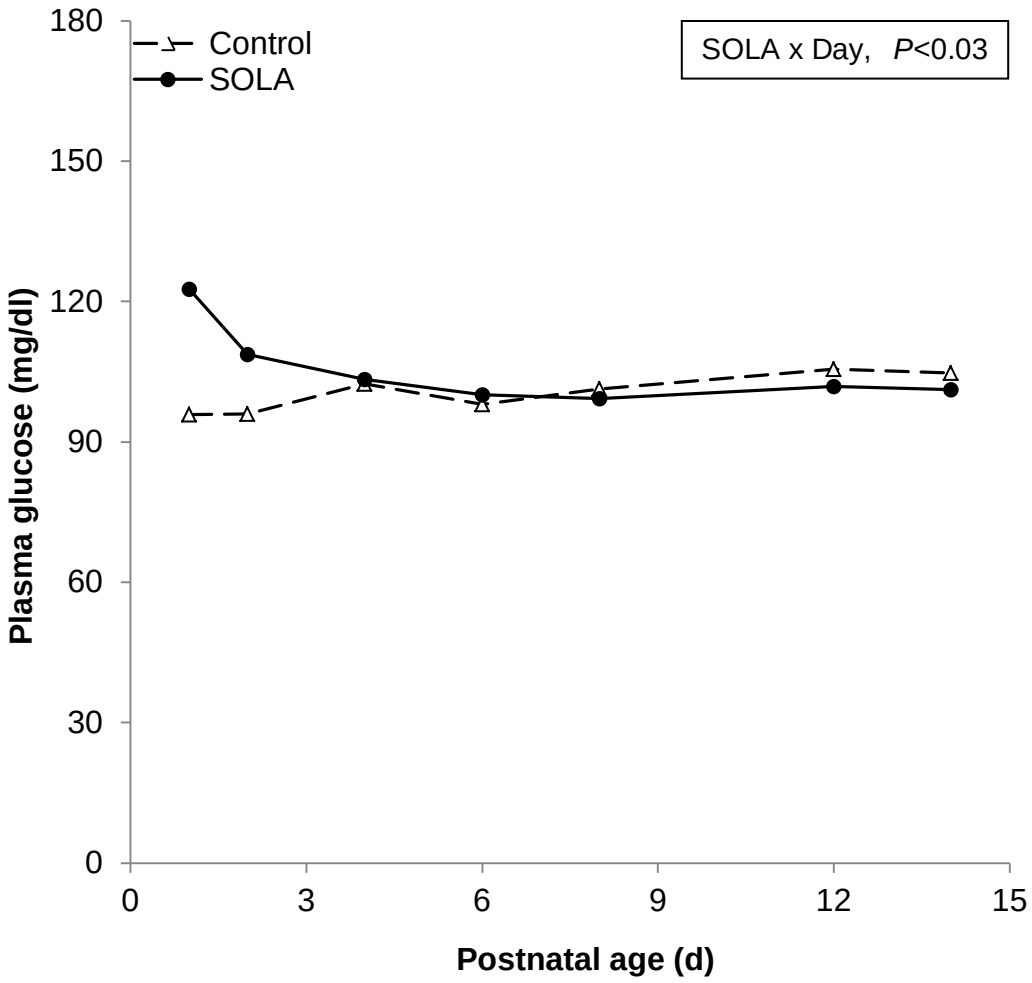

Figure 5.5 Effect of leptin antagonist therapy on plasma glucose in early postnatal life.

Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6). Blood samples were obtained in the indicated days at 1600 h, 8 h after the first daily IV injection. Treatment and post-treatment periods were analyzed separately. The *P*-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE during the treatment period was 5.7.

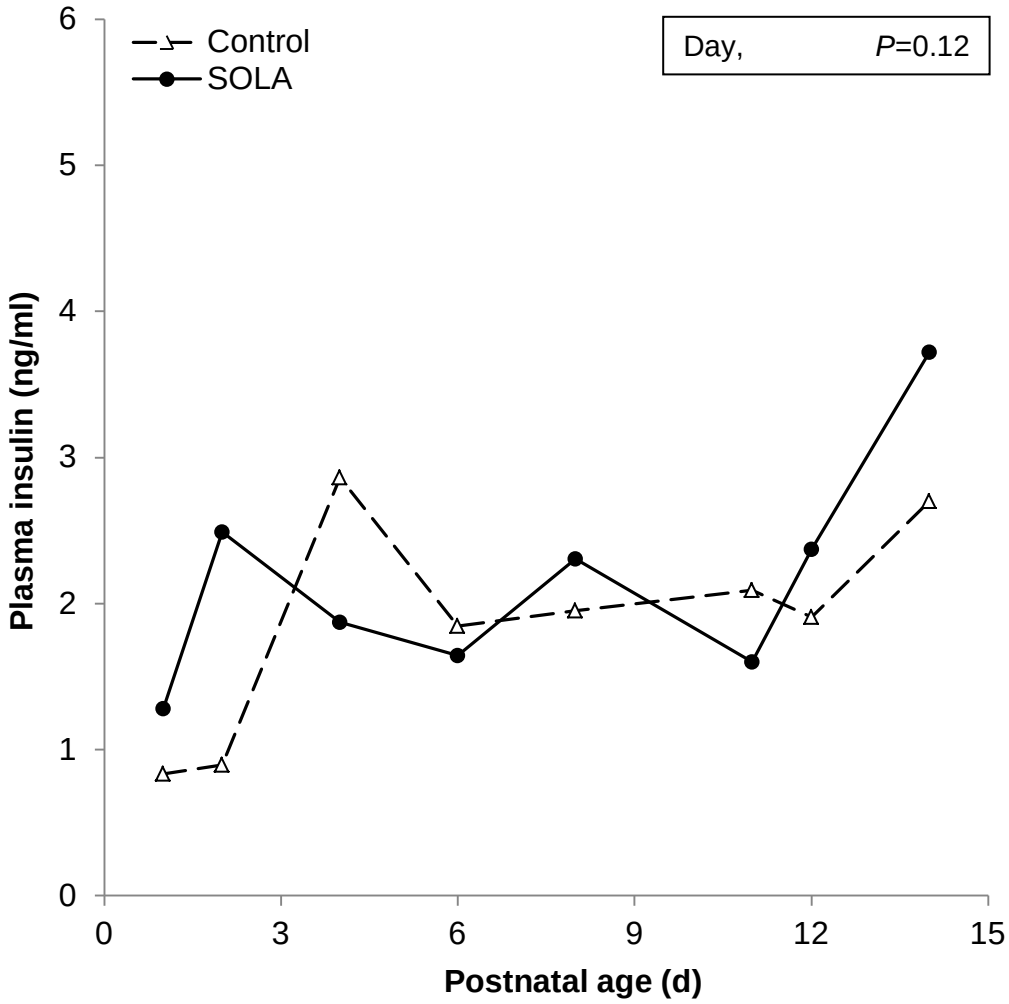

Figure 5.6 Effect of leptin antagonist therapy on plasma insulin in early postnatal life.

Lambs were treated between birth (before first feeding) and day 14 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 6). Blood samples were obtained in the indicated days at 1600 h, 8 h after the first daily IV injection. Treatment and post-treatment periods were analyzed separately. The *P*-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE during the treatment period was 1.8.

Effect of leptin antagonist therapy from day 30 to 35 of postnatal life.

Intake and Growth. A second group of lambs received twice daily IV injection of SOLA between day 30 and 35 of postnatal age, and were compared to the original Control group injected with saline solution. This second group of SOLA-treated lambs tended to be heavier than Control lambs at birth (Control vs. Late SOLA, 3.6 vs. 4.0 kg, P=0.12), and significantly heavier at both day 15 (6.7 vs. 8.0 kg, P<0.02) and before treatment on day 29 (10.4 vs. 12.6, P<0.01). These differences occurred even though they were subjected to the same exact experimental procedures as the original Control group, including twice daily IV saline injection between birth and day 14. Accordingly the pre-treatment body weight (average of day 25 to 29) was used as a covariate for the analysis of treatment and post-treatment effects on weight dependent variables (intake, ADG and BW).

During treatment, SOLA had no effect on DM or energy intake variables averaged over the treatment period (Table 5.6) or analyzed over time (Fig. 5.7 and 5.8). This lack of effect was also seen for all growth variables (Table 5.6 and Fig. 5.9), except for the profile of body weight over time: SOLA-treated lambs showed a steeper increase in BW during the treatment period (Fig. 5.10, SOLA x Day, treatment period, P<0.05).

Performance was also measured for 5 days after last injection (day 36 to 39 of postnatal age). Dry matter intake was numerically lower for SOLA than control animals and tended towards statistical significance when corrected for LW (Table 5.7, P = 0.11). The profiles over time suggest that these effects were initiated at cessation of treatment and remained constant over the entire post-treatment period (Fig. 5.7 and 5.8). In particular, the carryover effect of SOLA on relative dry matter intake was significant when analyzed over time (Fig. 5.8, post-treatment, SOLA, P = 0.05). Consistent with carryover effects of SOLA on intake variables, FWG was lower for SOLA lambs when

Table 5.6 Effect of leptin antagonist therapy on intake and growth during the treatment period (day 30 to 35 of postnatal life).

	Treatment[c]			
Variable[a]	**Control**	**SOLA**	**SD**	**Probability level[d]**
Dry matter intake				
Absolute[b], g/d	442	431	59	NS
Relative to LW, g/kg • d	34	33	3	NS
Energy intake				
Absolute[b], kcal/d	2591.7	2527.8	344.3	NS
Relative to LW, kcal/kg • d	199.0	195.1	17.6	NS
Relative to MBW, kcal/kg$^{0.58}$• d	553.9	592.1	66.8	NS
Growth				
Weight on day 29, kg	10.36	12.58	1.28	<0.008
Weight gain[b], kg	2.76	2.84	0.60	NS
Weight on day 36[b], kg	14.35	14.49	0.66	NS
ADG[b], kg/day	0.34	0.43	0.11	NS
FWG, %/day	3.1	3.2	0.5	NS

[a]LW, live weight; MBW, metabolic body weight (kg$^{0.58}$); ADG, average daily gain; FWG, fractional weight gain.
[b]Adjusted by covariate analysis for pre-treatment body weight.
[c] [c]Lambs were treated from day 30 to 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8).
[d]Type I error probability for the effect of leptin antagonist. NS = *P*>0.15.

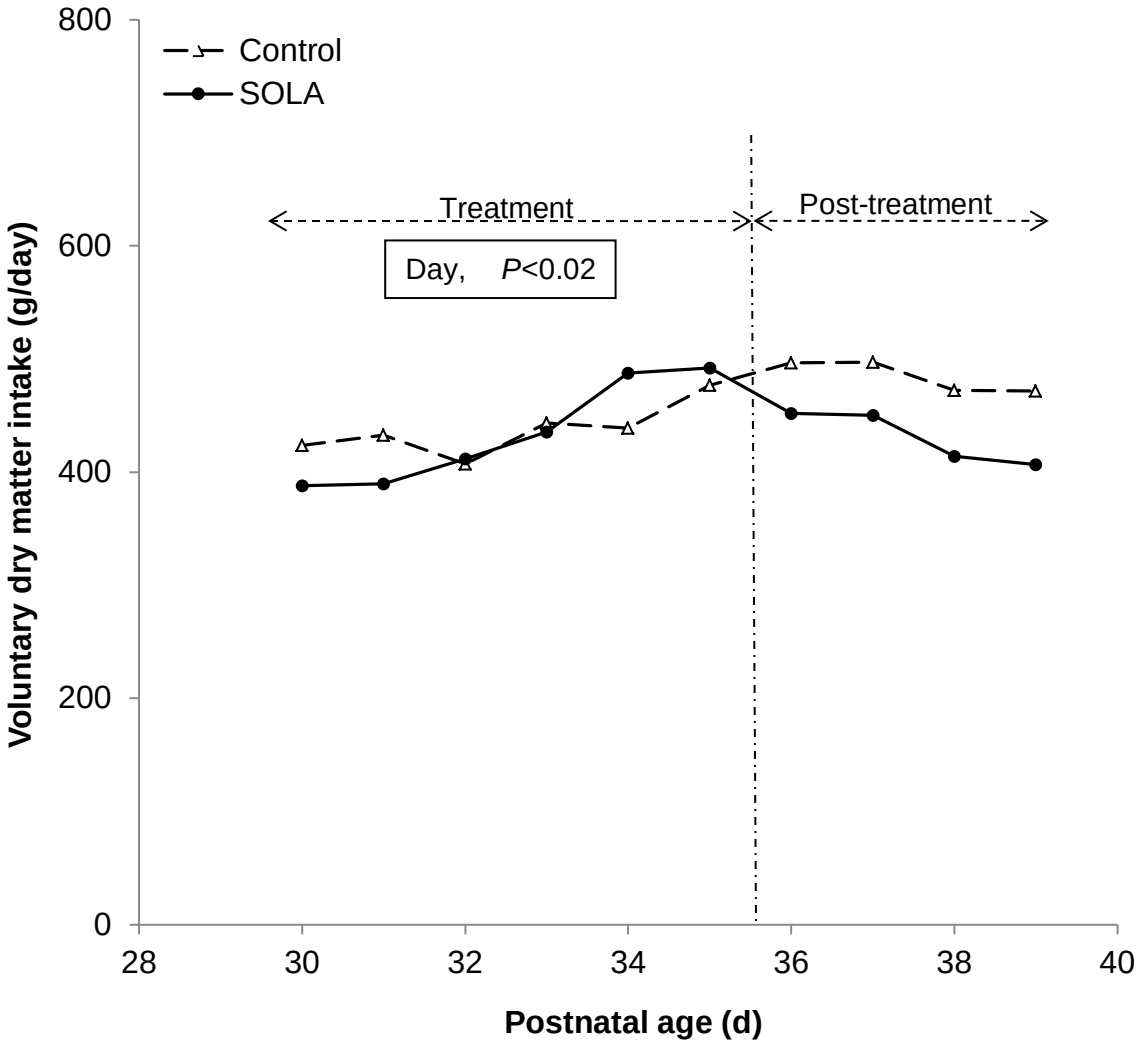

Figure 5.7 Effect of leptin antagonist therapy on voluntary dry matter intake in late postnatal life.

Lambs were treated between day 30 and 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8). Treatment and post-treatment periods were analyzed separately. The *P*-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE were 26.7 for n = 8 and 30.9 for n = 6 during the treatment period, and 34.4 for n = 8 and 40.3 for n = 6 during the post-treatment period.

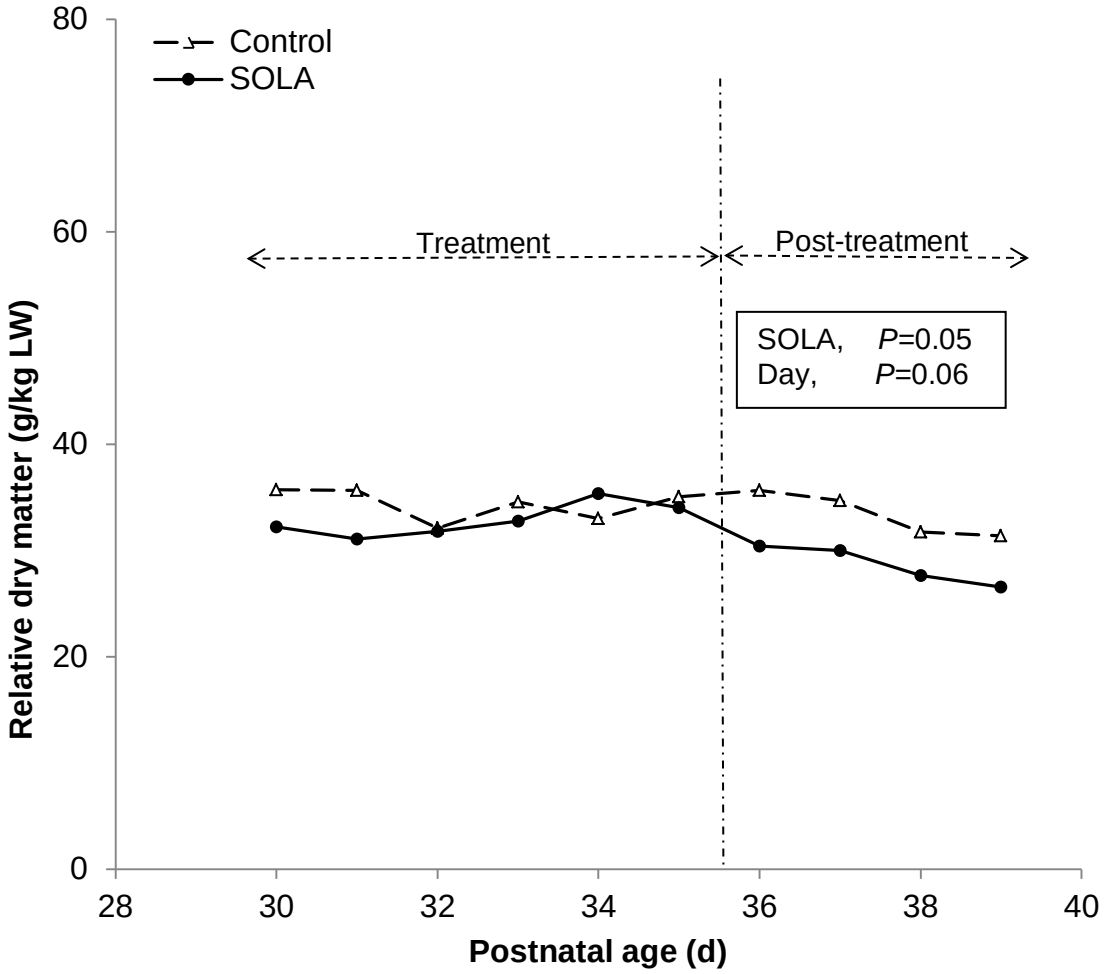

Figure 5.8 Effect of leptin antagonist therapy on relative dry matter intake in late postnatal life.

Lambs were treated between day 30 and 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8). Treatment and post-treatment periods were analyzed separately. The *P*-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE were 1.9 for n = 8 and 2.2 for n = 6 during the treatment period, and 2.0 for n = 8 and 2.3 for n = 6 during the post-treatment period.

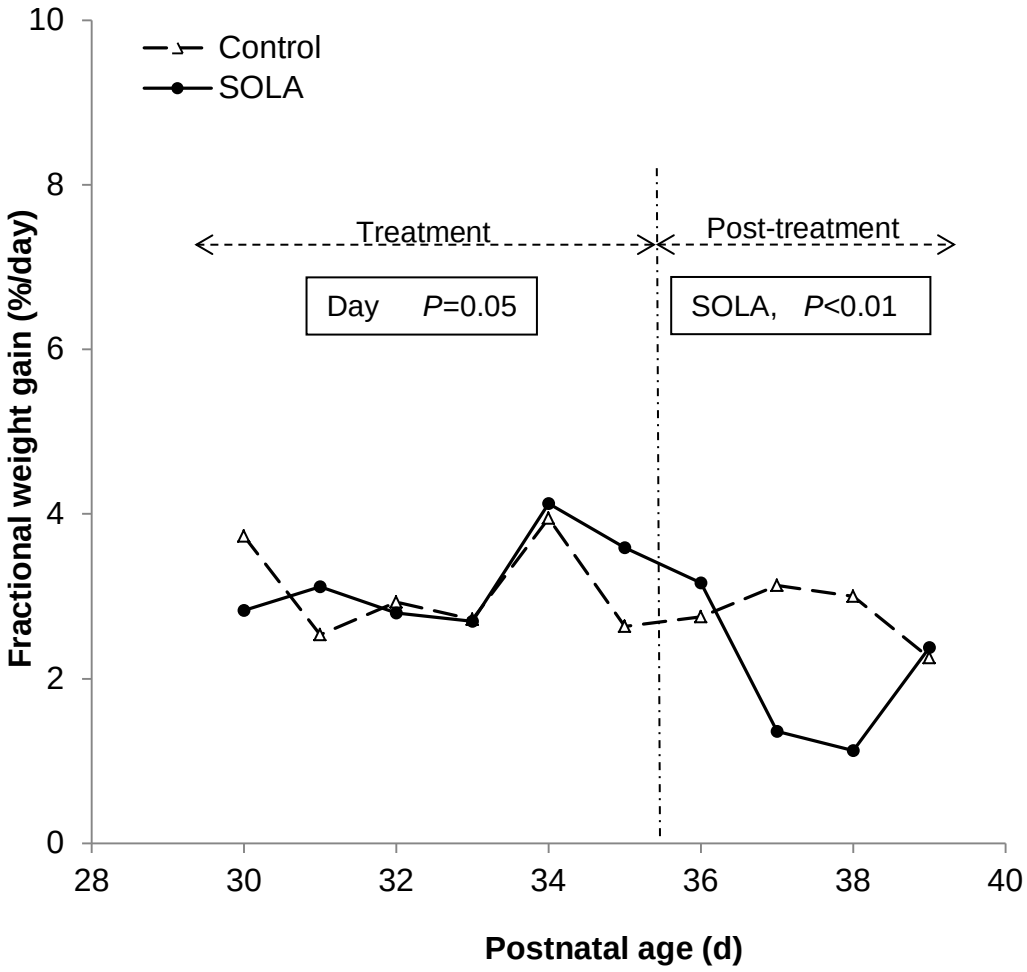

Figure 5.9 Effect of leptin antagonist therapy on fractional weight gain in late postnatal life.

Lambs were treated between day 30 and day 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8). Treatment and post-treatment periods were analyzed separately. The *P*-values are given only for effects that were significant (*P*<0.05) or tended to be significant (*P*<0.15). The pooled SE were 0.4 for n = 8 and 0.5 for n = 6 during the treatment period, and 0.6 for n = 8 and 0.7 for n = 6 during the post-treatment period.

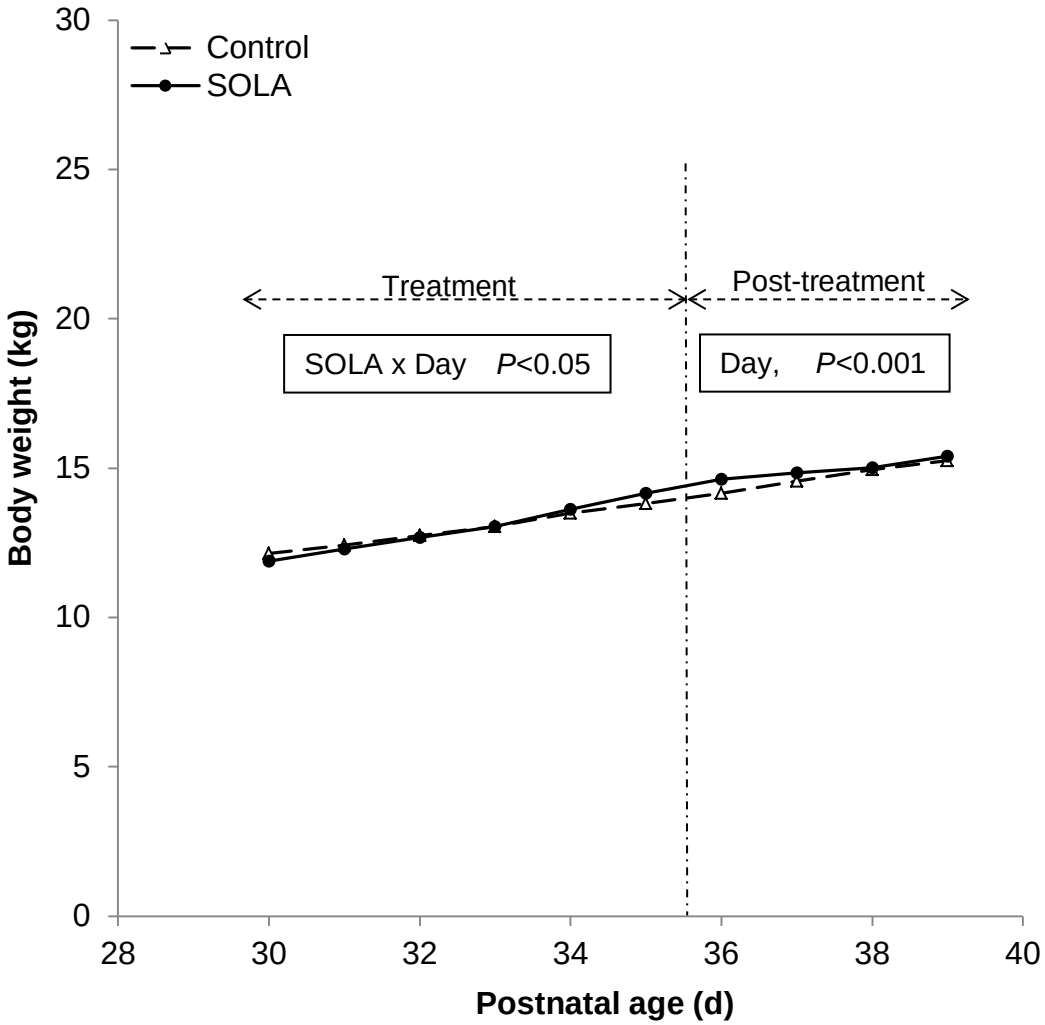

Figure 5.10 Effect of leptin antagonist therapy on body weight in late postnatal life.

Lambs were treated between day 30 and day 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8). Treatment and post-treatment periods were analyzed separately. The *P*-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE was 0.2 during the treatment period and 0.3 during the post-treatment period for both groups.

analyzed as an overall average (Table 5.7, SOLA, $P<0.05$) or over time (Fig. 5.9, post-treatment, SOLA $P<0.01$).

Plasma variables. Plasma SOLA progressively increased linearly over the first 3 days of treatment and plateaued to 1543 ng/ml over the last 3 day of treatment (Fig. 5.11). The plasma leptin concentration over the same period averaged 5 ng/ml in the Control animals (Fig. 5.11). Accordingly, plasma SOLA concentration was ~309-fold higher than endogenous leptin over the last 3 days of treatment (Fig. 5.11). The plasma SOLA concentration dropped to 423 and 87 ng/ml by 56 and 112 h after the last injection. Plasma concentrations of glucose and various metabolic hormones were similar before treatment (day 28 and 29 of age), with the exception of plasma T4, which tended to be higher in the SOLA group (Control vs. SOLA, 135 vs. 156 nmol/l, $P<0.10$). During treatment, SOLA had no effect on the profile of plasma concentration of glucose and insulin over time (results not shown) or on the average concentrations during the treatment period (Table 5.8). In contrast SOLA caused a progressive reduction in plasma T4 over the first 3 days of treatment followed by a maximal reduction over the last 3 days (Fig. 5.12, SOLA x Day, $P<0.007$).

Table 5.7 Effect of leptin antagonist therapy on intake and growth during the posttreatment period (day 36 to 39 of postnatal life).

	Treatment[c]			
Variable[a]	**Control**	**SOLA**	**SD**	**Probability level[d]**
Dry matter intake				
Absolute[b], g/d	482	433	82	NS
Relative to LW, g/kg • d	33	29	4	0.11
Energy intake				
Absolute[b], kcal/d	2826.4	2540.2	478.8	NS
Relative to LW, kcal/kg • d	193.0	170.2	24.7	0.11
Relative to MBW, kcal/$kg^{0.58}$• d	571.6	545.6	82.5	NS
Growth				
Weight on day 36 kg	14.35	14.48	0.66	NS
Weight gain[b], kg	1.35	0.99	0.56	NS
Weight on day 39[b], kg	15.71	15.48	0.95	NS
ADG[b], kg/day	0.33	0.27	0.14	NS
FWG, %/day	2.8	2.0	0.6	<0.05

[a]LW, live weight; MBW, metabolic body weight ($kg^{0.58}$); ADG, average daily gain; FWG, fractional weight gain.
[b]Adjusted by covariate analysis for pre-treatment body weight.
[c] Lambs were treated from day 30 to 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8).
[d]Type I error probability for the effect of leptin antagonist. NS = P>0.15.

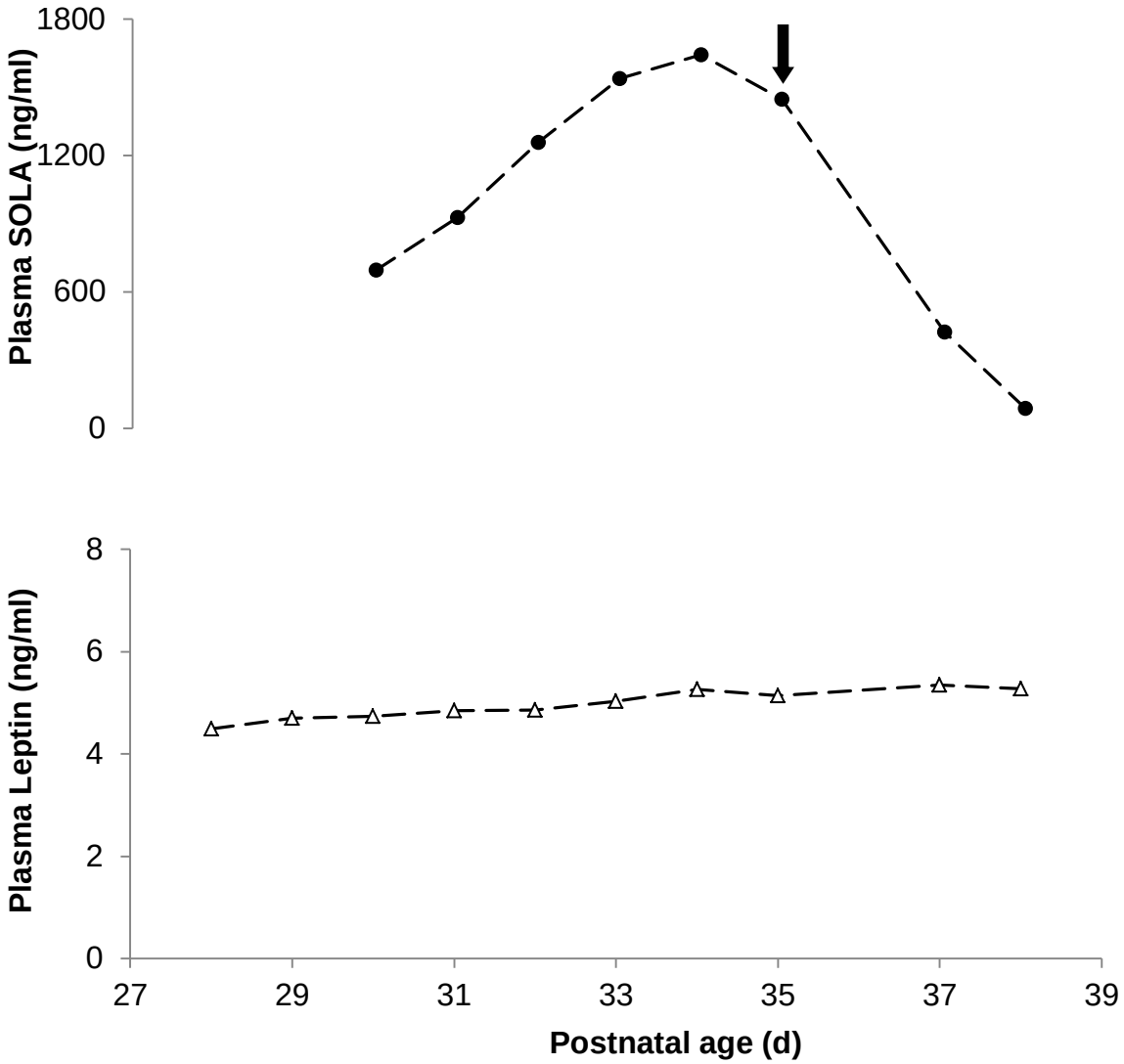

Figure 5.11 Concentration of endogenous plasma leptin or leptin antagonist during treatment.

Lambs were treated between day 30 and 35 of postnatal life with either excipient (Control, n=6) or leptin antagonist (SOLA, n=8). Blood samples were obtained in the indicated days at 1600 h, 8 hours after the first daily IV injection. Arrow indicates the last day of treatment. Plasma SOLA was measured only in SOLA-treated lambs (top panel), and endogenous plasma leptin was measured only in control animals (bottom panel). The pooled SE was 247 for SOLA and 0.5 for leptin.

Table 5.8 Effect of late leptin antagonist therapy on the plasma concentration of glucose and selected hormones during treatment period (day 30 to 35 of postnatal life).

Variable	Treatment[a]		SD	Probability level[b]
	Control	SOLA		
Insulin, ng/ml	3.7	5.3	1.8	NS
Glucose, mg/dl	110	105	6	NS
Leptin, ng/ml	5.0	---	0.2	---
SOLA, ng/ml	---	1252	371	---

[b]Lambs were treated between day 30 and day 35 of postnatal life with either excipient (Control, n=6) or leptin antagonist (SOLA, n=8).
[c]Type I error probability. NS = *P*>0.15.

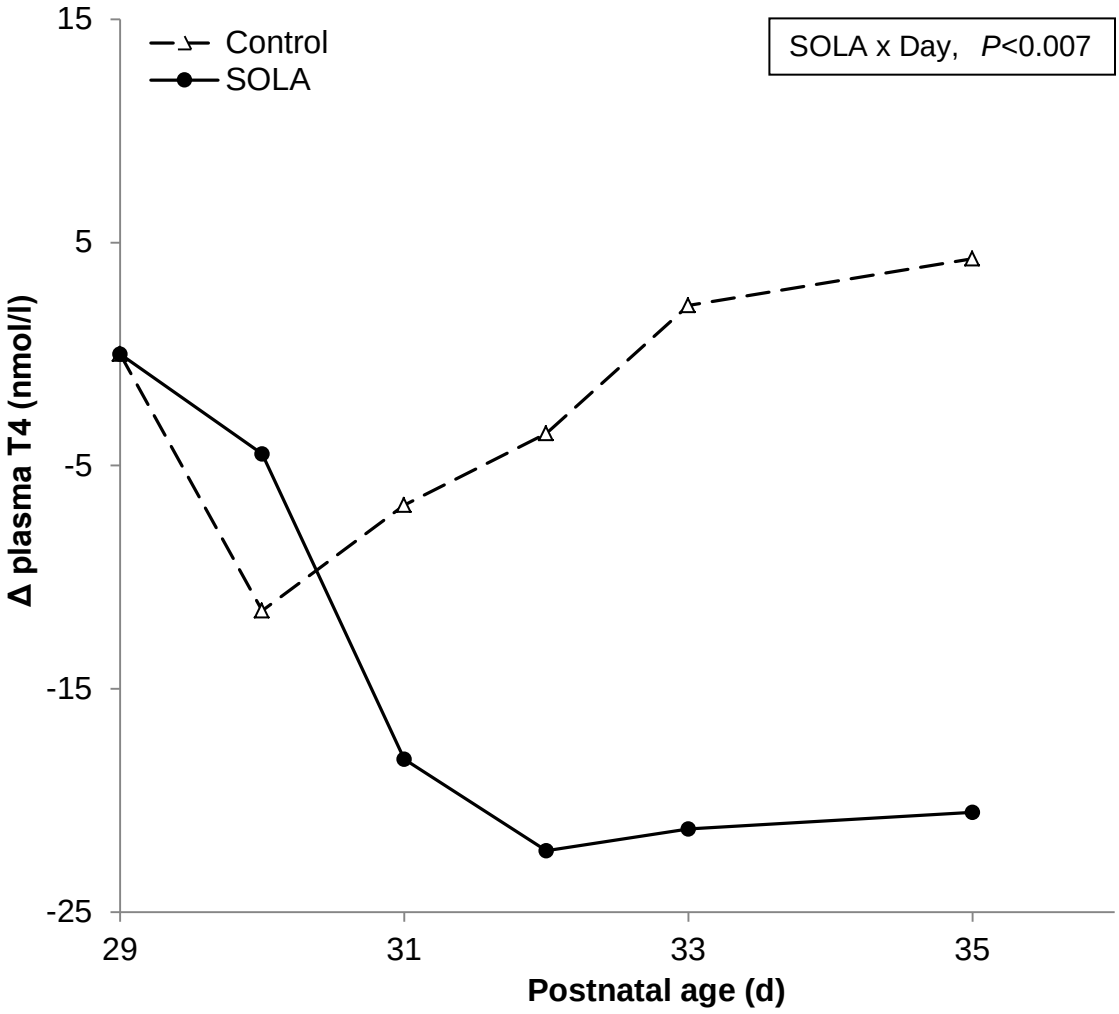

Figure 5.12 Effect of late leptin antagonist therapy on plasma delta T4 in late postnatal life.

Lambs were treated between day 30 and 35 of postnatal life with either excipient (Control, n = 6) or leptin antagonist (SOLA, n = 8). For each day of treatment, the change in plasma T4 concentration (Δ plasma T4) was calculated as the difference from pre-treatment plasma T4 (average of day 28 and 29 of age). The *P*-values are given only for effects that were significant ($P<0.05$) or tended to be significant ($P<0.15$). The pooled SE were 4.6 for n = 8 and 5.3 for n = 6.

Discussion

Leptin is known to induce hypothalamic signaling in mice during the first 16 days of life without changes in body weight or body fat content (15, 16). This is thought to reflect the immature state of axonal connections between the ARC and the more distal hypothalamic nuclei responsible for intake regulation (13, 15). Nevertheless, neonatal leptin is crucial because it acts as a neurotrophic factor driving the extension of ARC-derived axons towards their distal targets. In rodents, leptin appears to play this role within a small developmental window. First, rodents experience a spike in plasma leptin during the first two weeks of life (30). It is during this time window that leptin treatment efficiently promotes the growth of hypothalamic axonal projections in *ob*/*ob* mice (13). Interestingly, disruption of leptin concentration or its signaling during early life appears to be the mechanism whereby many fetal nutritional insults lead to an enhanced appetite and excessive fat deposition in postnatal life (5, 12, 31). For example, maternal undernutrition during gestation abolishes the leptin early postnatal spike in IUGR pups and appears to disrupt the normal development of the hypothalamic network responsible for energy homeostasis (12). In contrast, early leptin therapy of IUGR rat pups is sufficient to correct later disruption of energy metabolism including excessive appetite and obesity in later life (5). Whether leptin plays a similar sole in humans and other precocial animals remains largely undefined. The sheep is considered an appropriate model because the ontogeny of hypothalamic development is thought to approximate that of primates, including humans (17–19).

Studies of leptin action in the sheep have been limited to juvenile or adult animals. It has been demonstrated that intracerebroventricular infusion of leptin can reduce voluntary intake, increase muscle thermogenesis and GH secretion in well-fed animals, as well as normalize LH secretion in long-term food restricted ewes (32–34).

Remarkably, peripheral infusion of exogenous leptin is ineffective in affecting intake or endocrine changes in well-fed sheep (35) but is effective in correcting LH pulses of underfed ewes only when they are ovarectomized (36). This might indicate that in normal precocial animals, like sheep, leptin therapy is effective only when leptin is abnormally low. Accordingly, as a first step to assess a functional role of leptin in neonatal sheep, we opted for a strategy that would reduce naturally occurring leptin signaling.

Gertler et al. (37) initiated the development and evaluation of leptin antagonists. The first generation of leptin antagonists was based on the mutagenesis of amino acid residues 39 to 41 with alanine (L39A/D40A/F41A). This leptin mutant has the same affinity for the receptor as wild-type leptin but has no signaling activity (37). The second generation of leptin-antagonist incorporated the additional substitution of aspartic acid residue 23 by a non-charged amino-acid (D23L), leading to an 8-fold increase in binding affinity for the leptin receptor (22). Finally, a polyethylene glycol molecule was attached to the molecule (20). Pegylation reduced renal clearance of leptin and induced 16-fold increase in half-life compared to wild-type leptin (20), thereby, increasing substantially the biological antagonistic activity of SOLA. Studies performed in the mouse with the pegylated mouse version showed that its predominant mode of action was competitive inhibition of blood to brain leptin transport (20). Shpilman et al. (21) administered the mouse version of this antagonist by SC injection to growing mice and used body weight change as an end point. The mouse version antagonist was maximally effective at the dose of 6.7 mg/kg/d (43% greater weight change compared to controls) and 70% as effective at the dose of 2.2 mg/kg/d (21). The equivalent 70% effective dose on a metabolic BW basis for a newborn lamb is 0.91 $mg/kg^{0.75}$. Because we delivered this leptin antagonist via intravenous injection, we settled for a daily dose of 0.46 $mg/kg^{0.75}$

during the early period of administration. This resulted in a 237-fold excess of SOLA vs. wild-type leptin. We chose to double this dose during the late SOLA treatment to counteract the natural rise in endogenous leptin production and achieved a 309-fold excess.

We chose to treat lambs at two distinct times during early postnatal life. In or initial study, we treated lambs for the first 14 days of postnatal life given the observation that leptin promotes hypothalamic axonal growth immediately after birth in rodents and that such an effect could have long-term impact on energy metabolism (13, 15). SOLA treatment did not statistically increase dry matter intake over this period. The profile of dry matter intake over time was nevertheless suggestive of leptin regulation. SOLA-treated lambs had a numerical increase in feed intake for the entire 14-day treatment and this effect was lost upon cessation of treatment. This was somewhat surprising given the notion that gut limitation is a primary regulator of food intake in immediate postnatal life (Chapter 3). Moreover, SOLA treatment was associated with a significant increase of growth that was lost as soon as treatment was discontinued (Fig. 3). Abdennebi-Najar et al. (38) demonstrated that hypothalamic leptin signaling cascade was blocked after 45 min of a single IV injection of the rat version of this leptin antagonist in 1-day old pups. Moreover, Benoit et al. (39) evaluated the effect of leptin antagonist treatment in rat pups from postnatal day 2 to 13. In contrast to our observations in lambs, early leptin antagonist treatment induced long-term hyperphagia measured at P90 under regular chow regime and at P150 when challenged with a high fat diet (39). This hyperphagia was associated with higher rates of weight gain through adulthood on day 153 (39). The temporality of effects and the lack of differences in body composition at day 40 of life suggest that leptin signaling during early life in precocial

species does not have the same relevance and long lasting consequences than it does in rodents.

A previous experiment conducted in our lab demonstrated that newborn lambs during the first two weeks of life are incapable of reducing voluntary intake when caloric density of the diet is increased (Chapter 3). This suggested that dry matter intake in early life may be limited by physical constraints rather than by a central system responding to metabolic cues. Thereafter, we treated lambs between day 30 and 35 when GI tract capacity is presumably no longer limiting intake. To our surprise, chronic SOLA treatment did not increase voluntary intake during this later treatment period. However, weight gain was higher in SOLA-treated lambs during the same period. Similar observations were made in mice. Chronic treatment with pegylated mouse leptin antagonist for 8 days numerically increased food intake and significantly increased body weight (20). Interestingly, lambs treated with SOLA had a significant reduction in relative dry matter intake and a numerical reduction in weight gain relative to Control group during the post-treatment period. As suggested by Elinav et al. (20), this effect after chronic leptin antagonist treatment could be the result of a compensatory state of enhanced leptin sensitivity. It is possible that a longer treatment period with the leptin antagonist could induce an effect on dry matter intake. It is noteworthy that the leptin antagonist treatment reduced circulating T4 only when administered on day 30 and not during the immediate postnatal period. Previously, we demonstrated that plasma T4 concentration responds to changes in caloric content of the diet only after 7 to10 days after birth (Chapter 3). It is possible that there is a refractory period immediately after birth when the central regulation of circulating T4 does not operate. This would explain the lack of effect of SOLA on plasma T4 immediately after birth.

Previously, Levi et al. (40) demonstrated that glucose metabolism in 7-wk old mice can be altered by treatment with pegylated leptin for 3 days. Although fasting glucose and glucose tolerance were not different in treated animals, they showed higher fasting-insulin and glucose-dependent insulin secretion. Also, during hyperinsulinemic-euglycemic clamps, hepatic glucose output was suppressed 90% in control mice but only 39% in mice treated with the leptin antagonist (40). Although we were not able to detect differences in plasma insulin or glucose between groups, it is difficult to draw any conclusions because blood samples were not obtained under fasting conditions. On the other hand, it is well known that leptin can alter metabolic rate by increasing thyroid hormones concentration (41, 42). Upon leptin stimulation, neurons in the arcuate nucleus (ARC) increase the synthesis and secretion of thyrotropin-releasing hormone (41). This process takes place through the stimulation of melanocortin receptors in second order neurons distal from the ARC. For example, Rosenbaum et al. (42) demonstrated that exogenous leptin can increase circulating thyroid hormones levels in humans with low plasma leptin levels during sustained weight reductions of 10%. More importantly, such leptin treatment increased energy expenditure and further reduced fat mass (42). Accordingly, in our experiment, blockage of leptin signaling induced a significant decrease in plasma total T4 concentration when lambs were treated at day 30 of post-natal life. It is known that circulating thyroid hormones are associated with metabolic rate and energy expenditure (43). Therefore, the effects of leptin antagonist on T4 could explain the slightly higher rate of weight gain of treated lambs (Fig. 10) without significant changes in intake during the late treatment period.

In summary, our study suggests that leptin is functional after birth in sheep and can regulate food intake. Although, leptin antagonist appeared to increase voluntary intake and growth during the first two weeks of life, these effects were transient and did

not have long-lasting effects on body composition at 40 days of postnatal life. Interestingly, the effect of leptin on thyroid function was not observed during this period but was evident when animals were treated later in life. This is likely the consequence of a refractory period in the central regulation of thyroid hormones in early life. In conclusion, leptin signaling during the first two weeks of life does not have the same implications in lambs as it does in rodents. Also, the role of leptin on the ontogeny of hypothalamic development remains to be investigated. Due to the altricial nature of sheep, fetal life could offer a better window to explore this developmental process.

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CHAPTER 6: SUMMARY AND CONCLUSIONS

It has been discovered that the abnormal appetite and obesity of IUGR rodents is associated with defective hypothalamic centers regulating energy metabolism (1). In parallel, it was discovered that rodents develop a leptin surge that is not related to adiposity or appetite regulation during the first two weeks of life (2). During this period, leptin has essential neurotrophic effects in the hypothalamus, promoting axonal connections between neuronal centers responsible for energy homeostasis (3). The neonatal leptin peak is disrupted as a result of IUGR or other perinatal insults in rodents. This phenomenon induces a permanent disruption in the central control of energy homeostasis and a detrimental adult phenotype.

Although rodent models have been informative in exploring the postnatal consequences of the IUGR conditions and the mechanisms involved, the timing of final maturation of the hypothalamus differs between altricial rodents and precocial species like humans. For example, most of the hypothalamic development observed in rodents during neonatal life is believed to take place prenatally in precocial species (4–6). Additionally, rodent models offer experimental difficulties to study the effects of dietary caloric density on energy homeostasis during the pre-weaning period. Sheep models are an alternative to overcome these limitations. The ontogenesis of energy homeostasis in the sheep is believed to be similar to humans (7). Also, they can be artificially reared using milk replacer of different composition. Because of these advantages, we conducted studies using an IUGR sheep model to evaluate the role of dietary fat during early life. We also evaluated the role of the leptin-MC system in the control of energy homeostasis during neonatal life

Our first experiment investigated the interactions between birth size and fat content of the diet during the first 2 weeks of life of neonatal lambs (Chapter 3). Dietary fat content did not change dry matter intake of IUGR or Normal lambs during the first two weeks of life, suggesting that capacity of the GI tract is a primary factor limiting energy intake. By the second week of life, however, voluntary feed intake in animals receiving the HFD developed a curvilinear pattern whereas the pattern remained linear in animals fed a low-fat milk replacer. These data suggest that the homeostatic system regulating energy metabolism may become operational after the first week of life. Our data do not suggest that IUGR lambs differ from Normal lambs with respect to either mechanism (physical limitation or central regulation of feed intake). In agreement with results of others in sheep and rodent models, the IUGR condition favored greater lipid deposition. This effect was most obvious for the visceral fat depots. This effect suggests that the IUGR sheep model is very relevant to human health. Indeed, evidence in humans indicates that the IUGR condition is associated with higher visceral fatness in adulthood (8). Additionally the association between the IUGR condition, hypothyroid status and greater fat accretion indicate that lower metabolic rate might play an important role in the development of excess adiposity in these animals.

In future experiments, it will be important to determine whether IUGR and normal lambs differ in the development of hypothalamic networks during prenatal and postnatal life. Such data would provide information about the ontogeny of central control of energy homeostasis and effects of the IUGR condition. It will also be important to use experimental techniques to assess effects of the IUGR condition on energy expenditure. Indirect calorimetry, thermologgers and determination of respiratory exchange ratio could provide this type of information (9, 10). Experiments with IUGR lambs would also

benefit from the accurate and repeatable in vivo measurement of body composition to assess long-term effect of this condition.

The central MC system plays a critical role in regulating voluntary food intake and energy expenditure (11). In fact, its activation is one of the most important mechanisms whereby leptin regulates energy metabolism in postnatal life (12). Whether this system operates in early postnatal life was unknown. Our second experiment demonstrated that melanocortin receptor-4 signaling is functional in IUGR lambs as early as 4 days of life. Also we demonstrated that melanocortin activation could be an effective method to reduce relative intake and fatness associated with the IUGR condition, but the outset, we planned to have an additional experimental group of pair-fed IUGR lambs to determine whether melanocortin agonist treatment had effects above and beyond any intake effects. A lack of IUGR lambs prevented this. Nevertheless multivariate analysis suggested effects of the melanocortin agonist on fat deposition involving intake-independent effects. It was clear that these effects were associated with an increase in circulating T4 and lower fat retention in treated animals. Additional experiments are needed to ascertain this possibility. Moreover, it would be interesting to assess whether IUGR and Normal lambs have similar sensitivity and responsiveness to MC stimulation.

The neurotrophic effect of leptin in neonatal rodents has received lots of attention (3). In particular, disruption this effect has been regarded as a likely mechanism explaining the predisposition of obesity in IUGR animals, including humans (13). The timing of leptin action after birth in precocial species, including humans, however, remains unknown. In our final experiment, we explored the importance of leptin signaling in energy homeostasis during early life using a long-lasting leptin antagonist for two weeks after birth. This experiment also allowed us to examine the long-term consequences of disrupted leptin signaling during this period, a known sensitive

developmental window in rodents (3). The leptin antagonist numerically increased intake of newborn lambs but this effect disappeared at cessation of treatment. Although, the number of experimental units in our experiment was not sufficient to reach significance, these observations strongly suggest that leptin has a role in the regulation of voluntary intake during the immediate postnatal period. This is supported by a significant increase in growth rate of lambs treated with leptin antagonist exclusively during the treatment period. Additionally, this experiment demonstrated that disruption of leptin signaling during the first 14 days of life did not have carry-over effects on intake, growth or body composition up to day 40 of life. Therefore, variation in postnatal leptin signals immediately after birth does not seem to be as critical in precocial animals as it is in rodents. Based on the findings of our first experiment, we decided to analyze the effect of leptin antagonist treatment at an age when the GI tract would presumably no longer impose a limitation to intake. We tested the effect of a 5-day treatment starting at postnatal day 30. Although no differences in intake were detected, leptin antagonist decreased T4 concentration in treated lambs, presumably reducing metabolic rate. It is possible that a longer treatment period is necessary to induce a change in intake. Also, in the future, it is necessary to assess different and additional responses to leptin signaling disruption. For example: changes in hypothalamic networks, changes in body composition and their association with energy intake and expenditure as well as dynamic assessment of glucose metabolism particularly at the onset and termination at treatment. Overall, these experiments provide new information on the regulation of energy intake immediately after birth in an animal model that approximates the timing of hypothalamic development in humans.

References chapter 6

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