Intrauterine programming of leptin

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ABSTRACT

Many epidemiological studies published over the last ten years have indicated that environment during pregnancy affects adult phenotype and health of offspring. The permanent postnatal effects caused by environmental factors during human pregnancy have been termed *in utero* programming. Babies which are shorter or lighter at birth show catch-up growth during early postnatal life, develop reduced insulin sensitivity and increased risks of obesity. As adults, they also have higher incidence of diabetes and cardiovascular disease and increased concentrations of leptin in their blood. Leptin is a polypeptide produced by adipose tissue and secreted into blood, that acts to suppress appetite and increase energy expenditure. The guinea pig and pig were evaluated as experimental animal models in which to investigate mechanisms of *in utero* leptin programming in humans. Adipocyte development is more advanced at birth in guinea pigs, pigs and humans than in rodents.

The first aim was to determine whether leptin is expressed in adipose tissue of pigs and guinea pigs as is the case in humans. A leptin cDNA fragment was produced from guinea pig adipose RNA and found to have a nucleotide sequence with greater than 80% identity to leptin genes of human, rat, mouse, pig and cow. Leptin mRNA was detected in several adipose sites in the guinea pig and expression was higher in the adult than the fetus. A partial leptin cDNA was also produced from pig adipose tissue RNA and found to have a nucleotide sequence identical to that concurrently published for porcine leptin cDNA. Leptin mRNA was also detected in subcutaneous adipose tissue of pigs. The pig was chosen to investigate leptin programming because an assay for measuring leptin protein in plasma from this species was available whereas an equivalent assay in the guinea pig was not available.

Increased leptin production in humans is associated with obesity and larger adipocytes. Obesity associated with larger adipocytes in adult rats can be programmed by undernutrition during the first two-thirds of pregnancy. A study of long term outcome from the Dutch famine in the winter of 1944-1945 also found that nutritional restriction during the first half of pregnancy was associated with increased adult obesity in offspring. This lead to the concept that the fetus is susceptible to programming of tissues and endocrine systems during certain phases that affects the subsequent adult phenotype. The relationship between birth weight and adult leptin levels could be hypothetically due to altered adipocyte development in growth-retarded fetuses.

The second quarter of gestation is a critical period for adipocyte developent in the pig, a period of adipocyte commitment and development. A change in adipocyte numbers or characteristics during this period could lead to permanent changes in leptin production postnatally. I therefore hypothesised that the leptin axis in offspring is programmed by maternal nutrition during pregnancy and investigated whether leptin production in offspring is altered by maternal nutrition during the second quarter of pregnancy in pigs. I found that body weight at birth and at ~8.5 weeks of age was unaffected by the level of feed during this period of pregnancy. However, leptin mRNA abundance in adipose tissue ($p=0.015$) and plasma leptin concentration ($p=0.01$) were higher in progeny from mothers provided with more feed in the second quarter of pregnancy. Leptin protein concentration in plasma was correlated with leptin mRNA abundance in adipose tissue in these animals.

Growth hormone treatment during pregnancy alters maternal metabolism, especially increasing maternal glucose. This mimics diabetic pregnancy in humans which increases the transfer of glucose to the fetus. The increased glucose delivery to the fetus at a critical stage of adipocyte development might affect adipocyte development or endocrine systems that regulate leptin production. I hypothesised that maternal hyperglycemia would alter leptin programming. To determine whether maternal glucose or other ciculating metabolites are involved in intrauterine leptin programming, pigs were treated with growth hormone in the second quarter of pregnancy and the effects on maternal metabolites and progeny levels of leptin and leptin-regulating hormones were measured. Treatment with GH increased maternal plasma insulin, IGF-I and glucose concentrations. Weight of offspring at birth was not affected. GH treatment ($p<0.005$) during the second quarter of pregnancy increased plasma leptin concentrations in 61 day old progeny. Treatment with GH in pregnancy also increased triiodothyronine ($p=0.002$) and estradiol ($p=0.002$) and decreased IGF-II concentrations ($p=0.009$) in plasma from 61 day progeny.

Programming of postnatal leptin production by maternal environment in pregnancy is likely to be due to an increase in the availability of glucose to the fetus. A direct mechanism of programming leptin expression could be through glucose altering the UDP-*N*-acetylglucosamine pathway in the preadipocytes in the fetus or indirectly through the actions of fetal insulin or insulin-like growth factor-I on fetal adipocyte maturation. Also leptin may be programmed indirectly through the actions of fetal or placental leptin, insulin or insulin-like growth factor-I on fetal hypothalamic maturation.

STATEMENT

This thesis contains no material that has been presented for the award of any other degree or diploma in this or any other university or institution. To the best of my knowledge and belief it contains no material that has previously been published or written by any other person, except where specific reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the university library, being available for photocopying and loan.

Signed

Date

27/10/00

Jason Ekert

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ABBREVIATIONS

A	Spectrophotometric absorbance
ANOVA	analysis of variance
BMI	body mass index
cAMP	cyclic 3′, 5′-adenosine monophosphate
C/EBP	CCAAT/enhancer-binding protein
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DIG	digoxigenin
ds	double stranded
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
GH	growth hormone
ICV	intra-cerebroventricular
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-I
kb	kilobases
kDa	kilodalton
mRNA	messenger ribonucleic acid
mU	milliunit
NPY	neuropeptide Y
PPAR	peroxisome proliferator activated receptor
PCR	polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	reverse transcription
s.c	subcutaneously
sem	standard error of the mean
TBE	Tris-borate EDTA buffer
UTP	uridine-5′-triphosphate
UV	ultraviolet

PUBLICATIONS ARISING FROM THE RESEARCH IN THIS THESIS

Original research articles in refereed international scientific periodicals

Owens PC Ekert JE & Luxford BG (1999) Endogenous plasma leptin increases with age and is related to fatness and appetite. *In* Manipulating Pig Production VII, pp 176. Ed PD Cranwell. Melbourne: Frankland Pty Ltd.

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Ekert JE, Gatford KL & Owens PC (2000) Hyperglycaemia and hyperinsulinemia in pregnancy are associated with elevated leptin in offspring. International Congress of Endocrinology. 30th October-2nd November 2000, Sydney, Australia, OR14.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Epidemiological studies have shown that babies with a low body weight or reduced length at birth and during infancy will be at increased risk of obesity, coronary heart disease, stroke, diabetes or hypertension during adult life (Barker 1994; Barker 1998). Low birth weight is also associated with reduced insulin sensitivity as children (Law *et al.* 1995; Yajnick *et al.* 1995; Whincup *et al.* 1997; Chiarelli *et al.* 1999) and adults (Leger *et al.* 1997; Ravelli *et al.* 1998), impaired growth hormone secretion (Flanagan *et al.* 1999), increased rates of hypertension (Barker 1996) and elevated plasma leptin as adults (Lissner *et al.* 1999; Phillips *et al.* 1999). Fetal growth rate, and hence size at birth is significantly influenced by the supply of nutrients and oxygen from the mother via the placenta (Harding *et al.* 1995). Fetal adaptations to an adverse intrauterine environment, which may be advantageous during fetal life, may be disadvantageous postnatally, especially if associated with adult obesity (Godfrey *et al.* 1995). Obesity can be classified as a disease in which body fat has accumulated to such an extent that health may be adversely affected (Kopelman 2000). The mechanisms by which imbalances in energy intake and energy expenditure arise that produce obesity are poorly understood. Recently leptin, a protein secreted predominantly by adipose tissue, has been identified as an important regulator of appetite and energy expenditure.

1.2 REGULATION OF ADIPOCYTE DEVELOPMENT

1.2.1 Preadipocyte development

Adipocyte development and differentiation have been studied in established cell lines of adipose-derived stromal vascular and animal precursor cells as model systems. The adipocyte lineage is derived from an embryonic stem cell precursor with the ability to differentiate into the mesodermal cell types which include adipocytes, myocytes and chrondrocytes (Hwang *et al.* 1997). Commitment to the adipocyte lineage is through activation of regulatory genes triggered by external modulators which are currently unknown (Hwang *et al.* 1997). Once committed, preadipocytes are able to grow but must regress from the cell cycle before adipose conversion (Gregoire *et al.* 1998). Different genes are expressed in a sequential order during adipocyte differentiation in the attainment of the adipocyte phenotype. This is evident by the appearance of early, intermediate and late mRNA/protein markers and triglyceride accumulation (Figure 1.1). Growth arrest of preadipocyte cells is characterised by the expression of the early markers of adipocyte differentiation which include lipoprotein lipase (Cornelius *et al.* 1994; MacDougald *et al.* 1995b; Ailhaud 1996) and the mouse equivalent of the human α2 chain of the type VI collagen (designated A2Col6/pOb24). Growth arrest, not cell confluence or cell contact, seems to be necessary for adipocyte differentiation. The transcription factors CCAAT/enhancer-binding protein (C/EBP)-α and peroxisome proliferator activated receptor (PPAR)-γ appear to be involved in the growth arrest which is required for adipocyte differentiation (Gregoire *et al.* 1998).

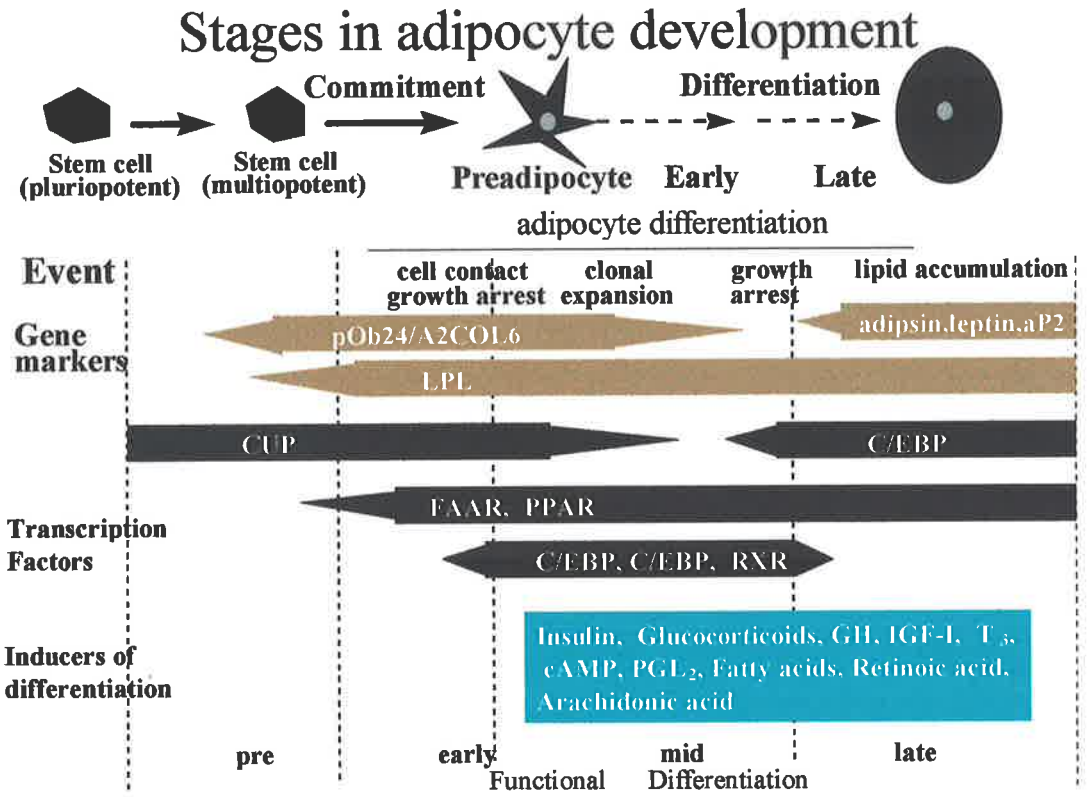


FIGURE 1.1. A model of in vitro adipocyte development (modified from Klaus, 1996)

1.2.2 Clonal expansion

C/EBPα has antimitotic activity by increasing p21/SDI-1 mRNA and protein levels and along with PPARγ induces growth arrest (TiMchenko *et al.* 1996). C/EBPα and PPARγ transcripts are expressed at low levels in preadipocytes at the clonal expansion stage but this may be sufficient to arrest growth in preadipocytes. Growth arrested preadipocytes require an appropriate combination of mitogenic and adipogenic signals to move into the differentiation process (Hwang *et al.* 1997). Preadipose cell lines undergo at least one round of mitotic clonal expansion during growth arrest in the early stages of adipocyte differentiation (Figure 1.1). DNA replication during mitotic division may increase the availability of *trans*-acting factors which transcriptionally activate (or depress) the regulatory genes that induce differentiation. This has been suggested to lead to the clonal expansion of committed cells. Endocrine factors which induce adipocyte differentiation in studies of cell systems include insulin, insulin-like growth factor-1 (IGF-I), glucocorticoids, triiodothyronine (T_3), cAMP, retinoic acid, prostaglandins and arachidonic acid (Hwang *et al.* 1997) (Figure 1.1).

1.2.3 Expression of adipocyte genes

Growth arrest and clonal expansion of preadipocytes in the early stage of adipocyte differentiation are associated with an elaborate change in the pattern of gene expression that can differ with cell culture models and *in vivo* (Figure 1.1). The earliest sign of adipocyte differentiation is marked by the appearance of lipoprotein lipase (LPL) mRNA. Mature adipocytes produce LPL to control lipid accumulation (Cornelius *et al.* 1994; MacDougald

et al. 1995b; Ailhaud 1996). Both C/EBP and PPAR classes of transcription factors are involved in early adipocyte differentiation and are expressed at low but detectable levels in preadipocytes(Hwang *et al.* 1997). PPARδ is activated before PPARγ. Expression of the isoforms C/EBPβ and C/EBPδ is transiently increased before the rise in PPARγ expression.

The subsequent decrease in C/EBPβ and C/EBPδ in early to mid stages of differentiation is accompanied by the induction of C/EBPα expression. C/EBPα undifferentiated protein (CUP) is decreasing through adipocyte development, as the SP1-like protein can bind to the C/EBPα promoter inhibiting transcription of C/EBPα. C/EBPα is expressed when clonal expansion decreases. The increase in C/EBPα coordinates the activation of several adipocyte genes by C/EBPα binding and transactivates the promoters of the 422/aP2, GLUT4, SCD1, PEPCK, leptin and insulin receptor genes which are involved in terminal differentiation (Figure 1.1). Sterol regulatory element binding protein-1c (SREBP-1c)/adipocyte determination and differentiation factor 1 (ADD1) is another transcription factor which is induced during early adipocyte differentiation. SREBP-1c/ADD1 is a bHLH-leucine zipper protein, which is involved in cholesterol metabolism and may regulate adipocyte gene expression (MacDougald *et al.* 1995b; Hwang *et al.* 1997; Gregoire *et al.* 1998).

Fat cells convert from a fibroblastic to a spherical shape during adipocyte differentiation and large changes occur in cell morphology, cytoskeletal components and the level and type of extracellular matrix components (Gregoire *et al.* 1998). Actin and tubulin expression are decreased early in adipocyte differentiation before changes in morphology and expression of adipocyte-specific genes (Spiegelman *et al.* 1982). Cell shape is altered due to the

differentiation process not from the accumulation of lipid stores. Blocking of triglyceride accumulation in 3T3 preadipocytes by depletion of biotin or by addition of lipolytic agents still allows the process of biochemical and morphological differentiation to proceed (Gregoire *et al.* 1998). Collagen expression is also an early occurrence in adipocyte differentiation (Dani *et al.* 1989). Preadipocyte factor-1 (pref-1) is involved in maintaining the preadipose phenotype and its expression decreases markedly through adipocyte differentiation such that pref-1 is undetectable in mature fat cells (Smas *et al.* 1993; Gregoire *et al.* 1998).

1.2.4 Terminal differentiation

Fat cells that enter the differentiation process for a short period of time can still dedifferentiate and re-enter the mitosis pathway. This occurs by disruption of cell-cell contact or exposure of cells to agents such as retinoic acid (Cornelius *et al.* 1994). Studies performed in BALB/c3T3 cells indicate cells beyond a certain stage in differentiation can not undergo dedifferentiation but are committed to terminal differentiation (Weir *et al.* 1986; Wang *et al.* 1993). Terminal differentiation is characterised by an increase in *de novo* lipogenesis. The activity, protein and mRNA levels of enzymes involved in triacylglycerol biosynthesis including those of ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, steroyl-CoA desaturase, glycerol-3-phosphate acetyl-transferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase and glyceraldehyde-3-phosphate dehydrogenase increase by 10 to 100 times during terminal differentiation (Spiegelman *et al.* 1983; Weiner *et al.* 1991). The number of glucose transporters and insulin receptors also increase as does insulin sensitivity (Gregoire *et al.* 1998). Adrenergic receptor numbers are increased during

terminal adipocyte differentiation through a loss of β_1-adrenergic receptors and an increase in β_2- and β_3-subtypes. There is an increase in mRNAs for proteins linked to lipid metabolism and committed adipocytes also express many other adipose specific genes including aP2, FAT/CD36 and perilipin. A number of secreted products are produced by adipocytes which include monobutyrin, adipsin, Acrp40/AdipoQ, PAI-1 and angiotensiongen II. During *in vitro* terminal differentiation expression of leptin mRNA is increased but its concentration is much lower than that detected in adipose tissue *in vivo*.

1.2.5 Adipocyte development in the fetal pig

In pigs, fetal subcutaneous adipose tissue development starts between 45 and 60 days of pregnancy (term is ~110 d). At day 45 of pregnancy the subcutaneous tissue consists of many short unorganised connective tissue fibres and many small lipid droplets scattered throughout the region. Lipid droplets are not evident in muscle stromal areas. Clusters of fat cells are first evident in the inner layer of subcutaneous adipose tissue at day 60 in the fetus and detected in the outer layer at day 75 (Hausman *et al.* 1986). C/EBPα measured by western blot and immunohistochemistry in porcine fetal adipose tissue is expressed at day 50 and increases by day 75 and 90. Neither C/EBPβ or C/EBPδ were detected at any of the developmental ages (50, 75 or 95 day) examined (Hausman *et al.* 1986). However, C/EBPβ or C/EBPδ were expressed in both 8 day postnatal and mature (180 day old) pigs. This contradicts the findings of *in vitro* cell culture studies of adipocyte development which shows the cascade of C/EBPβ, C/EBPδ and C/EBPα expression leading to development of fully differentiated adipocytes (Hausman *et al.* 1986). The difference between the *in vitro* and *in vivo* observations of the C/EBP expression cascade in adipocyte differentiation could

be due to the combination of methylisobutylxanthine (MIX) and dexamethasone (DEX) in most *in vitro* systems. C/EBPβ is induced *in vitro* in preadipocytes by MIX, while C/EBPδ is induced by DEX (Yeh *et al.* 1995). Cells *in vivo* that are developing into adipocytes in the fetus are not exposed to MIX and levels of insulin and cortisol are very low in the fetus. Thus during normal adipocyte development *in vivo* both C/EBPβ and C/EBPδ may not be induced but cells *in vitro* may express C/EBPβ and C/EBPδ if induced by MIX and DEX. Therefore the adipocyte differentiation process of cells determined by *in vitro* methods may not completely represent that which occurs *in vivo*.

1.3 DISCOVERY OF A SATIETY SIGNAL

Over 40 years ago, it was first proposed that a satiety factor is released into blood due to the extra fat accumulated when a mammal overeats in order to signal to the brain that the body is obese and further food intake is not needed. It was proposed that in response to this signal, the mammal would consume less food and increase energy expenditure. Experiments performed by Kennedy (1953) showed that substantial variation in food intake in young rats, associated with changes in heat loss to the environment, or in loss of energy through lactation, brought little change in weight. He also showed that lesions of the hypothalamus of lactating rats caused a slight rise in food intake and resulted in obesity. A satiety signal from fat stores to the hypothalamus was postulated to regulate body weight, through a "lipostatic" mechanism by which the amount of stored energy is perceived by the hypothalamus, which then varies food intake and energy expenditure to maintain energy reserves (Kennedy 1953).

This hypothesis was subsequently confirmed by parabiosis experiments using genetically obese (*ob/ob* and *db/db*) mice. The *ob/ob* mice are characterised by obesity, hyperphagia, transient hyperglycaemia and hyperinsulinemia. When the blood circulation of *ob/ob* mice was surgically linked to that of normal lean mice, allowing partial exchange of blood between them (parabiosis), no changes were observed in the lean mice, but the obese mice showed decreased food intake, and their blood levels of insulin and glucose fell (Coleman *et al.* 1969). Therefore, the blood of *ob/ob* mice must lack a factor that is present in the blood of normal mice. The *db/db* mice were characterised by obesity, hyperphagia, and severe diabetes with marked hyperglycemia and hyperinsulinemia. When *db/db* mice were parabiotically joined to normal (lean) mice, the normal mice showed decreased insulin and blood glucose levels and eventually died of starvation. Coleman *et al.* (1969) concluded the lean mice received a satiety factor causing inhibition of appetite. They also concluded that the *db/db* mice cannot respond to the satiety factor but contain abundant amounts of it in their blood.

More recently the *ob/ob* mice were found to have a mutation in the leptin gene rendering them leptin deficient (Zhang *et al.* 1994). The *db/db* mice have a mutant leptin receptor gene (Coleman *et al.* 1969; Tartaglia *et al.* 1995). Administration of leptin to *ob/ob* mice was found to decrease appetite and reduce obesity (Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995; Schwartz *et al.* 1996b; Mistry *et al.* 1997). A similar treatment applied to normal lean mice reduced body weight but had no effect on *db/db* mice (Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995; Schwartz *et al.* 1996b; Mistry *et al.* 1997).

The leptin gene in the mouse and human is expressed most highly in adipose tissue (Zhang *et al.* 1994; MacDougald *et al.* 1995a). The leptin receptor has been found to be expressed in the hypothalamus, suggesting that leptin protein acts on the hypothalamus (Ghilardi *et al.* 1996) as originally proposed by (Kennedy 1953) and (Coleman *et al.* 1969). Leptin treatment also normalises the elevated levels of neuropeptide Y (NPY) mRNA and alters the expression of a number of other genes for appetite regulating peptides in the hypothalamus of the *ob/ob* mouse (Stephens *et al.* 1995). High levels of NPY are associated with increased food intake and decreased energy expenditure (Stanley *et al.* 1986).

1.4 LEPTIN GENE AND PROTEIN

Zhang *et al.* (1994) cloned and sequenced the mouse leptin gene and its human homologue. Both have a highly conserved 167 amino-acid encoding open reading frame which produces a 4.5 kb mRNA in adipose tissue (Figure 1.2). The leptin gene has been physically mapped to human chromosome 7 (Green *et al.* 1995), mouse chromosome 6 (Zhang *et al.* 1994) and pig chromosome 18 (Neuenschwander *et al.* 1996). There is a single-copy of the leptin gene in the human genome (He *et al.* 1995).

Genomic organisation of the 5′ end of the mouse and human leptin genes have been investigated (He *et al.* 1995). The first intron occurs in the 5′-untranslated region. The protein coding sequence is contained in exons 2 and 3 separated by an intron at glutamine +49 in the protein. Two classes of cDNA in mice and humans have been shown, which differ by inclusion or exclusion of a single codon at +49. An AG splice-acceptor sequence can occur at the CAG codon of glutamine. The glutamine is found in a highly conserved area of the molecule but its significance is unknown. Therefore two leptin proteins in mice and humans can be theoretically made by an alternative splicing mechanism (Figure 1.2).

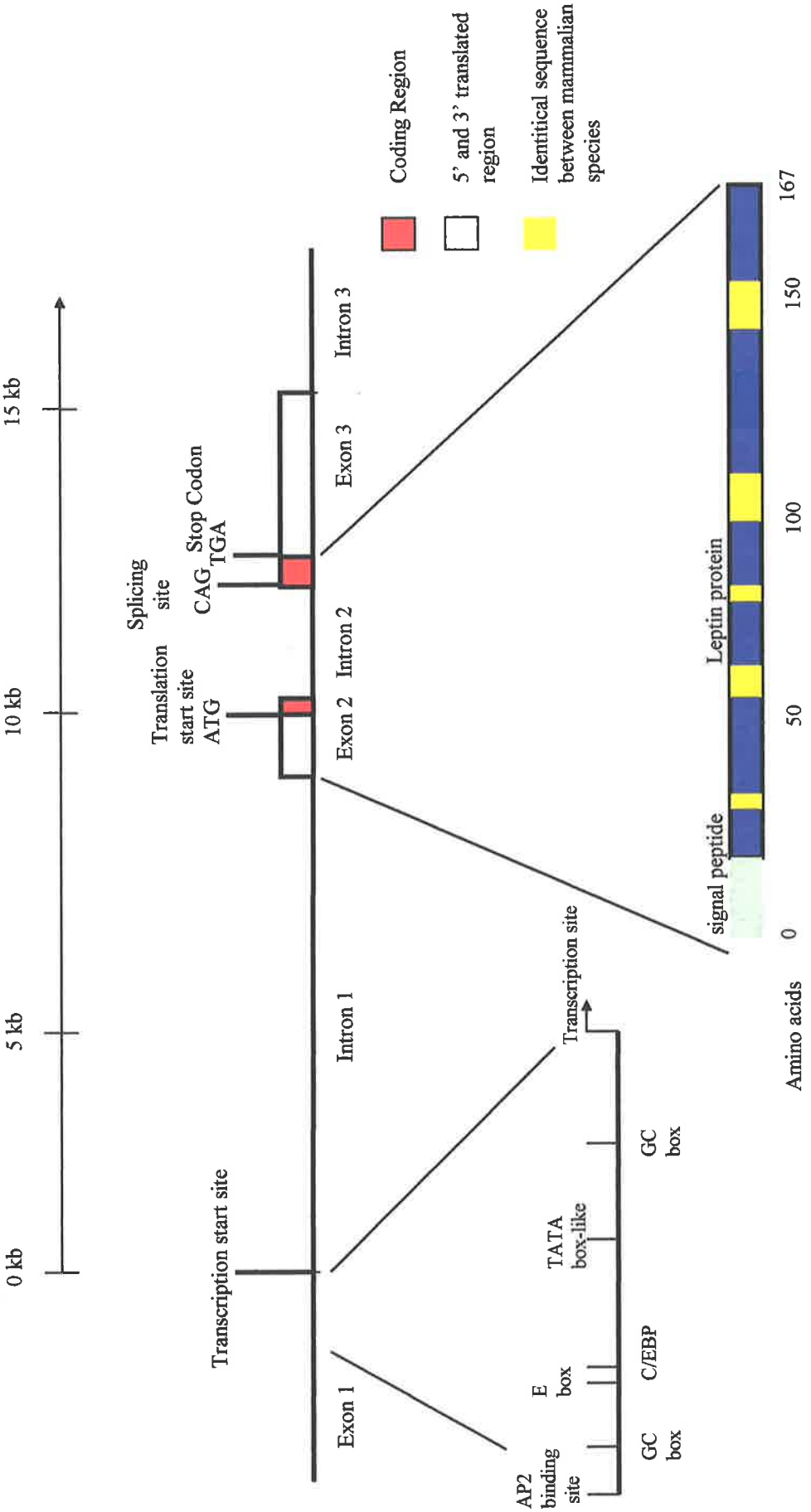


FIGURE 1.2 Genomic structure of the human leptin gene (Isse *et al.* 1995; Gong *et al.* 1996)

In the human leptin gene a single TATA- containing promoter is located 27-30 bp upstream of the transcription initiation sites of exon 1 (He *et al.* 1995). A number of potential cis-acting regulatory elements were identified. Three copies of the GC boxes or Sp1 consensus sequence (GGGCGG) at positions -79 to -74, -155 to -150 and -160 to -155. An E box (CANNTG, N=A/C/G/T) was found at positions -114 to -109 and an AP-2 binding site (CCCAGGCC) occurred at position -199 to -192 (Figure 1.2). A binding site for C/EBP occurs at -111 to -103 (Isse *et al.*, 1995). C/EBPα promotes adipocyte differentiation and transactivates the promoters of many adipose-specific genes (He *et al.* 1995). Co-transfection of a C/EBPα expression vector in primary rat adipocytes and 3T3-L1 preadipocytes induced leptin promoter activity significantly (He *et al.* 1995; Miller *et al.* 1996). Mutation of the leptin C/EBP site eliminated the activation of the leptin gene. The results showed that C/EBPα has transcriptional control over leptin mRNA expression which is compatible with the important role C/EBPα has in adipocyte differentiation. C/EBPα sites have been linked to pleiotropic transcriptional activator genes during preadipocyte differentiation. Although expression of C/EBPα is needed for adipocyte differentiation, it appears not enough to induce differentiation (Cornelius *et al.* 1994). Expression of C/EBPα is limited to tissues with high lipogenic capacity, including adipose, liver, intestine and lung (Figure 1.2).

Other potential transcription regulatory sites discovered include a glucocorticoid response element (TCTGATCT) at -1238 to -1246 and a cAMP response element-binding protein (CGTCA) at -1375 to -1380 (Gong *et al.* 1996). A novel, placental specific transcription factor site has also been detected. An enhancer located 1.9 kb upstream of the human leptin gene contains three sites designated placental leptin enhancer elements (PLE) 1-3. Proteins

binding to PLE3 are present in human placental extracts but not in extracts from non-placental sources (Bi *et al.* 1997) (Figure 1.2).

A nonsense mutation in codon 105 in a congenic C57BL/6J *ob/ob* mouse strain was found to cause a twenty-fold increase in leptin mRNA abundance, but the 16 kD leptin protein was not detected (Zhang *et al.* 1994). A co-isogenic SM/Ckc-$^{+Dac}ob^{2J}/ob^{2J}$ mouse strain was found to contain a different leptin gene mutation that does not synthesise leptin RNA. This mutation occurs approximately 7 kb upstream of the transcription start site, suggesting a structural alteration or sequence variation in the leptin gene promoter is the cause of obesity in *ob/ob* mice (Zhang *et al.* 1994).

The 3′ flanking regions of the leptin gene contain no polyadenylation sites in mouse, rat or human. A poly (A) stretch was found 3′ to cytosine +4183, suggesting that the C at +4183 is the 3′ end of the human leptin cDNA. There was no evidence of a polyadenylation signal (AATAAA) near this potential poly (A) addition site. The 3′ -untranslated region of the human leptin gene was found to be ~50 % identical to that of the mouse. There is a CT-rich sequence at +4417 to +4538 in the 3′ -flanking region of the human leptin gene (Isse *et al.* 1995).

Leptin from normal mouse plasma is present primarily as a monomer with a molecular size of approximately 16 kD. Immunoprecipitation has shown the leptin protein is present in plasma as a soluble hormone and has identical mobility to the 146 amino-acid human leptin protein expressed in yeast after signal peptide cleavage (Halaas *et al.* 1995).

1.5 REGULATION OF LEPTIN ACTION

1.5.1 Discovery of the leptin receptor

Parabiosis studies with *ob/ob* and *db/db* mice indicated that *db/db* mice are unable to respond to a soluble, circulating satiety factor (Coleman *et al.* 1969). This led to the suggestion that the *db* gene may encode a mutant receptor for this factor. A leptin receptor (R) cDNA was subsequently isolated from the choroid plexus and was shown to map to the same 5-cM interval on mouse chromosome 4 to which the *db* gene had been localised (Tartaglia *et al.* 1995).

In *db/db* mice no mutation in the coding region of the leptin-R cDNA was found and the leptin binding sites were unaltered. However, the cloned mouse leptin-R cDNA encoded a protein with a smaller cytoplasmic domain than the human homologue, suggesting that a longer mouse isoform may exist. From a wild-type mouse the longer isoform of leptin-R cDNA was cloned and found that the mRNA for this isoform is greatly reduced in *db/db* mice. A G to T mutation in *db* mice generates a new splice donor site and prevents the expression of the long isoform via competition for a splice acceptor. It is not clear whether the short isoform of leptin-R protein is expressed at high levels in *db/db* mice to stop the leptin-R signalling pathway by solely suppressing the activation pathway of the long isoform (Tartaglia *et al.* 1995).

1.5.2 Structure and activity of the leptin receptor

The primary structure of leptin-R shows homologies to the interleukin 6 (IL-6) type cytokine receptors. These receptors contain no enzymatic motifs in their cytoplasmic domains and instead associate with members of the *janus* kinase (Jak) family, a class of cytoplasmic tyrosine kinases. The binding of the ligand to the receptor activates the Jak kinase and causes phosphorylation of cytoplasmic proteins. Signal transducers and activators of transcription (STAT) are one class of such proteins. STAT proteins once phosphorylated induce dimerization of the leptin receptor and its translocation to the nucleus where it activates expression of specific genes (Bauman *et al.* 1996; Ghilardi *et al.* 1996).

The abilities of the short and long isoforms of leptin receptor to activate STAT proteins has been investigated (Ghilardi *et al.* 1996). The intron/ exon boundaries of the short and long isoforms of the leptin-R were sequenced, revealing the isoforms are generated by differential splicing within the last intron of the leptin-R gene. This is consistent with the data produced by Tartaglia *et al.* (1995).

The long isoform of leptin-R activated STAT-3, STAT-5, and STAT-6 upon ligand binding but STAT-1 and STAT-4 were not activated. The short form of the leptin-R was unable to activate any of the STAT proteins. This may explain why *db/db* mice show an obese phenotype despite abundant expression of the short form of the leptin-R. The short form of the leptin receptor has the capability of signal transduction as demonstrated by the induction of c-fos, c-jun and jun-B in cells expressing one of the short form receptors, OBRa (Murakami *et al.* 1997). The three STAT proteins identified to be activated through the long

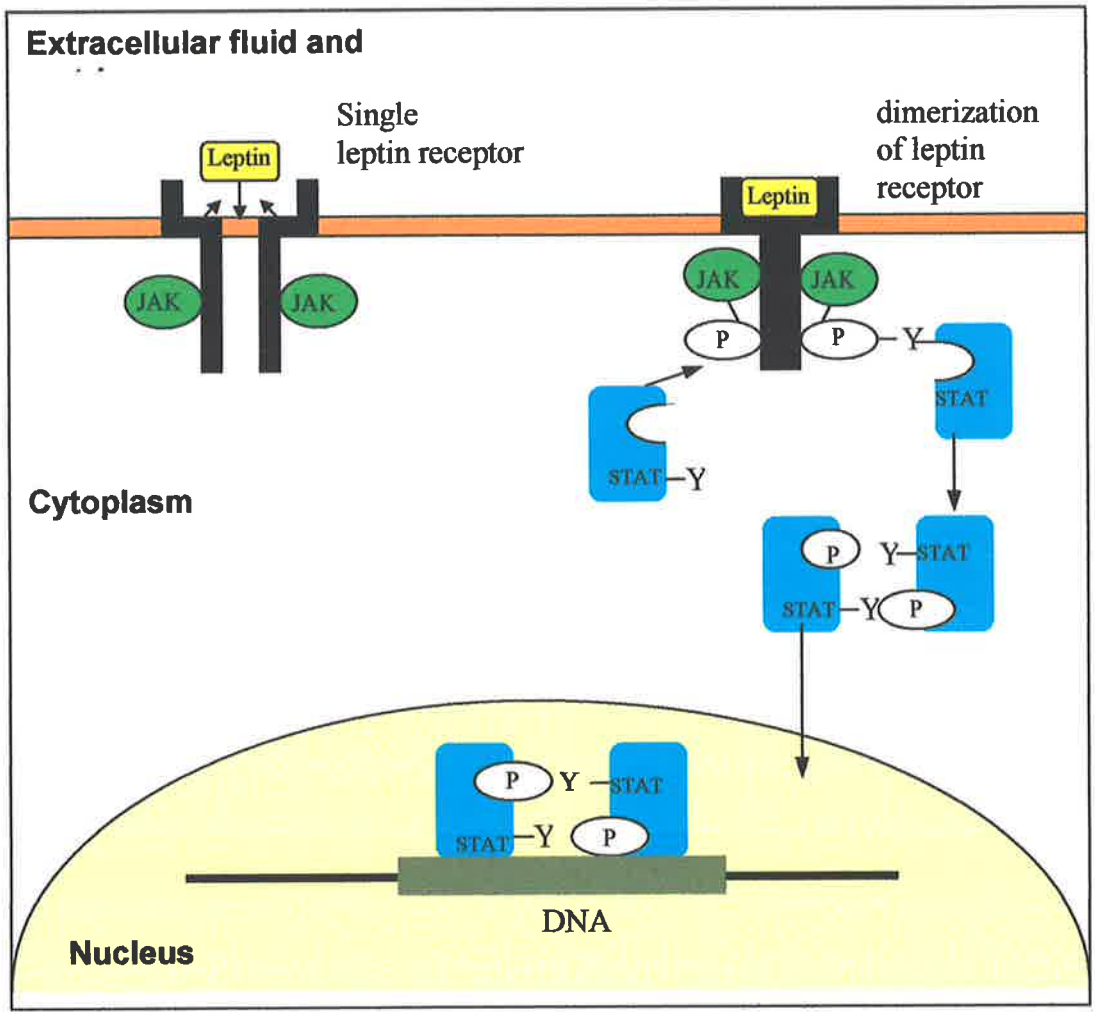


FIGURE 1.3. Leptin receptor
Binding of leptin to its receptor will induce dimerization of the receptor. Janus kinases (JAK) associated will be activated and will induce phosphorylation of tyrosine (Y) residues on the cytoplasmic domains of the receptor, producing phosphorylation docking sites for the STAT proteins. Once the STATs are phosphorylated they will dissociate from the receptor and form dimers which contribute to the active transcriptional regulators. After transport into the nucleus they will bind to STAT responsive elements in DNA and stimulate transcription of responsive target genes (Auwerx *et al.* 1998).
● - JAK; ⬭ -phosphorylation of tyrosine (Y); ▮ - STAT

leptin-R isoform may provide the basis for the pleiotropic effects of leptin. Therefore, the STAT proteins may represent potential mediators of the anti-obesity effects of leptin (Ghilardi *et al.* 1996) (Figure 1.3).

1.5.3 Expression of leptin receptor isoforms

Leptin receptors are expressed in most tissues. The long isoform of the receptor is predominantly expressed in the arcuate, lateral, ventromedial and dorsomedial nuclei of the hypothalamus (Ghilardi *et al.* 1996; Lee *et al.* 1996; Mercer *et al.* 1996). Both the long and short receptor forms are expressed in skeletal muscle (Tartaglia *et al.* 1995; Cioffi *et al.* 1996; Ghilardi *et al.* 1996) and hematopoietic tissues (Gainsford *et al.* 1996).

Short leptin receptor isoforms (OB-Ra, OB-Rc, OB-Rd and OB-Re) are thought to be involved in leptin transport in blood (DeVos *et al.* 1996). Expression of the short receptor isoforms has been found in the choroid plexus (Lynn *et al.* 1996) and the brain capillary endothelium (Golden *et al.* 1997). Short isoforms are also expressed in the lung and kidney, where they may be associated with leptin clearance (Cumin *et al.* 1996).

1.5.4 The movement of leptin across the blood-brain barrier

The long isoform of the leptin receptor is found in the arcuate nucleus of the hypothalamus which is isolated from the peripheral circulation by the blood-brain barrier. Leptin-specific binding has been observed in the choroid plexus, the site of cerebrospinal fluid (CSF) production and the location of the blood/cerebrospinal fluid barrier. The truncated leptin

receptor (soluble form) may act as a transporter for leptin across the blood/CSF barrier as there is evidence of active leptin uptake in the capillary endothelium and microvessels of the brains from humans and mice (Banks *et al.* 1996; Golden *et al.* 1997) (Figure 1.3). Caro *et al.* (1996) demonstrated that serum leptin and CSF leptin concentrations have a positive relationship in lean human individuals but not in obese humans whose serum leptin levels are higher than those of normal and lean individuals. This suggests that transport of leptin in the brain might be a rate-limiting step in leptin action in human obesity. This implies that above a certain leptin concentration in blood, an increase in leptin synthesis by the growing fat mass cannot effect appetite regulating centres in the brain. Schwartz *et al.* (1996a) proposed that the efficiency of leptin uptake by the brain, taken as the CSF/plasma concentration ratio, was lower in obese than in lean subjects. Both the above studies suggest that a saturable system transports leptin from blood to brain. Low efficiency brain leptin transport in obese individuals with high plasma leptin levels might therefore explain their apparent leptin resistance. It is possible that the central nervous system tissues of even obese individuals may be responsive if leptin were able to reach those sites.

Another potential mechanism of leptin resistance in obesity is reduced signal transduction by leptin receptors. Suppressor of cytokine signalling (SOC)-3 is a leptin-inducible inhibitor which may inhibit leptin signalling leading to leptin resistance. Interference with the interaction of leptin and its receptor by peripheral signals like glucocorticoids may also cause central leptin resistance (Ur *et al.* 1996; Schwartz *et al.* 1997).

The leptin receptor has been shown to occur in a number of isoforms, with the full-length isoform a signal transducing receptor with a mode of action related to the IL-6-type

cytokine receptors. The short isoform may be involved in the transport of leptin across the blood-brain barrier.

1.6 ACTIONS OF LEPTIN

Leptin is postulated to be a satiety factor released by peripheral adipocytes to affect appetite and hence fat deposition as part of a system of energy homeostasis. For adult weight to remain relatively constant there must be a balance between energy intake, energy expenditure and storage. The hypothalamus is the major centre of control of energy balance (Levin *et al.* 1996; Prins *et al.* 1997).

1.6.1 Leptin and regulation of body weight

The role of leptin in body weight maintenance in humans is not well understood. It has been proposed that as body fat mass increases, secretion of leptin by the adipocytes rises. Leptin signals to the brain that it is time to eat less and to increase energy expenditure so fat mass returns to a certain "set point". Circulating levels of leptin do indeed stay constant in response to a normal balanced diet in humans (Considine *et al.* 1996a). However, prolonged overfeeding (5 weeks) in humans caused a gain of 10% in body weight accompanied by an increase in circulating leptin and an increase in percentage of body fat (Kolaczynski *et al.* 1997). Leptin administration at high doses in lean and obese humans for 4 and 24 weeks produces a dose-dependent loss of weight and fat (Heymsfield *et al.* 1999).

Leptin is most likely a long-term regulator of body weight but may also contribute to a short-term regulatory system to control body weight. Normal (lean) mice fasted for 24 h

have much less leptin mRNA in their epididymal fat than do obese mice (Trayhurn *et al.* 1995). Intraperitoneal injection of mouse leptin twice daily for 48 hrs into fasted mice produced leptin levels similar to those of fed controls but did not alter weight loss or levels of glucose and insulin. Leptin infused rats have reduced food intake over the first 4 hours of refeeding and increased weight after 24 hrs (Ahima *et al.* 1996). Caloric restriction in humans for 5 days does not alter leptin mRNA levels in their adipose tissue (Vidal *et al.* 1996). However, overfeeding for 12 hrs in humans produces a slight increase in circulating levels of leptin which return to normal between meals (Kolaczynski *et al.* 1997). It appears that leptin can act as a short or long term regulator of body weight emulating meal consumption or dietary energy source in rats or mice but not in humans.

1.6.2 Leptin, neuropeptides and appetite regulation

1.6.2.1 Neuropeptide Y

Intracerebroventricular (ICV) administration of NPY leads to obesity due to stimulation of food intake and a reduction in energy expenditure (Figure 1.4). Administration of leptin to *ob/ob* mice for 30 days inhibited NPY biosynthesis and release by the hypothalamus causing a decrease in food intake, body weight, plasma insulin and corticosterone levels. Surgical removal of the ventromedial hypothalamus in normal rodents causes an increase in food intake, body weight, obesity and leptin gene expression and a decrease in energy expenditure. (Funahashi *et al.* 1995; Maffei *et al.* 1995b). This illustrates that leptin acts

through the hypothalamus and does not have a direct catabolic effect on adipocytes in mice because removal of the VMH does not lead to a reduction in obesity but increased adiposity.

ICV injection of leptin into the third cerebral ventricle of normal rats reduced food intake resulting in anorexia but this effect was not observed when leptin was injected intraperitoneally. This shows that leptin acts directly on brain centres to decrease voluntary food intake. NPY is expressed in the hypothalamic arcuate nucleus and also in extra-hypothalamic areas including the cerebral cortex, hippocampus and the zona incerta of the thalamus of rats (Schwartz *et al.* 1996b; Elmquist *et al.* 1998).

Fasting decreases corticotropin releasing hormone (CRH) gene expression and increases NPY gene expression in the hypothalamus of rats as well as leptin mRNA in adipose tissue and circulating leptin protein levels. ICV treatment with leptin reduces NPY mRNA levels in the arcuate nucleus of the hypothalamus and increases CRH mRNA levels in the paraventricular nucleus (MacDougald *et al.* 1995a; Ahima *et al.* 1996). Therefore, leptin reverses the effect of fasting on NPY production (Figure 1.4).

A normal leptin receptor is required for leptin to decrease NPY biosynthesis in the hypothalamus and to inhibit CRH gene expression, since these responses are not observed in *fa/fa* obese Zucker rats, which have a defective leptin receptor (Schwartz *et al.* 1996b). The effect leptin has on NPY is similar to that of insulin which lowered NPY mRNA in the arcuate nucleus during fasting in normal rats but not in obese *fa/fa* rats (Tartaglia *et al.* 1995; Schwartz *et al.* 1996b). This indicates that both insulin and leptin play a role in regulation of hypothalamic NPY gene expression during fasting.

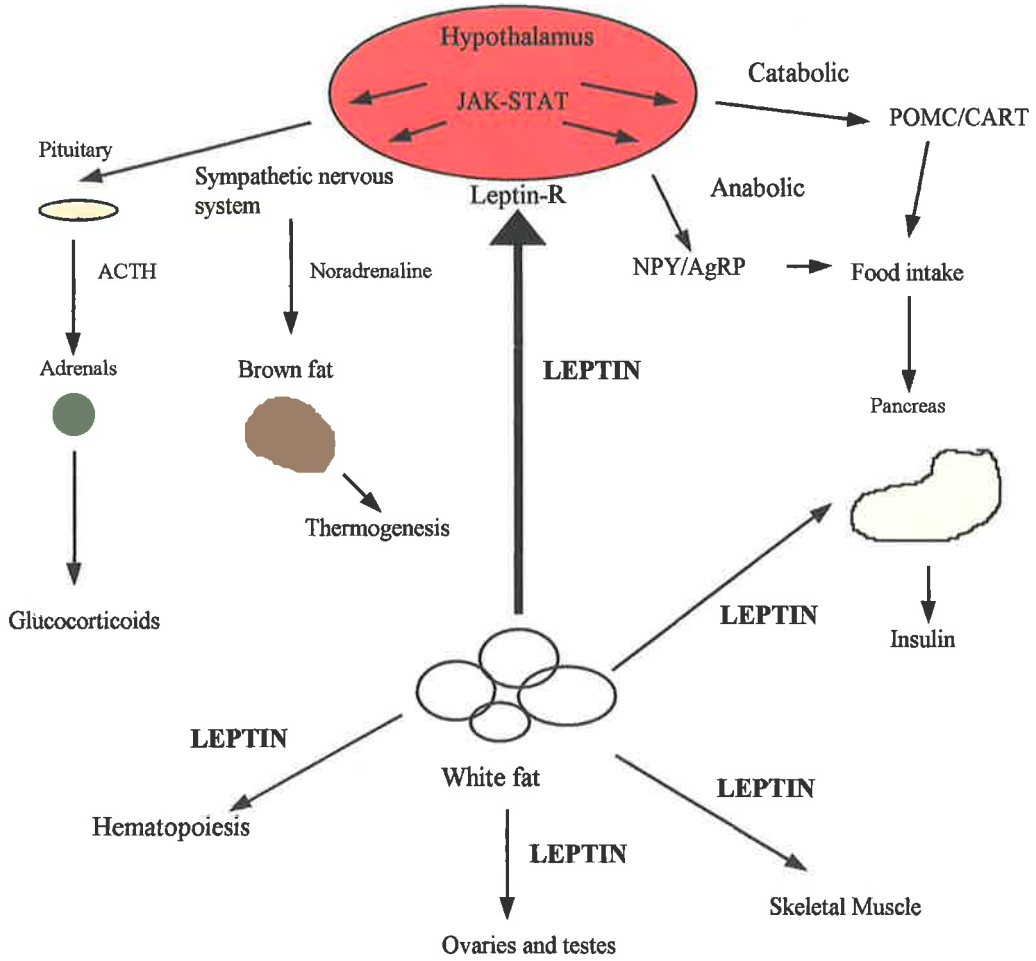


FIGURE 1.4. The potential targets of leptin actions.

JAK/STAT indicates the signalling pathway involving the Janus kinase (JAK) and the signal transducers and activators of transcription (STAT). NPY- is the hypothalamic neuropeptide-Y; ACTH- corticotropin; POMC- proopiomelanocortin, CART- cocaine- and amphetamine-regulated transcript, AgRP- agouti-related protein

Reductions of NPY in major hypothalamic regions (ARC, PVN and dorsomedial nuclei) coincide with a fall in NPY mRNA in fasted rats injected with leptin into the lateral ventricle (Wang *et al.* 1997). ICV NPY administration elevated plasma leptin levels, food intake and rate of weight gain. There was no significant difference in leptin mRNA levels between NPY-treated and saline-treated groups in epididymal fat. Leptin and NPY ARC neurons are important parts of the homeostatic pathway that regulates body fat mass. Hyperphagia, chronic leptin deficiency is therefore due to reduced inhibition of NPY-containing neural pathways (Wang *et al.* 1997).

Gene knockout of NPY reduces hyperphagia and obesity in *ob*/ob mice, indicating that the full response to leptin deficiency requires NPY signalling. However, mice that lack NPY still have intact feeding responses which suggests that more than one neural network is associated with regulating energy balance and indicates that some are capable of taking a major role in the absence of NPY (Erickson *et al.* 1996). Agouti-related protein (AgRP), orexin and melanin-concentrating hormone (MCH) are also effector signals of the leptin pathway.

1.6.2.2 Melanocortins

Melanocortins such as α-melanocyte-stimulating hormone (α-MSH) are potential leptin-sensitive mediators of adipose signalling to appetite centres in the brain.

Melanocortins are peptides cleaved from proopiomelanocortin (POMC) and act by binding to members of a family of melanocortin receptors (Cone *et al.* 1996). POMC expressing neurons are hypothalamic targets for leptin in the arcuate nucleus. Leptin receptor and POMC mRNA colocalize within the arcuate nucleus (Cheung *et al.* 1997a). POMC mRNA levels were found to be much lower in the arcuate nucleus of *ob/ob* mice than in lean normal mice. Treatment of *ob/ob* mice with leptin increased POMC mRNA to levels similar to the normal lean mice (Thornton *et al.* 1997). POMC gene expression in the hypothalamus is linked to regulation of food intake and gonadotropin secretion, inferring some of the effects of leptin on feeding and reproduction may be mediated by POMC neurons (Cheung *et al.* 1997a; Thornton *et al.* 1997).

AgRP is a melanocortin antagonist that is expressed in the arcuate nucleus of the hypothalamus where its mRNA colocalizes with NPY mRNA (Shutter *et al.* 1997). AGRP is increased by fasting (Hahn *et al.* 1998; Ebihara *et al.* 1999) and by leptin deficiency (Shutter *et al.* 1997; Ebihara *et al.* 1999) which indicates that antagonism of melanocortin receptors is important in body-weight regulation. ICV infusion of AGRP causes hyperphagia (Rossi *et al.* 1998) and a single ICV infusion of AGRP increases food intake by 30-fold (Ebihara *et al.* 1999).

CART neurons are found throughout the CNS, including the arcuate nucleus, and are another identified target of leptin and NPY action (Koylu *et al.* 1997; Kristensen *et al.* 1998). Low leptin levels in mice (starved or *ob/ob*) have low CART mRNA levels and this is increased by leptin administration (Kristensen *et al.* 1998). ICV injection of CART peptides into normal or starvation-induced feeding rats inhibits feeding and blocks the

feeding response stimulated by NPY. Antiserum that blocks CART peptide action increases feeding in rats (Kristensen *et al.* 1998).

1.6.3 Leptin, metabolic rate and thermogenesis

A study by (Wang *et al.* 1997) confirmed that leptin suppresses NPY biosynthesis, and also demonstrated that ICV administration of leptin to rats not only inhibited food intake but also stimulated brown adipose tissue expression of uncoupling protein (UCP), a measure of brown adipose tissue thermogenic capacity (Figure 1.4). The reduced arcuate NPY neuronal activity associated with increased thermogenesis is a vital part of energy balance regulation. The increase in brown adipose tissue mitochrondrial UCP mRNA and decrease in NPY release in the paraventricular nuclei in response to leptin may mediate leptin's hypophagic and thermogenic actions.

Leptin appears to increase the rate of metabolism and selectively inhibit the synthesis of fat as observed in leptin-R deficient *fa/fa* Zucker rats. Reduced thermogenesis and increased adiposity were observed in two day old pre-obese *fa/fa* Zucker rat pups. These pups also became fatter when given an identical diet to their normal lean littermates suggesting that metabolism of *fa/fa* rats is unresponsive to leptin (Moore *et al.* 1985; Markewicz *et al.* 1993).

Thermogenesis in brown adipose tissue is regulated by noradrenergic systems. Leptin administration has been shown to increase adrenaline turnover in interscapular brown adipose tissue but not interscapular white adipose tissue of mice (Collins *et al.* 1996).

Intravenous administration of leptin increases sympathetic nerve activity in brown adipose tissue, kidney, hindlimb and adrenal gland in normal lean rats. Obese Zucker (*fa/fa*) rats have no sympathic activation response to leptin suggesting that this action of leptin is mediated by leptin receptors (Haynes *et al.* 1997).

Mice injected subcutaneously with leptin have higher core body temperatures and metabolic rates than these injected with saline (Pelleymounter *et al.* 1995). Food-restricted mice treated with leptin show an increase in metabolic rate during circadian minima but no change during circadian maxima or after food consumption. *Ad libtum* fed mice treated with leptin exhibit a dose-dependent decrease in food intake without changing metabolic rate. When food supplies are low leptin's main role appears to be to control thermoregulatory energy expenditure. However, when food intake is abundant leptin changes food intake rather than energy expenditure (Doring *et al.* 1998).

1.6.4 Leptin and the adrenal system

Glucocorticoids are involved in the regulation of long-term energy balance as there is a relationship between nutritional status of mammals and the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Plasma corticosterone levels increase prior to food intake in nocturnally active rats with free access to food. Most strains of genetically obese mice have high levels of glucocorticoids and become glucocorticoid hypersensitive (Casanueva *et al.* 1999). Plasma levels of ACTH and cortisol have a negative relationship with plasma levels of leptin in adult humans (Licinio *et al.* 1997).

Regulation of the HPA axis by leptin is illustrated by the ability of leptin treatment to abolish both fasting and stress-induced increases in ACTH and/or corticosterone levels (Ahima *et al.* 1996; Heiman *et al.* 1997). These ACTH and glucocorticoid responses are mediated mainly by an increase in hypothalamic CRH secretion which indicates that leptin inhibits CRH release from the hypothalamus.

Administration of leptin to cultured rat, human or bovine adrenal cells decreases unstimulated and ACTH-stimulated adrenal glucocorticoid release (Bornstein *et al.* 1997; Pralong *et al.* 1998) (Figure 1.4). The long isoform of the leptin receptor is found in the adrenal cortex (Pralong *et al.* 1998).

Zakrzewska *et al.* (1997) have shown that glucocorticoids can restrain the effects of leptin. ICV injection of leptin into adrenalectomized rats caused a profound body weight loss and reduction in food intake. Administration of the synthetic glucocorticoid, dexamethasone, inhibited these effects of leptin. This is the reverse of the response to chronic ICV infusion of NPY in normal rats. These effects of NPY are also blocked by prior adrenalectomy (Sainsbury *et al.* 1997). Therefore, a key regulator of body weight and food intake appears to be glucocorticoids as they limit the central effects of leptin on NPY suppression.

Leptin increases corticotropin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus. CRH is a major stimulator of the HPA axis and glucocorticoid synthesis. However, leptin also acts peripherally to inhibit the synthesis of glucocorticoids and this reduces weight gain and insulin levels. A central activation of CRH and a peripheral

inhibition of glucocorticoid production by leptin could be complementary. Leptin may act at the adrenal gland to chronically depress steroidogenesis and reduce expression of steroid enzymes but in the hypothalamus leptin regulates steroidogenesis acutely. Therefore leptin is a multidimensional system that controls the hypothalamic-adrenal axis.

1.6.5 Leptin and skeletal muscle

Leptin mRNA is detected at low levels in skeletal muscle. Both the long and short isoforms of the leptin receptor are also found in muscle (Tartaglia *et al.* 1995; Cioffi *et al.* 1996; Ghilardi *et al.* 1996).

Leptin synthesis in skeletal muscle is regulated by nutrition. The hexosamine biosynthetic pathway in muscle is sensitive to energy availability and mediates the effects of glucose on the expression of a number of genes. An increase in tissue concentrations of the end product of the hexosamine pathway, UDP-*N*-acetyl-D-glucosamine, produced by infusion of glucosamine, uridine, glucose or lipid, increased leptin synthesis in skeletal muscle of rats. This suggests a positive biochemical link between nutrient availability and leptin expression in muscle (Wang *et al.* 1998). Peripheral infusion of leptin increases leptin mRNA expression in muscle but there was a greater increase in muscle leptin mRNA expression in calorie-restricted than *ad libitum* feed rats (Wang *et al.* 1999a) (Figure 1.4).

Leptin directly alters skeletal muscle lipid metabolism by reducing insulin stimulated increases in lipid storage (Muoio *et al.* 1997). Leptin does not affect muscle glucose

metabolism in normal lean mice but inhibits glycogen synthesis in muscle of *ob/ob* mice (Liu *et al.* 1997; Muoio *et al.* 1997). Defective glycogen synthesis in muscle is an early sign of insulin resistance which is commonly associated with obesity. The studies suggest that high leptin may contribute to insulin resistance in obesity. This implies that leptin's effects on fuel homeostasis, weight regulation and adiposity are partly mediated through direct actions on skeletal muscle (Muoio *et al.* 1997).

1.6.6 Leptin and hematopoiesis

Leptin and its receptor have cytokine characteristics. The leptin receptor has been found in hematopoietic tissues and the most abundant stromal cell type in adult human bone marrow is the adipocyte which expresses leptin (Gimble *et al.* 1996). Both leptin-R long and leptin-R short isoform have been found in fetal and adult murine hematopoietic tissues (Gainsford *et al.* 1996). Transfection studies show leptin enhances cytokine production and parasite phagocytosis. Leptin receptor long isoforms induce proliferation of Ba/F3 cells and differentiation of M1 cells into macrophages. Leptin has also been shown to stimulate fetal and adult eurythroid and myeloid development (Mikhail *et al.* 1997).

1.6.7 Leptin and reproduction

Human females have nearly twice the level of leptin in their blood as males, even when matched by BMI or amount of fat reserves (Rosenbaum *et al.* 1996). Estrogen can act directly on adipose tissue increasing leptin mRNA expression (Casabiell *et al.* 1998). The human ovary expresses leptin mRNA in granulosa and cumulus cells (Antczak *et al.* 1997;

Cioffi *et al.* 1997). Direct leptin administration to the ovary reduces IGF-induced progesterone and estradiol production by rat granulosa cells and in human ovaries (Spicer *et al.* 1997; Zachow *et al.* 1997). The effect is probably mediated by leptin receptors in the ovary (Cioffi *et al.* 1997; Karlsson *et al.* 1997).

1.6.7.1 Pregnancy

No difference is detected in the correlation between plasma leptin and BMI in pregnant and age-matched and BMI-matched non-pregnant women. In the first trimester of pregnancy there is an increase in maternal plasma leptin and a further significant elevation occurs in the second trimester. Leptin levels remain high during the third trimester (Chien *et al.* 1997; Hardie *et al.* 1997; Masuzaki *et al.* 1997b; Tamura *et al.* 1998). The increase in maternal leptin in pregnancy is unrelated to fetal growth (Tamura *et al.* 1998). Maternal plasma leptin levels 24 hours after delivery are below those observed during the first trimester (Hardie *et al.* 1997; Masuzaki *et al.* 1997b). The high leptin levels during the third trimester are probably not just from adipocyte stimulation but also from production of leptin by the ovaries and placenta, as estradiol and chorionic gonadotrophin levels within the pregnant mother correlate with the fluctuations in plasma leptin concentrations during pregnancy (Hardie *et al.* 1997).

High maternal leptin concentrations during pregnancy are associated with a leptin resistant state as there is no decrease in food intake or reduction in energy expenditure (Holness *et al.* 1999). Leptin resistance or a change in leptin bioavailability during pregnancy may be important in enhancing pregnancy outcome (Holness *et al.* 1999). Maternal leptin is not essential for implantation, development of the fetus, gestation or parturition. This was

shown by mating *ob/ob* female mice with *ob/ob* males and leptin administration at various stages of pregnancy (Mounzih *et al.* 1998).

1.6.7.2 Leptin at birth

Leptin is present in umbilical cord blood of humans at concentrations similar to those found in normal adults. Cord blood leptin seems to be produced from fetal and/or placental tissues, as maternal leptin serum levels are significantly higher than cord blood levels and do not correlate with leptin concentration in either arterial or venous cord blood. The leptin concentration in arterial cord blood is similar to but slightly greater than that of venous cord blood (Schubring *et al.* 1997; Sivan *et al.* 1997). This demonstrates that more leptin is secreted by the fetus than the placenta in humans. Amniotic fluid leptin levels do not correlate with venous or arterial cord blood leptin levels but correlate with leptin levels in maternal serum. This suggests that leptin in amniotic fluid is derived from the mother (Masuzaki *et al.* 1997b; Schubring *et al.* 1997; Sivan *et al.* 1997).

1.6.7.3 Leptin and the placenta

Human placental chorionic villi in the first trimester express leptin abundantly and in the third trimester chorion laeve and amnion also expresses leptin although at slightly lower levels (Masuzaki *et al.* 1997b; Senaris *et al.* 1997). Humans show elevated leptin levels in plasma and amniotic fluid during pregnancy (Schubring *et al.* 1996). Immunofluorescence and immunohistochemical techniques have demonstrated leptin in the human placenta,

particularly in the cytoplasm of syncytiotrophoblast cells (Masuzaki *et al.* 1997b; Senaris *et al.* 1997). In a number of species that differ in placentation leptin mRNA and protein has been found in the placenta (Hassink *et al.* 1997; Hoggard *et al.* 1997b; Masuzaki *et al.* 1997b; Senaris *et al.* 1997). Leptin is not found in the mesenchyme of the villi core or in blood vessels suggesting that leptin is synthesised and secreted locally and not circulated (Holness *et al.* 1999).

A positive correlation has been demonstrated between leptin levels in umbilical venous cord blood and birth weight of human neonates. Leptin may play a role in regulation of fetal weight and growth (Matsuda *et al.* 1997; Schubring *et al.* 1997; Sivan *et al.* 1997). This is supported by evidence of leptin and leptin receptor mRNA in murine placenta and in fetal cartilage and/or bone in mice (Hoggard *et al.* 1997b). Expression of the leptin receptor has been reported in the uterus but not the myometrium of pregnant rats (Chien *et al.* 1997). Leptin mRNA is present in adipose tissue of fetal sheep where its abundance is positively correlated with fetal body weight (Yuen *et al.* 1999).

Leptin may also play an important role in regulation of maternal and placental weight. Placental weight was shown to be negatively correlated with maternal leptin serum and amniotic fluid leptin concentration. In contrast, a positive association has been observed between serum leptin concentration in umbilical artery and placental weight. Fetal leptin might control maternal nutrient availability and fetal energy homeostasis through actions on the placenta (Schubring *et al.* 1997).

Leptin concentration in human cord blood has been shown to correlate with intrauterine growth patterns. Leptin concentrations in blood of newborns were higher in large for gestational age, but lower in small for gestational age than in average gestational age. These differences were probably due to variations in body fat (Koistinen *et al.* 1997). Leptin levels and adiposity have been shown to be closely correlated in adults (Considine *et al.* 1996a). Large for gestational age infants are generally fatter and small for gestational age newborns are generally leaner than normal (Koistinen *et al.* 1997).

1.6.7.4 Leptin and postnatal growth and development

Leptin mRNA expression in human babies correlates positively with their adiposity as humans are born with considerable amounts of body fat (Mantzoros *et al.* 1997; Jaquet *et al.* 1998). In healthy children and in small-for-age children leptin concentrations correlate positively with serum growth hormone-binding protein concentrations (Kratzsch *et al.* 1997). A negative relationship exits between leptin concentrations and growth hormone concentrations (Tuominen *et al.* 1997). Growth retarded babies are born with low leptin levels (Jaquet *et al.* 1999). These infants show catch-up growth for weight during the first year of life and by the end of this period have higher than normal plasma leptin levels (Jaquet *et al.* 1999). A negative association has been observed between leptin levels at birth and weight gain during the first few years in the infant (Ong *et al.* 1999, Jaquet *et al.* 1999). Therefore, leptin may play a role in regulation of infant catch-up growth.

Intracerebroventricular injection of leptin to lean and *ob/ob* mice affects food intake and

metabolic rate at different stages of neonatal development. Mice are responsive to leptin treatment from as early as between 7 and 11 days of age. Oxygen consumption is increased in lean and *ob/ob* mice but food intake was unaffected by leptin treatment. At 28 days leptin treatment decreased food intake and increased oxygen consumption in lean and *ob/ob* mice. This suggests a developmental sequence in the leptin signalling pathway is controlling metabolic rate and food intake (Mistry *et al.* 1999).

1.6.7.5 Leptin and puberty

Treatment with leptin advances the age of first ovulation in female mice (Chehab *et al.* 1997) In fed rats (Cheung *et al.* 1997b) and food-restricted rats (Gruaz *et al.* 1998) leptin treatment also prevents the starvation-induced delay in ovulation in female mice (Ahima *et al.* 1996; Cheung *et al.* 1997b). In human studies leptin levels in blood correlate with those of LH, FSH, estradiol and testosterone (Figure 1.4). Leptin levels in blood increase progressively with stage of pubertal development, reflecting adipose mass (Garcia-Mayor *et al.* 1997). A negative relationship exists between leptin levels and age at menarche in women (Matkovic *et al.* 1997). In boys the rise in testosterone through puberty is associated with reduced leptin expression (Garcia-Mayor *et al.* 1997) suggesting testosterone suppresses leptin levels (Blum *et al.* 1997; Palmert *et al.* 1998). Humans lacking functional leptin or leptin receptor genes do not go through puberty (Clement *et al.* 1998; Strobel *et al.* 1998).

1.7 REGULATION OF LEPTIN EXPRESSION

Leptin expression and circulating levels in the fed state increase in proportion to the amount of adipose stores in humans (Lonnqvist *et al.* 1995). Adipocyte size is a determinant of leptin mRNA expression and secretion (Sorensen *et al.* 1996; Lonnqvist *et al.* 1997). Leptin mRNA abundance may vary with adipose tissue site (Tritos *et al.* 1997). In subcutaneous fat leptin mRNA expression is higher than in visceral fat (Hube *et al.* 1996; Lonnqvist *et al.* 1997; Montague *et al.* 1997).

Leptin secretion by adipocytes is stimulated by glucocorticoids, estrogen and insulin and inhibited by androgens, drugs that increase intracellular cAMP, β_3-adrenergic receptor agonists and phorbol esters. Subcutaneous adipocytes are more receptive to insulin and less to glucocorticoids than omental adipocytes in terms of leptin production (Russell *et al.* 1998).

1.7.1 Nutrition

Leptin mRNA levels were increased in adipose tissue and plasma leptin levels were also increased when UDP-N-acetylglucosamine concentrations in rats were increased by infusion of glucose, glucosamine, uridine or lipid. Leptin mRNA expression was also increased in 3T3 preadipocytes by addition of glucosamine. Nutrient availability may regulate leptin gene expression and secretion through this hexosamine pathway (Wang *et al.* 1999a).

1.7.2 Sympathetic nervous system

Noradrenergic stimulation is required for thermogenesis in brown adipose tissue and is mediated by activation of adipose β-adrenergic receptors. Agents that increase intracellular cAMP, such as β-adrenergic agonists or Bt_2cAMP, suppress leptin expression in brown adipocytes (Slieker *et al.* 1996; Deng *et al.* 1997) (Figure 1.5). Increased sympathetic nerve activity via increased adrenalin inhibits leptin secretion in rat adipocytes (Gettys *et al.* 1996) and in humans (Fritsche *et al.* 1998). The catecholamine-induced inhibition of leptin secretion is a β_3-adrenergic receptor mediated response (Gettys *et al.* 1996; Trayhurn *et al.* 1996) (Figure 1.5).

1.7.3 Glucocorticoids

Identification of glucocorticoid response elements in the $5'$ flanking region of the leptin gene suggested that glucocorticoids could regulate leptin expression (Gong *et al.* 1996). Leptin mRNA expression and leptin secretion are increased by dexamethasone and hydrocortisone in cultured adipocytes (Slieker *et al.* 1996) (Figure 1.5). In humans, glucocorticoid administration increases circulating leptin levels (Kolaczynski *et al.* 1997).

1.7.4 Insulin

Insulin has long been implicated in the regulation of body weight. Insulin increases leptin

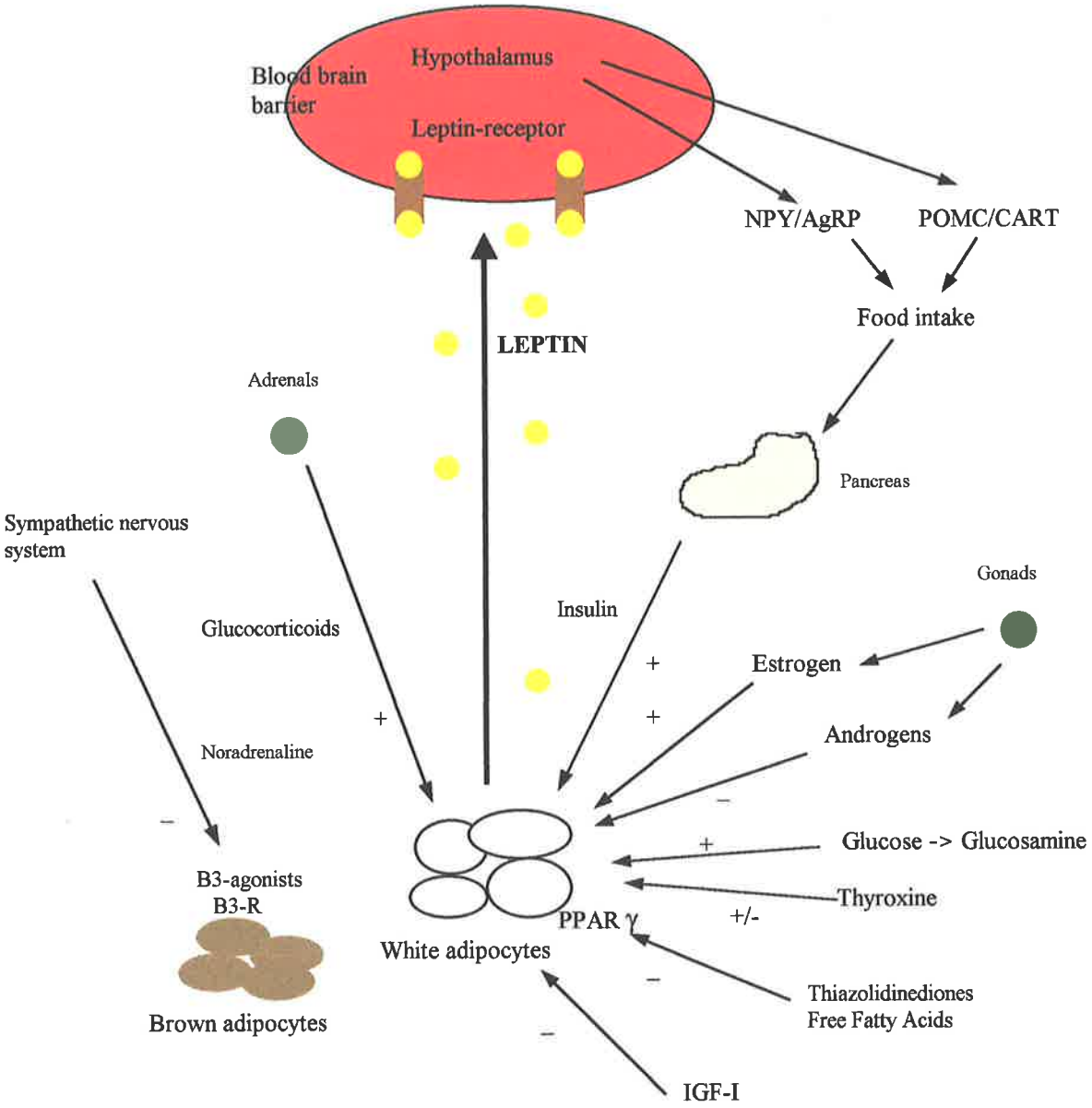


FIGURE 1.5. Schematic representation of the factors and regulatory pathways potentially involved in the control of the leptin gene expression in adipose cells.

β3- β_3-adrenergic receptors; PPARγ- peroxisome proliferator-activated receptors; NPY- Neuropeptide-Y. + and - indicates stimulation and inhibition of the leptin gene.

mRNA levels in cultured mouse (Rentsch *et al.* 1996), human (Wabitsch *et al.* 1996) and rat cells (Rentsch *et al.* 1996; Slieker *et al.* 1996) (Figure 1.5). Acute changes in circulating insulin might be responsible, at least in part, for the variations in leptin levels seen during the fasting-eating cycle. This is supported by studies in normal rats that show leptin mRNA is increased during hyperinsulinemia and reduced during hypoinsulinemia (Cusin *et al.* 1995).

Physiological levels of insulin (meal stimulated) in humans do not stimulate leptin secretion. Higher insulin levels sustained over a 5h period during a euglycemic-hyperinsulinaemic clamp also had no affect on plasma leptin levels (Dagogo Jack *et al.* 1996). However, prolonged exposure to insulin produced a delayed increase in plasma leptin concentrations in humans (Caro *et al.* 1996; Kolaczynski *et al.* 1996). Long term leptin levels are influenced by insulin independent of age (Ryan *et al.* 1996) or glucose tolerance (Malmstrom *et al.* 1996).

Plasma leptin levels in humans doubled after 8.5 h of insulin infusion compared to saline infusion (Malmstrom *et al.* 1996). This agrees with animal studies, where leptin mRNA expression increased in adipose tissue 4 hours after insulin injection and after 6 hours of euglycaemic-hyperinsulinemia (Saladin *et al.* 1995). This implies a role for insulin in chronic, but not acute, regulation of leptin concentration secretion in humans. There are several important interactions between insulin and leptin in which both may be involved in regulating the function of the other.

In humans, insulin resistance is associated with increased plasma leptin concentrations,

independent of adiposity (Haffner *et al.* 1997). Basal plasma leptin concentrations were found to be higher in insulin-resistant than in insulin-sensitive lean men. Obese men had a greater plasma leptin concentration than lean insulin-sensitive or insulin-resistant men (Segal *et al.* 1996). Insulin resistance may explain some of the variability in plasma leptin concentration, other than body fat mass contributing to the regulation of leptin production (Segal *et al.* 1996).

1.7.5 Peroxisome proliferator activated receptor γ

The relationship between insulin and leptin led Kallen *et al.* (1996) to investigate thiazolidinediones, a class of drugs that increase cellular responsiveness to insulin. They are also effective inducers of adipocyte differentiation. Thiazolidinediones have been shown to be selective ligands for PPARγ. Thiazolidinediones regulate the expression of adipocyte-specific genes through PPARγ, and also have an affect on leptin gene expression. PPARγ is a member of the thyroid/retinoid/steroid receptor super-family of transcription factors and regulates the transcription of many genes in adipocytes. Several thiazolidinediones repress leptin gene expression in differentiated 3T3-L1 adipocytes. The ED_{50} for inhibition of leptin expression by the thiazolidinediones BRL49653 is similar to their ED_{50} for binding to PPARγ (Figure 1.5).

A study by Rentsch *et al.* (1996) suggests this may be a physiologically significant pathway. They showed that the effect of free fatty acids (FFA) on leptin gene transcription is normally mediated by interactions with PPARγ. FFA caused a reduction in leptin expression in

cultured adipocytes similar to PPARγ suppressive action on leptin. This indicates that FFA interacting with PPARγ could play a critical role in the down-regulation of leptin expression.

1.7.6 Growth hormone and Insulin-like growth factor-I

GH and/or IGF-I are involved in the regulation of body composition. Hypophysectomised rats treated with IGF-I suppresses leptin mRNA expression while GH treatment has no effect. Insulin can stimulate and FFA can inhibit leptin mRNA expression levels are not significantly different between IGF-I or GH treated hypox rats. Therefore, IGF-I must be acting through an alternative pathway to suppress leptin mRNA expression (Boni Schnetzler *et al.* 1996). Further evidence of an IGF-I inhibitory effect on leptin was observed when healthy human subjects were chronically infused with recombinant IGF-I which caused a reduction in leptin concentration in blood (Dagogo Jack *et al.* 1998) (Figure 1.5).

1.7.7 Thyroid axis

Triiodothyronine treatment increases leptin mRNA abundance in cultured 3T3-L1 rat adipocytes but thyroxine had no effect (Yoshida *et al.* 1997). Humans that are in a hyperthyroid state have higher plasma leptin levels than normal or hypothyroid subjects (Yoshida *et al.* 1998). However, *in vivo* leptin mRNA expression was decreased in thyroidectomized rats infused with triiodothyronine or thyroxine (Escobar Morreale *et al.* 1997) and in hypothyroid rats infused with triiodothyronine (Fain *et al.* 1997).

1.7.8 Gonadal steroids

In humans, plasma leptin is significantly higher in females compared to males even when normalised to body fat mass. Testosterone and leptin are inversely related (Blum *et al.* 1997) which suggests that testosterone suppresses leptin levels. Administration of testosterone reduced serum leptin levels in hypogonadal males (Jockenhovel *et al.* 1997) and normal females (Elbers *et al.* 1997). Estrogen treatment in males given antiandrogens increases serum leptin levels (Elbers *et al.* 1997). Estrogen administration to female rats or humans increases leptin production (Shimizu *et al.* 1997).

1.8 INTRAUTERINE PROGRAMMING

1.8.1 Definition

Fetal development is affected by maternal diet and nutrient stores which provide glucose, amino acids and essential substrates for the conceptus. Fetal development is also controlled by delivery and transfer of these by the placenta to the umbilical circulation (Harding *et al.* 1995; Robinson *et al.* 1995).

Permanent effects on progeny caused by environmental factors during pregnancy has been termed "*in utero* programming" (Barker 1998). Animal studies have shown that the long-term effects of undernutrition depend on the stage of pregnancy at which it occurs. Tissues

and systems appear to be vulnerable to programming during certain phases of rapid cell division or differentiation and different tissues undergo these critical or sensitive phases of development at different stages of gestation (Barker 1994).

Four principles underlie fetal programming; 1) Undernutrition or adverse environment, leave life-long marks on the physiology and structure of the body; 2) Undernutrition at different times in early life has different effects; 3) Babies growing rapidly are more vulnerable to undernutrition during gestation. Growth rate can be suppressed early in gestation which may protect against consequences of undernutrition; 4) Programming of the body through fetal undernutrition itself, results from insufficient maternal intake of food or insufficient transport or transfer of nutrients. Nutritional status of the mother before conception is also important. Little is known about the cellular and molecular mechanisms which underlie programming and leave a permanent mark (Barker 1994).

1.8.2 Intrauterine programming of adiposity

Studies of an historical cohort of the Dutch famine of 1944-45 was one of the first to examine the relationship of adult obesity and early nutrition, which is thought to influence adipose cell number and cell size (Ravelli *et al.* 1976). Experiments in rats had previously shown that reduced nutrition during the suckling period followed by *ad libitum* feeding leads to smaller epididymal fat pads (Knittle *et al.* 1968). Others had hypothesised that early nutrition could affect appetite, growth and subsequent obesity during a critical period when differentiation of hypothalamic centres occurs (Widdowson *et al.* 1960). Rats given reduced

nutrient intake during suckling and subsequently fed *ad libitum* fail to catch up in growth with littermates feed *ad libitum* throughout development (Widdowson *et al.* 1960). The Dutch famine study found that nutritional restriction during the first half of pregnancy caused a significantly higher obesity rate in offspring compared to restriction of food during the last trimester and first few months of postnatal life (Ravelli *et al.* 1976). This finding is consistent with reduced nutrition affecting adipose cell number and hypothalamic centres regulating food intakes and growth.

Subsequent rat studies showed that mothers fed a ration containing 8% protein during pregnancy had offspring that were leaner and had smaller adipocytes as young adults than those from mothers fed a 20% protein diet (Shepherd *et al.* 1997). Adult rats whose mothers were fed 50% less than *ad libitum* in the first two-thirds of pregnancy grew faster, were hyperphagic and had greater cell diameters in their retroperitoneal and perigonadal fat pads than progeny of pregnant mothers provided unrestricted access to food (Jones *et al.* 1982). A similar experiment to that of Jones *et al.* (1982) in rats showed an increase in body weight of progeny from mothers fed a 50% restricted diet and during the first two weeks of pregnancy. There was however, no change in perirenal and gonadal fat pads or adipocyte size in male or female offspring (Pond *et al.* 1985). Both studies reported progeny of food-restricted dams grew faster during the first 16 weeks of postnatal growth (Jones *et al.* 1982; Pond *et al.* 1985). Feed restriction to one-third of recommended intake during the first 70 days of pregnancy in pigs led to progeny with reduced perirenal and subcutaneous backfat and smaller adipocytes in depot fat at 25 weeks of age (Pond *et al.* 1985).

The animal studies demonstrate that early postnatal growth rate is affected by maternal

nutrition during early pregnancy. A more recent study in rats demonstrated that food restriction during the last third of pregnancy produced progeny that postnatally were 20% fatter than non-restricted progeny. That study also found hyperphagia during the last third of pregnancy caused progeny to be 20% fatter in the intra-abdominal depot than progeny from mothers that were hyperphagic throughout pregnancy (Fiorotto *et al.* 1995).

1.8.3 Intrauterine programming of muscle development

GH treatment of dams before mid-pregnancy has been shown to alter fetal and postnatal muscle development with increased secondary myofibres in pigs (Rehfeldt *et al.* 1993; Kelley *et al.* 1995). Increased maternal nutrition during the second quarter of pregnancy in sows has a greater effect on progeny muscle development than similar treatment during the third quarter of pregnancy (Dwyer *et al.* 1994).

1.8.4 Intrauterine programming of endocrine systems

Low birthweight babies are more resistant to insulin and may also have a reduced ability to produce insulin leading to impaired glucose tolerance. Syndrome X, also known as the metabolic syndrome, is linked to thinness at birth which is measured by a low ponderal index (body weight /length^3). Babies born thin have less muscle. Also they have less fat and muscle in adult life which are peripheral sites of insulin action. Insulin is an important regulator of fetal growth (Barker *et al.* 1993a).

Recent epidemiological studies have shown that body weight at birth is associated with blood leptin concentrations in adults, independent of adiposity (Phillips *et al.* 1999; Lissner *et al.* 1999). This suggests that poor fetal growth may be associated with a heightened risk of leptin resistance and obesity in adult life (Lissner *et al.* 1999; Phillips *et al.* 1999).

1.8.5 Factors influencing fetal programming- programming mechanisms

Maternal metabolism during pregnancy can affect nutrient availability to the developing fetus. Maternal hormones such as insulin, insulin-like growth factor (IGF)-I, growth hormone (GH) and thyroid hormone may control nutrient partitioning between maternal, placental and fetal tissues (Brown *et al.* 1989). GH or GH-related hormones in the mother could influence nutrient availability to the fetus. GH treatment in pregnancy can affect substrate supply to the fetus and thus influence fetal growth. GH treatment induces insulin resistance in the mother increasing maternal glucose levels and therefore nutrient availability to the fetus (Kveragas *et al.* 1986). GH treatment may also increase placental transport of glucose to the fetus (Jenkinson *et al.* 1999). Circulating maternal metabolites and hormones have been manipulated in pregnant pigs by GH treatment. Pigs administered pGH during pregnancy have increased IGF-I (Sterle *et al.* 1995), insulin (Kveragas *et al.* 1986) glucose and free fatty acid levels (Spence *et al.* 1984) and decreased IGF-II (Sterle *et al.* 1995). Treatment of nonpregnant pigs with GH reduces fat deposition, promotes lean growth and increases blood levels of IGF-I (Machlin 1972; Chung *et al.* 1985; Campbell *et al.* 1988; Owens *et al.* 1990).

Increased maternal nutrition during the first trimester and then a subsequent decrease in maternal nutrition during the second trimester in sheep causes reduction in maternal glucose and insulin with no effect on IGF-I levels. The opposite occurred with an increase in maternal glucose and insulin when maternal nutrition was increased in the second trimester from a reduced diet in the first trimester (Wallace *et al.* 1996). Restricted feed intake in sheep during pregnancy reduced maternal triiodothyronine and thyroxine concentrations of IGF-I and insulin, (Wallace *et al.* 1997; Clarke *et al.* 1998) while mean maternal GH and progesterone concentrations were increased (Wallace *et al.* 1997).

1.9 AIMS OF THIS STUDY

The general aim of this study was to investigate the effect of maternal environment during pregnancy on postnatal programming of leptin and to identify potential mechanisms involved in this process.

Hypotheses:

(1) Leptin mRNA is expressed in adipose tissue of pig and guinea pig.

(2) Leptin mRNA abundance in offspring is programmed by maternal nutrition during the second quarter of pregnancy.

(3) Maternal metabolism during pregnancy programs leptin in offspring.

(4) Intrauterine programming of postnatal leptin is due to altered programming of adipocyte development or endocrine systems that regulate postnatal leptin production.

CHAPTER 2

EXPERIMENTAL ANIMAL MODELS OF LEPTIN PROGRAMMING IN HUMANS

2.1 INTRODUCTION

An experimental model was required in which mechanisms of human intrauterine programming of leptin could be studied. In mouse, rat, sheep, pig and human the leptin gene is expressed as a 4 to 5 kb mRNA in adipose tissue and produces a polypeptide of 167 amino acids. Both the mouse and rat have no significant adipose development before birth. In the mouse and rat critical periods of adipocyte development occur postnatally. Significant adipocyte development occurs before birth in the human. This makes it unlikely that intrauterine programming of adipocyte development occurs by the same mechanism in rodents and humans. The guinea pig and pig were evaluated as experimental models to study leptin programming because like humans, the guinea pig and pig deposit significant amounts of fat before birth. The pig also is a relatively obese animal postnatally. The guinea pig has a similar percentage of fat to that of a human baby at birth while the rat and pig are very lean at birth.

The aim of the following study was to determine whether leptin is expressed in adipose tissue in guinea pigs and pigs.

The reverse transcription polymerase chain reaction (RT PCR) was used to determine whether the leptin gene is expressed in adipose tissue of guinea pigs and pigs. Northern blotting analysis of RNA was used to ascertain whether leptin mRNA occurs in the guinea pig and pig at a similar size reported in other species.

2.2 MATERIALS AND METHODS

2.2.1 Identification of guinea pig leptin mRNA

2.2.1.1 Extraction of total RNA from guinea pig adipose tissue

Female guinea pigs 9 months of age *ad libitum* fed were sacrificed by intravenous overdose of sodium pentobarbitone. Adipose tissue was collected from epididymal, shoulder, retroperitoneal, parametrial, perirenal and dorsal fat pads. Tissue from the lung and liver were also collected. Dissected adipose tissue from fat pads and lung were snap frozen in liquid nitrogen. During the dissection of adipose tissue and lung, care was taken to avoid ribonuclease contamination. Instruments and working surfaces were cleaned regularly with alcohol. Adipose tissue and lung samples were stored at -80^{0}C until extraction of total RNA. Mouse epididymal fat and pig subcutaneous fat were collected as described above.

Total RNA was extracted with guanidinium isothiocyanate (Chomcynski *et al.* 1987) from the guinea pig fat pads, lung and liver. RNA was also extracted from mouse epididymal fat and pig subcutaneous fat by the same procedure. Approximately 200 mg of adipose tissue frozen at –80^{0}C was weighed and placed in 10 ml tubes (Falcon Tubes, Bectin Dickinson) and 1.2 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added. Tissue was homogenised using an Ultra-Turrax T25 homogeniser (Janke & Kunkel) with a dispersing tool. Before use the tool was cleaned thoroughly by washing with 70% ethanol and homogenising 1 M sodium hydroxide for 30 sec at ½ speed and followed by 7-10 ml of sterile water four times for 30 sec. Immediately before use the tool was homogenised in 5 ml of denaturing solution for 30

sec (Chomcynski *et al.* 1987). Washing of the dispersing tool was repeated between tissue samples. The adipose tissue was homogenised at full speed in three 30 sec bursts with a 30 sec break between each. The homogenate was transferred into a 2 ml Eppendorf tube and 120 µl of 2 M sodium acetate (pH 4) was added and mixed by repeated inversion. Phenol (Cat. No. P5566, Sigma, St Louis, IL, USA) (480 µl) was added and mixed by inversion. One hundred and twenty µl of a 1:1 mixture of chloroform (Cat. No. 18888, Ajax, Auburn, N.S.W., Australia) /isoamylalcohol (Cat. No. 1105, Ajax, Auburn, N.S.W., Australia) was added and mixed by repeated inversion. The samples were centrifuged at 13 000 rpm at 4^0C for 20 min. The aqueous layer was removed and added to an equal volume of isopropanol (Cat. No. 15252, BDH, England) and mixed by repeated inversion. Samples were incubated at -20^0C overnight to precipitate RNA. Samples were centrifuged at 4^0C for 20 min. The supernant was removed and the pellet was air dried for 15 min at room temperature. The pellet was dissolved in 50 µl of molecular grade water (Cat. No. 95284, Fluka, Switzerland) and stored at -20^0C.

2.2.1.2 Analysis of total RNA content

RNA integrity was determined by electrophoresis through a 1% agarose gel (5cm x 7.5 cm) containing 0.5 µg/ml ethidium bromide in 1x Tris-borate EDTA (TBE, 0.089 M Tris borate, 0.002 M EDTA) at 60 volts for 45 min in sterile 1x TBE buffer. The gel was transilluminated with ultraviolet light. The appearance of intact 28S and 18S ribosomal RNA was confirmed for each sample.

The absorbance of the RNA solution diluted 1:100 in sterile water was determined spectrophotometrically at 260 and 280 nm (Beckman Du-50 Spectrophotometer, Fullarton CA USA). An absorbance of 1.0 at 260 nm corresponds to 40 μg/ml RNA in solution. The ratio of A_{260}/ A_{280} was determined. RNA solutions that have a ratio between 1.8 and 2.0 are free of significant contamination with phenol and protein.

2.2.1.3 Reverse Transcription of guinea pig adipose RNA

Total RNA from guinea pig fat depots, guinea pig lung tissue as well as, pig and mouse adipose tissue were reverse transcribed into first strand cDNA using Superscript™ RNase H$^-$ (Cat. No. 18053-017, GIBCOBRL). 1 μg of RNA in 4 μl was added to 10 μl of molecular grade water (Cat. No. 95284, Fluka, Switzerland) and incubated with 2 μl of 100 μg/ml random hexanucleotides (Cat. No. RP-6, GeneWorks, Adelaide, Australia) at 70^{0}C for 10 min. The solution was chilled on ice for 5 min. 4 μl of 5x RT buffer (250 mM Tris-HCL [pH 8.3], 300 mM KCl, 15 mM $MgCl_2$), 2 μl of 0.1 M DTT and 2 μl 100mM Ultrapure dNTPs (Cat. No. 27-2035-01, Amrad Pharmica Biotech, Melbourne, Vic), 2 μl of 10 mM each dATP, dGTP, dCTP, dTTP was added. The solution was preincubated for 2 min at 43^{0}C before 2 μl of Superscript™ reverse transcriptase (200 units) was added. The reaction continued at 43^{0}C for 1.5 hrs before it was stopped by heating to 94^{0}C for 5 min. After 5 min at 0^{0}C, 26 μl of water was added for a final volume of 50 μl which was stored at -20^{0}C. A negative control (water containing no RNA as a template) for reverse transcription was processed with the samples.

2.2.1.4 DNA extraction

DNA was extracted from guinea pig lung and pig liver. DNA purification was performed with the Wizard genomic purification kit (Cat. No. A1120, Promega. Madison, WI, USA). 600 µl of nuclei lysis solution (Cat. No. A1120, Promega) was added to 100 mg of frozen tissue in a 6 ml tube (Falcon Tubes, Bectin Dickinson). The sample was homogenised as described in section 2.2.1. The homogenate was transferred to a 1.5 ml microcentrifuge tube and incubated at 65^0C for 15 min. 3 µl of RNase solution (Cat. No. A1120, Promega) was added to the homogenate and mixed by inversion and was incubated at 37^0C for 15 min. The homogenate was cooled at room temperature for 5 min. 200 µl of protein precipitation solution (Cat. No. A1120, Promega) was added and mixed by vortexing for 20 sec. The sample was centrifuged for 3 min at 13 000 g at room temperature. The supernant containing the DNA was removed and mixed with 600 µl of isopropanol in a sterile 1.5 ml Eppendorf tube. The solution was gently mixed until white thread-like strands appeared. The DNA solution was centrifuged for 1 min at 13 000 g at room temperature. The DNA was visible as a white pellet and the supernant was removed. 600 µl of 70% ethanol was added to the DNA pellet and mixed by inversion. The DNA suspension was centrifuged for 1 min at 13 000 g at room temperature. The ethanol was aspirated and the DNA pellet was air dried. 100 µl of DNA rehydration solution (Cat. No. A1120, Promega) was added to the DNA pellet and incubated at 65^0C for 1 hr. The DNA solution was stored at -20^0C.

2.2.1.5 PCR amplification of guinea pig leptin cDNA

The criteria for the design of PCR primers was that they were chosen to be complementary to one strand (5′ to 3′) upstream and complementary to the opposite strand (5′ to 3′) downstream from the sequence to be amplified. The 5′ ends of the primers define the ends of the amplified ds DNA PCR product. Primers should ideally contain a 40-60% G+C content and with no long stretches of any one base. The PCR primer pair should not contain complementary structures with each other longer than 2 bases to avoid formation of primer dimers (especially at the 3′ ends). Annealing of the 3′ ends of the two primers causes the formation of a "primer template" complex and primer extension produces a short duplex product called "primer dimer". Internal secondary structures such as hairpins should be avoided in primers (Williams 1989; Bej *et al.* 1991).

The published sequence of the pig leptin cDNA (Neuenschwander *et al.* 1996) was used to design oligonucleotide primer pairs. The first pair spanned nucleotides 144 (Lep 1- sense 5′ CAGTCTGTCTCCTCCAA 3′) and 310 (Lep 2- antisense 5′ GGTCTCCAGGTCATTC 3′) which were custom synthesised (Geneworks, Adelaide). An amplified fragment of 167 base pairs (bp) in length was expected (Figure 2.1). PCR was performed in a volume of 50 µl containing 10 µl of the reverse transcriptase (RT) reaction product (guinea pig cDNA), 10x buffer (670 mM Tris-HCl [pH 8.8 at 25^0C], 166 mM $[NH_4]_2SO_4$, 4.5% Triton X-100, 2 mg/ml Gelatin), 2 mM $MgCl_2$, 200 µM of each deoxynucleotide, 0.5 µM of each oligonucleotide primer pair and 1 unit of Taq DNA polymerase (Cat. No. TAQ-3, Biotech, Bentley, Western Australia). The PCR program consisted of 40 cycles of denaturation at

94^0C for 1 min, annealing at either 45 or 53^0C for 1 min, extension at 72^0C for 1min and a final extension at 72^0C for 10 min. A positive control (mouse cDNA instead of guinea pig cDNA) was processed in the PCR. Two negative controls (water containing no cDNA and guinea pig lung cDNA as template) for the PCR were processed with the samples.

The second set of primers designed from the published sequence of the porcine leptin cDNA (Neuenschwander *et al.* 1996) spanned nucleotides 142 (pOB 3- sense 5′ TGCAGTCTGTCTCCTCCAAA 3′) and 294 (pOB 4- antisense 5′ GATAATTGGATCACATTTCTG 3′) which were custom synthesised (Geneworks, Adelaide, Australia). The expected fragment length was 153 bp (Figure 2.1). PCR was performed as described above. Two positive controls (pig and mouse cDNA) were processed in the PCR. Two negative controls (water containing no cDNA and guinea pig lung cDNA as template) for the PCR were also processed with the samples.

A third set of primers were devised from published sequence of the leptin gene (Neuenschwander *et al.* 1996). The oligonucleotides spanned nucleotides 24 (pOB1- sense 5′ TTCYTGTGGCTTTGGYCC 3′) and 349 (pOB2- antisense 5′ AGCTCTTNGAGAAGGCCAGC 3′) of the cDNA, which generates a fragment of 325 bp (Figure 2.1). Degenerated bases were noted as Y= C or T and N= A, G, C, or T. The primer pair spans an intron so that products from contaminating genomic DNA could be detected. The PCR was performed as described above.

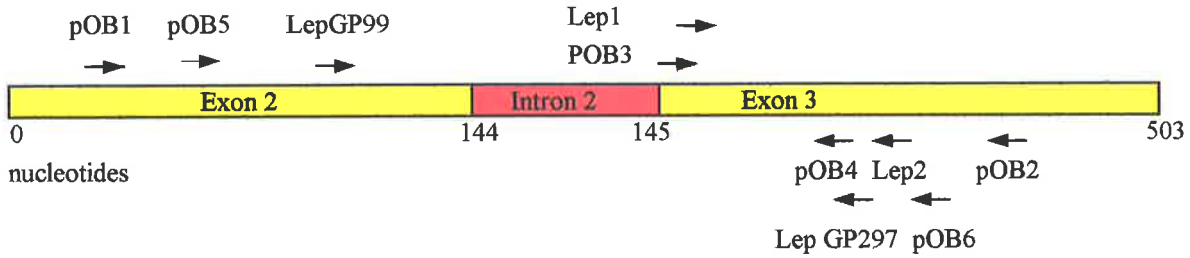


Figure 2.1 The porcine leptin gene.

Genomic DNA extracted from the guinea pig lung and pig liver as described in section 2.2.1.4 was added to the PCR as described above with primers pOB3- pOB4. This was performed to determine whether the length of the leptin amplicon from genomic DNA was of a similar length compared to guinea pig cDNA added to the PCR.

2.2.1.6 Gel electrophoresis of PCR products

The products of the above PCR amplifications were separated by electrophoresis on 2.0 % agarose gels at 80 volts for 1.25 hours. The gels were stained with ethidium bromide and then photographed under UV transillumination.

2.2.2 Sequencing of the guinea pig leptin PCR product

The PCR products obtained using primer pairs pOB1-pOB2 and pOB3-pOB4 with guinea pig adipose cDNA at template were purified by QIAquick PCR purification (Cat. No.

A1120, QIAGEN Pty. Ltd., Clifton Hill, Australia) to remove primers, nucleotides, polymerases and salts. The concentration of the leptin PCR product determined by spectrophotometer at 260 nm was 20 ng/µl. Sequencing was performed by the ABI PRISM Dye Terminator method (Perkin-Elmer, Foster City, California, USA). The sequencing mix contained 8.0 µl of terminator ready reaction mix, 20 ng/µl of PCR product, 3.2 pmole of primer in a final volume of 20 µl. The cycling profile consisted of 25 cycles at 96^0C for 30 sec, 50^0C for 15 sec and 60^0C for 4 min. The extension products were removed by ethanol precipitation. The sequencing products were analysed by the IMVS sequencing lab (Adelaide, Australia).

2.2.3 PCR amplification of guinea pig leptin cDNA using guinea pig specific primers

A set of primers were designed from the sequence of the partial guinea pig leptin cDNA isolated in section 2.2.2. The oligonucleotides spanned nucleotides 99 (LepGP99- sense 5′ GCAGTCGGTATCCTCCAAGCAC 3′) and 297 (LepGP297- antisense 5′ GGAAGGCAGGCTGGTGAGGA 3′) of the cDNA, which generates a fragment of 199 bp (Figure 2.1). The primer pair spans an intron present in human and mouse leptin genes so that products from contaminating genomic DNA could be detected. The PCR was performed as described in section 2.2.1.5 with an annealing temperature of 58^0C. A negative control (water containing no cDNA) for the PCR was processed with the samples.

2.2.4 Northern analysis of guinea pig and pig leptin mRNA

Quantity and purity of solutions of total RNA were determined as in section 2.2.2.2.

A digoxigenin-labelled DNA probe leptin mRNA was prepared using as template the 325 bp leptin PCR fragment obtained from guinea pig adipose tissue using primer pair pOB1-pOB2 followed by QIAquick PCR purification (Cat. No. A1120, QIAGEN) . Labelling of 0.5-1 μg of denatured leptin PCR product with digoxigenin was performed with the DNA labelling and detection kit (Cat. No. 1585 550, Roche Diagnostics, Mannheim, Germany) as described by Kessler *et al.* (1990). Digoxigenin labelled leptin DNA was purified by ethanol precipitation (Kessler *et al.* 1990).

Northern hybridisation was performed using 5 μg of total RNA in 20 μl isolated from fat and non-fat sites of guinea pig and pig (section 2.2.1.1). It was first denatured by incubating with 20 μl of 5x formaldehyde gel-running buffer (0.1 M (*N*-morpholino) propanesulfonic acid (pH 7.0), 40 mM sodium acetate, 5 mM EDTA), 3.5 μl of formaldehyde and 10 μl formamide for 15 min at 65^0C and then chilled on ice. 2 μl of sterile diethyl pyrocarbonate-treated formaldehyde gel-loading buffer (50% glycerol, 1mM EDTA (pH 8.0), 0.25% bromophenol blue; 0.25% xylene cyanol FF) was added to the denatured RNA sample. Duplicate denatured RNA samples of 20 μl were loaded onto different halves of a 1.5 % formaldehyde agarose gel (1x formaldehyde gel-running buffer and 2.2 M formaldhyde) and electrophoresed at 55 volts for 5 hours in 1x formaldehyde gel-running buffer and then soaked in 0.05 M NaOH for 20 min. The gel was rinsed briefly in 0.1% diethyl pyrocarbonate (DEPC) water and soaked for 45 min in 20x standard saline citrate (SSC) buffer (3M NaCl, 0.3 M $Na_3Citrate \cdot 2H_2O$, [pH 7.0]). The gel was cut in half and the RNA integrity and loading were evaluated by soaking one half in a solution of 0.5 μg/ml of ethidium bromide and photographed under ultraviolet transillumination. The distance of

migration of the 28S rRNA (5.1 kb) and 18S rRNA (1.9 kb) bands were measured. The $\log_{10}$ of the size of the bands of RNA were plotted against the distance migrated. The resulting calibration curve was used to determine the size of the RNA species detected by hybridisation after transfer from the second half the gel to a solid membrane.

The RNA was blotted from the second half of the gel onto a nylon membrane (Magnacharge, Geneworks) via standard capillary techniques using 20x SSC (Sambrook *et al.* 1989). After being soaked in 6x SSC for 5 min and dried for a least 30 min at room temperature, membranes were further dried at 70^0C for 1 hour. The membrane was prehybridised in a 'high SDS' hybridisation buffer (7% SDS, 50% formamide, 5x SSC, 50 mM sodium phosphate (pH7.0), 0.1% (w/v) N-lauroylsarcosine, 2% blocking reagent [1/5 volume of blocking solution containing DNA (Cat. No. 1585 614 Roche), [10x concentration]) for at least 1 hour at 50^0C. Hybridisation was at 50^0C overnight in 'high SDS' hybridisation buffer, together with the digoxigenin-labelled guinea pig leptin cDNA fragment (pOB1-pOB2) at 50 ng/ml. The membrane was washed twice for 5 min in 50 mls of 2x SSC, 0.1% SDS at room temperature, followed by two washes for 15 min in 0.5 SSC, 0.1% SDS at 68^0C under constant agitation (hybridisation oven, Ratek Instruments). The membrane was incubated for 30 min in 100 ml of 1x blocking solution (10x blocking solution [Cat. No. 1585 614, Roche] diluted 1:10 in maleic acid buffer [0.1M maleic acid; 0.15 M NaCl; pH 7.5]). The membrane was incubated for 30 min with 75 mU/ml of anti-(digoxigenin) serum/alkaline phosphatase conjugate (Cat. No. 1585 614 Roche). The membrane was washed twice for 15 min in 100 ml of washing buffer (maleic acid buffer [0.1M maleic acid; 0.15 M NaCl; pH 7.5], 0.3% [v/v] Tween® 20). The membrane was equilibrated for 5 min in 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCL, 50 mM

$MgCl_2$, pH 9.5). The membrane was placed (with DNA side facing up) in a plastic bag (10 cm x 20 cm) and 1 ml of CSPD® (Cat. No. 1585 614, Roche) was added as a chemiluminescence substrate. The membrane was preincubated for 5 min at room temperature and the hybridisation bag was squeezed to remove excess CSPD® liquid. The damp membrane was incubated for 15 min at 37^0C (hybridisation oven, Ratek Instruments) to enhance the luminescent reaction. The luminescence was detected by exposure of membrane to X-ray film for 20 min at room temperature (XAR, Kodak).

2.2.5 Identification of pig leptin mRNA

2.2.5.1 Extraction of RNA from porcine subcutaneous adipose tissue

Total RNA was extracted from pig adipose tissue with TRIzol according to the manufacturer's instructions (Cat. No. 15596, Gibco BRL Life Technologies International, Melbourne Vic). This method is a modification of the original procedure of Chomcynski *et al.* (1987). Adipose tissue was collected as described in section 2.2.1.1.

Frozen adipose tissue from -80^0C storage was preweighed into 10 ml (Falcon tubes, Becton Dickinson). 1.6 ml of TRIZOL reagent was added to 200 mg of adipose tissue. The tissue was homogenised exactly as described in section 2.2.1.1. Homogenised tissues stood in ice, while subsequent specimens were homogenised. The homogenates were incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.4 ml) was added to the homogenate. The tubes were shaken vigorously by hand for 15 seconds and the tubes were incubated at room temperature for 3 min. The tubes

were centrifuged at 12 000 g for 15 min at 4^0C. 800 µl of the aqueous phase that contains the RNA was removed and transferred into a fresh 2ml Eppendorf tube. RNA was precipitated from the aqueous phase by adding 0.8 ml of isopropyl alcohol. The tubes were incubated at room temperature for 10 min and centrifuged at 12 000 g for 10 min at 4^0C. The supernant was removed and the RNA precipitate was washed with 1ml of 75% ethanol. The sample was vortexed and centrifuged at 7 500 g for 5 min at 4^0C. The tube containing the RNA pellet was inverted on paper to drain completely and was then air dried at room temperature for 15 min. RNA was dissolved in 50 µl of molecular grade water and stored at -80^0C.

2.2.5.2 Integrity and concentration of porcine adipose RNA

Total RNA integrity was determined as described in section 2.2.1.2.

2.2.5.3 Reverse Transcription of porcine adipose RNA

Total RNA extracted from porcine adipose tissue as described in 2.2.5.1 was reverse transcribed into first strand cDNA using the Superscript™ RNase H^- reverse transcription kit (Cat. No. 18053-017, GIBCOBRL). Reverse transcription was performed as described in section 2.2.1.3. A negative control (water containing no cDNA) for the PCR was processed with the samples.

2.2.5.4 Polymerase chain reaction amplification of porcine adipose cDNA

pOB2 primer described in section 2.2.1.5 was used from published porcine data on the leptin gene (Neuenschwander *et al.* 1996; Ramsay *et al.* 1998) with a modification of pOB1. The oligonucleotides chosen spanned nucleotides 24 (pOB1A- 5′ TTCCTGTGGCTTTGGCCC 3′) and 349 (pOB2- 5′ AGCTCTTNGAGAAGGCCAGC 3′) of the cDNA, which should generate a fragment of 326 bp (Figure 2.1). A second set of leptin porcine primers spanned nucleotides 47 (pOB5- 5′ TGTCCTACGTTGAAGCCGTG 3′) and 314 (pOB6 5′ CGGAGGTTCTCCAGGTCATT 3′) were designed to produce an amplicon of 268 bp (Figure 2.1). The primer pairs spanned intron 2 so that products from contaminating genomic DNA could be detected. The PCR was performed as described in section 2.2.1.5. The PCR profile consisted of 40 cycles of denaturation at 94^0C for 30 sec, annealing at 58^0C for 30 sec and extension at 72^0C for 30 sec.

2.2.5.5 Gel electrophoresis of porcine RT PCR products

The products of the PCR amplification were separated by electrophoresis on 2.0 % agarose gels at 80 volts for 1.25 hours. The gels were stained with ethidium bromide and photographed under UV transillumination.

2.2.5.6 Sequencing of the porcine leptin RT PCR products

The products of RT PCR using porcine adipose cDNA as template and both pOB1A-pOb2 and pOB5-pOB6 primer pairs were purified by QIA-quick purification (Cat. No. A1120, QIAGEN Pty. Ltd., Clifton Hill, Australia). The concentration of the leptin PCR product

measured spectrophometerically at 260 nm was determined to be 30 ng/μl. Sequencing was performed as described in section 2.2.2.

2.3 RESULTS AND DISCUSSION

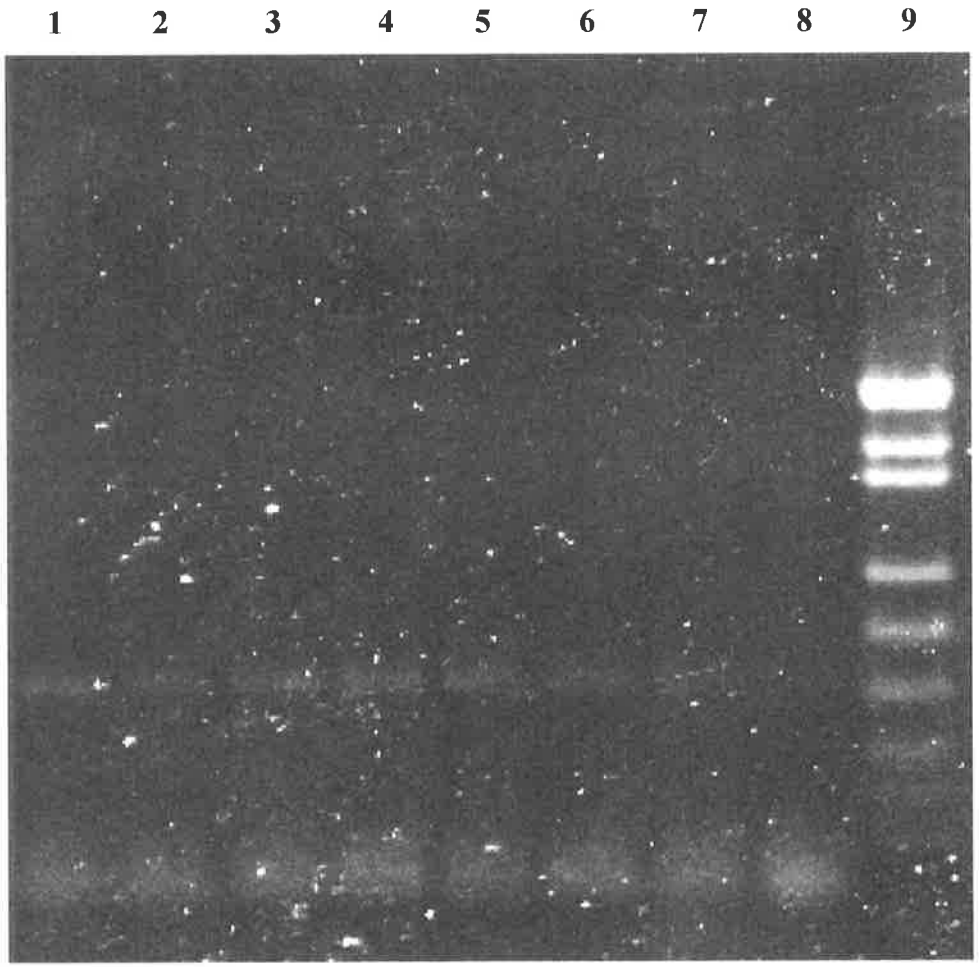
2.3.1 Detection of guinea pig leptin mRNA by RT PCR

Lep1-Lep2 primers should amplify a 167 bp fragment of guinea pig cDNA (Figure 2.1). RNA was reverse transcribed from total RNA extracted from epididymal, retroperitoneal and shoulder fat and lung from guinea pig as well as mouse epididymal fat. Amplified with Lep1-Lep2 primers and a magnesium concentration of 2 mM produced no detectable bands on a 2% agarose gel using ethidium bromide staining. The PCR conditions were varied by altering the magnesium concentration from 1.5 mM to 4 mM, oligonucleotide primer concentration from 0.5 μM to 2 μM and annealing temperatures of 45^0C to 53^0C. An oligonucleotide primer concentration (Lep1-Lep2) of 1 μM and magnesium concentration of either 1.5, 2.5, 3.5 and 4 mM amplified several bands but none of the predicted size. Annealing temperatures of 45^0C and 53^0C with magnesium concentration at 3 or 4 mM also failed to generate a 167 bp amplicon. The failure to produce the predicted amplicon could be due to either of the primers being partly homologous to another part of the guinea pig genome and binding to this site and amplifying non-specific segments of the genome.

Based on the reported porcine leptin nucleotide sequence (Neuenschwander *et al.* 1996) if leptin cDNA is similar in pig and guinea pig primers, pOB3-pOB4 should generate an amplicon of 153 bp. A faintly stained PCR product of approximately 150 bp was amplified

Figure 2.2 Size fractionation via 2% agarose gel electrophoresis of RT PCR product using pOB3-pOB4 primers and pig, mouse and guinea pig cDNA.

Lane 1- pig subcutaneous fat cDNA; 2- pig subcutaneous fat cDNA; 3- mouse epididymal fat cDNA; 4- guinea pig epididymal fat cDNA; 5- guinea pig shoulder fat cDNA; 6- guinea pig retroperitoneal fat cDNA; 7- guinea pig lung DNA; 8- Negative PCR (water instead of cDNA as template); 9- pUc19/Hpa II DNA.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6
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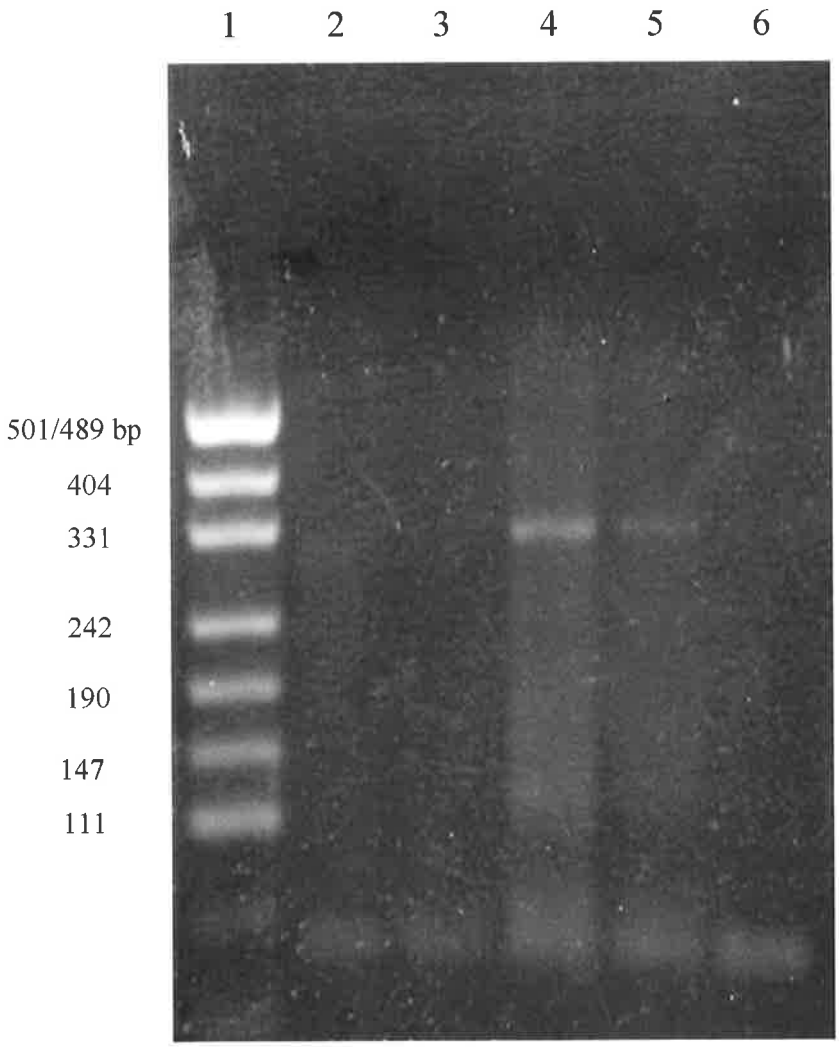
8
9
(bp)
501/489
404
331
242
190
147

Figure 2.3 Size fractionation via 2% agarose gel electrophoresis of RT PCR products using pOB1-pOB2 primers and guinea pig cDNA. Lane 1- pUC19 DNA/ Hp II marker; 2- Guinea pig epididymal fat cDNA; 3- Guinea pig shoulder fat cDNA; 4- Re-amplification of the product in lane 2; 5- 1:10 dilution of the PCR product in lane 4 6-Negative PCR (Water instead of cDNA as template)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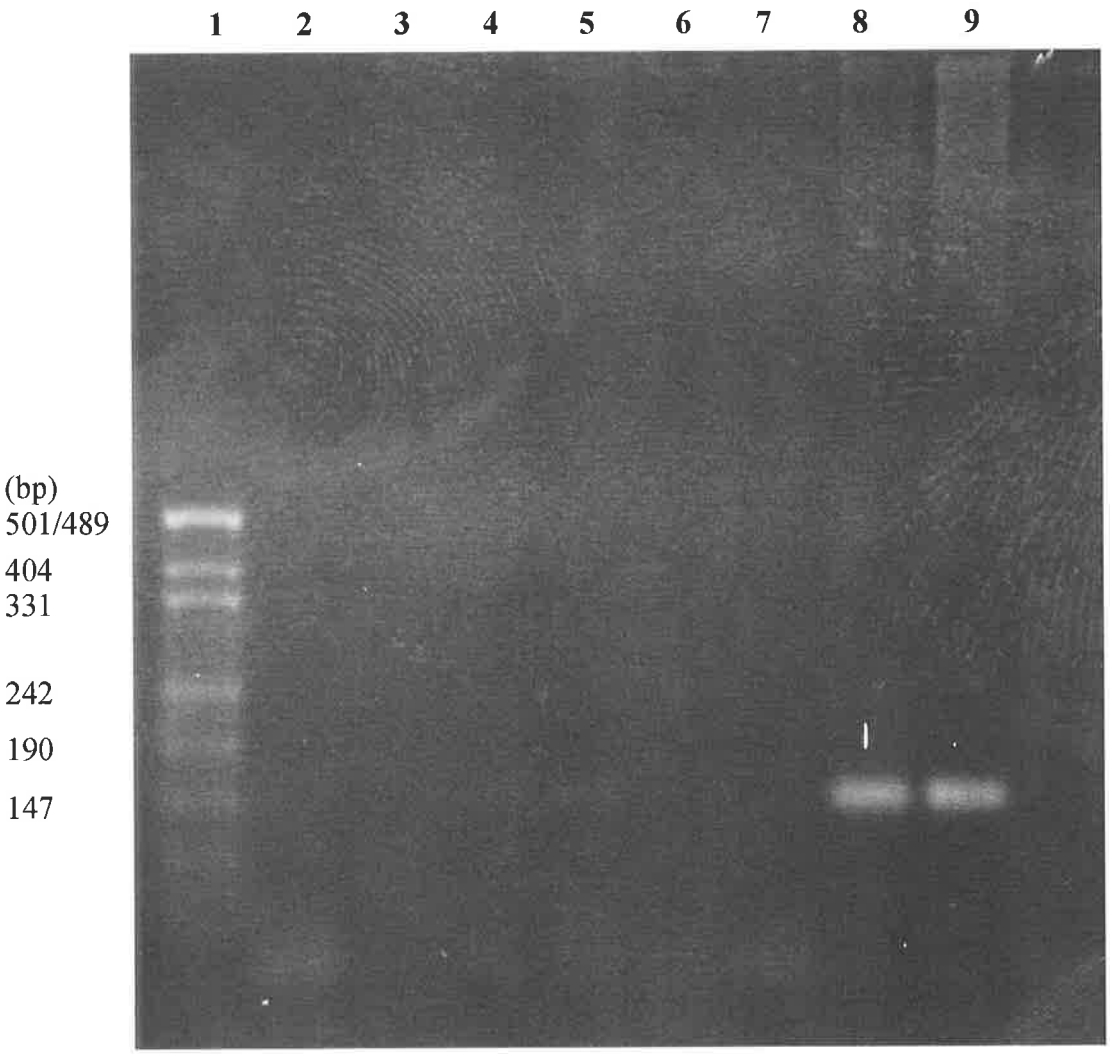
from total RNA of epididymal, shoulder and retro-peritoneal fat of the guinea pig using pOB3-pOB4 primers (Figure 2.2). A similar sized product was obtained from total RNA from pig subcutaneous adipose tissue and mouse epididymal fat (Figure 2.2). This is the first evidence that the leptin gene exists in the guinea pig. Sequencing was required to confirm this observation that guinea pig adipose expresses leptin mRNA.

pOB1-pOB2 primers should generate an amplicon of 325 bp and a fragment of approximately 330 bp was amplified by reverse transcribing total RNA from guinea pig adipose tissue and amplifying the cDNA. The band was only present in guinea pig epididymal fat. A stronger band was obtained by re-amplifying the epididymal PCR product (Figure 2.3). This result was not reproducible in any other tissue from guinea pig examined.

DNA extracted from guinea pig lung and pig liver produced the same PCR fragment of approximately 150 bp in length (Figure 2.4). There is no intron spanning the primer set as both the mRNA and DNA fragments were the same length. This is expected since pOB3-pOB4 should lead to amplification of a genomic DNA fragment in exon 3. The pOB3-pOB4 primers amplified guinea pig cDNA and DNA to produce a fragment of approximately equal length to that observed in the pig.

The pOB3-pOB4 primer pair were not suitable for the routine detection of mRNA as the primers do not span an intron. pOB1-pOB2 were used as they span intron 2 in the leptin gene and thus permit discrimination between products of cDNA genomic DNA amplification. pOB1-pOB2 primers were used in amplifying a fragment of ~330 bp from

Figure 2.4 Size fractionation via 2% agarose gel electrophoresis of RT PCR products and using pOB3-4 primers and mouse, pig and guinea pig cDNA. Also th fractionation of PCR products using pOB3-4 primers and pig and guinea pig DNA Lane 1- pUC19/ Hpa II marker; 2- mouse epididymal fat cDNA; 3- pig subcutaneous fa cDNA; 4- guinea pig retroperitoneal fat cDNA; 5- guinea pig epididymal fat cDNA; 6- guinea pig lung cDNA; 7- Negative PCR (water instead of cDNA as template); 8- pi adipose genomic DNA; 9- guinea pig lung genomic DNA.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guinea pig cDNA similar in size to the pig leptin cDNA fragment obtained here and reported by Neuenschwander *et al.* (1996).

2.3.2 Partial sequencing of the guinea pig leptin cDNA

The 153 bp product obtained with pOB3-pOB4 and the 325 bp product obtained with pOB1-pOB2 primers and guinea pig cDNA template were sequenced. The pOB1-pOB2 sequence had 100% identity with the pOB3-4 sequence.

Comparison of the guinea pig cDNA sequences generated with pOB3-pOB4 (153 bp) and pOB1-pOB2 (325 bp) with those published for the mouse, rat and human sequences were performed using the blast program package. Nucleotide sequence analysis of the 230 3'-bases of the pOB1-pOB2 sequence showed that this guinea pig cDNA was homologous to leptin cDNA from other species, exhibiting respectively 89, 85 and 83% identity with rat, mouse and human leptin cDNA (Figure 2.6) and encoded amino acids 16-95 of the leptin polypeptide (Figure 2.5).

The amino acid sequence of guinea pig leptin$^{16\text{-}95}$ polypeptide was homologous with that of rat, mouse, human, pig and cow leptin having, respectively 90, 86, 81, 86 and 85 % identity (Figure 2.6). The guinea pig codon for arginine 105 (CGA) is the same as that of the mouse and rat but bovine, pig and human have arginine encoded by CGG at 105. There are a number of large blocks of identical nucleotide sequence in the guinea pig and other mammalian species. Therefore, it can be concluded that the product amplified from guinea pig cDNA is derived from guinea pig leptin mRNA.

```
Mouse      162   A        T      A         CAAT          C       C                 G          221
Rat        106   A        T      A         CAAT          C       C                 G          165
Guinea pig 61    ATGACCACTGTCTCCAGGATTGTGGACATTTGACACAAGCAGTCGGTATCCTCCAAGCAC 120
Bovine     43    A      A T      A         CAAT        C   C       C         C   C          A   G 102
Pig        106   A      G T      A         CAGT          C       T         T   C          A       165
Human      106   A      A T      A         CAAT          C       C         A   C          A       165

Mouse      222                                                                                   281
Rat        166              C    T                    C                                        225
Guinea pig 121 AGGGTCACTGGCTTGGACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATG 180
Bovine     103                   T                              C       TC C                    162
Pig        166                   T                C             C   T   TG C                  225
Human      166   AA         C    T                              C           C      CC    A      225

Mouse      282                        G                                                          341
Rat        226              C         G                    A                T            C       285
Guinea pig181 GACCAGACTCTGGCATTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTG 240
Bovine     163              A         G        C           A                T            AG      222
Pig        226              C         G        C           A                T            AG      285
Human      226              A         G        C           A                TA           AG      285

Mouse      342                                                                                   401
Rat        286                TC                          C                                  345
Guinea pig 241 CAGATAGCCAATGACCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAG 300
Bovine     223              T                             C                T       C       GC      282
Pig        286     A        TG                            C        G       T       C       C       345
Human      286     A        T         C                   C        G   T   T   T   CG              T   345

Mouse      402              405
Rat        346              349
Guinea pig 301 AGCT         304
Bovine     283              286
Pig        346              349
Human      346              349
```

Figure 2.5 **Nucleotide sequence homology of mammalian leptin cDNA**

Figure 2.6 Amino acid homology of mammalian leptin

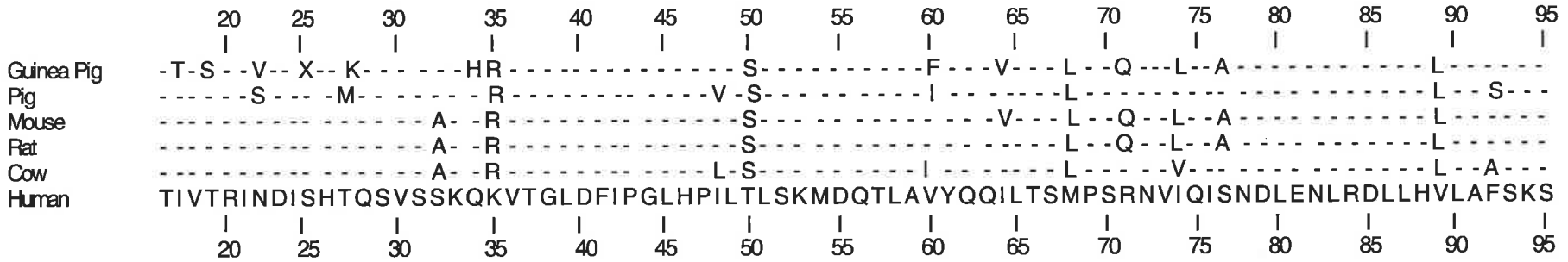
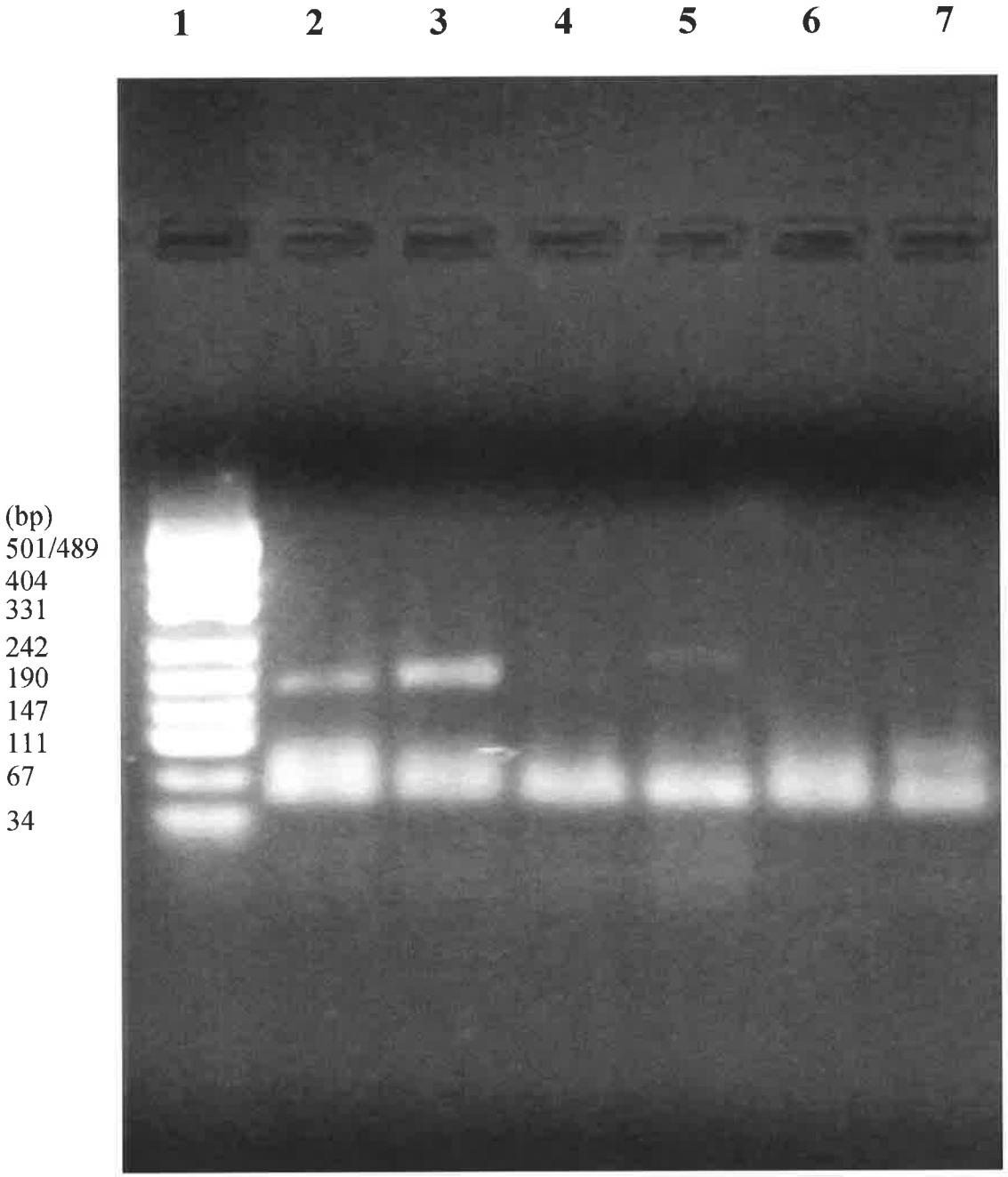

20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95
Guinea Pig -T-S--V--X--K-------HR----------S----------F---V---L--Q---L--A----------L-------
Pig ------S---M-------R---------V-S----------I----------L---------- ----------L--S---
Mouse ----------A--R---------S----------V---L--Q--L--A----------L-------
Rat ----------A--R---------S----------L--Q--L--A----------L-------
Cow ----------A--R---------L-S----------I----------L------V-- ----------L--A---
Human TIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKS
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95

Figure 2.7 Size fractionation via 2% agarose gel electrophoresis of RT PCR products using LepGP99-LepGP297 and adult and fetal guinea pig cDNA.

Lane 1- pUC19 DNA/Hpa II; 2-4 -Guinea pig adult dorsal fat; 5- Guinea pig fetal dorsal fat 6- Negative RT (Water instead of RNA); 7- Negative PCR (water instead of cDNA).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2.3.3 Detection of leptin mRNA in adipose tissue from adult and fetal guinea pigs

Based upon the partial nucleotide sequence of guinea pig leptin cDNA, primer pair LepGP99-LepGP297 should generate an amplicon of 199 bp. A fragment of approximately 200 bp was amplified by reverse transcribing total RNA from adult and fetal guinea pig adipose tissue and amplifying the cDNA. Dorsal adipose tissue expresses leptin mRNA in lower abundance in fetuses than adults (Figure 2.7). A product of approximately 60 bp was also present in RT PCR products from adipose cDNA template and in negative RT controls that contain no cDNA (Figure 2.7). The fragment is possibly a catimer of the primer pair. It may also be a non-specific PCR product due to contamination of one of the PCR reagents.

2.3. 4 Detection of guinea pig leptin mRNA by Northern analysis

RNA extracted from adipose was transcribed into cDNA. A guinea pig leptin cDNA fragment of 325 bp was amplified from the adipose cDNA using pOB1-pOB2 primers. The leptin PCR product where nucleotide sequence was confirmed to be guinea pig leptin was labelled with digoxigenin and used to probe Northern blots of guinea pig RNA. RNA extracted from guinea pig adipose tissue was denatured and electrophoresed through a formaldehyde gel. All adipose sites examined in the guinea pig expressed a leptin mRNA transcript as determined by northern blot analysis. The DIG-labelled leptin probe hybridised to a ~4.2 kb transcript that migrated between 28S (5.1 kb) and 18S rRNA (1.9 kb) (Figure 2.8). Total RNA extracted from pig subcutaneous adipose tissue produced a hybridising band which was approximately the same size length to the leptin mRNA transcript in the guinea pig RNA fat (Figure 2.8). The guinea pig leptin cDNA probe can cross-hybridise with

porcine leptin mRNA from adipose tissue. Experiments in section 2.3.2 showed that guinea leptin cDNA has high homology to porcine leptin cDNA. The ~4.2 kb leptin mRNA transcript in guinea pig and pig adipose tissue is similar in size to 4.4 kb leptin mRNA transcript estimated in pig adipose tissue found by Ramsay *et al.* (1998) and by Robert *et al.* (1998), but not the 3.1 kb leptin mRNA transcript reported by Bidwell *et al.* (1997). The guinea pig and pig leptin mRNA transcript of ~4.2 kb is also in accordance with the size of leptin mRNA reported in mice (Zhang *et al.* 1994), rats (Frederich *et al.* 1995) and humans (Green *et al.* 1995).

Expression of leptin mRNA was not detected in guinea pig lung but in liver RNA extracts a number of bands ranging from 4.5 kb and less were present (Figure 2.8). The dominant hybridising band of ~4.2 kb in guinea pig liver had been reported in the liver of chicken by RT PCR analysis (Taouis *et al.* 1998).

2.3.5 Detection of leptin mRNA in porcine adipose tissue by RT PCR

The predicted amplicon length with primers pOB1A and pOB2 is 325 bp using pig cDNA as template. A single RT PCR product of approximately 330 bp was detected in cDNA from pig subcutaneous fat with pOb1A and pOb2 primers (Figure 2.9). Primers pOB5 and pOB6 are predicted to generate an amplicon of 268 bp from this RT PCR template. A single fragment of approximately 270 bp was found from pig subcutaneous fat cDNA with pOb5 and pOb6 primers (Figure 2.9). The nucleotide sequences of both amplicons were determined and found to be >98% identical to porcine leptin cDNA sequences recently reported by others (Neuenschwander *et al.*, 1996; Ramsay *et al.*, 1998). Therefore, the

Figure 2.8 Leptin mRNA from fat and non fat sites of guinea pig and pig.

Northern blot of total RNA (5 μg) from guinea pig and pig. The blot was hybridised with guinea pig leptin cDNA probe and exposed overnight. Lane 1- Pig subcutaneous fat; 2 Guinea pig perirenal fat; 3-Guinea pig shoulder fat; 4-Guinea pig dorsal fat; 5-Guinea pi retroperitoneal fat; 6-Guinea pig lung; 7-Guinea pig liver

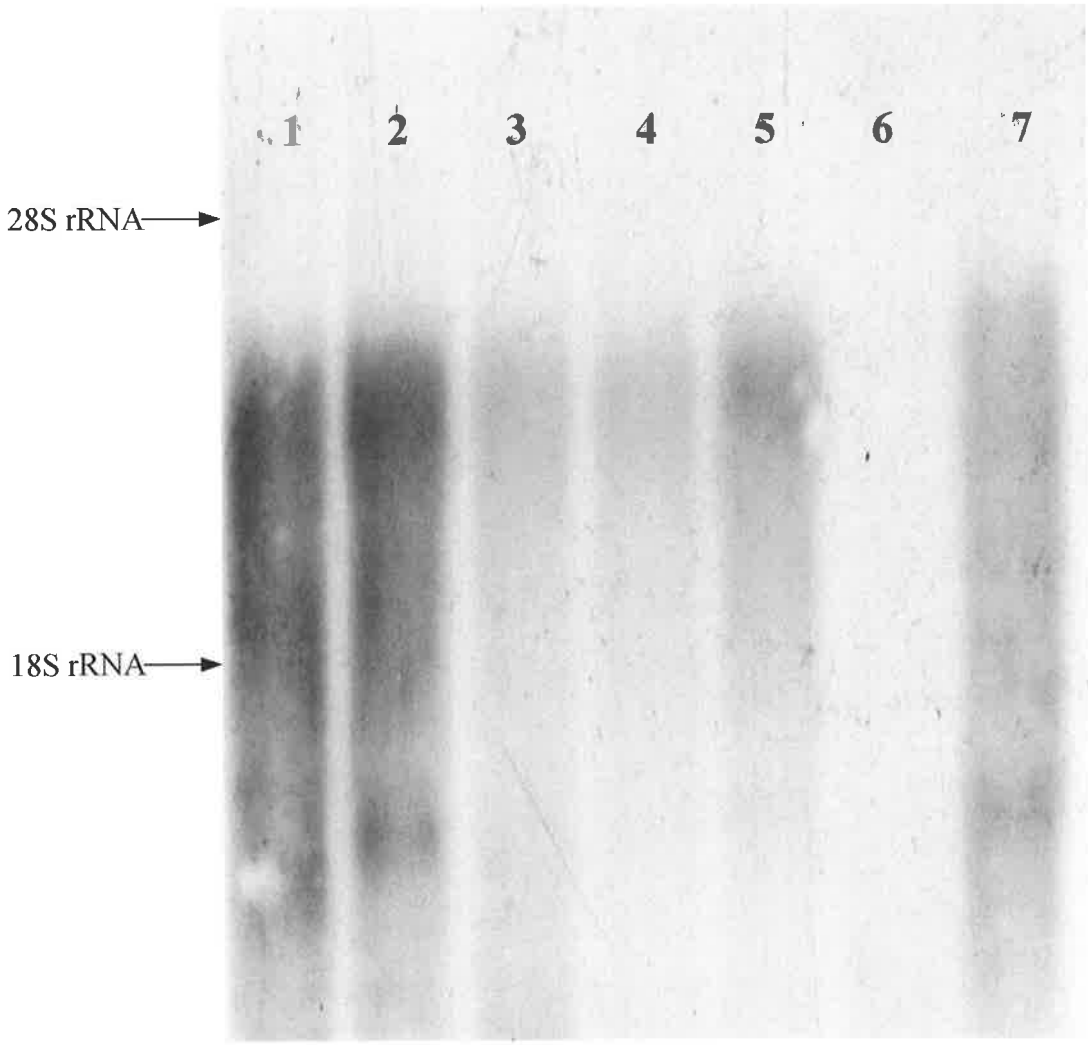
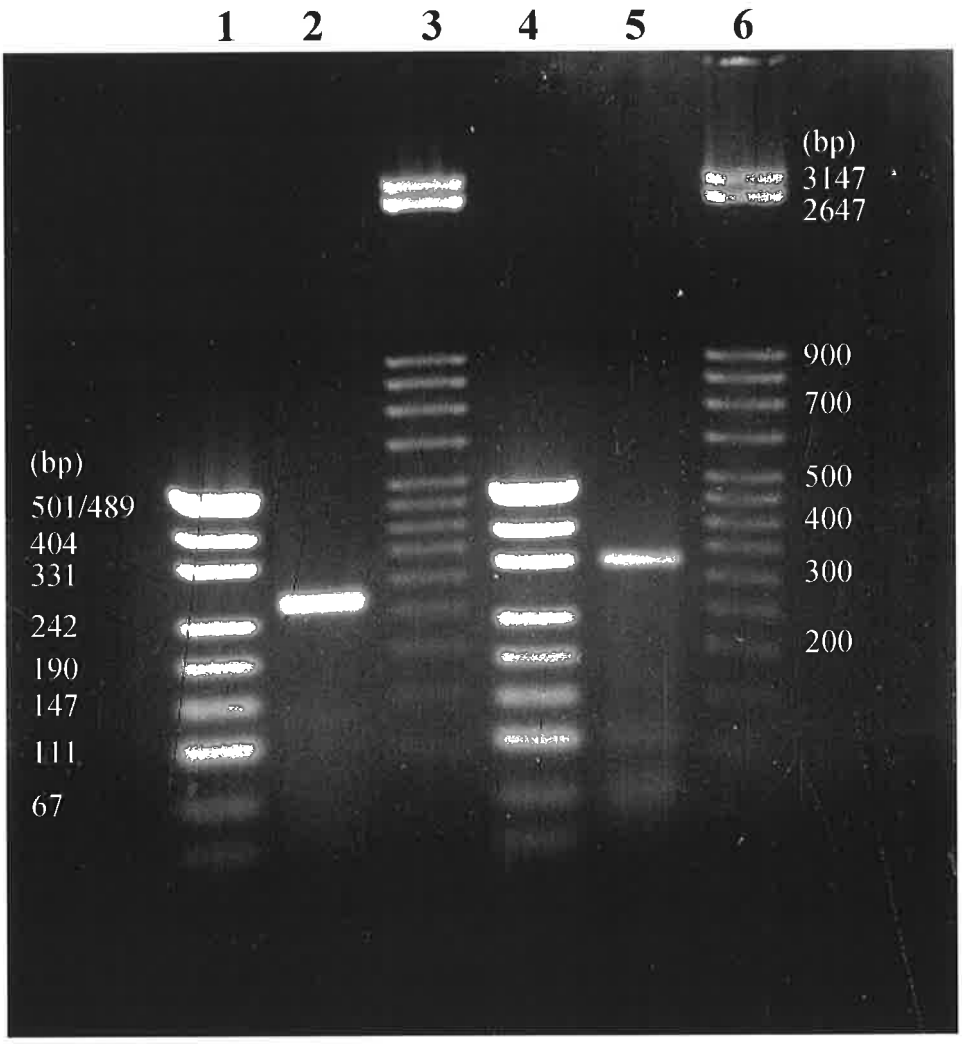

1
2
3
4
5
6
7
28S rRNA
18S rRNA

Figure 2.9 Size fractionation via 2% agarose gel electrophoresis of RT PCR products using leptin primers and from pig subcutaneous fat cDNA. Lane 1& 4-pUC19 DNA/Hpa II 2-pOb5-pOb6, 3 & 6-probase 50/ Eco RV, 5-pOb1A-pOb2.


1
2
3
4
5
6
(bp)
501/489
404
331
242
190
147
111
67
(bp)
3147
2647
900
700
500
400
300
200

results obtained here confirms reports from previous studies that the pig expresses leptin mRNA in subcutaneous adipose tissue (Neuenschwander *et al.*, 1996; Ramsay *et al.*, 1998).

2.4 CONCLUSION

The pig leptin RT-PCR amplified a single fragment of the predicted size for both pOB1A-pOB2 and also pOB5-pOB6 primers with no primer dimers, catimers or spurious bands. The nucleotide sequence confirmed their identity. The guinea pig leptin RT-PCR did not produce the reliable amplification of the predicted leptin cDNA fragment. However, this is the first study to show leptin mRNA is expressed in guinea pigs. A multi species assay for leptin protein in blood was available that detects leptin in pig plasma. The RIA did not react with leptin in guinea pig plasma. The pig was therefore chosen as the animal model with which to investigate intrauterine programming of leptin.

CHAPTER 3

DEVELOPMENT OF A QUANTITATIVE RT PCR ELISA TO MEASURE PORCINE ADIPOSE TISSUE LEPTIN MRNA

3.1 INTRODUCTION

Leptin mRNA is expressed in adipose tissue of the pig (Neuenschwander *et al.* 1996; Ramsay *et al.* 1998) and the porcine leptin gene has high homology to that of other mammalian species. Studies in Chapter 2 confirmed that subcutaneous adipose tissue in pigs expresses leptin mRNA. A quantitative method of measurement of porcine leptin mRNA has not been reported. A quantitative measure of leptin mRNA was needed to detect differences in leptin gene expression between animals.

Recent advancement in measuring mRNA allows RT PCR methods to be used with a non-isotopic DNA labelling system. PCR product is captured by streptavidin-coated plates with a biotinylated complementary oligonucleotide. The immobilised digoxigenin (DIG)-labelled PCR product can be measured by enzyme linked immunosorbant assay (ELISA) with peroxidase conjugated antibody to DIG.

The RT PCR DIG ELISA was chosen instead of Northern blot or RNAse protection assay. The ELISA has a higher throughput capacity as the assay can measure up to 36 samples in duplicate simultaneously. This is not possible by either Northern blot or RNAse protection methods. The aim of the studies described in this chapter was to develop an assay for leptin mRNA in porcine adipose tissue using the RT PCR DIG-ELISA system.

3.2 MATERIALS AND METHODS

3.2.1 RT PCR Digoxigenin Enzyme Linked Immunosorbant Assay (ELISA)

3.2.1.1 DIG-labelling of RT PCR products from pig adipose tissue

A DIG-labelled porcine leptin cDNA fragment was amplified with Taq* DNA polymerase (Cat. No. TAQ-3, Biotech, Bently, Western Australia) as described in 2.2.5.4 in a 70 µl volume containing 10 µl of porcine adipose tissue cDNA derived from 1 µg of RNA reverse transcribed as described in 2.2.5.3, 2 mM each of dATP, dCTP, dGTP and 1.9 mM dTTP and 0.1 mM DIG-dUTP (Cat. No. 1636120, Roche). The PCR procedure consisted of 30 cycles of 15 sec at 94 ^{0}C, 15 sec at 58 ^{0}C and 15 sec at 72 ^{0}C with pOB1A-pOB2 as primers. This produced 325 bp of DIG-labelled porcine leptin ds cDNA. A 349 bp fragment of DIG-labelled porcine β-actin ds cDNA was similarly amplified with 5′-TGT GAT GGT GGG TAT ATG GGT C-3′ and 5′-TAG ATG GGC ACA GTG TGG GT-3′ as primers.

3.2.1.2 General procedure for ELISA

DIG-labelled PCR product (20 µl) was pipetted into a sterile microfuge tube and 20 µl of denaturation solution (Cat. No. 1717 499, Roche) was added. The tube was centrifuged briefly and incubated for 10 min at room temperature. Hybridisation solution (Cat. No. 1717 472, Roche) containing the biotinylated complementary oligonucleotide at 50 ng/ml was added to make a final volume of 250 µl. The solution was vortexed and 200 µl was pipetted into a well of a streptavidin-coated microtiter plate strip (Cat. No. 1734 776, Roche). The

strips were incubated for 3 hours at 37^{0}C with constant shaking at 100 rpm (Orbital Mixer Incubator, Ratek Instruments). The solution was discarded and each well was washed 3 times with 250 µl of washing solution (Cat. No. 1717 502, Roche). The strips were tapped on a lint-free, dry absorbant cloth after the last wash to remove fluid. Anti-DIG-polymerized horse-radish peroxidase (200 µl of 10-50 mU/ml, Cat. No. 1633 716, Roche) was added to each well. The module was incubated at 37^{0}C for 30 min with constant shaking as above. The solution was discarded and each well was washed 3 times with 250 µl of washing solution. The modules were tapped on a lint-free, dry absorbant cloth after the last wash. 200 µl of 2,2- azino-di-[3-ethylbenzthiazoline sulphonate(6)] (ABTS, Cat. No. 1204 521, Roche), a substrate for peroxidase, was added to each well. An empty well had 200 µl of ABTS substrate solution added for measurement of the background absorbance of the ABTS solution. The strips were incubated at 37^{0}C for 30 min with constant shaking as above. The spectrophotometric absorbance at 405 nm was determined using a plate reader (Microplate autoreader EL 310, Bio-Tek Instruments).

3.2.1.3 Immobilisation of DIG-labelled PCR product by biotinylated oligonucleotide "capture probe"

DIG-labelled leptin PCR product was obtained as described in 3.2.1.1. The 5′-biotinylated oligonucleotide complementary to porcine leptin cDNA leptin oligo-1 (5′-Biotin GTC AGA CAG AGG AGG TTT-3′, Geneworks) was selected as it has a 50% GC content and has no hairpins (Figure 3.1). A concentration of 7.5 ng/ml of leptin oligonucleotide was used in the hybridisation solution and 10 µl of the leptin PCR product was measured by ELISA (section

AGG CAT CCT GAC CC 3'; Geneworks). Streptavidin-captured DIG-labelled β-actin PCR product was measured spectrophotometrically as in 3.2.1.2.

Detection of the DIG-labelled tissue plasminogen activator (gene from human genomic DNA, Cat No. 1636 120,Roche) PCR product by DIG-labelled ELISA method was used in the same ELISA with leptin and β-actin as a positive control for the ELISA.

3.2.2 Conditions for amplification of DIG-labelled leptin and β-actin cDNA fragments by PCR and detection by DIG-ELISA

DIG-labelled leptin and β-actin PCR products were amplified as in 3.2.1.1. For amplification of DIG-labelled leptin cDNA, the reaction was allowed to proceed for 21 to 39 cycles. For amplification of DIG-labelled β-actin cDNA, the reaction was allowed to proceed for 20 to 34 cycles. PCR tubes were removed from the thermal cycler after the appropriate number of cycles and stored at -20^{0}C before analysis by ELISA.

DIG-labelled leptin RT PCR product (10 µl) was added to each tube and 30 µl of β-actin RT PCR product was added to each tube. 20 µl of denaturation solution (Cat. No. 1717494, Roche) was added to each reaction tube and were centrifuged briefly and incubated for 10 min at room temperature. The hybridisation solution (25 ng/ml of leptin oligo-2) was added to make a final volume of 250 µl. The β-actin hybridisation solution (50 ng/ml of β-actin oligo) was added to make a final volume of 250 µl. Analysis of PCR products was performed as described in 3.2.1.2. A negative control for the ELISA was water instead of

PCR product which was processed with the samples and this measured the background of the DIG detection step.

3.2.3. Assay specificity

DIG-labelled leptin PCR product and β-actin PCR product were incubated on streptavidin-coated plates with one of the oligonucleotides complementary to leptin cDNA leptin oligo-2 to determine the specificity of the biotin-labelled oligonucleotides to capture their intended PCR product. The rest of the ELISA was performed as described in 3.2.2.

3.2.4 Anti-DIG- polymerized horse-radish peroxidase antibody in the ELISA

DIG-labelled leptin and β-actin PCR product were amplified as described in section 3.2.1.1. 10 µl of DIG-labelled PCR product was assayed in the ELISA. The anti-DIG-polymerized horse-radish peroxidase antibody was added at concentrations of 10, 20, 40 and 80 mU/ml was assayed as described in section 3.2.2. A negative control for the ELISA was water instead of PCR product which was processed with the samples and measured the background of the DIG detection step.

3.2.5 DIG-UTP in the PCR

DIG-labelled leptin cDNA was amplified as described in section 3.2.1.1 with either 75, 100 or 150 µM of DIG-labelled dNTPs in the PCR.

3.2.6 Effect of DIG-labelled PCR product in the ELISA

DIG-labelled leptin PCR product was amplified with 75, 100, 150 μM of DIG-labelled dNTPs in the PCR. 10 μl, 1μl or 0.5 μl of the leptin PCR product was assayed. The leptin hybridisation solution containing leptin oligo-2 at 25 ng/ml was added to make a final volume of 250 μl. The anti-DIG- polymerized horse-radish peroxidase antibody was at a concentration of 40 mU/ml and was assayed as described in section 3.2.2.

3.2.7 Amplification efficiency of the leptin and β-actin RT PCR DIG-ELISA

The yield of PCR product should double with each PCR cycle if the reaction occurs with 100% efficiency. However amplification efficiency is less than 100% due to primers and PCR conditions such as magnesium concentrations and amplification decreases dramatically when the reagents have been consumed. Amplification efficiency was estimated by examining the stage just after first detection of DIG-labelled PCR product and before the plateau stage was reached. Product yield is theoretically described by the general equation $Y= A(1+ R)^n$, where Y is the yield, A is the initial amount of template, R is the efficiency ($0< R < 1$) and n is the number of cycles. Y is measured by absorbance and R can be deduced from the slope of the curve after $\log_{10}$ transformation.

Pig adipose cDNA was amplified by PCR as described in section 3.2.1.1. For amplification of leptin cDNA, the reaction was allowed to proceed for 27, 30 or 33 cycles at which replicate PCR tubes were removed and stored at -20^0C before analysis by ELISA. For

amplification of β-actin cDNA, the reaction was allowed to proceed for 22, 25 or 28 cycles at which replicate PCR tubes were removed and stored at -20^{0}C before analysis by ELISA. An initial denaturation of the PCR product was for 3 min at 94^{0}C. At the end of each interval the PCR tube was removed and stored at -20^{0}C before analysis by ELISA.

The leptin RT PCR product was diluted 1 in 20 before 20 µl of the PCR product was added to each tube. 30 µl of β-actin RT PCR product was added to each tube. 20 µl of denaturation solution (Cat. No. 1717494, Roche) was added to each reaction tube and were centrifuged briefly and incubated for 10 min at room temperature. The leptin hybridisation solution was added to make a final volume of 250 µl which contains hybridisation buffer and the biotin labelled oligonucleotide leptin oligo-2 at 25 ng/ml. The β-actin hybridisation solution was added to make a final volume of 250 µl which contains hybridisation buffer and the biotin labelled oligonucleotide β-actin oligo at 50 ng/ml. The completion of the analysis of PCR products in the ELISA was described in section 3.2.2 and anti-DIG-polymerized horse-radish peroxidase (Cat. No. 1633716, Roche) was used at 40 mU/ml.

Each PCR reaction product was also analysed by electrophoresis in 2% agarose gel (Cat. No. A 9539 electrophoresis grade agarose, Sigma, St Louis, Missouri, USA) containing 0.5 µg/ml ethidium bromide in Tris borate/EDTA buffer. The size of the PCR products was determined by comparing their migration with molecular weight markers (pUC19 DNA/Hpa II, Cat No. DMW-P1, GeneWorks) under UV illumination. The logarithm of PCR product yield was plotted against reaction cycle.

3.2.8 Routine RT PCR DIG-ELISA quantitation

Routine quantitative RT-PCR was performed using a standard operating procedure at a cycle number that falls in the middle of the exponential curves for leptin and β-actin amplification. This was achieved by examining the exponential yield versus cycle number curves of the RNA controls for each RNA batch.

RT-PCR ELISA for leptin was routinely performed at 30 cycles for leptin and 26 cycles for β-actin. cDNA was amplified as described in section 3.2.1. For amplification of leptin cDNA, the reaction was allowed to proceed to 30 cycles. For amplification of β-actin cDNA, the reaction was allowed to proceed for 26 cycles. An initial denaturation of the PCR product was for 3 min at 94^0C. The amplification profile involved denaturation at 94^0C for 15 sec, annealing at 58^0C for 15 sec, extension at 72^0C for 15 sec. At the end of each interval the PCR tube was removed and stored at -20^0C before analysis by ELISA. Quantitation of leptin and β-actin RT PCR product was performed by DIG-ELISA.

3.2.8.1 Calibration of ELISA

Assays were calibrated using DIG-labelled leptin PCR product cDNA fragments for which the concentration of leptin or β-actin ds cDNA was firstly determined by comparing their ethidium bromide staining intensities with those of DNA standards of known mass (pUC19 DNA/Hpa II, Cat. No. DMW-P1, GeneWorks) after agarose gel electrophoresis. 0.5 to 10.0

ng/well of DIG-labelled leptin PCR product was measured by ELISA as described in section 3.2.8.

3.2.9 Assay Precision

To determine total variation within RT PCR ELISA system, total RNA was extracted in triplicate (section 2.2.5.1) from the same subcutaneous adipose tissue specimen. The RNA was reverse transcribed, amplified with leptin primers pOB1A-pOB2 and the DIG-labelled RT PCR product was measured by ELISA with leptin oligo-2 in the same run as described in section 3.2.8.

3.3 RESULTS AND DISCUSSION

3.3.1 Immobilisation of DIG-labelled PCR product by biotinylated oligonucleotide

Complementary biotinylated oligonucleotides were used to capture DIG-labelled leptin or β-actin PCR products on to the streptavidin coated plate. The concentration of the captured DIG-labelled leptin PCR was then determined by DIG-ELISA. The positive control (DIG-tPA) in the ELISA kit produced an A_{405} of 2.5. At both 7.5 and 50 ng/ml the biotinylated leptin oligo-1 produced an A_{405} equal to the negative control of approximately 0.2 after hybridisation to DIG-leptin cDNA. At 50 ng/ml leptin oligo-2 produced an A_{405} of 2.28. Therefore leptin oligo-2 but not leptin oligo-1 captured DIG-leptin PCR product. A

concentration of 50 ng/ml of β-actin oligonucleotide produced an A_{405} of 1.99 after hybridisation to DIG-β-actin cDNA.

The leptin oligo-2 and β-actin oligo were chosen as the biotinylated capture probes in the ELISA system as they were capable of capturing DIG-labelled RT PCR product.

3.3.2 PCR cycle profile of leptin and β-actin RT PCR DIG-ELISA

Measurement of DIG-labelled leptin and β-actin PCR product yield with increasing PCR cycle was required to determine the useable range of the system. The absorbance at 405 nm due to DIG-labelled leptin PCR product in the ELISA increased as the number of PCR cycles increased (Figure 3.2). Significant detection of leptin product occurred between PCR cycles 25 to 36 of amplification of leptin PCR product. After 36 cycles the leptin PCR plateaus (Figure 3.2).

Amplification of DIG-labelled β-actin PCR product measured by ELISA increased with PCR cycle number (Figure 3.3). Significant β-actin PCR product was detected after 23 amplification cycles and plateaued after 30 cycles.

Thirty cycles of PCR for leptin and 25 cycles for β-actin were chosen for subsequent routine analysis of leptin and β-actin mRNA.

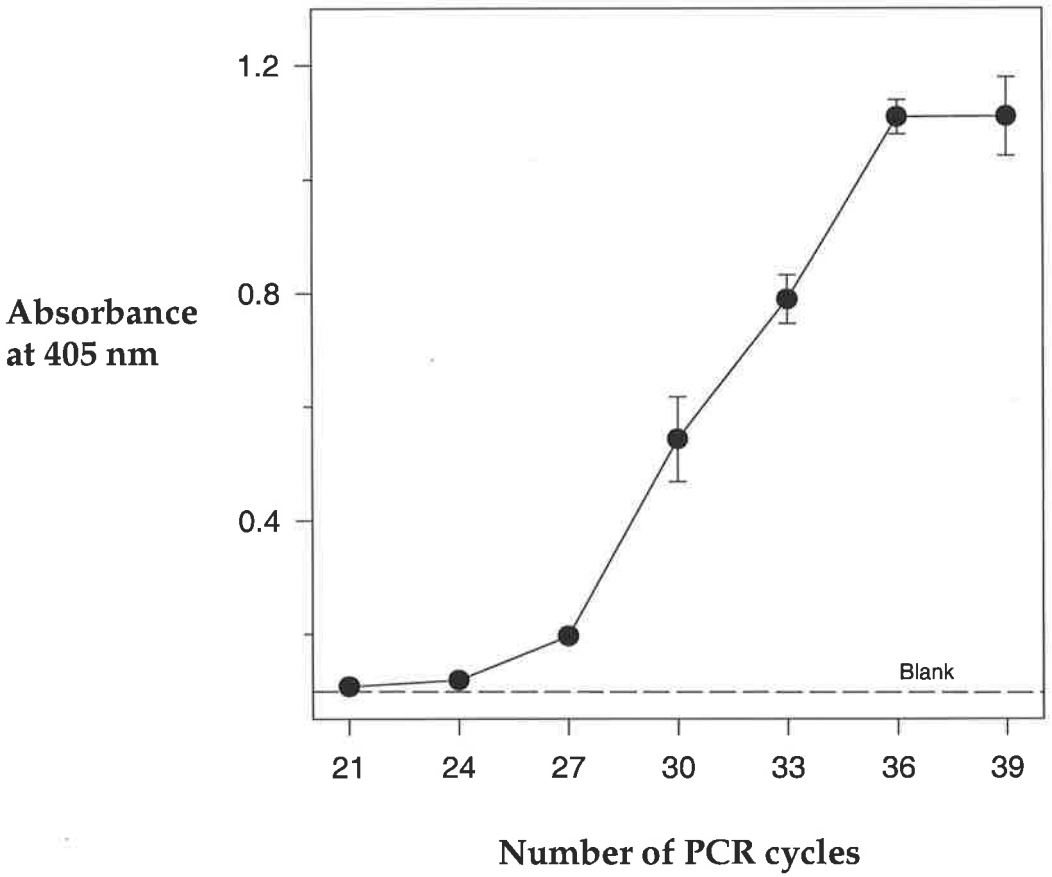


Figure 3.2 ELISA detection of DIG-labelled porcine leptin RT PCR product.

DIG-labelled leptin cDNA was amplified from 21 to 39 cycles and was detected in the ELISA with leptin oligo-2 capture probe. The blank for the ELISA is water instead of DIG-labelled PCR product.

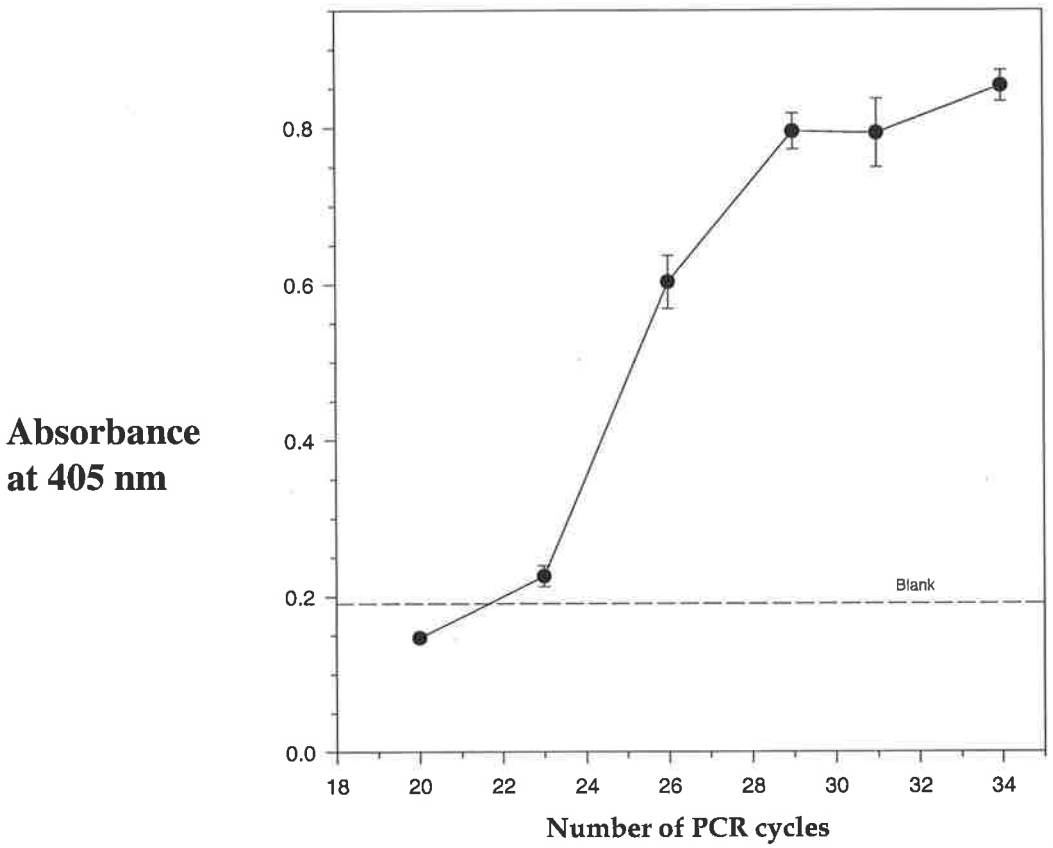


Figure 3.3 ELISA detection of DIG-labelled porcine β-actin RT PCR product.

DIG-labelled β-actin cDNA was amplified from 20 to 34 cycles and was detected in the ELISA with β-actin oligo capture probe. The blank for the ELISA is water instead of DIG-labelled PCR product.

3.3.3 Assay specificity

The biotinylated leptin oligo-2 when added with either the blank which contained water or DIG-labelled β-actin PCR product produced the same absorbance of 0.15 (Table 3.1). Biotinylated leptin oligo-2 added with DIG-labelled leptin PCR product produced an absorbance of 3.0 which is the maximum absorbance response. The biotin-labelled leptin oligo-2 did not promote capture of DIG-labelled β-actin DNA to streptavidin-coated plates as the absorbance is similar to the blank. The leptin capture probe is specific for DIG-labelled porcine leptin PCR product.

3.3.4 Anti-DIG-polymerized horse-radish peroxidase antibody in the ELISA

Anti-DIG-peroxidase binds to DIG-labelled PCR product which is captured to the streptavidin plate by the complementary biotinylated capture probe. A positive dose response to peroxidase-conjugated DIG antiserum was observed in the ELISA with immobilised DIG-leptin and β-actin PCR products (Figure 3.4). There was a sharp increase in A_{405} from 20 mU/ml to 40 mU/ml of anti-DIG with leptin PCR product. 40 mU/ml of anti-DIG added to the leptin PCR product produced a similar A_{405} measurement to 80 mU/ml of anti-DIG. Anti-DIG peroxidase at 40 mU/ml therefore binds to all the DIG-labelled PCR product in the ELISA. 40 mU/ml was chosen as the concentration of anti-DIG peroxidase in DIG-leptin ELISA.

An increase from 10 to 80 mU/ml of anti-DIG added to captured DIG-β-actin PCR product also increased the A_{405} in the ELISA (Figure 3.4). Anti-DIG peroxidase concentration of 40 mU/ml was chosen for the DIG-β-actin ELISA.

Table 3.1 Capture of DIG-labelled leptin or β-actin PCR product by biotinylated porcine leptin oligonucleotide.

DIG-labelled leptin, β-actin PCR product or water was captured with leptin oligo-2 capture probe in the ELISA.

	Blank (Water)	DIG- β-actin cDNA	DIG-leptin cDNA
$A_{405\ nm}$	0.15	0.15	3.0

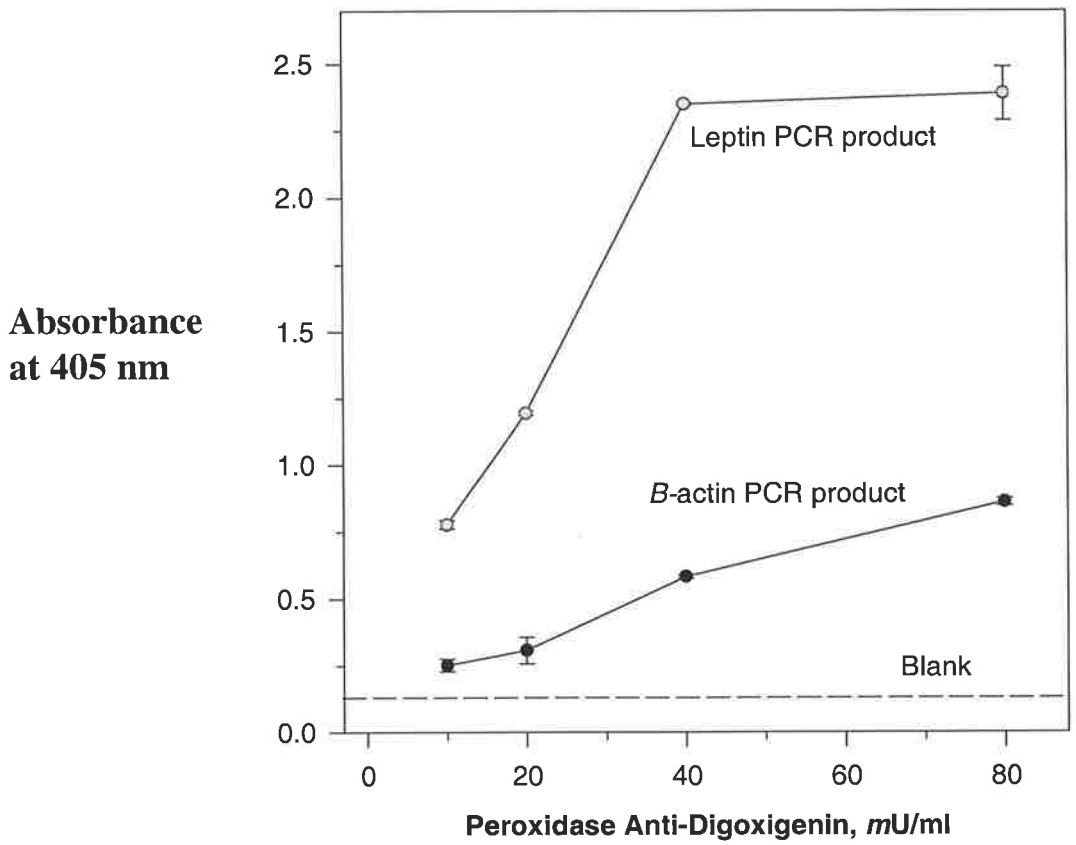


Figure 3.4 Effect of peroxidase anti-DIG in the PCR ELISA for the detection of leptin and β-actin DIG-labelled PCR product.

DIG-labelled leptin and β-actin PCR product were assayed in the ELISA with increasing concentration of anti-DIG-polymerized horse-radish peroxidase (section 3.2.2). The blank for the ELISA is water instead of DIG labelled PCR product.

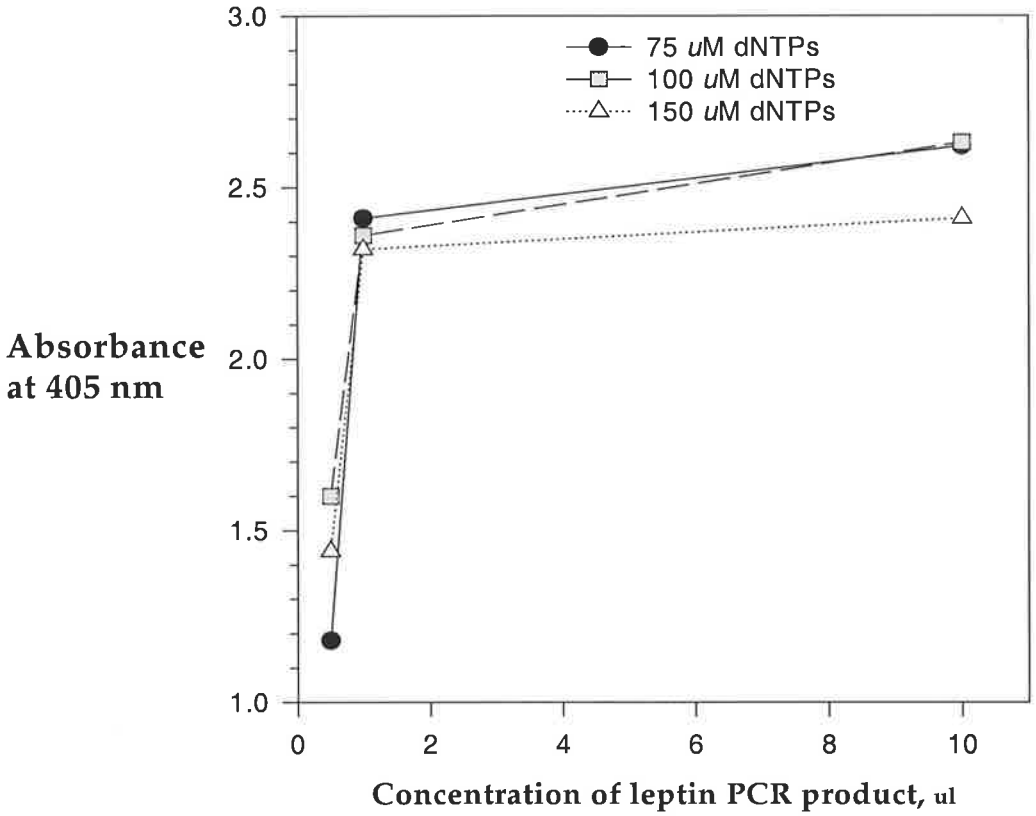


Figure 3.5 Effect of DIG-labelled leptin PCR and dNTPs in the PCR ELISA.

DIG-labelled leptin PCR product was amplified with either 75 μM dNTPs which contained 3.75 μM DIG-dUTP, or 100 μM dNTPs which contained 5 μM DIG-dUTP or 150 μM dNTPs which contained 7.5 μM DIG-dUTP. 10, 1 or 0.5 μl of DIG-labelled leptin PCR product amplified with different concentrations of dUTP were assayed with biotinylated leptin oligo-2 in the ELISA.

3.3.5 Effect of concentration of DIG-labelled leptin PCR product in the ELISA

The A_{405} response in the DIG-leptin ELISA increased with increasing amounts of DIG-labelled leptin PCR product added to the ELISA (Figure 3.5). A mixture of 1 µl of DIG-labelled leptin PCR product and biotinylated leptin oligo-2 after denaturation and hybridisation was selected for addition to streptavidin coated plates in subsequent routine ELISA of DIG-leptin.

3.2.6 Effect of concentration of dNTP mixture containing DIG-UTP in the leptin PCR ELISA

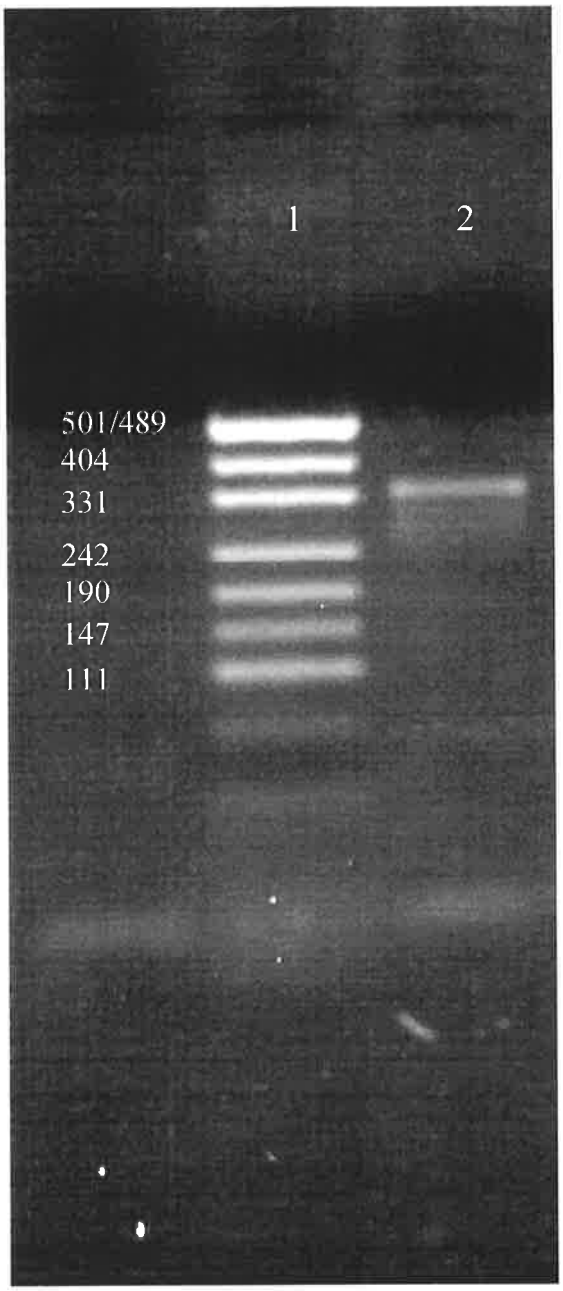
An increase in DIG-labelled dUTP and nonlabelled dNTPs in the amplification of DIG-labelled PCR did not significantly change the A_{405} measurement when the leptin PCR product was added to the plate at 10 µl or 1 µl (Figure 3.5). An increase in dNTPs containing DIG-dUTP increased A_{405} measurement when the leptin PCR product was at 0.5 µl. The mixture of unlabelled dNTPs containing DIG-dUTP was used at a concentration of 100 µM in the subsequent routine leptin ELISA.

3.3.7 Amplification efficiency of the leptin and β-actin RT PCR DIG-ELISA

The amplification efficiency for leptin detected by the ELISA is 41%. β-actin had an amplification efficiency of 45% when β-actin PCR product was detected in the ELISA.

Figure 3.6 Determination of concentration of DIG-labelled leptin standard.

Size fractionation via 2% agarose gel electrophoresis of RT PCR products using pOB1A-pOB2 leptin primers and pig subcutaneous cDNA. 1-pUC19 DNA/Hpa II, 2-leptin.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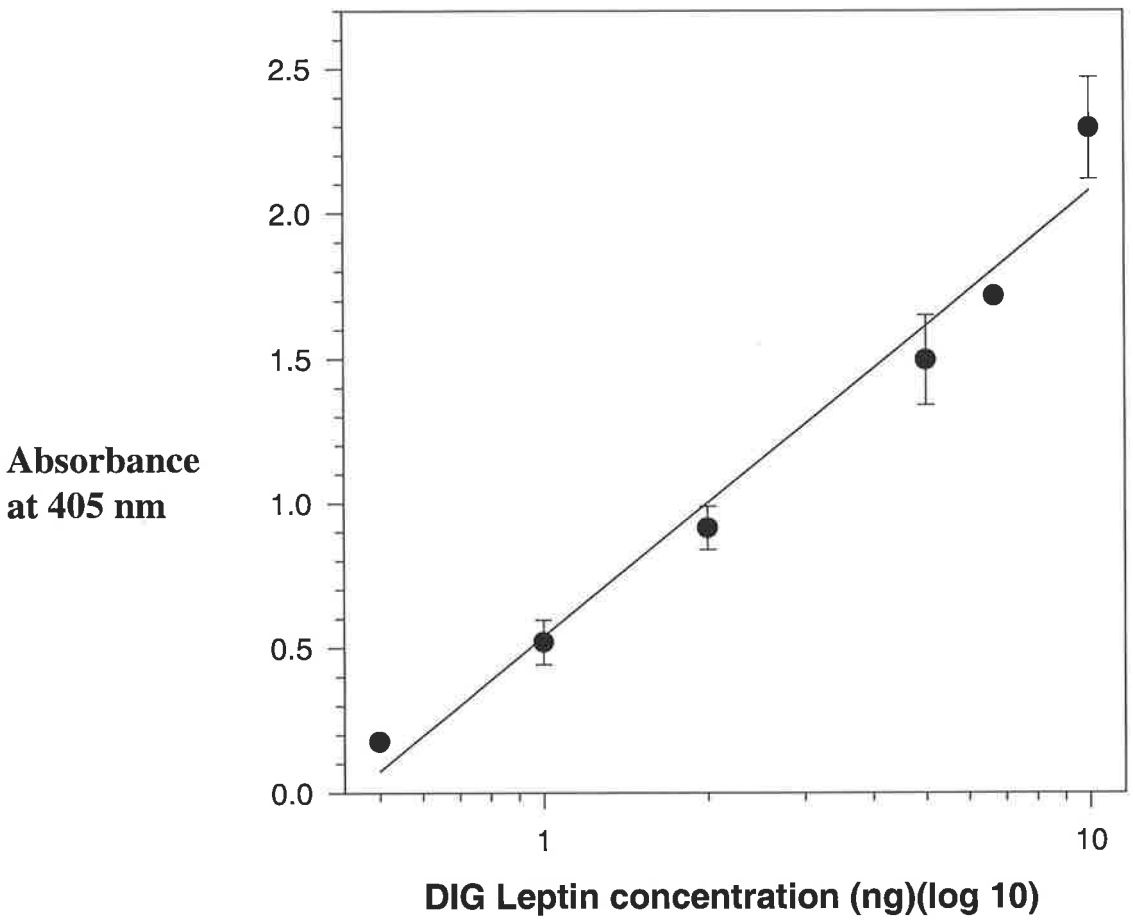

Figure 3.7 ELISA standard for DIG-labelled leptin PCR product.

0.5 to 10.0 ng/well of DIG-labelled leptin PCR product determined by ethidium bromide staining with known markers were assayed with leptin oligo-2 in the ELISA.

3.3.8 Calibration of ELISA

DIG-labelled leptin RT PCR produced an amplicon of approximately 340 bp which is 10 to 15 bp greater than the ds cDNA leptin PCR product obtained in the absence of DIG-dUTP (Figure 3.6). This is due to the incorporation of DIG-labelled dUTP which contains the bulky biotin side chain instead of dTTP into the leptin PCR amplicon. Concentration of DIG-labelled leptin PCR product was 15 ng/ml determined by comparison with the pUC19 markers whose concentrations are known.

This material was used as a standard for DIG-labelled leptin PCR ELISA at 0.5 to 10 ng/well (Figure 3.7).

3.3.9 Assay Precision

The overall variability of extracting RNA and measuring leptin or β-actin by RT PCR ELISA was examined by quantitatively assaying three RNA extracts in the same ELISA from the one pig subcutaneous adipose tissue. Total variation in the RNA RT PCR ELISA system was 16% for leptin mRNA and 34% for β-actin mRNA.

3.4 CONCLUSION

In conclusion, the results presented in this chapter show that a sensitive, specific quantitative non-isotopic assay to detect leptin mRNA in porcine adipose tissue has been developed. The

leptin RT PCR DIG-ELISA allows the quantitative comparison of leptin mRNA expression in a large number of pig adipose tissue samples.

CHAPTER 4

LEPTIN EXPRESION IN OFFSPRING IS PROGRAMMED BY NUTRITION IN PREGNANCY

4.1 INTRODUCTION

In adults, abundance of leptin mRNA in adipose tissue and concentrations of leptin protein in blood correlate positively with concurrent body weight and adiposity (Maffei *et al.* 1995b, Considine *et al.* 1996b). Leptin appears to be involved in the relationship between biomass and energy reserve from early in life because there are positive associations between the concentrations of leptin protein in human umbilical cord blood and both body weight and adiposity at birth (Schubring *et al.* 1997; Koistinen *et al.* 1997; Jaquet *et al.* 1999b; Ong *et al.* 1999). Furthermore, leptin mRNA abundance in fetal adipose tissue is positively correlated with fetal body weight in sheep (Yuen *et al.* 1999). Blood leptin levels in adults are also influenced by factors acting at or before birth. Independent studies of 502 men and women born in England in the 1920s (Phillips *et al.* 1999) and 1,462 women born in Sweden between 1908 and 1930 (Lissner *et al.* 1999) concluded that body weight at birth is related to blood leptin concentrations in adults, independent of adult adiposity. However, unlike the positive correlations seen in fetuses, newborns and adults between concurrent measures of leptin production and body weight or adiposity, the correlation between body weight at birth and leptin protein concentration in blood from adults is negative. Maternal nutrition during pregnancy affects body weight of the fetus (Lemons *et al.* 1986; Harding & Johnston 1995; Sohlstrom *et al.* 1998), newborn (Lechtig *et al.* 1975; Godfrey *et al.* 1996; Wallace *et al.* 1997) and adult (Barker 1997). A quantitative assay for porcine leptin mRNA in adipose had been developed as described in Chapter 3. The effect of nutrition during pregnancy on postnatal leptin expression in offspring was therefore examined.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The experiment was designed in accordance with the *Australian code of practice for the care and use of animals for scientific purposes* (6th edn. 1997, National Health and Medical Research Council) and approved by the Animal Experimentation Ethics Committee of Bunge Meat Industries Corowa. Large White gilts (primiparous sows, n=16) were fed a dry ration containing 13.5 MJ digestible energy and 150 g protein per kg at 2.2 $kg.d^{-1}$. From the 25th day of pregnancy (140.8 ± 2.37 *kg* live weight) to the 50th day, half were fed the same diet at 3.0 $kg.d^{-1}$ (term ~110 days). A female piglet of median birth weight for her litter was selected from each dam and killed by barbiturate overdose 59 days after birth. The study was confined to one sex to avoid the confounding effects of gender on growth phenotype (Tanner 1962, Bereskin *et al.* 1986) and plasma leptin concentration (Mantzoros *et al.* 1998). Subcutaneous fat was frozen in liquid N_2 and stored at -80°C for measurement of leptin and β-actin mRNA. Jugular venous EDTA-plasma was obtained and stored at -20 °C for assay of leptin protein.

4.2.2 Extraction of total RNA from porcine adipose tissue

Frozen adipose tissue 196 ± 9 mg was removed from -80°C storage into pre-weighed 10 ml tubes (Falcon tubes, Becton Dickinson). Adipose tissue was homogenised as described in

section 2.2.1.1. The TRIzol RNA extraction method was used as described in section 2.2.5.1.

4.2.3 Integrity and concentration of porcine adipose RNA

Total RNA integrity was determined as described in section 2.2.1.

4.2.4 Reverse Transcription of RNA from porcine adipose tissue

Total RNA was reverse transcribed into first strand cDNA using the Superscript™ RNase H$^-$ reverse transcription kit (Cat. No. 18053-017, GIBCOBRL). Reverse transcription was performed as described in section 2.2.1.

4.2.5 Polymerase chain reaction amplification of porcine adipose cDNA

A porcine leptin and β-actin cDNA fragment were amplified with Taq* DNA polymerase (Biotech International, Bently, Australia) as described in section 3.2.1 using 100 μM each of dATP, dCTP, dGTP and 80 μM dTTP and 20 μM DIG-dUTP (Cat. No. 1585 550, Roche).

For amplification of leptin cDNA, the reaction was allowed to proceed to 30 cycles. For amplification of β-actin cDNA, the reaction was allowed to proceed for 26. PCR products were stored at –20 ^{0}C before analysis by ELISA.

Quantitation of leptin and β-actin RTPCR product was performed by DIG-ELISA. The leptin RTPCR product was diluted 1 in 20 in molecular grade water before 20 µl of the PCR product was added to each tube. 30 µl of β-actin RTPCR product was added to each well. 20 *u*l of denaturation solution (Cat. No. 1717494, Roche) was added to each reaction tube and were then centrifuged briefly and incubated for 10 minutes at room temperature. The leptin hybridisation solution was added to make a final volume of 250 µl which contains hybridisation buffer and the biotin labelled oligonucleotide leptin oligo-2 at 25 ng/ml. The β-actin hybridisation solution was added to make a final volume of 250 µl which contains hybridisation buffer and the biotin labelled oligonucleotide β-actin at 50 ng/ml. The remainder of the ELISA procedure was performed as described in section 3.2.4.

The negative detection control used water instead of DIG-labelled PCR product which measured the background of the DIG detection step. A PCR ELISA negative control contained water instead of cDNA as a template in the DIG-labelling PCR which measured the background for the PCR ELISA.

Assays were calibrated with DIG-labelled leptin cDNA fragments for which the concentration of leptin ds cDNA was previously determined by comparing their ethidium bromide staining intensities with those of DNA standards of known mass (pUC19 DNA/Hpa II, Cat. No. DMW-P1, GeneWorks) after agarose gel electrophoresis as described in section 3.2.8.1.

4.2.6 Measurement of DNA content of progeny adipose tissue

Adipose tissue was delipidated prior to DNA extraction and measurement using the method of Colebrook *et al.* (1988). Adipose tissue (1.03 ±0.02 g) was thinly sliced and was homogenised with a Polytron tissue grinder in 10 mls of 2:1 chloroform:methanol in an ice-bath. The samples were sonicated for 1 hr at room temp before being rotated at room temperature overnight at 30 rpm. The samples were centrifuged at 3500 rpm for 10 min at 4 ^{0}C. The supernatant was aspirated and DNA extracted using the method of Gendimenico *et al.* (1988). The pellet was resuspended in 10 mls of 0.5 M perchloric acid (Cat. No. 101764B, AnalaR, England) and rotated for 10 min at 30 rpm at room temperature. The samples were centrifuged at 3500 rpm for 5 min at 4 ^{0}C. The aqueous and organic phases were removed and the pellet was resuspended in 2 mls of 0.5 M perchloric acid. DNA was hydrolysed by incubation at 90 ^{0}C for 30 min. DNA was recovered by centrifugation at 3500 rpm for 5 min at 4 ^{0}C and the supernatant removed which contains the DNA. Measurement of DNA was performed by the diphenylamine colourimetric method (Gendimenico *et al.* 1988, Burton, 1956). Calf thymus DNA (100 mg, Cat. No. D1501, Sigma, St Louis, IL, USA) was used as the standard (1.65 µg to 10.0 µg per well). Sample DNA or DNA standard (50 µl) was incubated at 50 ^{0}C for 3 hrs with 100 µl of diphenylamine solution (96.5% glacial acetic acid (Cat. No. 2789, APS Finechem, Seven Hills, N.S.W., Australia) 1.5% conc sulphuric acid (Cat. No. 1830, Ajax, Auburn, N.S.W., Australia), 0.008% acetaldehyde (Cat. No. 270034L, BDH, England) and 2% diphenylamine (Cat. No. D2385, Sigma,St Louis, IL, USA)) in triplicate in multiwell plates (Costar). Absorbance of the wells at 540 nm was determined with an Automated Microplate Reader (Bio-Tek).

4.2.7 Measurement of RNA content of progeny adipose tissue

RNA concentration was measured by homogenising 207 ± 8 mg of adipose tissue in 3 ml of 10 mmol/l sodium phosphate pH 6 and delipidating with 10 ml chloroform:methanol as above. After centrifugation, the cellular interphase was extracted with 10 ml of 0.5 mol/l perchloric acid by repeated inversion at 30 rpm for 10 min at 25 ^{0}C. The upper aqueous phase which contains the RNA was aspirated and lower phase contains chloroform:methanol residue. RNA was hydrolysed by addition of 4 ml of 0.3 mol/l KOH and incubated with 2.5 ml of ice-cold 0.5 mol/l perchloric acid. RNA content was estimated from the 260 nm absorbance of the supernatant after centrifugation.

4.2.8 Measurement of protein content of progeny adipose tissue

Tissue protein concentrations were assayed using the BCA Protein Assay Reagent (Cat. No. 23225X, Pierce, Rockford, USA). Adipose tissue was homogenised using an Ultra-Turrax T25 homogeniser (Janke & Kunkel) with a dispersing tool. Frozen adipose tissue 206.7 ± 7.66 mg was removed from -80^{0}C storage into pre-weighed 10 ml (Falcon tubes, Becton Dickinson) containing 3 ml of phosphate buffered Triton solution (0.01 M $NaH_2PO_2.2H_20$, Labchem Ajax; 0.002 % Trition X-100 AnalaR BDH; pH 6). The tissue was homogenised at full speed for a total of 90 s at ice slurry temperature. Homogenised tissues were stored at -20^{0}C.

10 µl homogenised solution or protein standard was incubated with 200 µl of BCA working reagent for 30 min in triplicate in multiwell plate (Falcon Microtest III flexible assay plate,

Becton Dickinson). The standard was prepared from RIA Grade Bovine Albumin (Cat. No. 23225X, Pierce, Rockford, USA) and the range was 2 to 20 μg per well. Absorbance of the wells at 540 nm was determined with an Automated Microplate Reader (Bio-Tek)

4.2.9 Measurement of water and lipid of progeny adipose tissue

Frozen adipose tissue 479 ± 14 mg was removed from -80^{0}C storage into pre-weighed glass vials. The adipose tissue was dessicated at 140^{0}C for 24 hours. The sample was re-weighed after being allowed to cool at room temperature. The dry-weight, water content and lipid content was then determined.

4.2.10 Plasma leptin radioimmunoassay

Leptin protein was measured in plasma by RIA using human [^{125}I]-leptin (Cat. No. 9081), guinea pig antiserum (Cat. No. XL-85K) to human leptin (both from Linco Research, St. Charles, Missouri) and human leptin (R&D Systems, Minneapolis, Minnesota) as standard. The dose-responses for pig plasma and human leptin are parallel (Figure 4.1). Leptin was measured in all plasma samples in a single assay (intra-assay CV= 4%) and is expressed in human leptin equivalents.

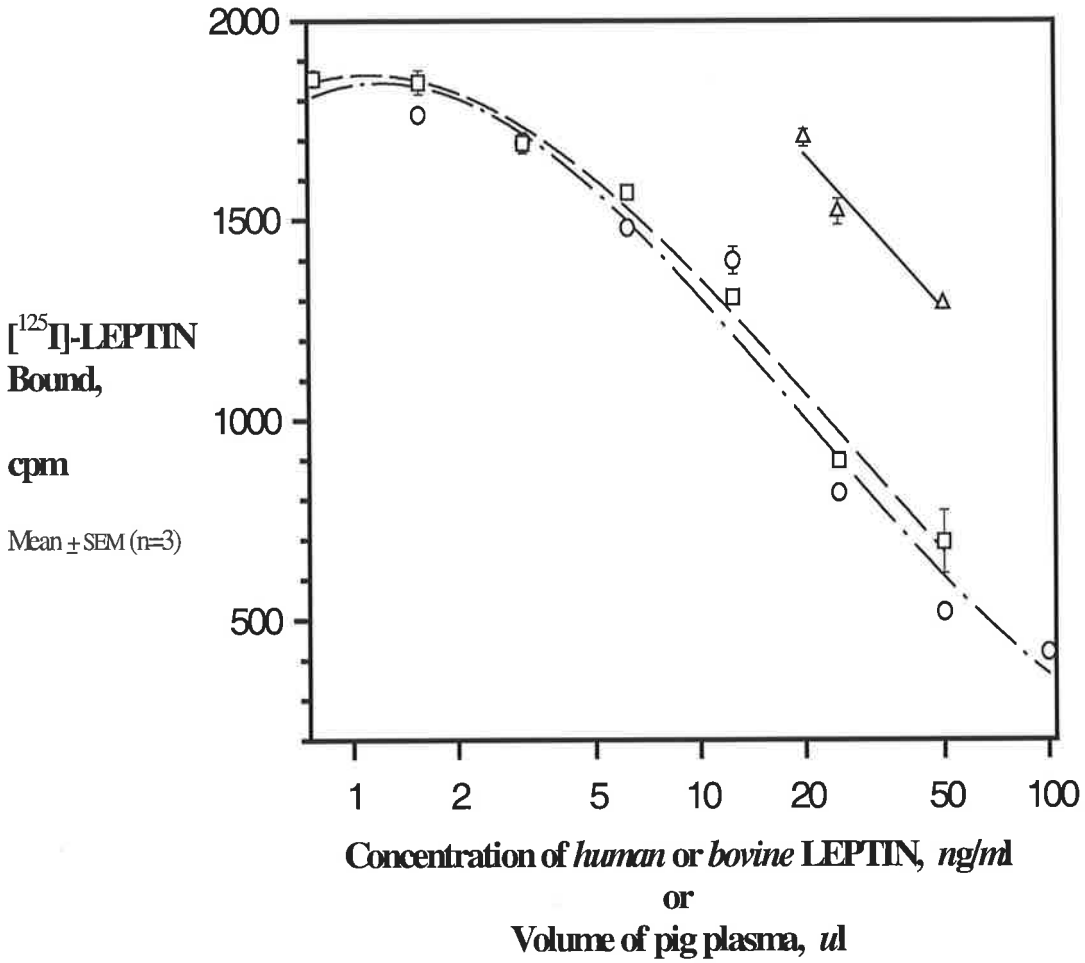

Figure 4.1 Leptin Radioimmunoassay. Inhibition of [^{125}I]-iodo-(*human*)-leptin binding to guinea pig anti-serum to *human* leptin by recombinant *human* (OPEN CIRCLE) and *bovine* (OPEN SQUARE) leptin and pig (OPEN TRIANGLE) plasma

4.2.11 Statistics

Results are presented as mean ± SEM (n = number of animals). Effects of maternal nutrition were assessed by t-test. Associations were evaluated by linear regression (SigmaStat V1, Jandel, San Rafael, California).

4.3 RESULTS

4.3.1 Effect of maternal nutrition during pregnancy on progeny

Maternal nutrition during the second quarter of pregnancy had no significant effect on the number of animals born alive per pregnancy (10.8 ± 0.4), the number of stillbirths per pregnancy (0.63 ± 0.27) or body weight at birth (1.43 ± 0.05 kg). Feed during pregnancy did not affect body weight at birth or at 59 days of age (20.8 ± 1.2 kg) for median birthweight females selected from each litter.

4.3.2 Effect of maternal nutrition during pregnancy on progeny adipocyte characteristics

Neither the content of lipid (500 ± 24 mg/g), water (478 ± 2 mg/g), protein (21.1 ± 2.3 mg/g), RNA (683 ± 49 μg/g) nor DNA (97.5 ± 8.5 μg/g) in subcutaneous adipose tissue at

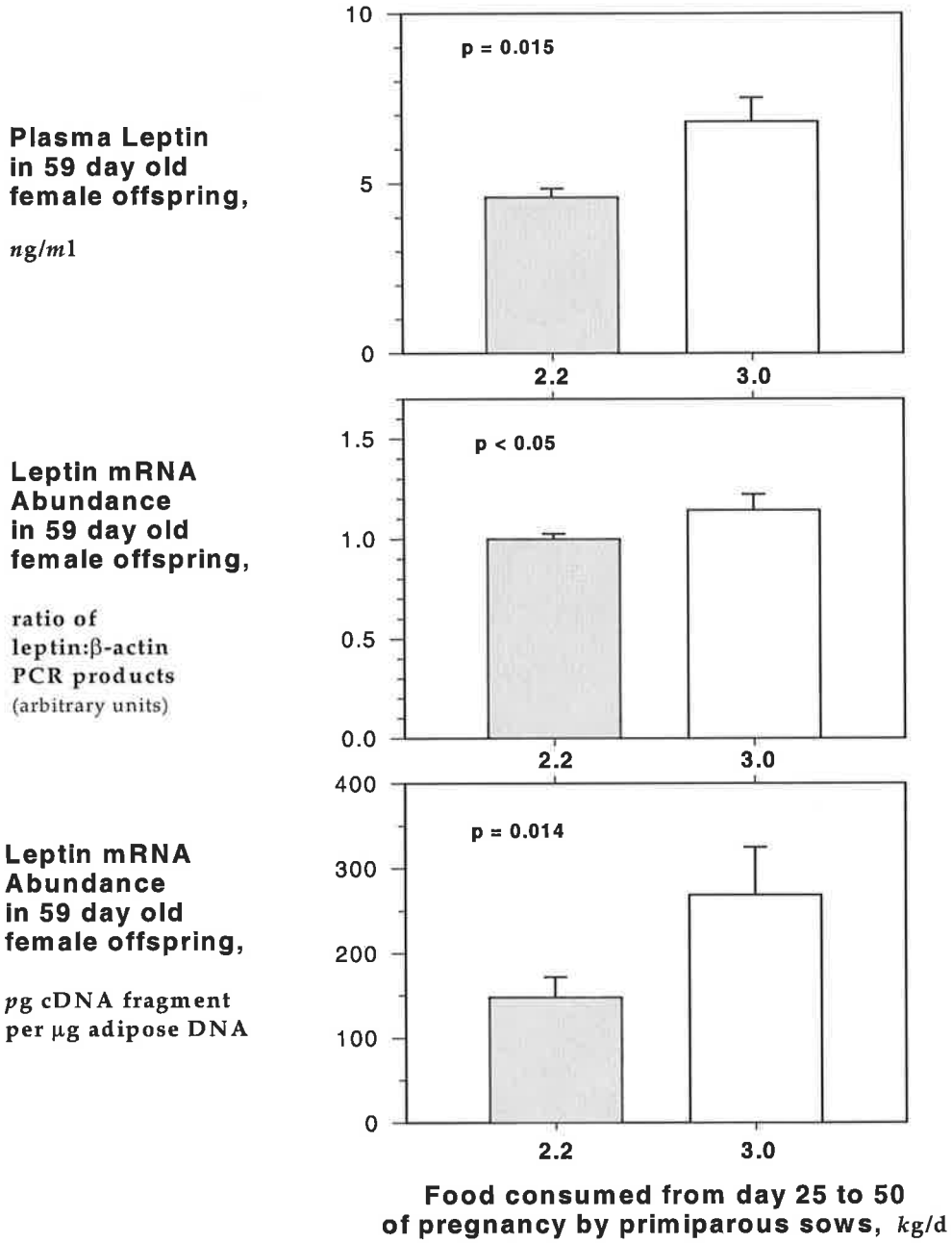

Figure 4.2. Maternal nutrition in the second quarter of pregnancy and leptin mRNA in 59 day old offspring (from Ekert *et al* [2000] J. Endocr. 165: R1-R6).

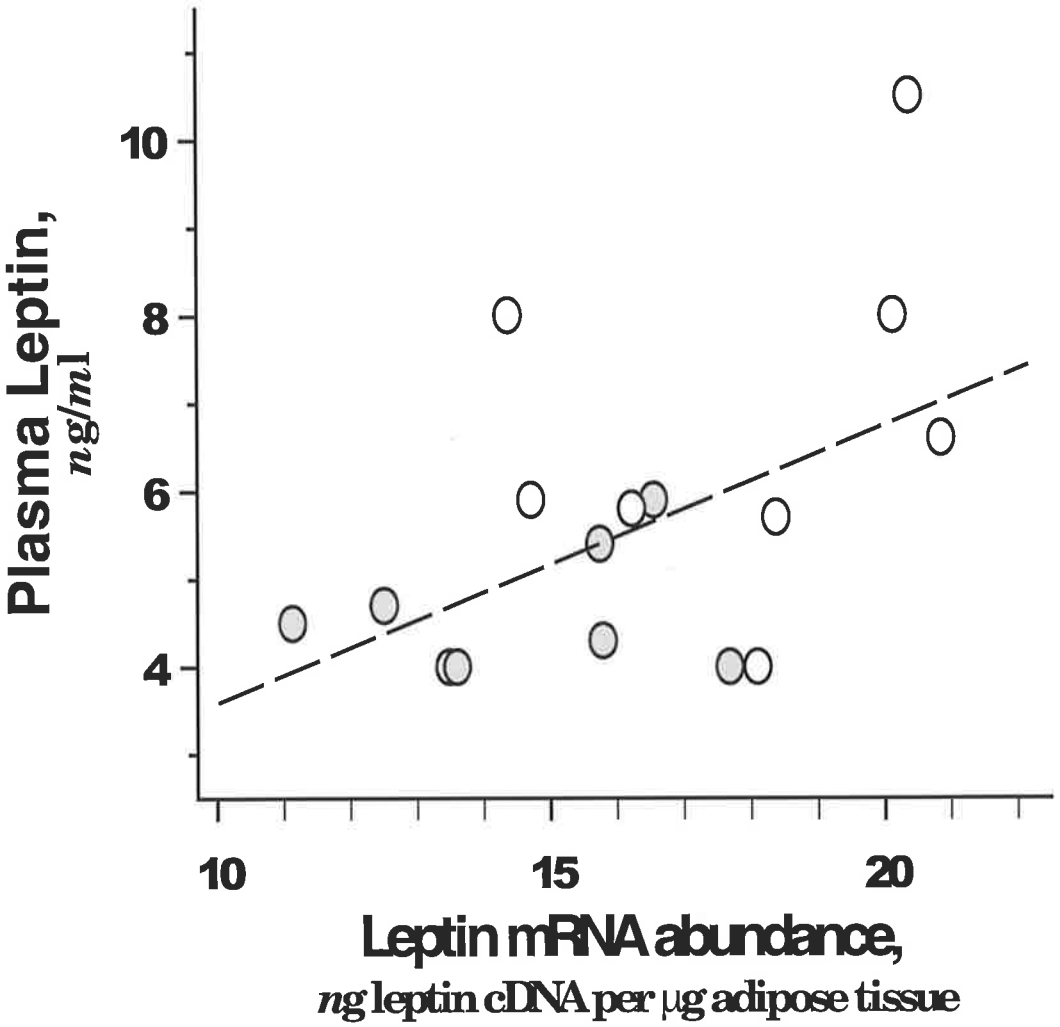

Figure 4.3. Relationship between leptin mRNA abundance in adipose tissue and leptin protein concentration in plasma 59 days after birth (from Ekert *et al* [2000] J. Endocr. 165: R1-R6). Primiparous sows were fed 2.2 kg/d (CLOSED SYMBOLS) through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS) (r=0.54, p=0.03).

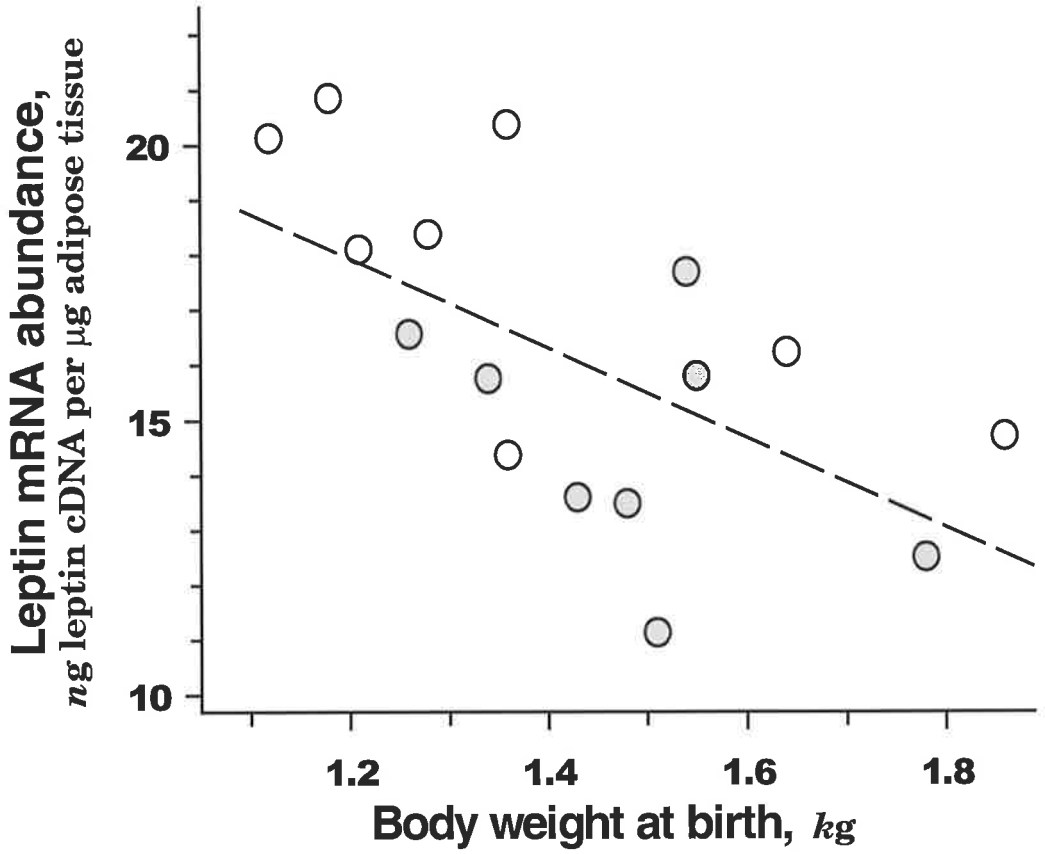

Figure 4.4. Relationship between weight at birth and leptin mRNA 59 days after birth (from Ekert *et al* [2000] J. Endocr. 165: R1-R6). Primiparous sows were fed 2.2 kg/d (CLOSED SYMBOLS) through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS) (r=-0.62, p=0.01).

59 d of age in the selected offspring was affected by maternal nutrition during pregnancy. Mass ratios (g/g) of RNA:DNA (8.2 ± 1.4), protein:DNA (280 ± 80) and lipid:DNA (5600 ± 402) were similarly unaffected.

4.3.3 Effect of maternal nutrition during pregnancy on leptin expression in progeny

Food availability during the second quarter of pregnancy altered both the concentrations of leptin protein in blood plasma and the abundance of leptin mRNA in subcutaneous adipose tissue of 59 day old progeny. Plasma leptin was ~30% higher, the ratio of leptin/β-actin mRNA in adipose tissue was ~15% higher, abundance of leptin mRNA per μg adipose DNA was ~80% higher (Figure 4.2). The amount of leptin cDNA fragment amplified per *n*g adipose RNA was ~20% higher (28.4 ± 1.4 *versus* 23.1 ± 1.3, p=0.015) in offspring from mothers provided more food. Nutrition in pregnancy did not affect abundance of β-actin mRNA in adipose tissue of progeny ($p > 0.2$).

Leptin protein concentration in plasma was positively correlated with leptin mRNA abundance per *n*g adipose RNA (Figure 4.3) and tended to correlate with leptin mRNA abundance per μg adipose DNA ($r=+0.45$, $p=0.09$) but was not related to the ratio of leptin/β-actin mRNA ($p>0.3$).

Abundance of leptin mRNA per cell (*i.e. p*g leptin cDNA fragment amplified per μg adipose DNA) was positively correlated with all measures of adipose cell size (RNA/DNA r=+0.96, p<0.0001; protein/DNA r=+0.88, p<0.0001; lipid/DNA r=+0.64, p<0.01).

4.3.4 Body weight at birth and postnatal leptin expression

Body weight at birth was negatively correlated with leptin mRNA abundance per *n*g adipose RNA (Figure 4.4) and with the ratio of leptin/β-actin mRNA ($r = -0.51$, $p < 0.05$) at 59 days of age.

4.4 Discussion

In the present study weight of progeny at birth and at 59 days of age were unaffected by the amount of feed provided to mothers during the second quarter of pregnancy. A feed rate of 3.0 $kg.d^{-1}$ is higher than the commercial feeding regime of 2.2 $kg.d^{-1}$ during pregnancy. Adipose cell size and subcutaneous adipose composition of DNA, RNA, protein and lipid were similarly unaffected.

Measures of leptin production in offspring were programmed by maternal nutrition. Greater food availability to pregnant mothers increased the concentration of leptin protein in blood and the abundance of leptin mRNA in adipose tissue. High abundance of leptin mRNA in adipose tissue and high plasma leptin concentrations are associated with greater adiposity in pigs (Robert *et al.* 1998; Spurlock *et al.* 1998; Owens *et al.* 1999) as in other mammals (Maffei *et al.* 1995b; Considine *et al.* 1996b; Lonnqvist *et al.* 1997). Maternal nutrition

during pregnancy in rats can program obesity associated with larger adipocytes (Jones *et al.* 1982; Shepherd *et al.* 1997). However, despite differences in leptin production there is no evidence of programmed obesity in the progeny analysed in the present study. Nutrition in pregnancy had no effect on body weight or adipose cell size. Differences in adiposity of the two groups of progeny might become evident at older ages, because leptin mRNA abundance in adipose tissue, plasma concentrations of leptin protein and body composition of fat increase with age in pigs (Spurlock *et al.* 1998; Barb *et al.* 1999; Owens *et al.* 1999).

The positive relationship observed in this study of adolescent female pigs between leptin mRNA abundance in adipose tissue and leptin protein concentration in blood is very similar to that reported for adult women (Lonnqvist *et al.* 1997). In mice, rats and humans, adipocytes appear to be the major sites of leptin expression and secretion into blood (Frederich *et al.* 1995; Maffei *et al.* 1995b). The results of this study indicate this is also the case in pigs.

The negative correlation between body weight at birth and subsequent expression of leptin in pigs is similar to recent observations in humans (Lissner *et al.* 1999; Phillips *et al.* 1999). The negative relationships in pigs and humans between body weight at birth and measures of leptin production in adolescents and adults are the opposite of the frequently reported positive correlations between concurrent measures of body weight or adiposity and leptin production seen in fetal sheep (Yuen *et al.* 1999), human infants (Ong *et al.* 1999) human adults (Considine *et al.* 1996b; Lonnqvist *et al.* 1997) and adult pigs (Owens *et al.* 1999). The negative relationship between birth weight and subsequent leptin production appears to be established in the first year of postnatal life in humans. While growth retarded newborn

infants have lower than normal concentrations of leptin in their blood at birth, by 12 months of age they have higher than normal plasma leptin levels (Jaquet *et al.* 1999b). This may be a consequence of "catch-up" growth. Infants with lower plasma leptin concentrations at birth and lower body weights at birth tend to gain weight more rapidly in the first months of postnatal life (Ong *et al.* 1999; Jaquet *et al.* 1999b). Lower birth weight infants may tend to have higher plasma leptin levels as juveniles and adults because "catch-up" growth may increase their risk of obesity.

Permanent effects on progeny caused by environmental factors during pregnancy has been termed "*in utero* programming" (Barker 1998). Blood pressure (Persson *et al.* 1992), (Woodall *et al.* 1996), cholesterol metabolism (Barker 1998, Lucas 1991), insulin response to glucose (Barker 1998; Lucas 1991), glucose and lipid metabolism (Desai *et al.* 1995) and plasma concentrations of insulin-like growth factors (Gallaher *et al.* 1998) in adults are all sensitive to influences acting before birth. Greater feed intake in the second quarter of pregnancy could theoretically increase postnatal expression of leptin by increasing the availability of glucose and/or other substrates to the fetus by a number of mechanisms (Lechtig *et al.* 1975; Lemons *et al.* 1986; Harding *et al.* 1995; Gluckman *et al.* 1997) during a critical stage of adipocyte development. Whether substrates such as glucose can directly program leptin expression, possibly acting on preadipocytes in the fetus through UDP-*N*-acetylglucosamine (Wang *et al.* 1998) or other pathways (Fukuda *et al.* 1999), or whether this occurs indirectly through actions of fetal hormones such as insulin or insulin-like growth factor-I that are sensitive to nutrition (Gluckman *et al.* 1997) and are capable of regulating fetal adipocyte maturation (Hausman *et al.* 1993; Martin *et al.* 1988) remains to be determined. Fetal tissues express leptin (Hoggard *et al.* 1997a), including adipose tissue

(Yuen *et al.* 1999), and leptin protein is detectable in fetal plasma from as early as 18 weeks gestation in humans (Jaquet *et al.* 1999b). Regardless of the mechanism by which leptin is programmed *in utero*, the present study shows that maternal nutrition during pregnancy is a determinant of postnatal expression of leptin.

CHAPTER 5

ENVIRONMENT DURING PREGNANCY PROGRAMS LEPTIN, INSULIN-LIKE GROWTH FACTOR-II, TRIIODOTHYRONINE AND ESTRADIOL IN PROGENY

5.1 INTRODUCTION

Epidemiological studies have shown that postnatal endocrine function is related to fetal growth, measured as weight or size at birth. Babies who are light or short at birth have reduced insulin sensitivity as children (Law *et al.* 1995; Yajnick *et al.* 1995; Whincup *et al.* 1997; Chiarelli *et al.* 1999) and adults (Leger *et al.* 1997; Ravelli *et al.* 1998), show catch-up growth during early postnatal life (Barker 1998; Ong *et al.* 2000) and elevated plasma leptin as adults (Lissner *et al.* 1999; Phillips *et al.* 1999). Environmental insults during fetal life that do not affect birth weight or size have also been associated with altered postnatal endocrine function (Ravelli *et al.* 1999) which has also been observed in Chapter 4.

Fetal growth involves the accretion and differentiation of tissues that are regulated by hormonal factors such as insulin, insulin-like growth factors and cytokines which can be affected by the nutrient supply from the mother (Fowden 1995; Robinson *et al.* 1995; Koistinen *et al.* 1997; Jaquet *et al.* 1998; Lepercq *et al.* 1998). Higher maternal nutrition during pregnancy alters maternal metabolism by increasing maternal glucose (Chandler *et al.* 1985; Wallace *et al.* 1997), free fatty acids and amino acids (Rees *et al.* 1999) which may affect nutrient delivery to the fetus thereby altering fetal growth. Maternal metabolism is altered in pregnancy by treatment with GH as plasma insulin (Kveragas *et al.* 1986), IGF-I (Sterle *et al.* 1995), glucose and free fatty acid levels are increased (Spence *et al.* 1984) which resembles a diabetic human pregnancy. This leads to increased fetal weight (Rehfeldt *et al.* 1993; Kelley *et al.* 1995; Sterle *et al.* 1995; Sterle *et al.* 1998; Gatford *et al.* 2000), placental weight (Sterle *et al.* 1995; Sterle *et al.* 1998), placental transport capacity (Harding

et al. 1997) and postnatal muscle development in pigs (Rehfeldt *et al.* 1993; Kelley *et al.* 1995).

Obesity associated with larger adipocytes and higher leptin production in adults (Lonnqvist *et al.* 1997; Mantzoros *et al.* 1998; Casanueva *et al.* 1999) can be programmed by undernutrition during early pregnancy (Jones *et al.* 1982). It is possible that the increased leptin levels in adults who were low birth weight babies is due to altered adipocyte development (Lissner *et al.* 1999; Phillips *et al.* 1999). The second quarter of gestation is a period of adipocyte maturation (Desnoyers *et al.* 1980; Hausman *et al.* 1986) in the pig and an increase in nutrient supply to the fetus during this period may change adipocyte characteristics during this period leading to permanent changes in leptin production postnatally. Fetal endocrine systems that regulate fat metabolism and leptin expression (Mantzoros *et al.* 1998; Casanueva *et al.* 1999) may also be sensitive to increased nutrient supply during the second quarter of gestation and may also be permanently altered. It is possible that postnatal leptin sensitivity can be programmed *in utero* (Mantzoros *et al.* 1998; Mistry *et al.* 1999; Ahima *et al.* 2000).

Altering maternal metabolites by GH treatment and nutrition during the second quarter of pregnancy (Spence *et al.* 1984) tests the hypothesis that maternal metabolism during pregnancy programs leptin in offspring. The aim of this experiment was to determine whether maternal metabolites are related to leptin programming. The second hypothesis tested was that intrauterine programming of postnatal leptin by change in maternal metabolites is due to altered programming of adipocyte development or endocrine systems

that regulate leptin production. The study determined whether indicators of adipocyte development or endocrine systems are associated with leptin programming.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Fifty three pregnant Large White primiparous sows were fed 2.2 $kg.d^{-1}$ of a dry ration containing 13.5 MJ digestible energy and 150 g crude protein per kg throughout pregnancy except that from the 25^{th} to the 50^{th} day of pregnancy half were fed at 3.0 $kg.d^{-1}$. From the 25^{th} day of pregnancy (mean ± SEM kg) animals on both levels of feed were injected daily for 25 days with 0, 4 or 8 mg of pGH (recombinant porcine growth hormone, Southern Cross Biotech Pty. Ltd., Melbourne, Australia) which delivered average daily doses of 0, 26.4 ± 0.5, or 54.3 ± 0.9 $\mu g.kg^{-1}$ (n = 6 to 11 dams per group). Pregnant sows were weighed and their depth of backfat measured 110 mm from the midline over the 13^{th} rib by β-mode ultrasound on days 25 and 50 of pregnancy. On day 40 of pregnancy a blood sample was collected from each mother by acute venepuncture into iced EDTA vacutaniers, centrifuged at 4 ^0C and stored at -20 ^0C. Within 12 hours of birth, litter size, individual piglet body weights and dimensions (body nose-to-tail length, biparietal head diameter, ponderal index, abdominal circumference) were recorded and each piglet was identified by ear tagging. Litter size was equalised by fostering piglets within treatment groups at this time. When piglets were measured 27 days after birth a single female of median birth weight for her litter was selected for further analysis. The selected female progeny were weighed at 27 d, 34 d and 61 d of age and blood was also collected at 61 days of age.

5.2.2 Analysis of metabolites and hormones

IGFs were separated from binding proteins by size exclusion chromatography of plasma at pH 2.5 (Owens *et al.* 1990). IGF-I and IGF-II concentrations were determined in neutralised chromatography fractions by RIA (Francis *et al.* 1989; Carr *et al.* 1995). Insulin (Insulin-CT kit, CIS bio international, France), cortisol (*Vitros* Cortisol assay, cat. No. 107 4053, Amersham, UK), estradiol (*Vitros* Estradiol assay, cat. no. 855 2630, Amersham), progesterone (*Vitros* Progesterone assay, cat. no. 193 8570, Amersham) and triiodothyronine (*Vitros* Total T3 assay, Amersham) were measured in plasma. Leptin was measured in plasma by RIA using a guinea pig antibody to human leptin (Linco Research, St Louis, MO, USA, Cat. 1085-K), human [^{125}I]-leptin (Linco Inc, Cat. 9081) and human leptin (R&D Systems, Cat. 398-LP) as described in Chapter 4. Plasma concentrations of glucose (GLUC HK, cat. 07 3672 4, Roche), urea (UREA, cat. 07 3685 6, Roche), free fatty acids (NEFA C, cat. 279-75402, Wako Pure Chemical Industries, Japan), triglycerides (TRIG, cat. 07 3679 1, Roche), lactate (LACT MPR1, cat. 149993, Roche) and cholesterol (CHOL, cat. 07 3663 5, Roche) were measured by semi-automated enzymatic analysis. Amino nitrogen concentrations were measured by colourimetric assay (Evans *et al.* 1993).

5.2.3 Measurement of DNA content of progeny adipose tissue

DNA content was measured by a modification of the diphenylamine method from 1.01 ± 0.02 g of thinly sliced homogenised adipose tissue as described in 4.2.6.

5.2.4 Measurement of RNA content of progeny adipose tissue

RNA concentration was determined from absorbance at 260 nm of RNA hydrolysates (Van den Hoff *et al.* 1997) prepared from delipidated homogenates of 201 ± 5 mg of adipose tissue in 3 ml of 10 mmol/l sodium phosphate pH 6 as described in 4.2.7.

5.2.5 Measurement of protein content of progeny adipose tissue

Frozen adipose tissue 205 ± 5 mg was removed from -80^{0}C storage into pre-weighed 10 ml tubes (Falcon tubes, Becton). As described in 4.2.8 tissue protein was measured using the BCA (bicinchoninic acid) Protein Assay Reagent according to the manufacturer's instructions (23225X, Pierce Chemical Co, Rockford, Illinios). Dry matter content was measured after dehydration at 140 ^{0}C for 20 hr.

5.2.6 Measurement of water and lipid of progeny adipose tissue

Frozen adipose tissue 716 ± 58 mg was removed from -80^{0}C storage into pre-weighed glass vials. Water and lipid content were determined as described in 4.2.9.

5.2.7 Statistical Analysis

Data is summarised as mean ± SEM (n = number of dams or progeny). Effects of treatments

were assessed by two-way analysis of variance. Associations between measurements were identified by Pearson correlation. Codependence of measurements were identified by backward stepwise regression and confirmed by multiple regression (SigmaStat V1, Jandel Scientific Software, San Rafael, California).

5.3 Results

5.3.1 Effects on pregnant dams

Primiparous sows fed 3.0 $kg.d^{-1}$ from the 25^{th} to the 50^{th} days of pregnancy gained more weight ($p<0.001$) and tended to be fatter ($p=0.08$) at day 50 than those fed 2.2 $kg.d^{-1}$ (Figure 5.1). Dams provided more feed also had higher concentrations of insulin ($p=0.05$) in their blood plasma on the 40^{th} day of pregnancy (Figure 5.2). Greater feed availability decreased the concentrations of free fatty acids ($p<0.05$) in maternal blood and tended to increase those of total IGF concentration, *i.e.* sum of IGF-I plus IGF-II ($p<0.06$), triglycerides ($p<0.06$) and urea ($p<0.1$, Figure 5.3). Maternal nutrition did not significantly affect maternal plasma concentrations of glucose (Figure 5.3), lactate (2.73 ± 0.23 mmol/l), cholesterol (2.34 ± 0.06 mmol/l) or α-amino-nitrogen (4.47 ± 0.07 mmol/l) during this period.

GH treatment of pregnant pigs in the second quarter of pregnancy reduced depth of back fat ($p<0.005$) and tended to increase maternal weight gain ($p<0.1$) during treatment (Figure 5.1). GH also increased maternal plasma concentrations of insulin ($p<0.005$) and IGF-I ($p<0.0001$), reduced those of IGF-II ($p<0.002$) and increased total IGF concentration ($p<0.0001$), on the 40^{th} day of pregnancy (Figure 5.2). GH treatment also increased the

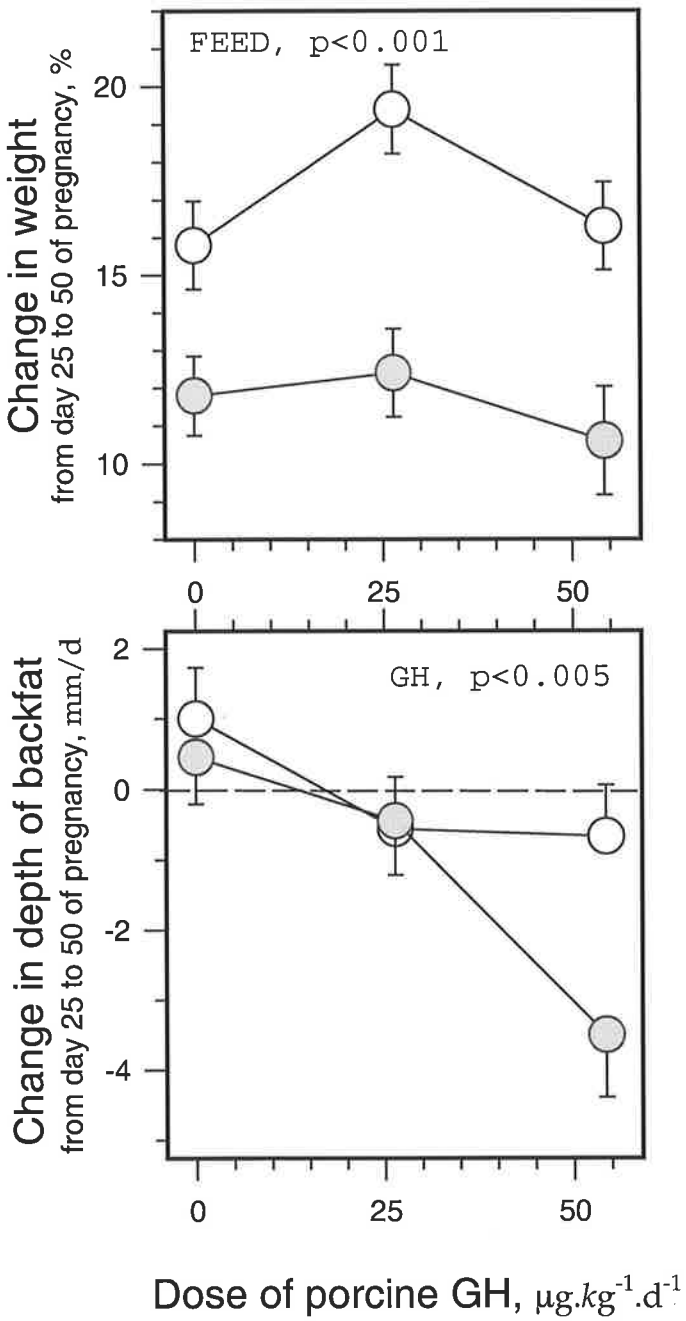

Figure 5.1. Effects of GH treatment and nutrition in the second quarter of pregnancy on maternal weight and fatness in pigs. Primiparous sows were fed 2.2 kg/d through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS). Gilts on both feed levels were injected s.c. daily with pGH.

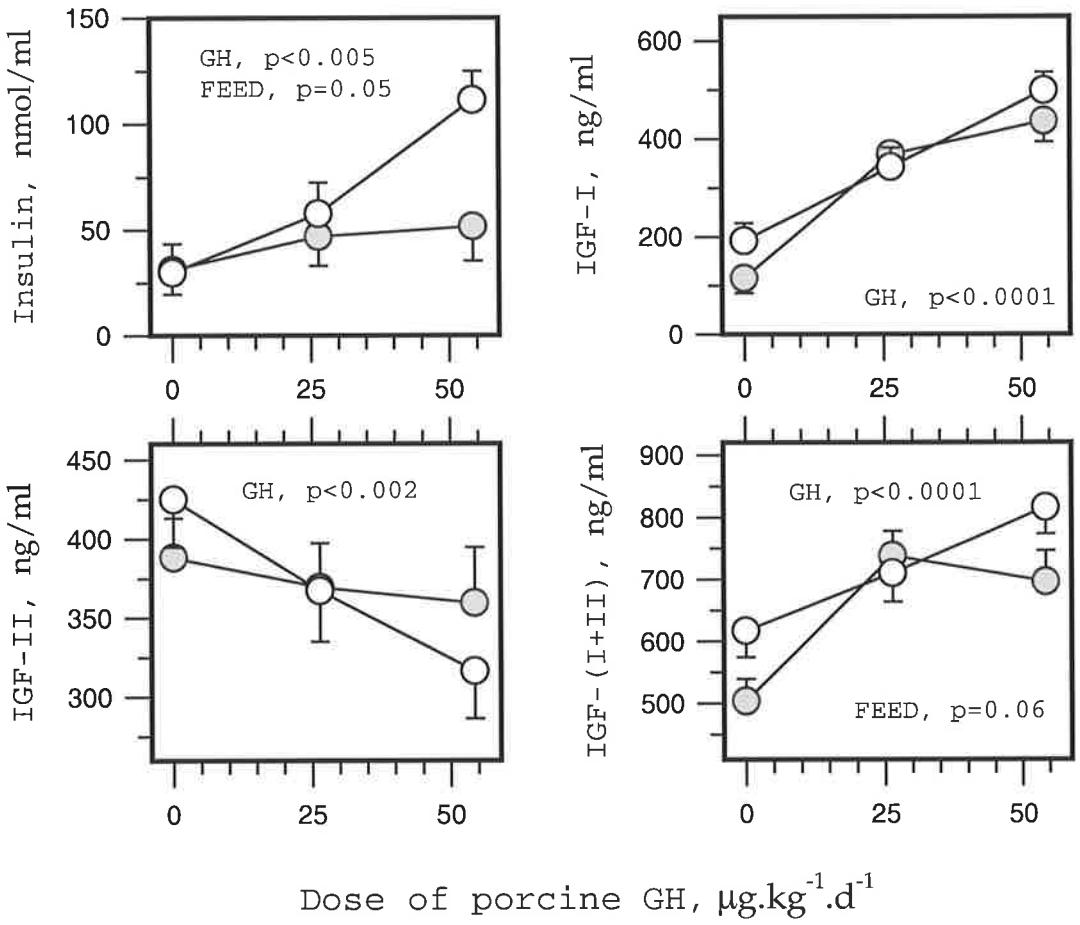

Figure 5.2. Effects of GH treatment and nutrition in the second quarter of pregnancy on insulin, IGF-I and IGF-II in maternal blood on day 40 of pregnancy. Primiparous sows were fed 2.2 kg/d through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS). Gilts on both feed levels were injected s.c. daily with pGH.

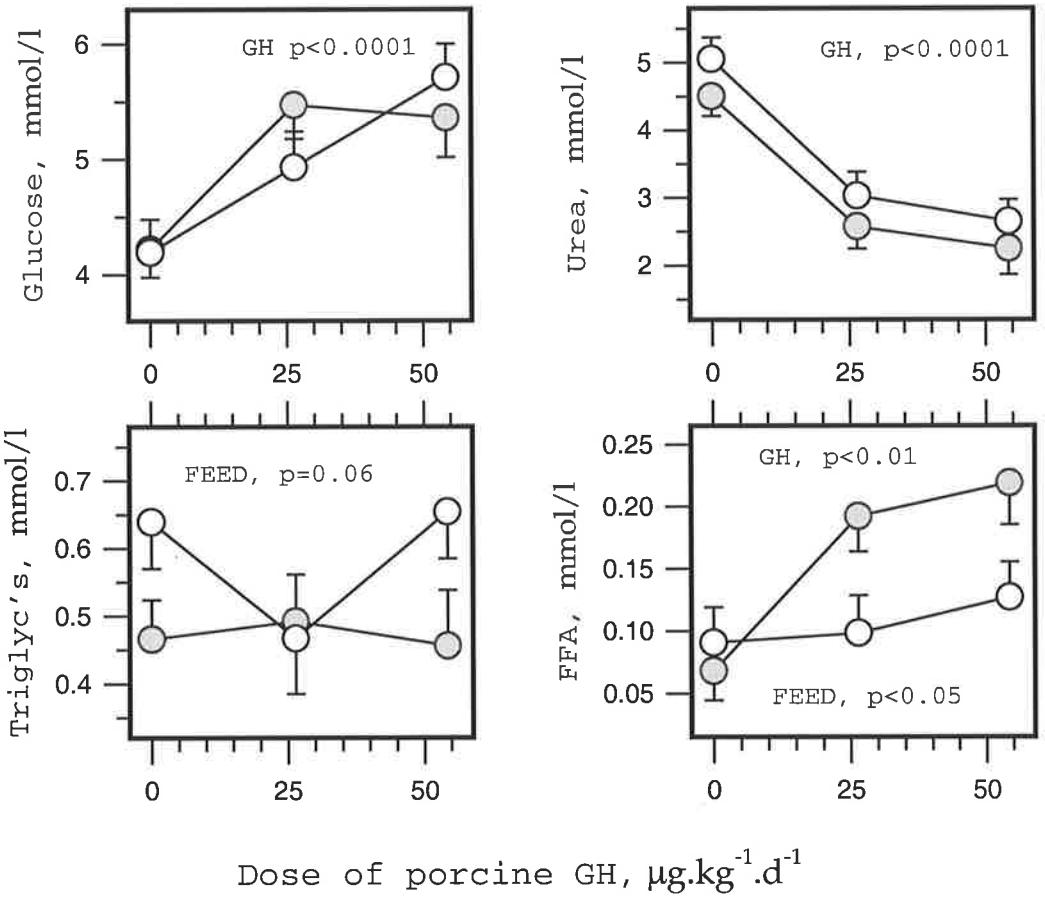

Figure 5.3. Effects of GH treatment and nutrition in the second quarter of pregnancy on glucose, urea, triglycerides and free fatty acids in maternal blood. Primiparous sows were fed 2.2 kg/d through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS). Gilts on both feed levels were injected s.c. daily with pGH.

concentrations of glucose ($p<0.0001$) and free fatty acids ($p<0.01$), and reduced those of urea ($p<0.0001$), in maternal plasma (Figure 5.3). Treatment with GH did not significantly affect maternal plasma concentrations of triglycerides (Figure 5.3), lactate, cholesterol or α-amino-nitrogen. No significant interactions between maternal nutrition and treatment with GH were observed for any maternal measurements.

5.3.2 Effects on progeny

5.3.2.1 Size at birth and growth of progeny

Neither maternal feed nor treatment with GH from day 25 to 50 of pregnancy affected the number of animals born alive (9.5 ± 0.3) or stillborn (0.5 ± 0.1) per pregnancy, or the average per litter of piglet body weight at birth (1.51 ± 0.03 kg). Similarly, neither maternal treatment affected body weight (1.55 ± 0.03 kg), biparietal head diameter (45.7 ± 0.2 mm), body nose-to-tail length (397 ± 3 mm), ponderal index (0.025 ± 0.0005) or abdominal circumference (257 ± 3 mm) at birth of the females selected for further study.

One female piglet whose body weight at birth was the median for the females in her litter was selected from each treated dam and weaned 27 days after delivery. Body weight at 27 days of age (detailed below) was positively correlated with their body weight at birth in median female progeny ($r= 0.32$, $p < 0.02$). Fractional rate of gain in their body weight (% per day) from birth to 27 days ($r=-0.37$, $p<0.01$), birth to 34 days ($r=-0.36$, $p<0.01$) and from birth to 61 days of age ($r=-0.44$, $p<0.001$) was negatively correlated with body weight at birth. Plasma concentrations of IGF-I ($r=0.28$, $p<0.05$) and cortisol ($r=0.28$, $p=0.05$) at 61

days of age were also negatively correlated with birth weight.

Higher level of feed but not treatment with GH of primiparous sows in the second quarter of pregnancy increased both body weight of offspring measured when they were weaned at 27 days of age ($p<0.05$) and rate of body weight gain (kg/d) for the first 27 days after birth ($p=0.05$, Figure 5.4). Neither maternal feed nor GH treatment in the second quarter of pregnancy affected body weights 34 days (8.1 ± 0.3 kg) or 61 days after birth (22.6 ± 0.7 kg) nor rates of weight gain (kg/d) from birth to 34 (127 ± 5 g/d) or from birth to 61 days of age (340 ± 10 g/d).

5.3.2.2 Adipose tissue content of progeny

Neither the content of lipid (469 ± 14 mg/g), water (512 ± 14 mg/g), protein (21.7 ± 1.9 mg/g), RNA (568 ± 47 μg/g) nor DNA (108 ± 4.6 μg/g) in subcutaneous adipose tissue at 61 d of age in the selected offspring was affected by maternal nutrition and GH treatment during pregnancy. Mass ratios (g/g) of RNA:DNA (5.2 ± 0.4), protein: DNA (193 ± 18) and lipid: DNA (4779 ± 266) were similarly unaffected.

5.3.2.3 Circulating metabolites and hormones of progeny

Females of median birth weight for the females in their litters selected from mothers fed more during the second quarter of pregnancy had significantly higher concentrations of leptin ($p<0.02$) in their blood plasma at 61 days of age. Maternal nutrition during pregnancy

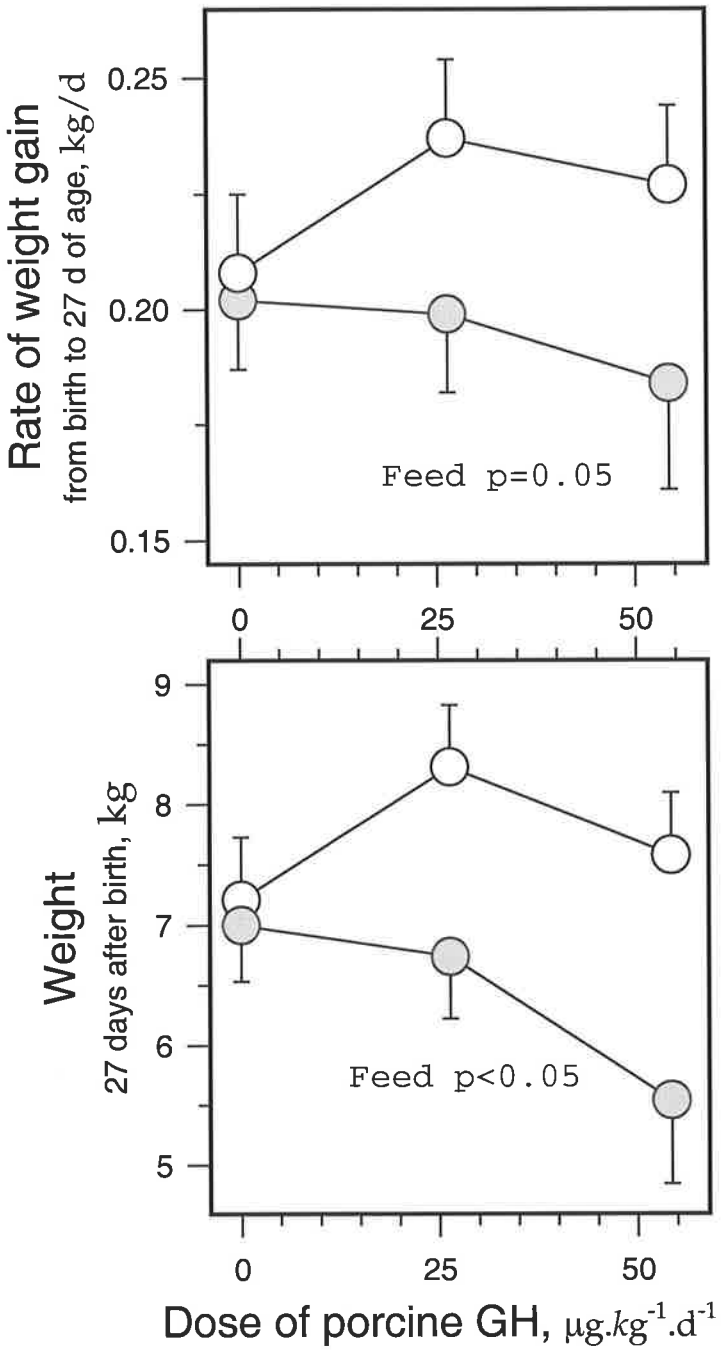

Figure 5.4. Effect of GH treatment and nutrition in the second quarter of pregnancy on postnatal growth of offspring in pigs. Primiparous sows were fed 2.2 kg/d through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS). Gilts on both feed levels were injected s.c. daily with pGH.

did not affect plasma concentrations of glucose (5.8 ± 0.6 mmol/l), insulin, (8.2 ± 0.2 ng/ml), IGF-I (180 ± 8 ng/ml), IGF-II, estradiol, progesterone (1.3 ± 0.2 nmol/l), cortisol (120 ± 7 ng/ml) or triiodothyronine in progeny (Figure 5.5).

Treatment of pregnant gilts with GH during the second quarter of pregnancy increased plasma concentrations of leptin ($p<0.002$), estradiol ($p<0.005$) and triiodothyronine ($p<0.001$) and reduced those of IGF-II ($p<0.02$) in progeny examined at 61 days of age (Figure 5.5). Maternal GH treatment did not affect plasma concentrations of glucose, insulin, IGF-I, progesterone or cortisol in progeny.

Plasma leptin concentrations in progeny were positively correlated with their plasma concentrations of IGF-I ($r=0.35$, $p<0.02$). No other individual measurements of progeny correlated with plasma leptin concentrations, but multiple regression showed that variance in plasma leptin concentrations in progeny could be best described by the independent contributions of plasma concentrations of IGF-I ($p=0.002$) and glucose ($p=0.026$) in the same progeny (*multiple linear regression*: leptin = 11.5 – (1.59 glucose) + (0.0183 IGF-I), $r=0.454$, $p<0.001$).

Concentrations of cortisol ($r=0.52$, $p<0.0002$) and IGF-I in 61 day old progeny ($r=0.44$, $p<0.001$) were both positively correlated with body weight at 61 days of age and with rates of body weight gain (kg/d) from birth to 61 days (cortisol $r=0.54$, $p=0.0001$; IGF-I $r=0.44$, $p=0.001$) and from 34 to 61 days of age (cortisol $r=0.56$, $p<0.0001$; IGF-I $r=0.46$, $p<0.001$). Plasma IGF-II concentrations in progeny were not related to their body weights, growth rates or plasma cortisol levels, but were positively correlated with their plasma IGF-I

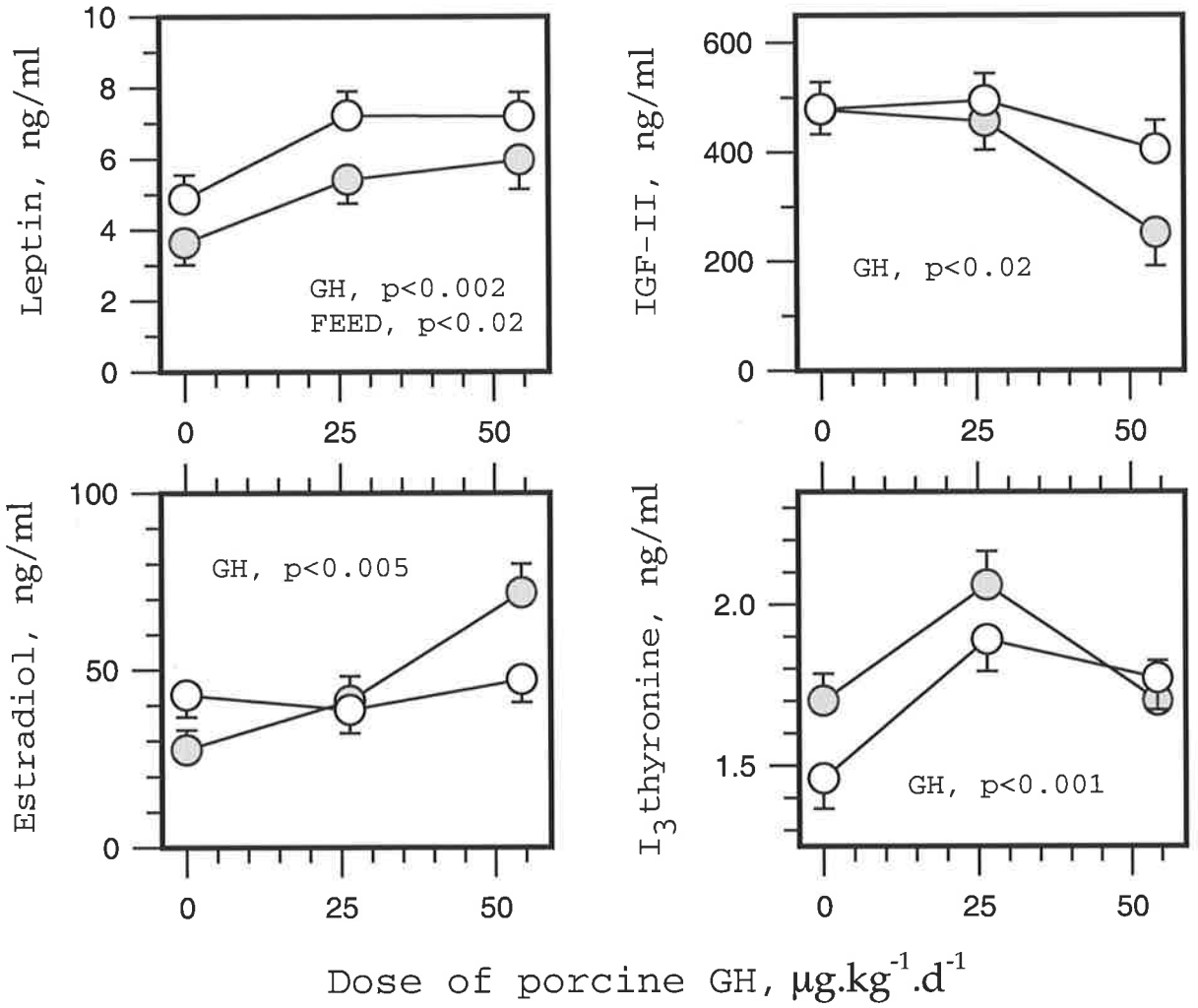

Figure 5.5. Effects of GH treatment and nutrition in the second quarter of pregnancy on leptin, IGF-II, estradiol and triiodothyronine in blood of offspring 61 days after birth. Primiparous sows were fed 2.2 kg/d through pregnancy (110 d) except half were fed 3.0 kg/d from day 25 to 50 (OPEN SYMBOLS). Gilts on both feed levels were injected s.c. daily with pGH.

concentrations ($r=0.41$, $p<0.005$). Of the measurements in 61 day old offspring, the combination of plasma concentrations of cortisol, IGF-I and IGF-II was the best correlate of both body weight at 61 days of age (*multiple linear regression*: weight = $17.3 + (3.4 \times 10^{-2}$ IGF-I) - (1.04×10^{-2} IGF-II) + (3.4×10^{-2} cortisol), $r=0.56$, overall $p<0.0001$ [IGF-I $p=0.025$, IGF-II $p<0.05$, cortisol $p<0.025$]) and rate of body weight gain (kg/d) from 34 to 61 days of age (*multiple linear regression*: growth rate = $0.38 + (7.6 \times 10^{-4}$ IGF-I) - (2.1×10^{-4} IGF-II) + (1.1×10^{-3} cortisol), $r=0.64$, $p<0.0001$ [IGF-I $p=0.02$, IGF-II $p=0.05$, cortisol $p<0.002$]).

5.3.2.4 Relationships between postnatal growth and maternal measures

Body weight at 27 days of age was correlated with dimensions at birth and maternal responses to treatment in pregnancy. Weight ($r=0.35$, $p<0.02$), nose to tail length ($r=0.34$, $p<0.02$) and abdominal circumference ($r=0.40$, $p<0.005$) of the body and biparietal diameter of the head ($r=0.40$, $p<0.005$) at birth were all positively correlated with body weight at 27 days, as were the changes in maternal body weight from day 25 to 50 of pregnancy ($r=0.30$, $p<0.05$) and the concentrations of insulin in maternal plasma on the 40^{th} day of pregnancy ($r=0.33$, $p<0.05$). Variance in body weight of progeny at 27 days of age could be best described by the independent contributions of plasma insulin in mothers on the 40^{th} day of pregnancy ($p<0.02$) and body weight of progeny at birth ($p<0.001$) (*multiple linear regression*: progeny body weight at weaning = $1.45 + (1.27 \times 10^{-2}$ maternal insulin) + (3.29 birth weight), $r=0.578$, overall $p<0.0005$). Rate of gain in body weight of progeny from birth to weaning was positively correlated with biparietal diameter of the head ($r=0.33$, $p<0.02$) and abdominal circumference ($r=0.36$, $p<0.01$) of the body at birth as well as the change in maternal body weight from day 25 to 50 of pregnancy ($r=0.30$, $p<0.05$) and maternal plasma

insulin on day 40 of pregnancy (r=0.41, p<0.01). The combination of maternal plasma insulin concentration on day 40 of pregnancy (p<0.01) and abdominal circumference at birth (p<0.005) was found to be the best correlate of rate of progeny weight gain from birth to 27 days of age (*multiple linear regression*: growth rate from birth to weaning = (4.46×10^{-4} maternal insulin) + (1.31×10^{-3} abdominal circumference at birth) - 0.15, r=0.59, p<0.0005).

5.3.2.5 Relationships between progeny circulating metabolites/hormones and maternal measures

Several measurements of pregnant dams correlated with plasma leptin concentrations in offspring. These include the increase in maternal backfat from day 25 to 50 of pregnancy (r=-0.33, p<0.02) and maternal plasma concentrations of glucose (r=0.47, p<0.002), IGF-I (r=0.41, p<0.01), urea (r=-0.36, p<0.025), insulin (r=0.35, p<0.025) and cholesterol (r=0.31, p<0.05). Maternal plasma concentrations of IGF-II, free fatty acids and the total concentration of IGFs were not individual correlates of plasma leptin levels in progeny. Multiple regression showed that plasma glucose in pregnancy was the major independent maternal correlate of plasma leptin concentration in progeny. Inclusion in multiple regression analyses of either plasma IGF-I or glucose concentrations in progeny, which are correlates of their plasma leptin concentrations, with maternal responses to treatments, showed that the combination between the plasma concentration of IGF-I (p<0.02), but not glucose, in progeny and the urea concentrations of their mothers during pregnancy (p<0.01) correlated with plasma leptin concentrations in offspring (*multiple linear regression*: progeny leptin = 5.53 - (0.687 maternal urea) + (0.014 progeny IGF-I), r=0.534, overall p<0.002). Inclusion of both progeny correlates of progeny leptin levels with maternal

responses to pregnancy treatments showed variation in the plasma concentrations of leptin in offspring was best accounted for by the combination of their plasma concentrations of glucose ($p=0.025$) and IGF-I ($p<0.05$) with the concentration of glucose in their mothers during treatment in pregnancy ($p<0.02$) (*multiple linear regression*: progeny leptin = 8.1 - (1.56 progeny glucose) + (0.013 progeny IGF-I) + (0.869 maternal glucose), $r=0.605$, overall $p<0.001$).

Plasma concentrations of estradiol in 61 day old offspring were negatively correlated with maternal plasma concentrations of urea ($r=-0.36$, $p<0.05$) and α-amino nitrogen ($r=-0.36$, $p<0.05$) and tended to be positively correlated with plasma levels of IGF-I ($r=0.29$, $p<0.08$) in their mothers on day 40 of pregnancy. The combination of maternal IGF-I ($p=0.026$) and triglyceride concentrations in pregnancy ($p=0.05$) was the best correlate of plasma estradiol concentrations in progeny (*multiple linear regression*: progeny estradiol = 45.1 - (33.1 maternal triglycerides) + (4.33×10^{-2} maternal IGF-I), $r=0.427$, overall $p=0.036$). Plasma triiodothyronine concentrations in 61 day old offspring were positively correlated with plasma concentrations of lactate ($r=0.47$, $p<0.005$) and cholesterol ($r=0.37$, $p<0.025$) in their mothers on day 40 of pregnancy. The strongest predictor of plasma concentrations of triiodothyronine in offspring was the combination of the concentrations of lactate ($p<0.001$) and free fatty acids in maternal plasma on day 40 of pregnancy ($p=0.06$) (*multiple linear regression*: progeny triiodothyronine = 1.3 + (0.129 maternal lactate) + (1.13 maternal free fatty acids), $r=0.552$, $p=0.0025$). Plasma concentrations of IGF-II in 61 day old offspring were positively correlated with those of urea ($r=0.35$, $p<0.05$), tended to be positively correlated with those of IGF-II ($r=0.29$, $p<0.07$) and tended to be negatively correlated with IGF-I concentrations ($r=-0.28$, $p<0.08$) in their mother's plasma on day 40 of pregnancy. Of

the measurements obtained during pregnancy and at birth, the best predictor of plasma IGF-II levels in progeny was the combination of IGF-I ($p<0.005$) and insulin levels in maternal blood on the 40th day of pregnancy ($p=0.02$) (*multiple linear regression*: progeny IGF-II = 523 - (0.56 maternal IGF-I) + (1.7 maternal insulin), $r=0.456$, overall $p<0.02$).

5.4 DISCUSSION

GH treatment had a greater effect than nutrition during the second quarter of pregnancy on altering maternal body composition and metabolism. Higher feed in the second quarter of pregnancy increased live weight, plasma insulin, total IGFs and decreased free fatty acid levels in the pregnant gilt. Total IGFs may be playing a minor role by increasing protein synthesis leading to increased live weight in the better fed pregnant dams. Increased maternal feed during early pregnancy reduced free fatty acid in pigs suggesting a reduction in lipolysis but the affect was not great enough to significantly change backfat depth. GH treatment reduced maternal backfat, plasma IGF-II, urea and increased plasma insulin, IGF-I, total IGFs, glucose, and free fatty acids. GH treatment through its lipolytic effect converted triglyceride into free fatty acids which was increased in maternal plasma. GH anabolic effects on protein metabolism were evident with reduced maternal urea levels but no change in α-amino nitrogen levels suggesting increased protein degradation and decreased protein synthesis (Harding *et al.* 1997) as there was no increase in maternal weight. In previous pig (Sterle *et al.* 1998; Gatford *et al.* 2000) and sheep (Harding *et al.* 1997) studies GH treatment during early pregnancy increased maternal plasma IGF-I which was demonstrated in this study.

Maternal insulin levels were raised in pregnant gilts both by GH treatment and higher feed during the second quarter of pregnancy. Maternal glucose on the high GH dose is not affected by maternal feed. However, maternal insulin levels are increased in the pregnant gilts on high GH dose and high feed suggesting that the pregnant dams had developed a higher state of insulin resistance at day 40 of pregnancy compared to dams on high GH dose and low feed. Insulin resistance is a characteristic of GH treatment in pregnant (Kveragas *et al.* 1986) and non-pregnant pigs (Chung *et al.* 1985).

GH treatment in pregnant dams in the second quarter of pregnancy significantly altered maternal metabolism consistent with previous studies and caused heavier fetuses (Sterle *et al.* 1998; Gatford *et al.* 2000), placenta, (Sterle *et al.* 1998) increased fetal length (Kelley *et al.* 1995) and altered fetal metabolism (Sterle *et al.* 1995; Gatford *et al.* 2000) at mid gestation and increased muscle fibre number postnatally (Kelley *et al.* 1995). While maternal nutrition which changes maternal metabolism less significantly than GH treatment in the second quarter of pregnancy only affected birth weight after day 80 of pregnancy (Noblet *et al.* 1985; Dwyer *et al.* 1994) but it affected postnatal muscle fibre development (Dwyer *et al.* 1994). Therefore, both GH treatment and maternal nutrition during the second quarter of pregnancy due to changes in maternal metabolism can affect fetal development and fetal endocrine systems which could be programmed in the fetus and permanently altered in the progeny.

Improved nutrition in early pregnancy but not GH treatment increased weight at 27 days and rate of weight gain between birth to 27 days in progeny. Greater feed intake in pregnancy could theoretically increase postnatal growth rate from birth to weaning by improving

maternal milk production during lactation or by increasing the programmed postnatal growth rate and appetite. Milk production can be increased by improved maternal nutrition throughout pregnancy in pigs but requires changes in maternal body composition of fat by the time of parturition (Revell *et al.* 1998). It is more likely that placental (Wallace *et al.* 1999) and fetal growth (Harding *et al.* 1995) may have been increased during the period of higher maternal feed intake and subsequently constrained during the remainder of pregnancy when maternal feed intake was lower. Due to the removal of the maternal constraint of pregnancy at birth, progeny whose mothers received more feed in the second quarter of pregnancy may therefore exhibit "catch up" growth in the first weeks after birth (Jaquet *et al.* 1999a). This interpretation is supported by a negative association observed between body weight at birth and fractional growth rate between birth and 27 days of age. It is also possible that maternal nutrition during pregnancy affects the appetite of newborn offspring, which in turn affects maternal milk production because it is partially driven by demand (Koketsu *et al.* 1996).

GH treatment and higher maternal nutrition during the second quarter of pregnancy increased postnatal plasma leptin, which confirms the observed programming of leptin by maternal nutrition in Chapter 4. GH treatment, which altered the pregnant dam's metabolism during pregnancy, had a greater effect on increasing postnatal plasma leptin levels than maternal nutrition. The mechanism of programming leptin in the fetus due to GH treatment and maternal nutrition could be different. Maternal correlates of plasma leptin in progeny were significantly altered during pregnancy by maternal feed rate (insulin) or GH (glucose, IGF-I, urea and insulin). Maternal plasma concentrations of IGF-II and free fatty acids, which were altered during GH treatment in pregnancy, and the total concentration of IGFs,

which was affected by both GH and nutrition in pregnancy, were not individual correlates of plasma leptin levels in progeny. Maternal glucose was the best maternal correlate with plasma leptin in progeny. Maternal urea was the best overall correlate with postnatal plasma leptin when combined with either progeny IGF-I or glucose which were the strongest postnatal correlates with plasma leptin. This suggests that maternal glucose or possibly maternal urea in the mother influences intrauterine programming of leptin. Maternal plasma glucose levels at day 40 of pregnancy were increased by GH treatment but not affected by improved maternal nutrition. Putnam and colleagues (Putnam *et al.* 1999) showed that a higher crude protein diet in late pregnancy in dairy cows increased glucose disposal rate but not glucose levels. In the GH treated mother, there may be more plasma glucose compared to the mother on an improved nutrition which causes a higher glucose gradient between the mother and the fetus. The gilts on the higher maternal feed potentially have a higher glucose disposal rate which allows more glucose to cross the placenta but not as great as the GH treated mothers (Putnam *et al.* 1999). Diabetes during pregnancy increases cord blood leptin levels in newborns (Gross *et al.* 1998; Shekhawat *et al.* 1998; Persson *et al.* 1999). The present results are also consistent with increased leptin production occurring in the fetus and persisting postnatally suggesting that maternal hyerglycaemia increases fetal leptin production.

Maternal glucose or possibly maternal urea might act either to alter adipocyte development in the fetus or endocrine systems that regulate leptin to cause increased postnatal leptin production. Neither GH treatment nor higher maternal nutrition during the second quarter of pregnancy altered adipocyte size postnatally. This suggests that the second quarter of

pregnancy is not a sensitive period in the pig to change adipocyte size that persists postnatally and does not increase progeny leptin via this mechanism.

Endocrine systems that regulate leptin expression in the adipocyte could have been altered by GH treatment or higher maternal nutrition during the second quarter of pregnancy. If these changes persisted postnatally they may cause higher postnatal leptin levels. Leptin expression in mature adipocytes is regulated by food intake, adiposity, energy balance, adipocyte factors, several cytokines and hormones including insulin, thyroxine, cortisol, IGFs, steroid hormones and catecholamines (Guerre-Millo 1997; Mantzoros *et al.* 1998; Trayhurn *et al.* 1999). Plasma leptin concentrations in progeny, which were programmed by both nutrition and GH treatment of their pregnant mothers, were positively correlated with their plasma concentrations of IGF-I which were not programmed by either maternal treatment. None of the hormones in the progeny that were programmed by GH treatment, which include estrogen, IGF-II and triiodothyronine were positively correlated with plasma leptin. None of the hormones in the progeny were programmed by maternal nutrition. This suggests that maternal nutrition and GH treatment do not increase progeny leptin via this mechanism. Other possible mechanisms not tested in this study that lead to intrauterine programming of leptin maybe due to higher maternal glucose during the second quarter of pregnancy increasing fetal glucose which will alter the hexosamine biosynthetic pathway (Wang *et al.* 1998), transcriptional pathways (Fukuda *et al.* 1999), or other systems (Mantzoros *et al.* 1998; Casanueva *et al.* 1999) increasing constitutive expression of leptin by fetal adipocytes that persists postnatally (Yuen *et al.* 1999; Atanassova *et al.* 2000).

Leptin resistance could be another potential explanation for increased postnatal plasma leptin as there was no change in body weight due to GH treatment or higher maternal nutrition. A study in boars showed that a positive relationship existed between plasma leptin at 20 weeks of age and the subsequent voluntary food intake in the following week (Owens *et al.* 1999). If the animals are leptin resistant or the metabolic actions of leptin have not been fully acquired at the stage of development studied (Devaskar *et al.* 1997; Mistry *et al.* 1999), the pigs with the greater plasma leptin might not yet have an altered appetite (Schwartz *et al.* 2000). The pigs with the high plasma leptin may demonstrate leptin insensitivity due to immature development of the leptin receptor or intracellular signalling pathways in the hypothalamus. Leptin resistance would be unable to downregulate NPY which has been demonstrated in aged rats that are unresponsive to leptin (Scarpace *et al.* 2000). In the rat, hypothalamic differentiation occurs postnatally and overfeeding during this period causes hyperphagia, elevated plasma insulin and leptin levels (Plagemann *et al.* 1999b; Vickers *et al.* 2000), an increase in percentage of NPY positive neurons per total number of neurons but no change in NPY content (Plagemann *et al.* 1999b). In the pig, NPY-like immunoreactivity (Pearson *et al.* 1996b) and galanin-like immunoreactivity (Pearson *et al.* 1996a) is evident at day 30 postnatally in cell bodies and fibres in the hypothalamus (Ma *et al.* 1994) suggesting that programming of the hypothalamus might occur earlier in the pig and hence affect leptin sensitivity. Programming of leptin resistance might occur via increased fetal plasma glucose concentration, since maternal diabetes in the last half of pregnancy in rats produced higher fetal plasma glucose concentrations that caused a reduction in fetal brain NPY mRNA and immunoreactive NPY levels (Singh *et al.* 1997).

Programming of triiodothyronine and estradiol in the progeny strengthens the argument of programming of the hypothalamus as both are regulated at the hypothalamus but cortisol and IGF-I that are also controlled at the hypothalamus were not programmed. Triiodothyronine is regulated via arcuate NPY neurons colocalized with AgRP neurons that connect with thyrotropin releasing hormone (TRH) neurons (Legradi *et al.* 1998; Legradi *et al.* 1999). Gonadotropin releasing hormone (GnRH) (Peilin *et al.* 1999) or luteinizing hormone releasing hormone (LHRH) neurons (Horvarth *et al.* 1996) interact with arcuate NPY neurons and galanin in the hypothalamus which are factors that regulate estradiol secretion from ovarian cells. Infusion of leptin (Ahima *et al.* 1999b) or NPY (McMinn *et al.* 1998) into the arcuate nucleus in the rat decreases NPY mRNA but has no effect on corticotropin releasing hormone (CRH) mRNA in the paraventricular hypothalamic region of the brain. Leptin infusion increases pituitary GH mRNA and hypothalamic growth hormone releasing hormone (GHRH) mRNA while leptin reduces somatostatin mRNA (Cocchi *et al.* 1999) and plasma IGF-I (LaPaglia *et al.* 1998; Vuagnat *et al.* 1998). Arcuate NPY neurons synapse with somatostatin neurons in the periventricular inhibiting GH secretion, while arcuate galanin neurons that colocalize with GHRH neuron stimulates GH secretion at the hypothalamic level which regulates IGF-I secretion from the liver. (Bluet-Pajot *et al.* 1998). This suggests that a hypothalamic pathway in which NPY/AgRP or galanin neurons connect with TRH or GnRH/LHRH but not CRH, GHRH or GH neurons were altered that lead to a permanent increase in triiodothyronine and estradiol secretion with no change in cortisol and IGF-I secretion.

Another explanation of intrauterine programming of triiodothyronine by GH treatment during the second quarter of pregnancy could be altered fetal pig thyroid gland development

due to increased abundance of triiodothyronine (T3) to the fetus from the mother (Fentener-van-Vlissingen *et al.* 1983; Kumar *et al.* 1989; Morell *et al.* 1994), or hepatic and brain 5′-deiodinase activity that converts thyroxine (T4) into T3 (Alaez *et al.* 1992). Thyroid activity in pigs starts to function at mid-gestation (Slebodzinski *et al.* 1994). Maternal thyroid hormones can cross the placenta in pigs during early gestation possibly affecting the thyroid status of the fetus (Krysin *et al.* 1997). GH treatment has been shown to increase T3 levels and decrease T4 producing an increase in the ratio of T3/T4 (Jorgensen *et al.* 1994). An increase in maternal thyroxine levels could increase the transfer of thyroxine across the placenta. An increase in fetal thyroxine could influence the fetal thyroid development or hypothalamic control of thyroxine that is permanently altered in the fetus that persists postnatally. Thyroxine treatment in hypophysectimized fetal pigs (Latimer *et al.* 1993) and sheep (Mesiano *et al.* 1989) does not increase tissue IGF-II levels but increases tissue and plasma IGF-I levels which probably explains the decreased IGF-II levels in the current study and increased thyroxine levels in the progeny.

In summary, intrauterine programming of leptin was caused by higher maternal nutrition and GH treatment during the second quarter of pregnancy. Adipocyte characteristics were unaffected while triiodothyronine, estradiol and IGF-II hormones were programmed by GH treatment but were not associated with programming of leptin. Maternal glucose is an important determinant of intrauterine programming of leptin and may be involved with other pathways regulating leptin or changes in hypothalamic function leading to the programming of leptin. Altered programming of the hypothalamus in control of triiodothyronine and estradiol would further support the theory of programming of leptin and leptin resistance due to a permanent alteration in hypothalamic regulation of leptin.

CHAPTER 6

GENERAL DISCUSSION

6.1 Experimental animal models of intrauterine programming of leptin in humans

Higher adult leptin levels independent of adult body weight and obesity have been associated with low birth weight in babies in independent studies (Phillips *et al.* 1999; Lissner *et al.* 1999) indicating intrauterine programming of adult leptin production. The general hypothesis of this thesis is that intrauterine environment programs leptin expression. No previous study had examined the effect of maternal environment during pregnancy on postnatal leptin production.

In this thesis, an animal model in which intrauterine programming of leptin occurs was established. Leptin mRNA expression in subcutaneous adipose tissue was confirmed in the pig by RT PCR and Northern analysis. In addition, this is the first study to discover leptin mRNA expression in adipose tissue in the adult and in fetal guinea pig. The presence of leptin mRNA in guinea pig adipose tissue was confirmed by nucleotide sequencing of RT PCR product. Northern analysis detected leptin mRNA expression in the guinea pig liver which has been demonstrated in chicken liver (Taouis *et al.* 1998). The guinea pig could be used in future studies investigating intrauterine programming of leptin once a reliable method of quantitation of guinea pig leptin mRNA is established. A quantitative RT PCR assay of porcine leptin mRNA was designed and validated. The pig was used as the model of intrauterine programming of leptin and to investigate mechanisms of leptin programming. A number of studies recently have used the rat as a model to study programming of leptin (Plagemann *et al.* 1999b; Vickers *et al.* 2000).

Different stages of fetal and neonatal development of the brain and adipocytes may cause animals to respond to changes in nutritional environment and lead to postnatal obesity in different ways. The Dutch famine study showed that exposure to undernutrition in the first half of pregnancy produced no change in birthweight but resulted in an increase in fatness as adults (Ravelli *et al.* 1976; Ravelli *et al.* 1999). Undernutrition may have an effect on the differentiation of the hypothalamic centres in the fetus which in adults regulate food intake and growth and hence lead to obesity. Conversely, undernutrition in late pregnancy in humans caused male adults to have a reduced rate of obesity. Adipocyte development was suggested to be retarded reducing the number of adipocytes (Ravelli *et al.* 1976). This suggests that the timing of the insult is a critical factor in affecting adipocyte or hypothalamic centres which can lead to programming of obesity postnatally.

Brain development in the pig occurs prenatally and continues into early postnatal life (Pond *et al.* 2000). Brain neuropeptides including NPY and POMC control feeding and growth. At day 30 of gestation NPY-like immunoreactivity (Pearson *et al.* 1996b), galanin-like immunoreactivity (Pearson *et al.* 1996a) and proopiomelanocortin (POMC) mRNA (Ma *et al.* 1994) have been detected in many parts of the fetal pig brain. Permanent changes in expression of NPY, galanin and POMC may be involved in the intrauterine programming of leptin (Sahu 1998; Jureus *et al.* 2000; Schwartz *et al.* 2000).

There are differences in the ontogeny of adiposity between the rat, human and pig. The rat is the leanest of the three species, depositing fat after birth and continues to do so in postnatal life. The human infant is born fatter than neonates of most other species with a large amount of lipid accumulating in fetal adipose tissue in the last trimester of pregnancy (Hirsch 1972).

The pig has a similar adipocyte development pattern to the human but is leaner than the human baby at birth. The first reported adipose tissue appears at day 50 of gestation in the fetal pig (Desnoyers *et al.* 1980) and fat cell cluster differentiation begins between day 45 and 60 in fetal life (Hausman *et al.* 1986). Preadipocyte differentiation and recruitment in fetal subcutaneous tissues increases considerably between 80 days and term in the pig (Hausman *et al.* 1993). Therefore, the time of programming of specific tissues such as the brain and adipocytes are likely to be different between species making it difficult to compare programming events across species.

6.2 Intrauterine programming of leptin by maternal nutrition

Maternal nutrition during pregnancy programmed postnatal leptin production in the pig. Higher leptin mRNA expression and plasma leptin postnatally was found in progeny of mothers having a higher feed intake in the second quarter of pregnancy. However, there was no change in adipocyte size or number to account for the higher leptin levels postnatally. Several studies in rats have reported that higher nutrition from birth to weaning (Plagemann *et al.* 1999b) and in the post-weaning period (Vickers *et al.* 2000) lead to a permanent increase in plasma leptin. The rats with higher plasma leptin were fatter at 21 days (Plagemann *et al.* 1999b) and 105 days (Vickers *et al.* 2000) after birth which was not observed in the postnatal pigs with higher leptin in the present study. Early signs of obesity are associated with increased number of NPY immunoresponsive neurons in the hypothalamus (Plagemann *et al.* 1999b), increased galanin (hypothalamic stimulator of food intake) neurons in the paraventricular nucleus (Plagemann *et al.* 1999a) and reduced number

of cholecystokinin (modulates food intake at the hypothalamus) positive neurons in the paraventricular nucleus (Plagemann *et al.* 1998).

Vickers et al. (2000) showed that undernutrition throughout pregnancy in rats produced progeny with higher plasma leptin and larger retroperitioneal fat pads. When the rat progeny from undernourished mothers were overfed postnatally the effects of undernutrition on plasma leptin were amplified. This shows that the effects of nutrition on leptin are different at different stages development.

Animal studies of the programming of obesity have provided supporting evidence that critical periods of development of hypothalamic centres and adipocyte development influence appetite, growth, body composition and fat patterning. McCance *et al.* (1974) showed runts in pig litters produced by restriction in maternal nutrient supply have similar growth rates but do not attain the size of their littermates of normal birth weight. They suggested that maternal diet during early fetal life affected hypothalamic function during a critical period in early gestation that influences the rate of growth and development at later stages. In the rat, restriction of dietary protein in early development when brain cells are rapidly multiplying irreversibly reduces the number of brain cells (Winick *et al.* 1966) supporting McCance's idea. This suggests a particular tissue is most sensitive to programming when an insult occurs during a period of rapid tissue division.

Rat studies looking at programming of obesity have examined the effect of feed restriction during the first two-thirds of pregnancy and have reported inconsistent results. Jones *et al.* (1982) reported that male but not female progeny of rats restricted in feed intake during

pregnancy grew faster and were hyperphagic. However, in another study, both female and male progeny of feed restricted dams grew faster and showed no evidence of hyperphagia (Pond *et al.* 1985). Enns *et al.* (1983) found no increase in food intake, overall growth or growth of adipose tissue in male progeny of food restricted dams. Anguita *et al.* (1993) repeated the above experiments finding opposite effects of early intrauterine malnutrition in male and female progeny. Female progeny had normal growth and higher fat accumulation but the males had reduced weight gain and lower plasma triiodothyronine (Anguita *et al.* 1993). The reduction in triiodothyronine could be due to altered hypothalamic control of energy regulation. These studies show that malnutrition during early pregnancy affects adiposity and growth postnatally which could be due to altered adipocyte development or changes in hypothalamic centres.

Neonatal nutrition also affects hypothalamic function and adiposity in rats. Rats on a low caloric diet (large litter size) before weaning are lighter in weight and are relatively hyperphagic in adulthood (Oscai *et al.* 1978; Beck *et al.* 1983). The permanent change in appetite may be due to programming of hypothalamic centres. There is a growth spurt in the rat brain from days 7 to 20 of postnatal life and myelination occurs during this period. This may make this a sensitive period for programming the hypothalamus in the rat. Yeh (1988) found rats undernourished during the suckling period showed no permanent alterations in myelin and myelin lipids but had irreversible stunting of whole-body and brain growth. Myelination therefore does not affect growth of the rat. More recently rats undernourished at weaning showed postnatal growth restriction, increased NPY in the paraventricular nucleus and no change in plasma leptin (Plagemann *et al.* 2000). Conversely, overfeeding during the suckling period in rats causes increased weight, hyperglycemia, hyperinsulinaemia and

insulin resistance. Thus, under- or over- nutrition during the neonatal period in the rat influences the differentiation and functional maturation of hypothalamic nuclei which control body weight and food intake leading to permanent changes in the hypothalamus.

Nutrition during fetal and neonatal development in the rat affects differentiation of the hypothalamus and adipocytes. In the present study in pigs there was no change in adipocyte development (size and number) associated with leptin programming in the offspring.

6.3 Maternal metabolism is associated with intrauterine programming of leptin in pigs

The changes in maternal metabolism in the second quarter of pregnancy due to GH treatment resemble similar alterations in metabolism of a diabetic mother or gestational diabetes in humans during pregnancy. Maternal glucose was the strongest maternal predictor of plasma leptin postnatally in progeny. Gestational diabetes in humans causes hyperglycaemia and hyperinsulineamia which are sustained throughout pregnancy leading to insulin resistance in the mother, while the effects of GH treatment during the second quarter of pregnancy on maternal glucose and insulin persist only for the treatment period. Gestational diabetes causes fetal hyperinsulinism that leads to increased birth weight (Lepercq *et al.* 1999). Fetal hyperinsulinaemia in pigs has no affect on fetal bodyweight but the fat content of the fetus rises (Garssen *et al.* 1983). GH treatment in pregnant pigs during the second quarter of pregnancy causes increased fetal growth (Gatford *et al.* 2000). There was no change in birth weight from the piglets whose mothers were treated with GH but the growth pattern of the fetus may have been altered.

Maternal glucose concentration is a major determinant of fetal glucose uptake (Hay *et al.* 1985). Increased glucose in the fetus will affect fetal development impacting on adipocyte development and brain development, which are in turn linked to regulation of postnatal leptin production. Maternal diabetes in rats causes fetal hyperglycaemia and high plasma insulin levels while reducing fetal brain NPY mRNA and plasma NPY (Singh *et al.* 1997). Babies whose mothers had gestational diabetes have been found to have greater placental and cord blood leptin levels compared to non-diabetic mothers (Lepercq *et al.* 1998). The decrease in NPY due to high glucose in the fetus could be due to leptin inhibiting NPY which has been shown to occur in adult life. The increased leptin from the placenta and(or) fetus may also be influencing brain development as leptin increases neuronal and glial proteins (Ahima *et al.* 1999a) and increases brain growth (Steppan *et al.* 1998; Ahima *et al.* 1999a; Steppan *et al.* 1999). Hyperinsulineamia may also affect brain growth, since insulin is a regulator of neuronal differentiation and maturation (Brennan 1988; Schechter *et al.* 1999). Therefore, leptin or insulin may be affecting the central nervous development and causing dysfunction of brain and adipocyte development which leads to higher leptin production postnatally.

6.4 Potential mechanisms of intrauterine programming of leptin

While higher maternal nutrition only programmed plasma leptin in the progeny, programming of plasma triiodothyronine, IGF-II, estrogen and leptin concentrations in offspring by GH treatment of pregnant mothers suggests that maternal GH treatment has significant effect on multiple endocrine axes of the fetus that persisted postnatally.

High leptin levels should theoretically decrease appetite and body weight while increasing energy expenditure (Mantzoros *et al.* 1998). Although progeny with high plasma leptin would be expected to be more obese there was no difference in current body weight of progeny with high and low leptin. High leptin is thought to be an indicator of leptin resistance which may lead to the development of obesity. The progeny with high leptin in the present study may have developed leptin resistance.

Adipocyte size in the progeny in this study was not altered by higher maternal nutrition or GH treatment of pregnant mothers. None of the possible regulators of leptin including triiodothyronine, IGF-II, estrogen, IGF-I, glucose, insulin, cortisol and progesterone were co-programmed with leptin suggesting no involvement in the increased leptin levels. However, leptin mRNA in the study in Chapter 4 was increased due to higher maternal nutrition during the second quarter of pregnancy. Increased fetal glucose due to higher maternal glucose may increase the activity of the glucosamine pathway in adipocytes which increases leptin mRNA expression. Also higher fetal glucose may induce transcription factors such as C/EBPα or PPAR-γ that regulate leptin mRNA expression (MacDougald *et al.* 1995b; Hwang *et al.* 1997).

An increase in plasma triiodothyronine and estradiol that are both regulated by neurons connected to NPY neurons in the hypothalamus (Horvarth *et al.* 1996; Legradi *et al.* 1998; Legradi *et al.* 1999; Peilin *et al.* 1999) without a change in adipocyte development is consistent with hypothalamic changes leading to leptin resistance and a permanent increase in plasma leptin in progeny. Leptin crosses the blood-brain barrier by a saturable mechanism (Banks *et al.* 1996). Individuals with diet induced obesity have reduced sensitivity to

elevated leptin, probably due to a limitation in leptin transport into cerebrospinal fluid (CSF), indicated by increased plasma: CSF leptin ratio in obesity (Caro *et al.* 1996; Schwartz *et al.* 1996a). *Fa/fa* and Koletsky rats also have decreased plasma: CSF leptin ratios due to defective membrane leptin receptor expression. Therefore, leptin resistance in progeny may be due to changes in receptor-mediated leptin transport into the brain (Wu Peng *et al.* 1997). Leptin resistance in progeny may also occur at steps downstream from the initial stage of receptor binding (El-Haschimi *et al.* 2000). Changes in suppressor of cytokine signalling (SOC-3) and cytokine-inducible SH2-containing protein (CIS) in hypothalamic neurons are also potentially involved in mechanisms of leptin resistance. Obesity is associated with increased amounts of SOC-3 and CIS which inhibit leptin signalling (Bjorbaek *et al.* 1999; Emilsson *et al.* 1999). Progeny may also have altered neuropeptide signalling in the brain, since agouti (Ay/a) mice defective in the melanocortin (MC4) receptor in the brain are resistant to both peripheral and central leptin injection (Halaas *et al.* 1997).

6.5 Implications of high postnatal plasma leptin levels in progeny

Leptin resistance in progeny with high plasma leptin and normal adipocyte size may only be apparent. At the developmental stage at which the progeny were studied the leptin sensitvity may not be fully acquired. In neonatal mice high plasma leptin with increased bodyweight and appetite suggests the animals are insensitive to leptin (Devaskar *et al.* 1997; Mistry *et al.* 1999). Leptin injected intrapertioneally into lean and *ob/ob* mice does not decrease body weight or appetite until day 28 postnatally. However, leptin treatment increased O_2 consumption, an indicator of metabolic action, by day 17 of postnatal life in mice (Mistry *et*

al. 1999). The full actions of leptin in pigs may not be acquired until an older age than that studied, so that leptin may not affect appetite in 8 to 9 week old female pigs.

Regardless of leptin's central actions, persistently high leptin levels may elicit peripheral actions. Leptin suppresses the secretion of insulin from pancreatic β-cells (Poitout *et al.* 1998) which contain leptin receptors (Kieffer *et al.* 1996) and leptin also inhibits insulin receptor signalling pathways in HepG2 liver cells (Cohen *et al.* 1996). Leptin treatment of adipocytes inhibits insulin stimulation of carbohydrate and lipid metabolism as well as insulin stimulation of protein synthesis (Muller *et al.* 1997). High plasma leptin concentrations in female progeny may thus cause peripheral insulin resistance.

Leptin induces lipolysis (Wang *et al.* 1999b), alters lipid partitioning in skeletal muscle (Muoio *et al.* 1997) and increases fatty acid synthesis in the liver (Cohen *et al.* 1998). High plasma leptin may therefore have an effect on peripheral lipid metabolism causing a decrease in triacylglycerol synthesis and increase lipolytic rates and lipid oxidation.

Leptin also increases sympathetic nerve activity in brown adipose tissue, kidney, adrenal gland and hindlimb skeletal muscle (Haynes *et al.* 1997; Mizuno *et al.* 1998). Cardiovascular and renal function via the central nervous system may also be affected by high plasma leptin.

Leptin interacts with the hypothalamic-pituitary-gonadal axis. Leptin enhances gonadotropin secretion (Barash *et al.* 1996) which implies that female pigs with elevated leptin could have advanced onset of puberty due to increased hypothalamic release of gonadotropin releasing

hormone. Leptin also directly stimulates LH release and inhibits insulin-like growth factor-mediated secretion of estradiol in ovarian follicular cells.

High leptin in the progeny may also affect the hypothalamic-pituitary-adrenal axis. Leptin directly inhibits glucocorticoid synthesis and ACTH-stimulated glucocorticoid secretion in adrenal cortical cells (Bornstein *et al.* 1997; Pralong *et al.* 1998). Leptin also regulates CRH synthesis and secretion by the hypothalamus (Costa *et al.* 1997; Heiman *et al.* 1997).

6.5 Clinical significance

Barker and colleagues were the first to show that low birthweight babies or babies that are small in relation to the size of their placentas have increased rates of cardiovascular disease and non-insulin-dependent diabetes as adults (Barker *et al.* 1993b). The fetal growth trajectory is set at an early stage of development which determines the size at birth but the maternoplacental capacity to supply sufficient nutrients to maintain trajectory can alter the birthweight (Godfrey *et al.* 2000). Maternal nutrition and GH treatment during the second quarter of pregnancy had no effect on birthweight but led to a change in endocrine status of the progeny which affected leptin, estradiol, triiodothyronine and IGF-II. Fetal weight was increased with GH treatment at similar doses to those used in the present study during the second quarter of pregnancy in gilts (Gatford *et al.* 2000) which suggests that the GH treated fetuses grew faster in the second quarter of gestation in this study. The fetuses that were growing faster by midpregnancy must therefore have experienced reduced growth rates in the second half of pregnancy because of their similar birth weights. Maternal undernutrition in the last trimester in fetal sheep causes fetal wasting and placental oxidation of fetal amino

acids to maintain lactate output to the fetus which leads to thinness at birth (Harding *et al.* 1992). Also in late pregnancy stressful conditions such as placental insufficiency, undernutrition, or restricted blood flow prematurely activate the fetal hypothalamic-pituitary axis with increases in NPY mRNA in the hypothalamus (Warnes *et al.* 1998) and earlier than normal rises in fetal cortisol levels (Fowden *et al.* 1998). High glucocorticoids cause a change from cell proliferation to differentiation which can lead to an inappropriate pattern of growth for the developmental stage, with long term-effects later in life such as hypertension and glucose intolerance (Barker *et al.* 1993b; Fowden *et al.* 1998).

There was no difference in weight and size at birth in the progeny in this study. This demonstrates that intrauterine programming of endocrine axes can occur without a change in birth weight or birth characteristics, possibly via changes in fetal development such as hypothalamic function. This is consistent with the results of the Dutch famine study where undernutrition during early pregnancy had no effect on birthweight but caused increased incidence of obesity in adults (Ravelli *et al.* 1976; Ravelli *et al.* 1999). The timing and influence of change in maternal nutrition is an important factor affecting the juvenile endocrine system.

6.6 Animal production implications

Animal agriculture is not highly concerned with leptin but is concerned with improvement of productive efficiency, carcass composition and animal health. The second quarter of pregnancy is a sensitive period for the programming of these characteristics in the pig as endocrine systems and growth pattern were altered postnatally. Understanding when

sensitive periods of development in the fetal pig occur would enhance the progress of programs designed to hinder fat development postnatally. Beneficial effects of increased maternal nutrition (or GH treatment) during the second quarter of pregnancy in the progeny is the development of leptin resistance which will make the progeny incapable of inhibiting adiposity and appetite leading to an increase in the feed to gain ratio in offspring and postnatal increase in fatness. The second possibility is that sensitivity to leptin will develop at an older age causing a reduction in adiposity and appetite. Higher leptin in the female progeny may induce an early onset of puberty reducing the time between birth and first pregnancy in the pig.

6.7 Future directions

The studies in this thesis show that intrauterine environment programs postnatal leptin. Maternal glucose is an important determinant of plasma leptin postnatally. Maternal glucose may have programmed leptin resistance, altering the regulation of leptin, fetal leptin expression of other transcriptional pathways regulating leptin or programming of hypothalamic pathways of leptin action.

The following future studies should be considered.

1) A direct mechanism of programming leptin expression could be through the UDP-*N*-acetylglucosamine pathway or leptin transcriptional pathways in the preadipocytes in the fetus. An indirect mechanism could be through fetal insulin or insulin-like growth factor-I induced changes in fetal adipocyte maturation. This could be tested by infusing glucose into pregnant animals such as the pig or guinea pig and measuring UDP-*N*-

acetylglucosamine in the adipocytes of fetuses and progeny. Infusion of insulin and/or IGF-I into fetuses to examine the same end points should also be considered. To determine whether leptin transcriptional pathways have been programmed C/EBPα, PPARα and SP1 transcription factors should be measured in the adipocytes of fetus and postnatal progeny.

2) Programming of leptin could be indirectly through changes in the development of the hypothalamus. GH treatment of the mother or infusion of glucose into the pregnant animal should be evaluated for effects on brain growth and hypothalamic expression of NPY, AgRP, galanin, POMC, SOC-3, CIS and leptin receptor in the fetus and progeny.

3) To determine whether the progeny were leptin resistant due to defective brain leptin transport a similar experiment should be performed and the appetite and hypothalamic expression response of the progeny to peripheral leptin administration measured.

4) To determine whether the progeny develop obesity after acquiring leptin resistance a similar GH experiment in the mothers would be performed with the end point being 25 weeks of age in the progeny with adiposity, appetite and the appetite and hypothalamic expression response to peripheral leptin administration measured.

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