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Long-Term Outcomes After Intrauterine Therapy of the
Growth-Restricted Fetus

Ana-Mishel Spiroski

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Science, The University of Auckland, 2014.
Abstract

The experiments described in this thesis investigated the effects of intra-amniotic insulin-like growth factor-1 (IGF1) treatment of fetal growth restriction (FGR) in the ovine fetus on perinatal morbidity and mortality, on growth from birth through 18 months of age, and on metabolic and endocrine function at 18 months of age. Ewes carrying singleton lambs (n=196) were randomised to un-operated Controls (n=41) or to FGR (n=144), induced by uteroplacental embolisation. FGR fetuses were further randomised to receive either 360 μg recombinant human IGF1 (FGR-IGF1, n=61) or saline (FGR-Saline, n=66) intra-amniotically once weekly for 5 weeks.

IGF1 treatment of FGR did not affect fetal or perinatal morbidity or mortality. FGR-Saline lambs (n=31) were 15-20% lighter at birth than Controls (n=37). Growth velocity for weight in the first week after birth was 18% greater (p<0.05) in FGR-Saline lambs compared with Controls, but in the second week was 15% greater (p<0.01) in FGR-IGF1 lambs (n=32) compared with Controls. FGR-IGF1 females had ~10% increase in relative lean mass and ~10% decrease in relative visceral adipose (both, p<0.05) compared with Controls at 18 months of age. One week after birth, FGR-IGF1 females had greater GHR and IGF1 mRNA expression than FGR-Saline (p<0.01), and FGR-IGF1 males had greater IGF1 mRNA expression (p<0.01), in skeletal muscle. Up-regulated skeletal muscle IGF1 mRNA expression persisted in FGR-IGF1 males and females at 18 months of age following growth hormone stimulation compared with Control and FGR-Saline offspring (p<0.01). In FGR-Saline adult females, insulin sensitivity (S_i) and glucose disposition (D_i) tended to be decreased by ~50% and ~24% respectively compared with Controls. These changes were reversed in FGR-IGF1 females. Plasma insulin secretion in the first 15 minutes of a glucose tolerance test and D_i were ~80% greater in FGR-IGF1 females compared to FGR-Saline. In FGR-IGF1 males the maximal change
in L-Arginine-stimulated insulin secretion was increased by 40% and S_i tended to be greater (by ~67%) than FGR-Saline. FGR-IGF1 adult males had a 34% greater increase in plasma ACTH concentration (p<0.05) in response to corticotrophic stimulation compared to FGR-Saline males. In contrast, in response to Metyrapone FGR-IGF1 females’ peak ACTH response and 11-deoxycortisol area under the curve tended to be decreased (20% and ~40%, respectively), compared with Controls.

These data demonstrate that, although intra-amniotic IGF1 treatment of the FGR fetus did not alter fetal or perinatal mortality, it prolonged the accelerated growth velocity for weight in the perinatal period and was associated with decreased relative visceral adipose and increased relative lean mass in adult females. Sex-specific effects on HPA axis function could have implications for wellbeing as age progresses. There were few other significant effects on adult endocrine or metabolic function indicating that IGF1 treatment of FGR is not associated with significant adverse effects through young adulthood, but does not provide dramatic benefits, suggesting that this is unlikely to be utilised as a clinical intervention.
Dedication

This work is lovingly dedicated to my mother, Nancy Short-Logan, whose strength, generosity and encouragement sustains me, and to the memory of my father, Andrija Spiroski (22 August 1949–31 October, 2013), cija veljikadusnost, nesebicnost, I posvetenost kon negovata familija I prijateli e mojata najgolema inspiracija.
Acknowledgments

I gratefully acknowledge the support of my primary supervisor, Professor Frank Bloomfield. I am indebted to him for the opportunity to pursue doctoral studies with the LiFePATH Group. I appreciate his perseverance and sincere encouragement throughout. To my co-supervisor, Dr Mark Oliver, I offer my heartfelt thanks for his tireless support whilst finding my feet at Liggins Ngapouri, and for his excellence in teaching ovine fetal surgical techniques. To my co-supervisor, Dr Anne Jaquiery, who taught me the art of lambing, and without whom the weak and feeble lambs of the study would not have made it past the first night, I greatly appreciate her efforts and support throughout.

Travis Gunn, the most amazing research assistant in the history of fetal growth restriction research at Liggins Ngapouri, has my enduring gratitude for putting up with me when nothing was going according to plan, and for helping fix it. I am immensely appreciative of Maggie Worthington and Anita Wylie who provided stellar technical assistance at Ngapouri and cared for my sheep as if they were their own. Special thanks to Gerald Pinckney and Fanny Leduc for exemplary veterinary care throughout the study. My genuine thanks go to Hui Hui Phua who, after Frank banned me from the laboratory following a rather unfortunate equestrian-related finger amputation, assisted with the adult post-growth hormone stimulation test mRNA analyses. To Eric Thorstensen, Chris Keven, and all former LiFePATH technicians from both the Liggins Institute and Liggins Ngapouri who contributed to this work, Colleen Shaw who helps keep the Liggins laboratory running, and to the administrative teams who keep everything ticking over: many, many thanks.

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awarded me with a scholarship that enabled me to complete this work, and the Perinatal Society of New Zealand for travel funding.

My sincerest gratitude goes to my lovely family and friends from around the world who travelled the doctoral path with me, offering support and guidance throughout. Thank you all for making this accomplishment possible.
# Table of Contents

Abstract ........................................................................................................................................ ii  
Dedication ................................................................................................................................... iv  
Acknowledgments ....................................................................................................................... v  
List of Figures ............................................................................................................................ xiii  
List of Tables ................................................................................................................................ ii  
Abbreviations ............................................................................................................................... ii  

Chapter 1. Literature Review ..................................................................................................... 12  
1.1. Fetal Growth and Development ...................................................................................... 12  
1.2. Fetal Growth Restriction ................................................................................................. 13  
1.2.1. Fetal Growth Restriction and the Brain .................................................................... 16  
1.2.2. Fetal Growth Restriction and Neuroendocrine Adaptation ..................................... 17  
1.2.3. Fetal Growth Restriction and Hepatic Growth ......................................................... 19  
1.2.4. Fetal Growth Restriction and Myogenesis ............................................................... 20  
1.2.5. Fetal Growth Restriction and Cardiac Development ............................................... 22  
1.2.6. Postnatal Consequences of Fetal Growth Restriction .............................................. 23  
1.2.7. The Developmental Origins of Health and Disease .................................................. 25  
1.3. Interventions to Improve Outcomes in the Growth-Restricted Fetus ............................ 26  
1.4. Nutrient Intake in the Growth-Restricted Neonate ........................................................ 27  
1.5. Experimental Paradigms of Fetal Growth Restriction ................................................... 28  
1.6. Experimental Interventions to Improve Outcomes after Fetal Growth Restriction ...... 29  
1.6.1. Maternal l-Arginine Supplementation ..................................................................... 30  
1.6.2. Maternal Sildenafil Citrate ....................................................................................... 32  
1.6.3. Maternal Growth Hormone ...................................................................................... 34  
1.6.4. Insulin-Like Growth Factor-1 .................................................................................... 35  
1.7. Summary ......................................................................................................................... 39  

Chapter 2. Methods ................................................................................................................... 40  
2.1. Ethics Approval ................................................................................................................ 40  
2.2. Generation of Singleton-Bearing Ovine Pregnancies ..................................................... 40  
2.3. Feto-Maternal Surgical Catheterisation .......................................................................... 42  
2.3.1. Sedation .................................................................................................................... 42  
2.3.2. Surgical Preparation ................................................................................................. 43
3.3.3. Morbidity .................................................................................................................. 93
3.4. Discussion ....................................................................................................................... 93
3.5. Conclusions ..................................................................................................................... 94

Chapter 4. Plasma Hormone and Metabolite Concentrations, Growth and Body Composition in the Perinatal Period ................................................................. 95
4.1. Introduction ..................................................................................................................... 95
4.2. Methods ......................................................................................................................... 96
4.3. Results ............................................................................................................................ 96
4.3.1. Fetal Characteristics ................................................................................................. 96
4.3.2. Lambing of Chronically Catheterised Ewes .............................................................. 97
4.3.3. Birth Characteristics ................................................................................................. 98
4.3.4. Perinatal Characteristics ......................................................................................... 98
4.3.5. Plasma Hormone and Metabolite Concentrations .................................................. 107
4.3.6. Milk Intake .............................................................................................................. 108
4.4. Discussion ...................................................................................................................... 112
4.4.1. Effect of IGF1 Treatment on the Growth-Restricted Singleton Ovine Fetus .......... 112
4.4.2. Effect of FGR ........................................................................................................... 114
4.4.3. Hormone Ontogeny in the Growth-Restricted Ovine Fetus .................................. 115
4.5. Limitations .................................................................................................................. 117
4.6. Conclusions ................................................................................................................... 117

Chapter 5. Hepatic and Skeletal Muscle mRNA Expression at One Week of Age .............. 119
5.1. Introduction .................................................................................................................. 119
5.2. Methods ....................................................................................................................... 120
5.3. Results ........................................................................................................................ 121
5.3.1. Effects of FGR and Intra-amniotic IGF1 Treatment of FGR on Skeletal Muscle mRNA Expression at One Week of Age ......................................................... 121
5.3.2. Effects of FGR and Intra-amniotic IGF1 Treatment of FGR on Hepatic mRNA Expression at One Week of Age ................................................................. 126
5.4. Discussion ................................................................................................................... 131
5.4.1. Effects of FGR and Intra-Amniotic IGF1 Treatment on mRNA Expression at One Week of Age ........................................................ 131
5.5. Limitations ................................................................................................................ 133
5.6. Conclusions ................................................................................................................. 134
Chapter 6. Postnatal Plasma Hormone and Metabolites, Growth, Body Composition, and Post Mortem Carcass and Organ Characteristics .................................................................135

6.1. Introduction ..................................................................................................................135
6.2. Methods .....................................................................................................................137
6.3. Results .........................................................................................................................137
  6.3.1. Postnatal Growth Through 18 Months of Age .......................................................137
  6.3.2. Body Composition ...............................................................................................138
  6.3.3. Plasma Hormone and Metabolite Concentrations Through 18 Months of Age ....145
  6.3.4. Post Mortem Analysis of Carcass Characteristics and Organ Size .........................148
6.4. Discussion ..................................................................................................................151
  6.4.1. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Growth .................151
  6.4.2. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Hepatic Growth ....153
  6.4.3. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Brain Growth ........154
  6.4.4. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Thyroid Growth .......155
  6.4.5. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Cardiac Growth .......155
6.5. Limitations ..................................................................................................................156
6.6. Conclusions .............................................................................................................156

Chapter 7. The Somatotrophic Axis at 18 Months of Age .......................................................158

7.1. Introduction .............................................................................................................158
7.2. Methods .....................................................................................................................159
7.3. Results .......................................................................................................................160
  7.3.1. Plasma Glucose Concentration ............................................................................160
  7.3.2. Plasma Insulin Concentration .............................................................................160
  7.3.3. Plasma Glucose to Insulin Ratio .........................................................................160
  7.3.4. Plasma Free Fatty Acid Concentration .................................................................161
  7.3.5. Plasma Insulin-Like Growth Factor-1 Concentration .............................................162
  7.3.6. Feed Intake .............................................................................................................163
  7.3.7. Effects of FGR and Intra-Amniotic IGF1 Treatment of FGR on Skeletal Muscle mRNA Expression Following a Growth Hormone Test in adult sheep .........................166
7.4. Discussion ..................................................................................................................169
  7.4.1. Effect of FGR and Intra-Amniotic IGF1 Treatment of FGR on Plasma Hormone and Metabolite Response to a Growth Hormone Stimulation Test ........................169
  7.4.2. Effect of FGR and Intra-Amniotic IGF1 Treatment of FGR on the Somatotrophic Response to a Growth Hormone Stimulation Test ........................................172
Chapter 8. Physiological Tests .............................................................................................................177

8.1. Introduction ........................................................................................................................................177

8.2. Methods ..............................................................................................................................................178

8.2.1. Glucose Tolerance Test ..................................................................................................................179

8.2.2. Hyperglycaemic Clamp ..................................................................................................................179

8.2.3. Adrenaline Stimulation Test .........................................................................................................180

8.2.4. Metyrapone® Test .........................................................................................................................181

8.2.5. Arginine Vasopressin/Corticotropin-Releasing Hormone Stimulation Test.........................181

8.3. Results ..............................................................................................................................................182

8.3.1. Glucose Tolerance Test ..................................................................................................................182

8.3.2. Hyperglycaemic Clamp ..................................................................................................................185

8.3.3. Adrenaline Stimulation Test .........................................................................................................189

8.3.4. Metyrapone® test ........................................................................................................................192

8.3.5. Arginine Vasopressin-Corticotropin-Releasing Hormone Stimulation Test.........................196

8.4. Discussion .........................................................................................................................................199

8.4.1. Glucose and Insulin Axis Response at 18 Months of Age in Females .......................................199

8.4.2. Adrenergic Response at 18 Months of Age in Females ..............................................................201

8.4.3. Hypothalamic-Pituitary-Adrenal Axis Response at 18 Months of Age in Females .................202

8.4.4. Glucose and Insulin Axis Response at 18 Months of Age in Males .............................................203

8.4.5. Adrenergic Response at 18 Months of Age in Males .................................................................205

8.4.6. Hypothalamic-Pituitary-Adrenal Axis Response at 18 Months of Age in Males .................205

8.5. Limitations ........................................................................................................................................206

8.6. Conclusions .......................................................................................................................................207

Chapter 9. Conclusions ............................................................................................................................208

Chapter 10. References ............................................................................................................................217
List of Figures

Figure 1.1 Routes of administration and potential effects of intrauterine interventions for the treatment of FGR ....................................................................................................................... 30

Figure 2.1 Anatomical location of linear measures ........................................................................ 54

Figure 2.2 Representative illustration of defined areas for dual X-ray absorptiometry analyses ................................................................................................................................................... 59

Figure 2.3 Representative quantitative polymerase chain reaction amplification curves........ 86

Figure 2.4 Representative standard curve ................................................................................. 87

Figure 3.1 Characteristics of experimental groups and animal use ................................................. 91

Figure 4.1 Fetal whole blood measures and fetal mortality ...................................................101

Figure 4.2 Effect of FGR and intra-amniotic IGF1 treatment on early postnatal growth in females ......................................................................................................................................................103

Figure 4.3 Effect of FGR and intra-amniotic IGF1 treatment on early postnatal growth in males ......................................................................................................................................................104

Figure 4.4 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations in the early postnatal period in female lambs.........................110

Figure 4.5 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations in the early postnatal period in male lambs.........................111

Figure 4.6 Effect of FGR and intra-amniotic IGF1 treatment on plasma insulin-like growth factor-1 concentrations in the early postnatal period in lambs.............................................112

Figure 5.1 Skeletal muscle somatotrophic mRNA expression at one week of age in females122

Figure 5.2 Skeletal muscle mRNA expression at one week of age in females ........................123

Figure 5.3 Skeletal muscle somatotrophic mRNA expression at one week of age in males...124

Figure 5.4 Skeletal muscle mRNA expression at one week of age in males............................125
Figure 5.5 Hepatic somatotrophic mRNA expression at one week of age in females ..............127
Figure 5.6 Hepatic mRNA expression at one week of age in females ..................................128
Figure 5.7 Hepatic somatotrophic mRNA expression at one week of age in males .............129
Figure 5.8 Hepatic mRNA expression at one week of age in males ....................................130
Figure 6.1 Effect of FGR and intra-amniotic IGF1 treatment on growth through to 18 months of age ................................................................................................................................139
Figure 6.2 Effect of FGR and intra-amniotic IGF1 treatment on plasma IGF1 from weaning to 18 months of age ................................................................................................................................145
Figure 6.3 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations from the perinatal period to weaning in females ......................146
Figure 6.4 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations from the perinatal period to weaning in males.........................147
Figure 7.1 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations during a growth hormone stimulation test in female sheep at 18 months of age ................................................................................................................................161
Figure 7.2 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations during a growth hormone stimulation test in male sheep at 18 months of age ................................................................................................................................162
Figure 7.3 Effect of FGR and intra-amniotic IGF1 treatment on plasma IGF1 and during a growth hormone test in adult sheep ................................................................................................................................163
Figure 7.4 Effect of FGR and intra-amniotic IGF1 treatment on the correlation between weight and basal plasma IGF1 concentration in adult sheep .................................................164
Figure 7.5 Effect of FGR and intra-amniotic IGF1 treatment of FGR on skeletal muscle mRNA expression following a growth hormone test in females ..............................................167
Figure 7.6 Effect of FGR and intra-amniotic IGF1 treatment of FGR on skeletal muscle mRNA expression following a growth hormone test in males ...........................................................168

Figure 8.1 Plasma glucose and insulin during a glucose tolerance test at 18 months of age.183

Figure 8.2 Area under the curve of glucose following a glucose tolerance test at 18 months of age relative to glucose to insulin ratio at birth........................................................................183

Figure 8.3 Whole blood glucose and plasma insulin responses during a hyperglycaemic clamp at 18 months of age.................................................................................................................187

Figure 8.4 Plasma glucose, free fatty acids and β-hydroxybutyric acid during an adrenaline stimulation test at 18 months of age ........................................................................................................190

Figure 8.5 Peak plasma free fatty acid concentration during an adrenaline stimulation test relative to abdominal fat tissue at 18 months of age .............................................................192

Figure 8.6 Plasma cortisol, 11-deoxycortisol, cortisone and adrenocorticotropic hormone during a Metyrapone® test at 18 months of age .................................................................194

Figure 8.7 Plasma cortisol and adrenocorticotropic hormone during an arginine vasopressin corticotropin-releasing hormone stimulation test at 18 months of age.................................197
### List of Tables

Table 2.1 Composition of UniC concentrate feed pellet ........................................................... 41
Table 2.2 Physiological testing series for adult sheep ........................................................................ 68
Table 2.3 Quantitative polymerase chain reaction target genes .............................................. 84
Table 2.4 Quantitative polymerase chain reaction housekeeping genes ................................. 85
Table 3.1 Deaths during the prenatal period ............................................................................ 92
Table 3.2 Postnatal exclusions, deaths and euthanasia ............................................................. 93
Table 4.1 Characteristics at birth according to sex and experimental group ..........................102
Table 4.2 Growth velocity in the first two weeks after birth ..................................................105
Table 4.3 Effect of FGR and intra-amniotic IGF1 treatment on body composition at one week of age .......................................................................................................................................106
Table 4.4 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormones in the early perinatal period .......................................................................................................................................109
Table 6.1 Effect of FGR and intra-amniotic IGF1 treatment on growth in females ..............140
Table 6.2 Effect of FGR and intra-amniotic IGF1 treatment on growth in males ..................141
Table 6.3 Effect of FGR and intra-amniotic IGF1 treatment on body composition at 4 months of age .......................................................................................................................................142
Table 6.4 Effect of FGR and intra-amniotic IGF1 treatment on body composition at 12 months of age .......................................................................................................................................143
Table 6.5 Effect of FGR and intra-amniotic IGF1 treatment on body composition at 18 months of age .......................................................................................................................................144
Table 6.6 Effect of FGR and intra-amniotic IGF1 treatment on carcass characteristics and organ size at 18 months of age .......................................................................................................................................150
Table 7.1 Effect of FGR and intra-amniotic IGF1 treatment on FGR on plasma hormone and metabolite concentrations, areas under the curve and feed intake during a growth hormone stimulation test...

Table 7.2 Effect of sex on skeletal muscle mRNA expression following a growth hormone stimulation test...

Table 8.1 Physiological testing series at 18 months of age...

Table 8.2 Plasma glucose and insulin response to a glucose tolerance test at 18 months of age...

Table 8.3 Plasma glucose and insulin responses during a hyperglycaemic clamp at 18 months of age...

Table 8.4 Plasma glucose, free fatty acids and β-hydroxybutyric acid response to an adrenaline stimulation test at 18 months of age...

Table 8.5 Plasma cortisol, cortisone, 11-deoxycortisol, and adrenocorticotropic hormone in response to a Metyrapone® test at 18 months of age...

Table 8.6 Plasma cortisol and adrenocorticotropic hormone during an arginine vasopressin/corticotropin-releasing hormone test at 18 months of age...
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µL</td>
<td>microlitre</td>
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<tr>
<td>µm</td>
<td>micrometre</td>
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<tr>
<td>11BHSD</td>
<td>11-β hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>18S</td>
<td>18S ribosomal 1</td>
</tr>
<tr>
<td>Abdo</td>
<td>abdominal girth</td>
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<tr>
<td>ACTB</td>
<td>beta actin</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<td>ADR</td>
<td>adrenaline stimulation test</td>
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<td>ADR-AUC\textsubscript{BHBA15}</td>
<td>β-hydroxybutyric acid area under the curve 15 minutes after an adrenaline infusion</td>
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<td>β-hydroxybutyric acid area under the curve 60 minutes after an adrenaline infusion</td>
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<tr>
<td>AGA</td>
<td>appropriate for gestational age</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related peptide</td>
</tr>
<tr>
<td>AIR\textsubscript{15}</td>
<td>acute insulin response</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
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<tr>
<td>ARC</td>
<td>arcuate nucleus of the hypothalamus</td>
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<tr>
<td>ARG</td>
<td>L-Arginine</td>
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<tr>
<td>ART</td>
<td>assisted reproductive technology</td>
</tr>
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<td>AU</td>
<td>Australia</td>
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<td>AUC</td>
<td>area under the curve</td>
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<tr>
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</tr>
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<td>biparietal diameter</td>
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<td>BS</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSA</td>
<td>body surface area</td>
</tr>
<tr>
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<td>birthweight</td>
</tr>
<tr>
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<td>body weight</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>Cath</td>
<td>catheter</td>
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<tr>
<td>CBS</td>
<td>carbonate-buffered saline</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHEST</td>
<td>chest girth</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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CIDR  controlled internal drug release device

cm  centimetre

cm$^2$  square centimetre

cm$^3$  cubic centimetre

CNP  c-natriuretic peptide

CO$_2$  carbon dioxide

Comt$^{-/-}$  catechol-O-methyl transferase knockout

CpG  cytosine-guanine dinucleotide

CRH  corticotropin-releasing hormone

CRL  crown-to-rump length

C$_t$  cycle threshold

C$_{td}$  deuterium oxide concentration

CV  coefficient of variation

CYP21a1  cytochrome P450 21-hydroxylase

d  day

D$_1$  day 1

D$_2$O  deuterium oxide

dGA  days gestational age

DI$_{GI}$  disposition index of glucose

D$_n$  day after birth

DOHaD  developmental origins of health and disease

DRWG  daily relative weight gain

DTT  dithiothreitol

DXA  dual-energy X-ray absorptiometry

E  cortisone

EDTA  ethylenediaminetetraacetic acid

$e^{-kt}$  exponential decay over time

ERK  extracellular-signal regulated kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>F</td>
<td>cortisol</td>
</tr>
<tr>
<td>F:E</td>
<td>cortisol to cortisone ratio</td>
</tr>
<tr>
<td>FAM</td>
<td>carboxyfluorescein</td>
</tr>
<tr>
<td>FE</td>
<td>facial eczema</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FGR</td>
<td>fetal growth restriction</td>
</tr>
<tr>
<td>FL</td>
<td>forelimb length</td>
</tr>
<tr>
<td>FM</td>
<td>fat mass</td>
</tr>
<tr>
<td>G</td>
<td>glucose</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose-6-phosphatase</td>
</tr>
<tr>
<td>G:I</td>
<td>glucose to insulin ratio</td>
</tr>
<tr>
<td>GA</td>
<td>gestational age</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GEO</td>
<td>geometric mean</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transferase</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHR</td>
<td>growth hormone receptor</td>
</tr>
<tr>
<td>GHT</td>
<td>growth hormone test</td>
</tr>
<tr>
<td>GHT-AUC&lt;sub&gt;FFA&lt;/sub&gt;</td>
<td>free fatty acid area under the curve after a growth hormone stimulation test</td>
</tr>
<tr>
<td>GHT-AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>glucose area under the curve after a growth hormone stimulation test</td>
</tr>
<tr>
<td>GHT-AUC&lt;sub&gt;I&lt;/sub&gt;</td>
<td>insulin area under the curve after a growth hormone stimulation test</td>
</tr>
<tr>
<td>GHT-AUC&lt;sub&gt;IGF1&lt;/sub&gt;</td>
<td>insulin-like growth factor 1 area under the curve after a growth hormone stimulation test</td>
</tr>
<tr>
<td>GI</td>
<td>G index</td>
</tr>
<tr>
<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
</tr>
<tr>
<td>GTT</td>
<td>glucose tolerance test</td>
</tr>
</tbody>
</table>
GTT-AUC_{G15} glucose area under the curve in the 15 minutes after a glucose bolus
GTT-AUC_{GT} glucose area under the curve 180 minutes after glucose bolus
GTT-AUC_{I15} insulin area under the curve in the 15 minutes after glucose bolus
GTT-AUC_{IT} insulin area under the curve 180 minutes after glucose bolus
GV growth velocity
GV_{0.5-4} growth velocity from two weeks to four months of age
GV_{4-8} growth velocity from four to eight months of age
GV_{8-12} growth velocity from eight months to twelve months of age
GV_{12-18} growth velocity from twelve to eighteen months of age
HCl hydrochloric acid
HDAC histone deacetylase
HGC hyperglycaemic clamp
HGC-AUC_{IARG} insulin area under the curve after an L-Arginine bolus
HGC-AUC_{ISS} insulin area under the curve during the steady state period
HIF1A hypoxia-inducible factor-1-α
HKGs housekeeping genes
HL hindlimb length
HOMA-IR homeostatic model assessment for insulin resistance
HPA hypothalamic pituitary adrenal
HPRT1 hypoxanthine phosphoribosyltransferase 1
hr hour
HT hock to toe
I Insulin
I_{ARG} peak plasma insulin concentration after an L-Arginine bolus
during a hyperglycaemic clamp
I_{G} Mean glucose infusion
I_{GARG} mean glucose infusion after an L-Arginine bolus
during a hyperglycaemic clamp
IGF1 insulin-like growth factor-1
IGF1R  insulin-like growth factor-1 receptor
IGF2  insulin-like growth factor-2
Igf2/−  insulin-like growth factor-2 knockout
IGFALS  insulin-like growth factor acid labile subunit
IGFBP  insulin-like growth factor binding protein
IGS  mean glucose infusion during the steady state period of a hyperglycaemic clamp
IHC  Immunohistochemistry
IMTG  intramuscular triglyceride
IR  insulin receptor
IRS  insulin receptor substrate
IS  Insulin secretion
ISS  mean plasma insulin concentration during the steady state period of a hyperglycaemic clamp
IUGR  intrauterine growth restriction
kg  kilogram
L  litre
LBW  low birth weight
LD  latissimus dorsi
LM  lean mass
ln  natural log transformation
LV  left ventricle
M  moles
m  metre
MAPK  mitogen-activated protein kinase
MET  Metyrapone® test
MET-AUC_{ACTH}  adrenocorticotropic hormone area under the curve after a Metyrapone® bolus
MET-AUC_{F}  cortisol area under the curve after a Metyrapone bolus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>MET-AUC₅</td>
<td>11-deoxycortisol area under the curve after a Metyrapone bolus</td>
</tr>
<tr>
<td>MET-SUPPᵢ</td>
<td>suppression of cortisol after a Metyrapone bolus</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>mmHg</td>
<td>millimetre mercury</td>
</tr>
<tr>
<td>mmol</td>
<td>millimole</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>(Na)₂CO₃</td>
<td>sodium carbonate</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nmol</td>
<td>nanomole</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>NR3C1</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature compound</td>
</tr>
<tr>
<td>P&amp;P</td>
<td>primer and probe</td>
</tr>
<tr>
<td>P70S6K</td>
<td>ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>p85α</td>
<td>phosphatidylinositol 3-kinase regulatory subunit p85α</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PaO₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>PAR</td>
<td>predictive adaptive response</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen</td>
</tr>
<tr>
<td>PI</td>
<td>ponderal index</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td>PPIA</td>
<td>peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RDS</td>
<td>respiratory distress syndrome</td>
</tr>
<tr>
<td>rhIGF1</td>
<td>recombinant human insulin-like growth factor-1</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RM ANOVA</td>
<td>repeated measures analysis of variance</td>
</tr>
<tr>
<td>RPL19</td>
<td>ribosomal protein L 19</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>S</td>
<td>11-deoxycortisol</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SGA</td>
<td>small for gestational age</td>
</tr>
<tr>
<td>S_i</td>
<td>insulin sensitivity</td>
</tr>
</tbody>
</table>
SLC2A2  glucose transporter, solute carrier family, member 2
SLC2A4  glucose transporter, solute carrier family, member 4
SS      steady state
STAT5   signal transducer and activator of transcription 5
T₀      time zero
T₃      L-3,3',5'-triiodothyronine
T₄      L-3,5,3',5'-tetraiodothyronine
TBS     tris-buffered saline
TBST    tris-buffered saline with 0.1% Tween-20
TG      target gene
Tm      melting temperature
TNES-6U 10 mmol Tris HCl pH 7.5, 125 mmol NaCl, 10 mmol EDTA, 1.0% SDS, 6 M urea
TRA1    thyroid receptor α-1
TRE     thyroid response element
TSC     tuberous sclerosis complex
Tsc2⁻/⁻ tuberous sclerosis complex-knockout
TSH     thyroid-stimulating hormone
U       units
UK      United Kingdom
USA     United States of America
USP     United States pharmacopeia
V₀      volume on day zero
VLBW    very low birthweight
VMN     ventromedial nucleus of the hypothalamus
W₁      weight on day 1
WHO     World Health Organisation
Wk      week
Wₙ      weight on a given day after birth
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>YWHAZ</td>
<td>tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>alpha</td>
</tr>
<tr>
<td>( \beta )</td>
<td>beta</td>
</tr>
<tr>
<td>( \beta_{HBA} )</td>
<td>beta-hydroxybutyrate</td>
</tr>
<tr>
<td>( \Delta )</td>
<td>delta (change)</td>
</tr>
<tr>
<td>( \Delta I_5 )</td>
<td>the change in plasma insulin concentration following the ( \ell )-Arginine bolus from steady state during a hyperglycaemic clamp</td>
</tr>
<tr>
<td>( \Delta I_{515} )</td>
<td>change in insulin secretion from baseline 15 minutes after glucose bolus</td>
</tr>
<tr>
<td>( \Delta R_n )</td>
<td>the log magnitude of the signal generated</td>
</tr>
<tr>
<td>( \Delta V_t )</td>
<td>change in volume due to growth over the experiment</td>
</tr>
</tbody>
</table>
Chapter 1. Literature Review

Fetal growth and, thus, size at birth affect perinatal morbidity and mortality and influence life-long health. Adversity in utero compromises the fetal development of appropriate physiological processes, which, thus, restrict growth and result in distinct phenotypic adaptations. Babies born following fetal growth restriction are at an increased risk of neonatal complications which may contribute to increased disease incidence later in life.

1.1. Fetal Growth and Development

The placental transfer of nutrients and hormones provides the fetus with critical information regarding maternal resources and the ex utero environment (Sandovici, Hoelle, Angiolini, & Constância, 2012). Adequate fetal blood flow, and thus nutrient and oxygen, supply is necessary for the appropriate development of the fetal glucose and insulin axis, and of the nutrient-mediated somatotrophic axis (Bloomfield, Spiroski, & Harding, 2013). In the sheep, as in the human, glucose, a primary substrate necessary for fetal oxidative metabolism (Battaglia & Meschia, 1978; Hay, 2006) is tightly regulated to provide a constant nutrient supply to the fetus, enabling appropriate growth throughout gestation. As conceptus size (Koong, Garrett, & Rattray, 1975) and thus glucose requirements (Molina, Meschia, Battaglia, & Hay, 1991) increase, placental transfer capacity must also increase (Hay, 2006).

Research in humans shows that the control of insulin-dependent processes in the placenta shifts from mother to fetus through gestation (Hiden et al., 2006). In the late gestation ovine fetus, production of insulin by the developing pancreas (Hay, 2006) and increased peripheral insulin sensitivity (Aldoretta, Carver, & Hay, 1998) enhance glucose utilisation. Thus, the development of late-gestation maternal insulin resistance (George et al., 2010), which facilitates glucose delivery to the conceptus (Fowden & Moore, 2012), safeguards fetal
glucose delivery leading up to parturition and prepares the fetus for \textit{ex utero} survival. Alterations in the glucose-insulin axis associated with reduced fetal growth establish the potential for postnatal dysregulation (Bazaes et al., 2003), which can result in the development of metabolic disease states.

Various maternal factors can reduce fetal growth trajectory by limiting the transfer of adequate levels of oxygen and nutrients to the fetus thereby altering the appropriate development of metabolic processes. Reduced fetal nutrient allocation can be caused by poor maternal nutritional status (Henriksen, 1999), chronic placental and maternal disease (Lin & Santolaya-Forgas, 1998), and the inappropriate activation of inflammatory cascades (Challis et al., 2009). Resultant fetal deprivation during critical periods of development can induce physiological and phenotypic adaptation which alter \textit{in utero} growth trajectory, thus resulting in fetal growth restriction.

\textbf{1.2. Fetal Growth Restriction}

Fetal growth restriction (FGR) is defined as decreased intrauterine growth velocity (Ergaz, Avgil, & Ornoy, 2005); the growth-restricted fetus is differentiated from the constitutionally-small fetus in that there is a failure to attain growth potential due to pathological conditions. In the developed world, the pathology of fetal growth restriction originates primarily from placental insufficiency (Aviram, Biron-Shental, & Kidron, 2010; Galan & Groban, 2013). Placental aetiologies and pathological processes which compromise fetal blood supply include shallow trophoblast invasion (Teasdale, 1984), reduced placental villous development (Jackson et al., 1995), decreased capillarisation (Almasry & Elfayomy, 2012) with fewer or shorter capillary segments (Mayhew, Wijesekara, Baker, & Ong, 2004), villous morphological abnormalities, infarcts, parenchymal lesions and uteroplacental vasculopathy (Mifsud & Sebire, 2014). Although the placenta is a plastic organ with significant capacity for
adaptation, placental injury compromises fetomaternal exchange secondary to defective uteroplacental perfusion. Placental insufficiency restricts blood flow to the fetus resulting in constrained fetal growth. FGR is reflected in abnormal Doppler velocimetry (Galan & Grobman, 2013; Morris et al., 2010) and reduced fetal growth velocity. In humans, hepatic blood flow in utero is positively correlated with fat mass at birth (Godfrey et al., 2012), which suggests reduced peripheral tissue accretion due to haemodynamic compromise.

Haemodynamic redistribution in the growth-restricted ovine fetus occurs via reduced peripheral circulation concurrent with dilatation of vasculature in organs such as the brain (Giussani, Spencer, & Hanson, 1994). Godfrey and colleagues report that redistribution of hepatic blood flow to favour resource allocation to the developing brain in conditions of limited supply of essential nutrients occurs at the expense of adipose deposition (Godfrey, et al., 2012). This circulatory redistribution, termed “brain-sparing”, is associated with disproportionate, or asymmetric, growth and a raft of well-defined physiological adaptations (Fowden, Giussani, & Forhead, 2006). In the hypoxaemic growth-restricted fetus, the proportion of umbilical venous blood that is shunted through the ductus venosus, thus, bypassing the liver increases (Battaglia, 2007). The more severe the umbilical haemodynamic compromise, the greater the shunting (Kiserud, Kessler, Ebbing, & Rasmussen, 2006). Hepatic venous hypoperfusion, the severity of which is relative to the amount of placental vascular resistance, induces a disproportionate prioritisation of blood supply and resultant right lobe hypoxaemia (Kessler, Rasmussen, Godfrey, Hanson, & Kiserud, 2009). This shunting is reproducible at 125 days gestational age in the sheep (term, 145 days) with materno-fetal hypoxia defined as an overall reduction of fetal liver oxygenation by ~50% as measured by blood oxygen level-dependent magnetic resonance imaging (Sørensen et al., 2011). Both fetal hypoxia (Bristow, Rudolph, Itskovitz, & Barnes, 1983) and placental insufficiency (Thorn,
Brown, Rozance, Hay, & Friedman, 2013) in the sheep are associated with increased hepatic
gluconeogenesis to maintain adequate glucose supply to vital organs. Reduced fetal blood
and, thus, nutrient supply constrains tissue accretion (Sferruzzi-Perri, Vaughan, Forhead, &
Fowden, 2013), compromising fetal growth trajectory.

Growth-restricted fetuses who fail to attain their growth potential in utero are defined as
small for gestational age (SGA), or ≤10th centile for growth, and display increased peripheral
insulin sensitivity at birth (Diderholm, 2009; Ibáñez et al., 2008) and glucose disposal at 48
hours of age (Bazaes, et al., 2003). This glucose-insulin axis dysregulation promotes the rapid
restoration of body weight toward the mean via accelerated postnatal growth (Beltrand et
al., 2009). However, due to insufficient energy stores and increased brain to body weight
ratio and insulin-sensitivity, there is also a propensity towards neonatal hypoglycaemia
(Diderholm, 2009). FGR is associated with an increased incidence of morbidity and mortality
in neonates (Bloomfield, Oliver, & Harding, 2006; Mandruzzato et al., 2008) and with an
increased propensity to develop specific postnatal physical and physiological characteristics,
such as stunted growth, impaired cognitive functioning and increased risk of morbidity
throughout life (Barker, 1998; Ross & Beall, 2008). Pregnancies complicated by FGR can
produce both appropriate for gestational age (AGA) and SGA neonates. However, parents
that are below average in body size are predisposed to produce innately small-for-
gestational-age SGA infants (Milovanovic et al., 2012). Although these infants are smaller and
lighter than their appropriate-for-gestational-age AGA counterparts, they do not display
dysregulated hormonal or metabolic parameters, which characterise the growth-restricted
infant (Diderholm, 2009).
1.2.1. Fetal Growth Restriction and the Brain

In the human, non-human primate, and sheep, precocial species which have a high level of physiological maturity at birth compared with altricial species, such as rodents, prenatal brain development ensures relative independence at birth. Global neuronal hyperplasia, specialisation, cellular migration and myelination occur throughout gestation. Thus, fetal deprivation can impact on neurogenesis at distinct periods in addition to resulting in a global deficit. In the sheep, as volume and thus metabolic requirements of the brain increase throughout gestation, cerebral blood flow also increases (Rudolph & Heymann, 1970). Whilst brief uteroplacental restriction in the fetal sheep at 84 days gestational age induces white matter damage and neuronal death in the hippocampus (Rees, Stringer, Just, Hooper, & Harding, 1997), chronic ovine placental restriction is associated with reduced thickness in both motor and visual cortices, decreased cerebellar Purkinje cell density, and constrained hippocampal growth (Rees, Bocking, & Harding, 1988). Similarly, late gestation (120-140 dGA) embolisation-induced uteroplacental insufficiency reduces subcortical white matter myelination and Purkinje cell dendritic growth and results in severe gliosis in the cerebral cortex at 140 dGA (Mallard, Rees, Stringer, Cock, & Harding, 1998).

Preliminary data in the FGR human brain suggest that mitochondrial metabolic capacity is compromised (Story et al., 2011), whilst cortical cellularity is reduced (Samuelsen et al., 2007). Similarly, in the growth-restricted human there is reduced cortical complexity (Esteban et al., 2010), lesser grey matter as a proportion of brain volume and reduced intelligence which persists into adolescence in individuals born with very low birth weight (<1500 grams) who are born preterm (Martinussen et al., 2005). Interestingly, moderate nutrient restriction in the non-human primate which does not result in apparent fetal growth restriction, nonetheless reduces subventricular zone thickness and increases apoptotic cells despite greater cell proliferation at mid-gestation (Antonow-Schlorke et al., 2011).
1.2.2. Fetal Growth Restriction and Neuroendocrine Adaptation

Sheep studies have been invaluable in elucidating hypothalamic-pituitary-adrenal (HPA) axis ontogeny and the mechanistic adaptations to deprivation in the fetus. Integrative hypothalamic regulatory networks are intact in the ovine fetus from mid-gestation (Adam et al., 2008) to term (Keller-Wood, Powers, Gersting, Ali, & Wood, 2006; Mühlhäusler et al., 2004). Anterior pituitary corticotrophic cells are arginine vasopressin (AVP), corticotrophic-releasing hormone (CRH) and cortisol-responsive (Butler, Schwartz, & McMillen, 1999), and display a progressive increase in CRH responsiveness throughout gestation (Lü, Yang, & Challis, 1991). Adrenal steroidogenesis, which is quiescent from 90-120 days gestation (Wintour, Crawford, Mcfarlane, Moritz, & Tangalakis, 1995), increases near term to contribute to preparturient tissue maturation (Fowden, Li, & Forhead, 1998). Pituitary proopiomelanocortin (POMC) and glucocorticoid receptor expression are inversely associated with adrenal corticosteroid production (Keller-Wood, et al., 2006); thus, as corticosteroid secretion increases around parturition (Alexander et al., 1968), negative-feedback sensitivity is decreased.

Whilst spontaneous variations in fetal growth do not alter HPA axis function in the chronically-catheterised ovine fetus (Probyn, Stacy, Desai, Ross, & Harding, 2008), growth restriction induces fetal adaptation (Morrison, 2008). Periconceptional undernutrition increases glucocorticoid receptor-mediated regulation of POMC and NPY in the hypothalamus (Stevens et al., 2010), whilst modest maternal undernutrition in the first half of pregnancy reduces pituitary and adrenal responsiveness in late gestation (Hawkins, Hanson, & Matthews, 2001; Hawkins et al., 1999). Placental restriction-mediated chronic hypoxaemia induces the development of non-CRH-sensitive pituitary cells which secrete ACTH at a significantly increased rate (Butler, Schwartz, & McMillen, 2002), whilst stimulating fetal adrenal growth and steroidogenesis (Ross et al., 2000). Increased adrenal...
responsiveness (Fraser, Braems, & Challis, 2001; Giussani, Fletcher, & Gardner, 2011) and reduced glucocorticoid sensitivity (Gagnon, Challis, Johnston, & Fraher, 1994; Myers, Hyatt, Mlynarczyk, Bird, & Ducsay, 2005) in the hypoxaemic fetus could compromise sensitivity to the preparturient rise in cortisol, which is well-documented in chronically-catheterised (Bassett & Thornburn, 1969) and un-operated sheep (Alexander, et al., 1968). A rise in L-3,3’,5-triiodothyronine (T3) around parturition (Nwosu, Kaplan, Utiger, & Delivoria-Papadopulos, 1978) is thought to be concomitant with the preparturient cortisol surge (Fraser & Liggins, 1989), and is also suggested to mediate the maturational effects of cortisol (Forhead, Cutts, Matthews, & Fowden, 2009). Although thyroid hormone bioactivity varies throughout fetal development (Darras, Hume, & Visser, 1999), T3 has been shown to regulate gluconeogenesis (Fowden, Mapstone, & Forhead, 2001), promote nutrient storage (Forhead, et al., 2009) and increase hepatic somatotrophic gene expression (Forhead et al., 2000) in late gestation, whilst placental insufficiency in sheep is associated with reduced plasma T3 and L-3,5,3’,5’-tetraiodothyronine (T4) (Cabello & Levieux, 1981) possibly via lesser extrathyroidal conversion (Wrutniak, Veyre, & Cabello, 1990). Appropriate development of thyroid function is important in mediating the maturation of the somatotrophic axis around parturition. Thyroidectomy resulting in reduced plasma T3 concentrations in the ovine fetus abolishes the pre-partum increase in hepatic (Forhead, et al., 2000) and skeletal muscle (Forhead, Li, Gilmour, Dauncey, & Fowden, 2002) growth hormone (GH) receptor (GHR) abundance, which could delay the onset of postnatal GH-dependent growth. Impaired fetal growth in humans and sheep resulting in a small for gestational age neonate is associated with depressed thyroid function (Bagnoli, Laura, Sara, & Salvatore, 2013) and could compromise postnatal growth (De Blasio, Gatford, Robinson, & Owens, 2006).
Perturbed placental supply creates an adverse environment which alters the development of the fetal stress response (Phillips & Jones, 2006), and accelerates fetal maturation (Allen, 2005) at the expense of growth. During pregnancy, both maternal and fetal factors can contribute to impaired development and activation of the nutrient-mediated somatotrophic axis (Bloomfield, et al., 2013). Deprivation-mediated somatotrophic insensitivity in utero (Jensen, Harding, Bauer, & Gluckman, 1999) and postnatally (Kyriakakou et al., 2009), thus can result in persistent alterations in somatic growth.

1.2.3. Fetal Growth Restriction and Hepatic Growth

In the sheep, hepatic hyperplasia dominates during early fetal life whilst hypertrophic growth and differentiation of the liver into a metabolic organ begins around 84 days gestation (Liman, 1996). Brief, mid-gestation placental embolisation-induced fetal growth restriction has been shown to reduce extramedullary haematopoietic progenitor cells and liver weight suggesting the potential to alter both hyperplastic and hypertrophic growth (Cheung, Bogic, Gagnon, Harding, & Brace, 2004). Indeed, the growth-restricted ovine liver is smaller (Eremia, de Boo, Bloomfield, Oliver, & Harding, 2007) with reduced hepatic IGF1 concentration around birth (Darp et al., 2010). Whilst glucose utilisation in the growth-restricted ovine fetus is comparable to that of controls, mRNA expression of gluconeogenic enzymes (Limesand, Rozance, Smith, & Hay, 2007) and skeletal muscle insulin signalling and gluconeogenic protein expression (Thorn et al., 2009) increase in order to maintain sufficient glucose supply. Hepatic compensatory adaptation to constrained fetal growth, such as a bias towards hepatic gluconeogenesis during fetal life, predisposes the offspring to altered postnatal metabolic capacity.
1.2.4. Fetal Growth Restriction and Myogenesis

Skeletal muscle is the largest insulin-sensitive organ in the body and the primary site of glucose disposal (Shulman et al., 1990). Early gestation maternal nutrient restriction in sheep promotes the development of a more glycolytic fibre type distribution concurrent with greater capacity for adipose stores (Daniel, Brameld, Craigon, Scollan, & Buttery, 2007) and fewer secondary myofibres (Zhu, Ford, Nathanielsz, & Du, 2004). Maternal nutrient restriction in mid-to-late gestation reduces oxidative, mitochondrial-dense myofibres which results in a concomitant reduction in skeletal muscle insulin receptor and insulin-sensitive glucose transporter SLC2A4 mRNA expression (Costello et al., 2008). Both insulin and IGF1 promote mitogenesis in a cell-specific context via the mammalian target of rapamycin (MTOR) signalling pathway. Insulin is necessary for amino acid-mediated upregulation of translation initiation in skeletal muscle (Brown, Rozance, Barry, Friedman, & Hay, 2008) and is sufficient to stimulate cellular hyperplasia (Stephens et al., 2001) via the mitogen-activated protein kinase (MAPK) and extracellular-signal regulated kinase (ERK) pathways in the ovine fetus. Concurrent with fetal hypoinsulinaemia, the growth-restricted fetus displays 80% greater skeletal muscle insulin receptor mRNA expression and reduced skeletal muscle amino acid uptake compared with control (Thorn, et al., 2009), suggesting an insulin-dependent mechanism in skeletal muscle accretion. However, amino acid infusion in hyperthermia-induced growth-restricted fetal sheep suppresses protein catabolism and increases protein accretion by 150% concurrent with increased plasma IGF1 concentrations (Brown, Rozance, Thorn, Friedman, & Hay, 2012), which suggests that increased amino acid supply can promote somatic growth regardless of insulin dysregulation.

Functional adaptation in skeletal muscles to fetal deprivation is concomitant with alteration of the intrinsic metabolic properties of the myofibres, which adapt to environmental constraints (Guth & Yellin, 1971). Myofibre types range from anaerobic fibres with high...
glycolytic capacity to aerobic fibres with high mitochondrial density (Peinado et al., 2004), and dictate the metabolic capacity of the muscle (He, Watkins, & Kelley, 2001). Myofibrillar insulin sensitivity and capacity for glucose utilisation relative to the insulin-regulated glucose transporter solute carrier family, member 4 (SLC2A4) content (Marette et al., 1992) is defined in relation to oxidative capacity: Type I ≥ Type IIA ≥ IIB. Type I fibre distribution increases from birth. The fibre-type ratio, which continues to shift towards a more oxidative phenotype until a few weeks after birth (Costello, et al., 2008; Greenwood, Slepetis, Bell, & Hermanson, 1999; Maier et al., 1992), stays relatively constant through adulthood (Peinado, et al., 2004). However, hypertrophy of Type II fibres, and thus an inclination towards anaerobic metabolism, contributes to increased muscularity (Maier, et al., 1992) and altered postnatal metabolic capacity. Although investigations into the molecular mechanisms of postnatal adaptation are incomplete, common pathways are emerging as being associated with metabolic programming.

In sheep, placental restriction is associated with a persistent postnatal increase in skeletal muscle insulin receptor abundance, and in phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT) and glucose transporter SLC2A4 protein expression, suggesting a potential mechanism for accelerated early postnatal growth after birth via increased peripheral insulin sensitivity (Muhlhausler et al., 2009). By 30 days postnatal age, reduced whole-body insulin sensitivity and mRNA expression of insulin-signalling molecules in the skeletal muscle suggest that skeletal muscle-mediated insulin resistance (De Blasio, Gatford, Harland, Robinson, & Owens, 2012) could contribute to the rapid and excessive accumulation of excess adipose tissue (Louey, Cock, & Harding, 2005). Additionally, insulin-sensitive fibres, which are more susceptible to fetal programming (Jørgensen et al., 2009) are reduced in adults with metabolic syndrome, resulting in reduced capacity for insulin-stimulated glucose transport.
(Stuart et al., 2013) and, thus peripheral insulin sensitivity. A compromised fetal environment which mediates persistent phenotypic change and intrinsic dysregulation at the tissue level could contribute to compromised adult metabolism.

1.2.5. Fetal Growth Restriction and Cardiac Development

Similar to skeletal muscle, cardiomyocyte hyperplasia occurs in the fetal ovine heart primarily during early gestation whilst terminal differentiation and hypertrophy occurs later in gestation (Jonker et al., 2007; Reini, Wood, & Keller-Wood, 2009). During the last trimester, cardiomyocyte proliferative capacity decreases whilst functional maturation ensues. From ~115 days gestation until term, mononuclear cardiomyocytes undergo terminal differentiation, fusing to form binucleated and multinucleated cells, thus increasing in cross-sectional area (Jonker, et al., 2007). Increased ventricular mass near birth is primarily due to greater cell volume resulting from terminal differentiation and hypertrophy, rather than hyperplasia (Reini, et al., 2009). In fetal ovine myocytes, IGF1 stimulates hyperplasia and hypertrophy in fetal ovine myocytes via the MAPK/ERK and PI3K and AKT pathways (Sundgren et al., 2003). A paradoxical oxygen-dependent hypoxia-inducible factor-1-α (HIF1A)-mediated mechanism (Ren, Accili, & Duan, 2010) modulates the predominance of either mitogenesis or myogenesis, depending on environmental oxygen availability.

Early investigations suggest that chronic mid-gestation embolisation-induced FGR is associated with increased protein to DNA ratio in the right ventricle concurrent with increased left ventricle thickness and heart weight (Murotsuki, Challis, Han, Fraher, & Gagnon, 1997). Indeed, reduced cardiomyocyte binucleation in the left ventricle and decreased cardiomyocyte maturation is apparent near term in the growth-restricted fetus (Bubb et al., 2007). T3 has been shown to suppress hyperplasia in vitro in mid-gestation ovine fetal cardiomyocytes (Chattergoon, Louey, Stork, Giraud, & Thornburg, 2012), whilst T3
infusion in thyroidectomised ovine fetuses reduces cardiomyocyte proliferation via cell cycle inhibition, augmenting both hypertrophy and terminal differentiation to promote structural cardiac maturation (Chattergoon et al., 2012). Whilst the GH gene contains a thyroid response element (TRE) in the promoter, the IGF1 gene does not (Adams, 1995). Adams suggests that in the ovine fetus, maturation of thyroid metabolism at ~80 days gestation (Polk, 1995) is coincident with myocyte hypertrophy. Additionally, increased expression of thyroid receptor α-1 (TRA1), as seen during late gestation is associated with perinatal mitochondrial maturation (Buroker, Ning, & Portman, 2008), suggesting thyroid-mediated modulation of maturation and metabolic processes.

1.2.6. Postnatal Consequences of Fetal Growth Restriction

Developmental plasticity provides the opportunity for an organism to adapt to an adverse intrauterine environment. Persistent fetal genetic and epigenetic dysregulation can increase susceptibility to chronic disease (Bloomfield, 2011). Although constrained growth in the face of deprivation is immediately beneficial for survival (Diderholm, Ewald, Ahlsson, & Gustafsson, 2007; D. Patel & Kalhan, 1992), in the case of fetal deprivation, immune function (Ferguson, 1978), cardiorespiratory systems (Crispi et al., 2010; Pellanda et al., 2009), neurological development (Allen, 2005), capacity for growth (Modi et al., 2006), and metabolic competence (Chiesa et al., 2008; Diderholm, 2009) can be compromised. The resultant fetal phenotype is one which has adapted to survive in a less favourable environment, but at the expense of not attaining its full genetic potential for growth. Thus, whilst reduced size at birth is associated with increased disease risk in adulthood (Gluckman, Hanson, Cooper, & Thornburg, 2008), adaptation to fetal deprivation is of critical importance to the predisposition of chronic disease development.
The terms FGR and IUGR (intrauterine growth restriction) are often used interchangeably with the term SGA. However, the latter is defined as a birth weight below a certain centile, usually the 10th or 5th centile, whilst FGR refers to slowing of fetal growth and the failure to achieve growth potential. Growth-restricted babies can be either AGA (above the centile cut-off for SGA) or SGA. Fetal growth restriction reduces lean mass and subcutaneous adipose at birth (Thorn, Rozance, Brown, & Hay, 2011); small size at birth and thinness associated with rapid catch-up growth in the FGR neonate results in increased visceral adiposity (Damsky & Fisher, 1998; Singhal et al., 2010) and increased leptin concentrations in childhood (Beltrand, et al., 2009). A persistent increase in circulating leptin concentrations may favour a positive energy balance and promote the development of obesity-related metabolic disorders (Wajchenberg, Giannella-Neto, da Silva, & Santos, 2002), whilst rapidly increased fat deposition concurrent with altered physiology as a consequence of growth restriction can increase the risk for chronic disease development in adulthood (Gluckman, Hanson, & Pinal, 2005).

Although the “fat accumulation hypothesis” suggests that higher levels of adiposity resulting from an increased postnatal growth trajectory are of more importance in the determination of adult chronic disease risk than is size at birth (Robertson, 1976), data support the importance of intrauterine and early postnatal growth as major factors associated with the risk of adult disease (Gluckman, et al., 2008; Hales & Ozanne, 2003; Ross & Beall, 2008). A recent study of 17,046 children reports that SGA children remain shorter and thinner than AGA counterparts irrespective of postnatal growth velocity (Kramer et al., 2014). This could suggest a failure of SGA children to “catch up” in body size by 11.5 years of age in the study population. Interestingly, a recent systematic review and meta-analysis also suggests that individuals born with low birth weight are at reduced risk for long-term overweight risk
(Schellong, Schulz, Harder, & Plagemann, 2012). However, Schellong and colleagues report that the majority of longitudinal human studies (~80%) utilise body mass index to define overweight, rather than visceral adiposity which is an accurate predictor of cardio-metabolic dysfunction (Lee, Huxley, Wildman, & Woodward, 2008). Adults born with low birth weight have decreased insulin secretory capacity (Jensen et al., 2002), decreased insulin-stimulated glucose uptake (Hermann et al., 2003), and decreased insulin-signalling in skeletal muscle (Jensen et al., 2008; Ozanne et al., 2005), which could contribute to the development of insulin resistance later in life. Additionally, it has been suggested that small for gestational age individuals display apparent pancreatic β-cell dysfunction relative to peripheral insulin resistance, which can promote the development of metabolic disease states (Green, Rozance, & Limesand, 2010). As growth-restricted individuals are predisposed to increased risk of metabolic disease, surveillance of metabolic state is necessary to determine if physiologically relevant adipose depots contribute to compromised health status. The fetal origin of adult disease has serious implications for world-wide public health (Bateson et al., 2004), and requires the consideration of early intervention to mitigate future burden on the healthcare system.

### 1.2.7. The Developmental Origins of Health and Disease

The pioneering observation that individuals of low socioeconomic status in early life who consequently transition to affluence have increased susceptibility to chronic disease (Barker & Osmond, 1986) is re-shaping the landscape of public health. David Barker hypothesised that fetal and infant adversities influence the development of chronic disease in adulthood. The Developmental Origins of Health and Disease (DOHaD) hypothesis describes the contribution of early life adaptations of adversity to the development of non-communicable disease (Barker, 1990). Whilst the ability to adapt to an adverse environment facilitates
survival, these adaptations can become a hindrance if the *ex utero* environment does not reflect that which was experienced *in utero*. Thus, developmental plasticity during fetal life which enables adaptation to unfavourable *in utero* conditions can predispose the organism to increased incidence of morbidity and mortality. Environmentally-induced modifications of gene expression, termed fetal “programming”, can, thus result in life-long persistence of increased disease risk.

1.2.7.1. Mechanisms of Fetal Programming

Epigenetics seek to elucidate how structural changes occur within DNA without intrinsically altering the sequence, yet resulting in modified gene expression. Epigenetic modifications include DNA methylation, which occurs within cytosine-guanine dinucleotide (CpG) islands (Woodfine, Huddleston, & Murrell, 2011), the transcriptional co-repressor histone deacetylases (HDACs), and microRNAs. FGR is associated with extensive epigenetic modifications in methylation (Chernausek, 2012; Einstein et al., 2010) and microRNA expression (Laker, Wlodek, Connelly, & Yan, 2013). Key mechanisms underlying the early life epigenetic programming contribute to increased susceptibility to disease. These epigenetic marks alter post-translational modification and resultant gene expression in the individual and subsequent generations (Roseboom & Watson, 2012).

1.3. Interventions to Improve Outcomes in the Growth-Restricted Fetus

There are currently no effective clinical interventions which improve outcomes in the growth-restricted fetus. Clinical measures focus on the detection of FGR, and monitoring of fetal wellbeing to judge the timing of delivery to balance the risk of intrauterine death with complications of preterm birth. Regular assessment of the growth-restricted fetus is necessary to determine the balance between optimising intrauterine development and minimising the risk of intrauterine death, thereby maximising overall survival (Baschat,
Gembruch, & Harman, 2001; Hecher, Bilardo, & Stigter, 2001; The GRIT Study Group, 2004). Although there is a collective uncertainty about the optimal timing of delivery based on commonly used measures of fetal well-being (The GRIT Study Group, 1996), postnatal management emphasizes nutrition to ensure an appropriate growth trajectory (Clayton et al., 2007). FGR is associated with increased risk in immediate and long-term morbidity and mortality (Alberry & Soothill, 2007). Whilst ongoing research aims to reduce perinatal morbidity and mortality in the growth-restricted (Boers et al., 2007) and small for gestational age (Boers, van der Post, Mol, van Lith, & Scherjon, 2011) infant, persistent organ plasticity in the neonatal period lengthens the critical window of susceptibility in the programming of adversity and, thus, should be considered when determining treatment protocols.

1.4. Nutrient Intake in the Growth-Restricted Neonate

Reduced capacity for lipolysis and gluconeogenesis (Diderholm, et al., 2007) and predisposition to neonatal hypoglycaemia (Diderholm, 2009) in the growth-restricted neonate is of concern for immediate health and well-being (Tudehope, Vento, Bhutta, & Pachi, 2013). Recently, the modulation of infant nutrition to reduce postnatal growth acceleration also has been suggested in humans in order to reduce the future risk of obesity (Chomtho et al., 2008). However, reduced nutrient intake in the FGR neonate can contribute to postnatal growth restriction, which can negatively affect postnatal developmental outcomes (Sakurai, Itabashi, Sato, Hibino, & Mizuno, 2008) and reduce glucose tolerance (Eriksson, Osmond, Kajantie, Forsén, & Barker, 2006). Additionally, in growth-restricted preterm infants, reduced fetal growth velocity influences postnatal catch-up growth irrespective of birth weight (Roggero et al., 2011) suggesting that fetal, rather than postnatal, intervention needs to be considered. Rather than relying on postnatal interventions to
mitigate poor outcomes in the growth-restricted neonate, improving growth prior to the
development of adverse postnatal conditions seems the most logical approach.

Intrauterine intervention provides an ontological opportunity to alter the growth potential of
affected individuals, which is not possible postnatally. Additionally, intrauterine intervention
for FGR prior to irreversible growth failure could provide the best option for improving the
long-term health status of fetuses experiencing restricted growth (Harding & Bloomfield,
2004), and could abrogate the development of disease states associated with fetal growth
restriction in the neonate. Research conducted at The Liggins Institute utilises mammalian
paradigms of fetal growth restriction, which have been integral to the assessment of
maternal (Liu, Harding, Evans, & Gluckman, 1994), fetal (Jensen, et al., 1999), and amniotic
(Bloomfield, Bauer, Van Zijl, Gluckman, & Harding, 2002; Bloomfield, van Zijl, Bauer, &
Harding, 2002; Darp, et al., 2010) routes of nutrient and hormone administration to improve
fetal growth, with varying levels of success.

1.5. Experimental Paradigms of Fetal Growth Restriction

Discrete human investigations provide various pieces of information regarding disease
processes, whilst epidemiological studies emphasize the associations between early life
experiences and long-term outcomes. It is not feasible to elucidate precise causality from
these data. The utilisation of animal paradigms enables the direct and expansive
investigation of compromised fetal growth and development under controlled conditions.
The development of numerous mammalian paradigms of fetal deprivation provides robust
data which can help establish causative mechanisms and the opportunity to evaluate novel
interventions.

Various mammalian paradigms have been utilised to help determine the underlying
mechanisms of acute and persistent adaptations to fetal growth restriction (Bol, Reusens, &
Remacle, 2008; De Blasio, Gatford, Robinson, & Owens, 2007; Kappeler et al., 2009; Owens et al., 2007; Tosh et al., 2010). Extensive studies in rodents have investigated both long-term and molecular mechanistic pathways associated with fetal growth restriction and accelerated postnatal growth (Bol, et al., 2008; Owens, et al., 2007; Tosh, et al., 2010). However, due to the relative immaturity at birth of the offspring, it is not feasible to apply data from altricial species to those of precocial. Like the human, the sheep has a high level of maturity at birth. Additionally, whilst rodent pregnancies are multiparous, singleton pregnancies are common in the sheep. Multiparity affects the development of HPA (Rumball et al., 2008) and somatotrophic (De Zegher et al., 1989) axes; thus, controlling for parity in translational studies is of significant importance.

Ovine paradigms of fetal growth restriction are well-described and provide conclusive data regarding physiological and molecular mechanisms of adaptation during fetal life (Morrison, 2008). The relatively long gestation (term ~145 days) and large fetal size enable direct physiological assessment at various stages of pregnancy. The robustness of the sheep has also allowed the application of various experimental fetal interventions to treat the growth-restricted fetus (Bauer et al., 2003; Boyle et al., 1998; Brown, et al., 2012; de Boo, Eremia, Bloomfield, Oliver, & Harding, 2008; Gadhia et al., 2013; Jensen, et al., 1999; Wali et al., 2012).

1.6. Experimental Interventions to Improve Outcomes after Fetal Growth Restriction

Various experimental interventions for FGR have been utilized to investigate potential therapies (Figure 1.1): direct intravenous and enteral fetal supplementation of nutrients (Charlton & Johengen, 1987) and growth factors (Bloomfield, van Zijl, Bauer, Phua, & Harding, 2006; Kimble, Breier, Gluckman, & Harding, 1999; Kind et al., 1996), maternal GH (de Boo, et al., 2008; Harding, Evans, & Gluckman, 1997) and sildenafil citrate (Miller, Loose,
Jenkin, & Wallace, 2009) have been investigated. Data are far from complete, and results are variable. Due to the poor efficacy and potentially deleterious physiological effects of various experimental treatments, these therapies are not currently recommended for human intervention. Experimental intrauterine interventions reviewed here aim to alter growth trajectory in the growth-restricted fetus through increased uteroplacental perfusion, improved placental functional capacity, or increased fetal nutrient and oxygen delivery.

![Figure 1.1 Routes of administration and potential effects of intrauterine interventions for the treatment of FGR](image)

 Nitric oxide (NO), growth hormone (GH), insulin-like growth factor-1 (IGF1).

1.6.1. Maternal \(\text{L-Arginine} \) Supplementation

Maternal vascular diseases, including preeclampsia, are relatively common causes of FGR. Nitric oxide (NO) is a vasodilator produced through enzymatic oxidation of \(\text{L-Arginine} \) by NO synthase (NOS) (de Pace, Chiossi, & Facchinetti, 2007). NO oxidation results in the production of nitrites and nitrates, stable metabolites which are excreted in the urine. Decreased levels
of amniotic NO in the early second trimester, and increased levels in the third trimester, are associated with pregnancies complicated by FGR (Tranquilli et al., 2003). Increased circulating concentrations of an endogenous inhibitor of endothelial NOS, asymmetric dimethylarginine, and decreased brachial artery vasodilatation, have been reported in women who become pre-eclamptic (Savvidou et al., 2003). NO donors such as \( \text{L-Arginine} \) have, therefore, been investigated as a potential therapy for FGR complicated by placental vascular pathology.

Early case-studies of \( \text{L-Arginine} \) treatment in FGR-complicated pregnancies suggested increased length of gestation (Lampariello et al., 1997) and improved growth (Xiao & Li, 2005). A non-randomized clinical trial of 20 g·d\(^{-1}\) maternal intravenous \( \text{L-Arginine} \) infusion for 7 days at an unspecified gestational age, and in combination with unspecified “routine therapies”, resulted in a significant increase in birth weight of FGR neonates compared to FGR neonates of mothers who did not receive \( \text{L-Arginine} \) (Xiao & Li, 2005), whilst neonates from \( \text{L-Arginine} \)-treated mothers remained significantly lighter than non-FGR controls. This increase in birth weight was accompanied by an increased gestational age at delivery.

Although these early results seemed promising, other data do not support these findings. In FGR-complicated pregnancies, a 30-minute maternal intravenous infusion of 30 g of \( \text{L-Arginine} \) around 33 weeks’ gestation increased maternal serum nitrite and growth hormone levels but did not result in increased uteroplacental perfusion, birth weight, or gestational age at birth (Neri, Mazza, Galassi, Volpe, & Facchnetti, 1996). Furthermore, recent data from a randomized double-blind controlled trial in FGR-complicated pregnancies found no benefit for gestational age at birth, birth weight, need for neonatal oxygen supplementation, or duration of parenteral nutrition following 14 g daily maternal oral \( \text{L-Arginine} \) supplementation from 28 weeks gestation to term (Winer et al., 2009). Therefore, although
\[ \text{Arginine is readily available and appears to be safe in pregnant women, it is not currently recommended for the treatment of FGR.} \]

**1.6.2. Maternal Sildenafil Citrate**

Sildenafil citrate, another NO donor, also acts via the NO pathway to cause temporary smooth muscle relaxation in endothelial vessels (Maharaj et al., 2009; Villanueva-Garcia et al., 2007). In nulliparous women, a single dose of either 25 g or 100 g oral sildenafil given during the luteal phase resulted in significant short-term increased uterine volumetric flow in subjects with high basal flow (Hale et al., 2010). Small myometrial arteries dissected from myometrial biopsies at the time of Caesarean section in pregnancies complicated by FGR demonstrated increased vasoconstriction compared with arteries from control biopsies; addition of sildenafil significantly decreased vasoconstriction and increased relaxation in arteries from FGR-complicated pregnancies (Wareing, Myers, O'Hara, & Baker, 2005). Animal data also suggest that sildenafil may have beneficial effects for placental perfusion and fetal growth. In the catechol-O-methyl transferase knockout mouse (Comt\(^{-/-}\)), in which there is abnormal uterine artery resistance and FGR, treatment with sildenafil between 12.5 and 18.5 days of a 21-day pregnancy restored umbilical Doppler waveforms to normal and also normalised fetal growth (Stanley et al., 2012). In the placenta-specific insulin-like growth factor-2 knockout mouse (Igf2\(^{-/-}\)), treatment with 0.4 mg·mL\(^{-1}\) sildenafil in drinking water between 12.5 and 18.5 days increased fetal abdominal circumference with a trend towards increased fetal weight (Dilworth et al., 2013).

In rat studies of FGR induced by maternal hypoxia, late-gestation maternal sildenafil supplementation increased pup size; however, in rat dams housed in non-hypoxic control conditions sildenafil decreased pup size, while both hypoxia and sildenafil resulted in increased maternal cyclic guanosine monophosphate (cGMP) levels (Refuerzo et al., 2006). In
guinea-pigs treated with sildenafil for approximately the last third of gestation, pup size was increased with high dose treatment (500 µg·kg·d⁻¹) and newborn pups were more resistant to perinatal asphyxia (Sanchez-Aparicio et al., 2008). However, data from the sheep are contradictory.

In a study of FGR induced by single uterine artery ligation, a 48 hour intravenous infusion of sildenafil to the ewe resulted in decreased uterine artery blood flow, with fetal hypoxaemia and tachycardia (Miller, et al., 2009). In contrast, chronic subcutaneous sildenafil administration to nutrient-restricted ewes from 115-128 days of a 145 day pregnancy increased fetal weight (Satterfield, Bazer, Spencer, & Wu, 2010). However, presumably the reduced fetal growth in this paradigm was secondary to impaired nutrient supply rather than impaired placental function, and fetuses are therefore unlikely to have had the classical FGR phenotype including hypoxaemia and polycythaemia.

Limited data on sildenafil treatment for FGR-complicated pregnancies in humans is beginning to emerge; however, interpretation is difficult due to the combined effects of other concurrent interventions such as L-Arginine, oxygen supplementation, and corticosteroid treatment (Villanueva-Garcia, et al., 2007). In a non-blinded, non-randomised study of early-onset, severe FGR (abdominal circumference <5th percentile; <25 weeks’ gestation or estimated fetal weight <600 g), thrice daily maternal treatment with 25 mg sildenafil until delivery resulted in increased abdominal circumference growth velocity compared to fetuses of “sildenafil-naive” women who either refused, or were not offered treatment (von Dadelszen et al., 2011). However, a greater proportion of sildenafil-treated mothers developed secondary pre-eclampsia, although this was not a statistically significant finding. There currently is a randomised placebo controlled trial of maternal sildenafil treatment of women with severe, early-onset dismal prognosis FGR (the STRIDER trial, ACTRN
12612000584831 2013-2017), with the primary outcome of being alive at term without serious adverse neonatal outcomes, and secondary outcomes being improved fetal growth velocity (Ganzevoort et al., 2014). This trial should answer the question of whether maternal sildenafil treatment in severe fetal growth restriction improves fetal outcomes in humans.

1.6.3. Maternal Growth Hormone

Maternal GH supplementation (0.14 mg·kg·d⁻¹) from day 95-125 gestation in an over-nourished adolescent ewe paradigm of FGR resulted in altered nutrient partitioning and increased fetal growth velocity (Wallace, Matsuzaki, Milne, & Aitken, 2006). Earlier treatment (35-65 days gestation) also improved fetal growth, but this was a result of significantly increased adipose tissue (Adam et al., 2011), which was associated with alterations in hypothalamic development and leptin signalling. Maternal GH treatment in normal ovine pregnancies (twice-daily 0.1 mg·kg⁻¹ GH from day 125-134 gestation) has also been shown to significantly increase placental diffusion capacity and fetal protein oxidation, but without increased fetal growth (Harding, et al., 1997).

Similarly, in growth-restricted fetuses (induced by uteroplacental embolisation), a continuous fetal GH infusion (baseline of 2.5 mg·d⁻¹ superimposed with 1.0 mg·d⁻¹ pulses from 117-127 days gestation) increased fetal circulating IGF1 concentrations but without an increase in fetal growth rate (Bauer, et al., 2003). In fact, kidney and small intestine weight were reduced compared to untreated FGR fetuses, with evidence of tissue-specific changes in gene expression levels of the GH and IGF1 receptor (Bloomfield, van Zijl, et al., 2006). In contrast, twice-daily 0.1 mg·kg⁻¹ maternal GH supplementation from 100-128 days gestation in ovine pregnancies with FGR induced by placental embolisation did result in significantly increased fetal growth rate and fat deposition, but with significantly decreased circulating fetal insulin and IGF1 concentrations compared to controls (de Boo, et al., 2008). However, maternal GH
treatment was associated with hydranencephalic brain lesions in some fetuses. Thus, data on GH treatment for FGR are inconsistent and may suggest a significant risk of adverse effects.

1.6.4. Insulin-Like Growth Factor-1

Structurally homologous to proinsulin, IGFs bind to receptors, ligand-specific binding proteins (IGFBPs) and insulin receptors (Rinderknecht & Humbel, 1978) producing pleiotropic immune (Law, Tu, Liu, & Lau, 2008), endocrine (Gluckman, 1997), autocrine, and paracrine effects (Holt, 2002). Widely present in the developing human fetus (Han, Lund, Lee, & D'ercole, 1988), IGF1 is implicated in the regulation of normal placental function and appropriate fetal growth (Gluckman, 1997; Owens, 1991). IGF1 is necessary for appropriate fetal and postnatal growth and development (Tsoi, Cale, Bird, & Kay, 2003), and is positively associated with neonatal birth weight (Geary, Pringle, Rodeck, Kingdom, & Hindmarsh, 2003). In SGA and AGA growth-restricted infants, cord IGF1 concentrations are significantly decreased when compared to AGA controls (Verkauskiene et al., 2007). Altered circulating profiles of IGFs and their binding proteins persist into childhood in FGR subjects (Cutfield et al., 2002); this may be part of a more widespread somatotrophic axis dysregulation which includes reduced IGF1 production and sensitivity. In humans, dysregulation of the somatotrophic axis, as seen in response to FGR, is implicated in the development of chronic disease states (Fowden & Forhead, 2004).

Decreased circulating fetal concentrations of IGF1 and tissue-specific alterations in IGF1 and IGF1R mRNA expression are also found in experimental paradigms of FGR, such as uteroplacental embolisation (Shaikh et al., 2005) or carunclectomy in sheep (Gentili, Morrison, & McMillen, 2009). In pregnant guinea pig dams, chronic hypoxia induces up-regulation of pro-inflammatory cytokines (Guo et al., 2010), which have been shown to induce a state of IGF1 resistance (O'Connor et al., 2008). The phenotype of the growth-
restricted fetuses in these experimental paradigms is similar to that of human FGR with hypoxaemia, hypoglycaemia and alterations in amino acid metabolism (Eremia, et al., 2007; Jensen, et al., 1999). Both maternal and fetal administration of IGF1 in various mammalian paradigms has been tested, with differing effects on both the mother and the developing fetus.

1.6.4.1. Maternal Insulin-Like Growth Factor-1

In pregnant guinea pigs, maternal infusion of 0.1 mg·kg^{-1}·d^{-1} IGF1 from 20-38 days gestation (term 69 days) increased fetal crown-to-rump length, abdominal circumference, carcass weight, kidney weight, caecum, gastrointestinal tract, adipose tissue, and muscle mass but reduced spleen, liver, and brain weights (Sferruzzi-Perri, Owens, Pringle, Robinson, & Roberts, 2006). This appeared to be secondary to increased placental mass and functional capacity and improved placental carbohydrate transport near term (Sferruzzi-Perri, et al., 2006; Sferruzzi-Perri, Owens, Standen, Taylor, Robinson, et al., 2007). Maternal metabolic substrate uptake in the dams was increased (Sferruzzi-Perri, Owens, Standen, Taylor, Heinemann, et al., 2007) and maternal adipose mass reduced by about 30% (Sferruzzi-Perri, et al., 2006). These findings suggest altered substrate availability in mid- to late-gestation with a potential catabolic effect throughout pregnancy. Although maternal IGF1 treatment has been shown to increase fetal growth in guinea-pigs, significant effects on maternal physiology, including competitive anabolism during fetal development, requires additional investigation to determine the postnatal metabolic ramifications of this treatment on offspring.

1.6.4.2. Fetal Insulin-Like Growth Factor-1

In normally-grown fetal sheep, acute IGF1 infusion results in increased fetal amino acid utilisation and fractional synthetic rate for protein in skeletal muscle (Shen et al., 2003).
However, if the mother was fasted for 120 hours prior to IGF1 infusion, fractional synthetic rate was not increased and the utilisation of amino acids was increased to a much lesser extent than in the fed state (Shen, Wisniowski, Denne, Boyle, & Liechty, 2005). This indicates that adequate substrate supply is necessary for effective tissue growth. In chronically catheterized fetal sheep, 26±4 µg·h⁻¹·kg⁻¹ IGF1 infusion for 10 days beginning at 120 days gestation had organ specific effects on growth (Lok, Owens, Mundy, Robinson, & Owens, 1996) and significantly increased plasma IGF1, IGFBP-1 and -3 concentrations, and decreased liver IGF1 and IGF1 mRNA abundance (Kind, et al., 1996). Acute studies investigating the effects of intravenous fetal infusion of IGF1 (50 µg·kg·hr⁻¹) on metabolism in placental embolisation-induced FGR sheep showed a reduction of fetal blood glucose, oxygen, and amino acid concentrations (Jensen, et al., 1999) and decreased tissue catabolism (Jensen, van Zijl, Evans, & Harding, 2000), suggesting an anabolic effect on both the control and FGR fetus. The fall in fetal blood oxygen and metabolite concentrations in the FGR fetus at this dose are a potential concern, as this could indicate an obligatory increase in oxidation of alternative substrates to facilitate protein accretion, which could have detrimental effects on the fetus.

In a small cohort of non-human primates, an 80 µg injection of IGF1 in healthy fetuses every other day for 10 days at 110 or 130 days gestation (term 165 days) resulted in increased weight and mass of the small intestine, thymus, spleen, and kidney as well as a significant increase in circulating B lymphocytes, and the CD4/CD8 T lymphocyte ratio in fetal lymph node immune cell populations (Tarantal, Hunter, & Gargosky, 1997) suggesting a direct effect on lymphoid tissue proliferation. Both short-term and longer-term infusions of high dose IGF1 (80 µg·h⁻¹) to chronically catheterized fetal sheep led to organ specific increases in growth, with both hypertrophy and hyperplasia (Lok, et al., 1996; Lumbers et al., 2009); however, there was no significant effect on body size or weight. Thus, direct fetal
administration has been shown to alter fetal protein accretion and to have organ specific
effects, but there is little evidence for a sustained effect on fetal growth and direct fetal
administration is unlikely to ever be a feasible approach in the human fetus.

1.6.4.3. Intra-Amniotic Insulin-Like Growth Factor-1

The amniotic sac, also a fetal compartment, is routinely accessed in high-risk human
pregnancies. Fetal swallowing is the main route of amniotic fluid turnover, and the
importance of fetal swallowing in the growth and development of fetal sheep was
highlighted by Bradley and Mistretta (1973). Fetal intake of amniotic fluid has been shown in
both humans (Pritchard, 1966) and sheep (Harding, Bocking, Sigger, & Wickham, 1984) and
fetal uptake into the portal vein of intra-amniotically administered IGF1 has been
demonstrated in the sheep (Bloomfield, Breier, & Harding, 2002). In oesophageal-ligated
fetal sheep, a procedure which impairs amniotic fluid intake by the fetus, a constant low-
dose infusion of 1-8 µg·d⁻¹ of IGF1 beyond the ligation from 90-147 days gestation prevented
both ligation-induced growth restriction and the decrease in bowel thickness seen in saline-
infused ligated controls (Kimble, et al., 1999). This study showed that IGF1 administered
directly into the proximal gastrointestinal tract improved fetal gut growth in the FGR fetal
sheep and led to experiments pursuing administration of IGF1 into amniotic fluid, with the
hypothesis that fetal swallowing would lead to direct fetal uptake.

Indeed, intra-amniotic IGF1 supplementation (20 µg·d⁻¹ for 10 days in mid- to late-gestation)
had direct effects on IGF1 mRNA and protein expression in the gut but also decreased
endogenous fetal IGF1 expression in liver and skeletal muscle, suggesting a systemic effect
causing negative feedback (Shaikh, et al., 2005). Longer-term but less frequent treatment,
with thrice-weekly injections of 120 µg IGF1 from 100-128 days gestation (term 145 days)
significantly increased fetal growth rate and organ growth in FGR secondary to placental
embolisation (Eremia, et al., 2007); extending the dosing interval to 360 µg once weekly still had a beneficial effect on fetal growth (Wali, et al., 2012). Thus, the amniotic route seems a promising approach. With the potential to modify intrauterine fetal adaptations to a suboptimal environment, intra-amniotic IGF1 has the potential to attenuate the development of postnatal characteristics associated with FGR. The development of a prenatal intervention to treat the growth-restricted fetus could serve to abrogate postnatal complications of the disease by improving growth and development, metabolism, and endocrine function in those affected. As research up to this point is limited to fetal outcomes, longer-term studies of the postnatal consequences of increasing fetal growth rates in FGR fetuses are essential before any clinical utility is investigated.

1.7. Summary

Fetal insults alter the developmental trajectory and life path of an individual. The perinatal environment can serve to perpetuate these physiological adaptations and, thus, promote the development of disease states. Although factors which potentiate enduring maladaptation to the postnatal environment are complex and integrated, it may be possible to beneficially influence postnatal health through in utero intervention. The experiments conducted in this thesis were designed to investigate the long-term postnatal adaptations to an in utero intervention in a well-established ovine paradigm of fetal growth restriction. The focus of the research assesses outcomes related to postnatal hypothalamic-pituitary-adrenal and somatotrophic axis regulation, metabolic function, growth and body composition in a systematic and randomised manner.
Chapter 2. Methods

2.1. Ethics Approval
The justifications for the experimental manipulations used in this study were approved by the University of Auckland Animal Ethics Committee (AEC/02/2008/R628 and AEC/03/2011/R874).

2.2. Generation of Singleton-Bearing Ovine Pregnancies
Multiparous Romney ewes 4 to 5 years of age were bred in three-week intervals from January to July in 2010, and January to April in 2011 at Ngapouri Research Farm, Reporoa, New Zealand (NZ). Ewes were bred outdoors in a single flock on an adequate nutritional plane of 3-4% dry matter kg of bodyweight (WT)⁻¹·day⁻¹. To synchronise estrous, intravaginal controlled internal drug release devices (CIDRs) (Pfizer, Auckland, NZ) containing 0.03 g progesterone were inserted into ewes of acceptable condition (body condition score 2.5-3.0 on a 5-point scale) (Russel, Doney, & Gunn, 1969). After 14 days, CIDRs were removed. Ovulation was induced in ewes outside of the natural breeding season with 100-500 µg (according to the time of year) intramuscular equine chorionic gonadotropin (Bioniche Animal Health, Armidale, New South Wales, Australia (AU)). Ewes were placed into a breeding paddock for two days prior to addition of proven Poll Dorset rams. Rams remained with the ewes for 2 days. Gestational age is calculated from the day the rams were introduced; therefore, for the purposes of this study, gestational age is ±1 day.

Ewes were ultrasound scanned at ~40 and ~70 days after ram entry to confirm singleton pregnancy, and were shorn between 70 and 80 days gestation. Following paddock-based feed, sheep were acclimated to a complete concentrate pellet feed (UniC) comprised of
86.8% dry matter with 10.5 MJ metabolisable energy per kg dry matter (Table 2.1, Dunstan Nutrition Ltd, Hamilton, NZ) by pasture supplementation with UniC feed pellets from day 70 to 82. Following paddock acclimatisation, ewes were brought into group pens in a feedlot with 12 hour-controlled photoperiods for further acclimatisation.

<table>
<thead>
<tr>
<th>Gross Composition</th>
<th>g·100g⁻¹ dry matter</th>
</tr>
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<tbody>
<tr>
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<tr>
<td>Lipid</td>
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</tr>
<tr>
<td>Ash</td>
<td>3.8</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
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<tr>
<td>Neutral detergent fibre</td>
<td>40.8</td>
</tr>
<tr>
<td>Soluble sugars and starch</td>
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</table>

<table>
<thead>
<tr>
<th>Macro Element Composition</th>
<th>g·100g⁻¹ dry matter</th>
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<tr>
<td>Magnesium</td>
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<tr>
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<td>Sulphur</td>
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</tr>
<tr>
<td>Sodium</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Table 2.1 Composition of UniC concentrate feed pellet

Due to a high prevalence of facial eczema (FE) in the local area during the 2011 breeding season, plasma gamma-glutamyl transferase (GGT) was assayed on gestational day ~82 to determine potential sporidesmin-induced liver damage. Periconceptional sporidesmin exposure in ewes is associated with altered placental morphometry and reduced fetal pancreatic and thyroid growth (Oliver & Harding, 2009). Although not displaying visual symptoms of FE (facial lesions), ewes with plasma GGT ≥100 U·L⁻¹ (normal range 32-70 U·L⁻¹)
were excluded from the study to eliminate the confounding effects of gestational sporidesmin exposure on fetoplacental development (Oliver & Harding, 2009).

Ewe weight was measured and body condition score assessed upon entry into the feedlot and following the 2-week feedlot acclimatisation period. Ewes which developed lameness over the acclimatisation period were excluded. Ewes which maintained a body condition score of 2.5-3.0 (Thompson & Meyer, 1994) and weight throughout acclimatisation were moved to 1.4 x 1.2 m individual pens with free access to water and once-daily concentrate feeding. From day 93 to 96 ewes were desensitised to regular human interaction through twice-daily contact in individual pens. Ewes were electronically randomised to either Control or fetal growth restriction (FGR) groups at 96 days gestation.

2.3. Feto-Maternal Surgical Catheterisation

2.3.1. Sedation

Ewes randomised to the FGR group underwent surgery between 97 and 100 days’ gestation. Ewes were fasted 24 hours prior to surgery but allowed access to water. Anaesthesia of ewes was induced in the individual housing pen with 5 mg·kg⁻¹ intravenous Propofol (Health Support Limited, Auckland, NZ). If a second dose was required to ensure appropriate induction, this was noted. Ewes were placed supine on a surgical trolley and legs secured with soft ropes looped above the fetlock. Ewes were intubated with a size 9 endotracheal tube (Shoof, Auckland, NZ) and ventilated with 2% isofluorane (MedSource, Auckland, NZ) in 100% oxygen at 1-2 L·min⁻¹. Isofluorane was titrated throughout the procedure by monitoring palpebral reflex and arousal state. Sheep with inadequate respiratory drive were mechanically ventilated at 10-12 breaths·min⁻¹ under a reduced anaesthetic dose (whilst
maintaining adequate anaesthesia as above) until independent respiration resumed. If necessary, mechanical ventilation was continued throughout the procedure.

### 2.3.2. Surgical Preparation

Ewes were given 3.0 mL intramuscular Duplocillin (150 mg·mL⁻¹ procaine penicillin, 115 mg·mL⁻¹ benzathine penicillin, Intervet, Auckland, NZ) in the inner thigh. The abdomen and left flank were shaved, scrubbed with an iodine surgical prep solution (Provet, Auckland, NZ) and sprayed liberally with both ethanolic Hibitaine (Johnson & Johnson, Auckland, NZ) and 10% ethanolic iodine (Vetpak, Te Awamutu, NZ). The surgical field was prepared with sterilised surgical drapes.

### 2.3.3. Surgical Procedure

A midline laparotomy incision approximately 15 cm in length was made from below the umbilicus towards the mammary and haemostasis achieved. Presence of a singleton fetus was verified manually and the pregnant horn noted. The greater omentum was carefully displaced and a 20 mm lancet point cannula with a sharpened trocar guided via the abdominal cavity to the left flank. The flank was punctured cleanly. The trocar was removed from the cannula and catheters were guided via the cannula into the abdomen. Catheters were flushed with 0.9% sterile saline (Baxter Healthcare, Auckland, NZ).

When necessary, the orientation of the fetus was gently altered to enable manual identification of a hindlimb. The fetal hindlimb was held through a portion of the uterus free of placentomes and major vasculature. A 1-2 cm incision was made through the uterine wall and membranes. The fetal hindlimb was externalised, loosely-wrapped with sterile saline-soaked gauze and fixed in a stable position; amniotic fluid loss was minimised by opposing uterine and membrane tissues around the exteriorised limb with Babcock clamps. Fetal hock-
to-toe (HT) of both hindlimbs was measured during catheterisation and general developmental observations were noted.

2.3.4. Fetal and Amniotic Catheterisation

Polyvinylchloride catheters were used throughout the procedure. An incision was made and the tarsal artery was identified. A 0.9 mm catheter with a 0.5 mm internal diameter (SteriHealth, Victoria, AU) approximately 6.0 cm in length was advanced into the descending aorta (Murotsuki, Gagnon, Matthews, & Challis, 1996) via the tarsal artery. The catheter was secured with 4/0 United States Pharmacopeia (USP) silk sutures and the incision closed with 4/0 USP silk suture on a round cutting needle (Cooper Medical, Auckland, NZ). Catheters were gently flushed with 0.9% sterile saline to assess patency and avoid clotting. If catheterisation of the artery was not feasible, the inferior vena cava was catheterised via the saphenous vein instead. Following fetal hindlimb catheterisation, a saturation catheter which has multiple orifices distributed up to ~10 cm proximal from the tip to facilitate drug delivery should blockage occur was coiled, secured with 2/0 USP Resorba® silk and placed in the amniotic cavity and 2.0 mL gentamicin sulphate (40 mg·mL⁻¹, Pfizer, Auckland, NZ) injected into the amniotic fluid as a precautionary measure. The fetus was returned to its original position and the uterine layers closed with 2/0 USP silk on a round needle using a continuous interlocking blanket stitch and a purse string-type closure to minimize catheter movement and amniotic leakage. All catheters were sampled and flushed to ensure patency following closure.

2.3.5. Maternal Uterine Artery Catheterisation and Closure

Bilaterally, a 1.27 mm catheter with 0.86 mm internal diameter (SteriHealth, Victoria, AU) 12-20 cm in length, was introduced into the distal uterine arteries, advanced proximally and
secured with 4/0 USP silk sutures. The uterus was closed with 4/0 USP Resorba® silk on a half-circle, round-bodied needle and haemostasis verified. The uterus and greater omentum were returned to an appropriate position within the abdomen and the tunica closed tightly with a continuous suture of cotton umbilical tape. The skin was closed with 1/0 USP silk in a continuous suture. 4.0 mL of a 2.5 mg·mL⁻¹ bupivacaine, 4.5 µg·mL⁻¹ adrenaline mixture (AstraZeneca, Auckland, NZ) was injected subcutaneously along the abdominal incision. The flank-wound was closed with 2/0 USP Resorba® silk to minimise catheter movement and 1.0 mL bupivacaine/adrenaline was injected subcutaneously around the wound. All catheters were sampled and flushed with 10 U·mL⁻¹ sodium heparinised 0.9% sterile saline (sodium heparin, Hospira, Victoria, AU) and patency noted upon closure.

2.3.6. External Catheter Stabilisation and Ewe Recovery

Catheters were placed in a Ziploc® bag sutured with umbilical tape approximately 10 cm distal to the shoulder midline on the ewe’s back. All wounds were sprayed with both ethanolic hibitaine and 10% ethanolic iodine. A body stocking spanning the abdomen from the thoracic limb to the pelvic limb was placed on the ewe. To minimise the risk of infection absorbent gauze was placed over the flank wound.

At the completion of the procedure, isoflurane was discontinued, and palpebral reflex and arousal state were monitored until the ewe began licking and chewing. Upon arousal from anaesthesia, the ewe was extubated and respiration monitored. When an appropriate level of arousal was reached the ewe was returned to the individual pen and covered with a surgical drape to protect the catheters from damage during the initial attempts at standing. The ewe was kept under supervision and aided as necessary until sufficient balance and
ability to stand independently was achieved. Once ewes were independently mobile and able to lick, chew and vocalise, feed was provided.

2.3.7. Post-Surgical Care and Fetal Monitoring

Flank wounds were monitored for infection and cleaned on a regular basis until sufficiently-healed. Catheters were sampled and flushed daily with heparinised sterile saline to establish and maintain patency. Fetal whole blood was sampled 1-2 days following surgery to measure fetal blood gas status and to assess fetal wellbeing, and at 102 days gestational age (dGA) to establish basal measures. Basal partial pressure of carbon dioxide (\(\text{PaCO}_2, \text{mmHg}\)), partial pressure of oxygen (\(\text{PaO}_2, \text{mmHg}\)) and pH were measured on an i-STAT® (Abbott Point of Care, Inc., Princeton, New Jersey, USA) with CG3+ or CG4+ i-STAT® cartridges. Glucose (mmol·L\(^{-1}\)) and lactate (mmol·L\(^{-1}\)) were measured on a YSI 2300 SELECT™ Biochemistry Analyser (Yellow Springs Instruments, Queensland, AU).

2.4. Uterine Artery Embolisation

Fetal growth restriction (FGR) was induced via bilateral uterine artery embolisation with 20-50 µm Superose® 12 polymerised polysaccharide microspheres (GE Healthcare, Auckland, NZ) over a 5-day period (103-107 dGA).

2.4.1. Plasma Collection

Prior to embolisation, maternal uterine whole blood samples were collected to measure plasma c-natriuretic peptide (CNP) and pro-CNP in either a 10.0 mL heparinised or 5.0 mL ethylenediaminetetraacetic acid (EDTA) vacutainer tube (BD Bioscience, Auckland, NZ). Blood was centrifuged at 3,220 \(g\) at 4°C for 10 minutes and the resultant plasma was collected and stored at -80°C prior to shipping to collaborators in Christchurch.
2.4.2. Preparation of Microsphere Injection

A flask of microspheres was mixed with a magnetic stirrer until fully re-suspended. 1.0 mL of microspheres was added to 100 mL 0.9% sterile saline in an autoclaved 250 mL Schott bottle on a magnetic stirrer. The 1% microsphere dilution was aliquotted into autoclaved 5.0 mL glass scintillation vials. Microspheres were autoclaved, sealed with Parafilm (ThermoFisher Scientific, Auckland, NZ) and stored at 4°C until use. Microsphere infusions were drawn up to the required amount in sterile 3.0 mL syringes.

2.4.3. Titration of Uterine Artery Embolisation

Twice-daily, 0.2 mL fetal blood was collected in heparinised 1.0 mL sterile syringes (BD Bioscience, Auckland, NZ) for analysis of pH, PaCO₂, PaO₂, glucose and lactate concentrations. Embolisation was undertaken up to twice daily, titrated against fetal blood gas results. Embolisation was gradually increased until an appropriate level of hypoxia, PaO₂ \( \leq 18 \text{ mmHg} \), was attained. Embolisations were followed by a 5.0 mL 0.9% sterile saline flush. If fetal blood collection was not possible due to non-functioning catheters the uterine arteries were embolised with the mean overall dose of the breeding group. If a uterine artery catheter became clotted during the embolisation week, the full embolisation amount was ingested into the patent catheter and noted. If the fetus became acidaemic (pH\( \leq 7.29 \)), hypoglycaemic (\( \leq 0.6 \text{ mmol·L}^{-1} \)), or hyperlactaemic (\( \geq 2.0 \text{ mmol·L}^{-1} \)), this was noted. Fetal blood gas measures incompatible with life were the first indication of fetal death. Blood samples were not available in all fetuses; thus, fetal death was defined as the date of abortion in those which fetal blood gas measurements were unavailable. If fetal death was apparent the ewe was immediately euthanised and post mortem conducted as deemed necessary. From 110-133 days gestational age, maternal uterine arteries were flushed once-weekly with heparinised 0.9% sterile saline to maintain patency.
2.5. Intra-Amniotic Treatment

At 107 days gestational age surgical ewes were electronically randomised to treatment groups. The FGR-IGF1 group received 3.6 mL intra-amniotic injections of 100 µg·mL⁻¹ human recombinant insulin-like growth factor 1 (IGF1, Genetech, San Francisco, California, United States (US)) once-weekly to day 135 of gestation. The FGR-Saline group received an intra-amniotic injection of 3.6 mL 0.9% sterile saline. Prior to the intra-amniotic injection, a 5.0 mL EDTA maternal uterine artery blood sample was collected as previously described.

2.5.1. Preparation of Insulin-Like Growth Factor-1

Acetic acid (99 mL of 0.01 M) was filtered through a 0.2 µm Supor polyethersulfone membrane Acrodisc® (PALL Life Sciences, New York, US) into a sterile pottle. 1.0 mL of 10 mg·mL⁻¹ IGF1 was added to the filtered acetic acid and the solution was gently mixed. The 100 µg·mL⁻¹ IGF1 solution was aliquoted into autoclaved 5.0 mL glass scintillation vials, sealed with Parafilm® and frozen at -20°C until required.

2.5.2. Intra-Amniotic Injection

100 µg·mL⁻¹ IGF1 was thawed overnight at 4°C. 3.6 mL of either 0.9% sterile saline or 100 µg·mL⁻¹ IGF1 was drawn into a sterile 5.0 mL syringe and kept on ice until required. Amniotic catheters were flushed with 0.9% sterile saline to verify patency. Amniotic fluid was drawn into the dosing syringe and characteristics of the fluid noted (e.g. turbidity, general colouration, presence of meconium, fresh blood, or clotted blood). The amniotic fluid was gently mixed with the dosing solution and slowly injected into the catheter followed by a 5.0 mL 0.9% sterile saline flush.
2.6. Care of Chronically-Instrumented Ewes

From 110-145 days gestation ewe pens were opened into the central aisle to allow individual ewes to move freely for 30-45 minutes per day. Flank wounds were tended to, as previously described. If arterial catheters were chewed, became entangled or torn, this was noted and the catheters were closed. If the amniotic catheter was damaged, the external portion of the catheter was sterilised with ethanolic hibitaine and iodine and joined with a sterile portion of catheter tubing. A stopcock was affixed and 2.0 mL of 40 mg·mL⁻¹ gentamicin sulphate was injected into the amniotic fluid as a precautionary measure followed by a saline flush.

2.7. Catheter Removal from Chronically-Instrumented Ewes

On gestational day 135, following collection of the final uterine blood sample and intra-amniotic injection, stopcocks and excess catheters were removed. The flank wound was shaved, condition of the wound noted and granular tissue removed. The area was thoroughly soaked with both ethanolic hibitaine and ethanolic iodine. Excess catheter length was gently withdrawn through the flank wound until resistance was met. If a catheter pulled free from the wound in its entirety, this was noted. The catheter bundle was clamped with a large smooth-grip needle holder approximately 10 cm from the skin surface to minimise catheter contamination and drainage. Stopcocks and excess catheter length were cut away and discarded.

Catheters were individually removed from the bundle and, using mosquito clamps, gently pulled until firm resistance was met. Catheters were knotted, excess material cut away, and the remaining length of catheter tucked subcutaneously. If the wound was well-healed and showed no signs of infection it was sealed with SuperGlue™ (Bostik, Auckland, NZ). If infection was apparent the flank wound was debrided, bandaged and allowed to drain until
discharge ceased. The back suture was removed and sprayed with ethanolic iodine. Following catheter removal ewes were monitored for signs discomfort and/or preterm delivery.

2.8. Lambing of Chronically-Instrumented and Ewes

Ewes were monitored with the help of other research staff 24-hours a day from 138 days gestation until the completion of lambing. Ewes were monitored during labour to detect malpresentation or ewe exhaustion. If after 30 minutes, labour was unproductive, or if the lamb became stuck in the birth canal (nose and front hooves visible, thoracic limbs locked against pelvis), dystocia was assumed, and lambing was assisted. If catheters failed to separate from either the uterus or lamb during birth and the lamb became suspended, catheters were cut. Immediately upon delivery, protruding catheters were removed from the lamb hindlimbs. The chest was gently squeezed from the base of the ribs to the sternum. Mucus was removed from the head and aspirated from the oral and nasal passages. The lamb was placed on a woollen lamb coat laid on a towel, and left to bond with the ewe.

The pair was observed from a distance for signs of distress. Lamb feeding behaviour and mobility were noted. If within 1 hour the lamb was unable to latch on to the teat and feed, the ewe was hand-milked and the lamb was manually-fed via a rubber teat affixed to a 50 mL catheter-tip syringe. This was repeated as necessary until 2 hours post-birth or until the lamb was sufficiently-mobile and displayed successful feeding behaviour.

2.9. Animal Husbandry

2.9.1. Feto-Maternal Care

Ewe feed intake was monitored and adjusted throughout gestation: ewes were fed 3.0-4.5 kg·day$^{-1}$ depending on gestational stage, and up to 5.0 kg·day$^{-1}$ during lactation per farm protocol.
Signs of fetal unwellness such as irregular ewe behaviour including reduced feed intake, abnormal body posture, lethargy or hyperactivity, inappropriate vocalisation and general malaise were noted. In ewes presenting with vulvar discharge or frank signs of abortion with no signs of apparent distress, delivery of the abortus was allowed. When possible, aborted fetuses were weighed, measured and any conformational irregularities or catheter displacement noted. Abortive ewes were euthanised with 100-120 mg·kg"^{-1} sodium pentobarbitone (300 mg·mL"^{-1}, Provet, Auckland, NZ). If the ewe was unable to deliver the abortus, or was in apparent distress and/or pain prior to or during delivery, the ewe was euthanised and the abortus manually removed. If intact with identifiable limbs, weight and linear measures were collected.

2.9.2. General Care

Complications of pregnancy and labour and the need for assisted delivery were recorded in all groups. Lambing began in May and continued through November. Although lambing was conducted in a feedlot the ambient temperature was not controlled. To reduce the risk of hypothermia in neonatal lambs, pens were lined with gardening shade cloth and filled to an appropriate depth with pine shavings. When necessary, towel-wrapped hot water bottles were placed in the pen and heat-lamps were suspended 50 cm above the pen, out of reach of the ewe.

2.10. Veterinary Interventions

2.10.1. Maternal Veterinary Interventions

Surgical ewes were given 3.0 mL Duplocillin after spontaneous delivery of lambs and monitored until placental expulsion. The placenta was checked to ensure delivery in its entirety, and for signs of infection. In the case of placental retention, ewes were given 100
mg oxytocin (Ethical Agents, Manukau City, NZ) subcutaneously, and monitored until the placenta was expelled. If the placenta was affixed to the uterus by the catheters and expulsion was not attained, the placenta was manually removed and the ewe kept under observation for 3 days to monitor for bleeding, vulvar discharge and infection.

Maternal veterinary intervention was conducted as needed to ensure optimal health following delivery of the chronically-catheterised lamb: flank wound infections were cleaned and treated with an appropriate course of antibiotics, maternal uterine infections were flushed with a dilute iodine solution and treated with intramuscular 2.0 mg·kg$^{-1}$ ceftiofur hydrochloride (Pfizer, Auckland, NZ) for 7-10 days. Catheters protruding from the vulva were removed manually and ewes were observed for signs of infection.

The well-recognised symptoms of hypocalcaemia and hypomagnesaemia were treated with 0.2 mL·kg$^{-1}$ subcutaneous Calci TAD 50® (132 mg calcium hydroxide, 310 mg calcium gluconate monohydrate, 4,290 mg calcium borogluconate, 65 mg magnesium chloride-hexahydrate, 60 mg phosphorylethanolamine·mL$^{-1}$, Ethical Agents, Manukau City, NZ) and a slow intravenous infusion of 1.0 mL·kg$^{-1}$ warmed 0.9% sterile saline. Veterinary-diagnosed pregnancy toxaemia (acetonaemia) was treated as above and a slow infusion of 1.0 mL·kg$^{-1}$ warmed 25% dextrose (Baxter Healthcare, Auckland, NZ). In pregnancy toxaemia, temperature and hydration status were monitored throughout. If the ewe displayed a diminished (feed intake reduced by 50%) or absent appetite, 0.02 mg·kg$^{-1}$ intravenous brotizolam (Boehringer Ingelheim Ltd., Manukau City, NZ), 0.5 mL·kg$^{-1}$ Headstart Gold® (22.5 mg calcium, 4.6 mg magnesium in stabilised thixotropic propionate, Virbac, Manukau City, NZ) and 0.5 mL·kg$^{-1}$ ketol (0.8 mL·mL$^{-1}$ propylene glycol, with mineral glycerophosphates, choline, cobalt and iodine, Bomac, Manukau City, NZ) were given and feed intake monitored.
2.10.2. Neonatal Care

Veterinary interventions for treatment of abnormalities and deformations were conducted as necessary to safeguard lamb survival and long-term health. Catheter and suture remnants were removed from the hindlimbs, ear tags were inserted and EDTA and heparinised blood samples were taken. The umbilicus and all puncture wounds were inspected and sprayed with both ethanolic hibitaine and ethanolic iodine.

Abnormalities that could be treated were: fused eyelids, which were coaxed open shortly after birth; inverted eyelids, which were externalised and affixed with a 4/0 USP silk suture for 3-5 days; hindlimb instability due to either soft tissue laxity or bony deformations, which was treated by immobilisation in a well-padded hard plastic splint 12 hours a day for 7-10 days; excess mucus in the respiratory tract which restricted breathing and caused distress was cleared with steam inhalation with eucalyptus oil and gentle chest percussion.

2.11. Biometry

2.11.1. Weight and Linear Measures

All lambs were treated in the same manner. Two hours post-delivery the lamb was removed from the ewe but kept within line-of-sight and within audible range of maternal vocalisations. Lamb weight was recorded and the lamb was placed in a padded “cradle” with limbs free-hanging. Crown-to-rump length (CRL) from the mid-orbital peak to the ischial tuberosity, chest girth (CHEST) spanning the peak of the spinous processes of the thoracic vertebrae immediately posterior to the forelimb, abdominal girth (ABDO) at the largest portion between the last rib and the anterior aspect of the pelvic limb, forelimb length (FL) from the joint space between the glenoid cavity and greater tubercle to the ulnar tuberosity and then to the toe, hindlimb length (HL) from the femoral trochanter to the tibiofemoral
joint line to the hock to the toe, hock-to-toe length (HT), and biparietal diameter (BPD) width immediately posterior to the orbita were recorded (Figure 2.1). Abnormalities such as inadequate wool cover, fused, inverted or malformed eyelids, jaw deformations, FL:HL asymmetry, limb deformation, or increased joint laxity were noted. Relative weight per unit body length was calculated with body mass index (BMI, kg·CRL⁻²), G index (GI, kg·CRL⁻¹.₅) (Gootwine, 2013), and ponderal index (PI, kg·CRL⁻³) (Rohrer, 1921).

Lamb weight and linear measures were collected on the third day of life to monitor early neonatal growth. Experimental sheep were measured weekly through 4 weeks and monthly thereafter through 12 months of age. From 13-17 months of age WT, CRL, CHEST, ABDO and HT length were measured.

Figure 2.1 Anatomical location of linear measures
Adapted from the University of Pennsylvania School of Veterinary Medicine ruminant gross anatomy illustrations (http://cal.vet.upenn.edu/projects/grossanat/index.htm).
2.11.2. Calculations

Growth velocity was calculated using an exponential method validated in humans (Patel, Engstrom, Meier, & Kimura, 2005):

\[
GV \left( \text{g.kg}^{-1} \cdot \text{d}^{-1} \right) = 1000 \times \ln \left( \frac{W_n}{W_1} \right) \frac{1}{D_n - D_1}
\]

Where \( W_1 \) represents WT in kg at \( D_1 \) and \( W_n \) represent weight on any given day after \( D_1, D_n \).

Growth velocity of linear measures were calculated in the same manner.

2.12. Postnatal Blood and Plasma Assays

2.12.1. Postnatal Blood Collection

A 10.0 mL heparinised blood sample and 4.0 mL EDTA blood sample were collected with a vacutainer concurrent with postnatal measures and processed as previously described at 2 hours post-birth.

2.12.2. Hormone and Metabolite Assays

Hormone and metabolite assays were performed by LiFePATH technicians at The Liggins Institute, University of Auckland.

Metabolite concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High-Technologies Corporation, Tokyo, Japan). Glucose (Roche, Manheim, Germany), lactate and free fatty acid (FFA) (Randox Laboratories, Crumlin, UK) concentrations were measured by enzymatic colorimetric assay. Urea (Roche) and \( \beta \)-hydroxybutyric acid (BHBA, Randox) concentrations were measured by kinetic UV assay. Mean inter- and intra-assay coefficients of variation (CV) were 5.1% and 3.7%, respectively, for lactate; 3.4% and 2.4%, respectively,
for FFA; 4.3% and 3.8%, respectively, for urea; 4.0% and 1.1%, respectively, for BHBA, and mean intra-assay CV for glucose was 1.6%.

11-deoxycortisol (S), cortisol (F) and cortisone (E) concentrations were analysed with HPLC tandem mass-spectrometry (Rumball et al., 2008). Mean inter- and intra-assay CVs were 5.1% and 10.7%, respectively, for 11-deoxycortisol, 5.9% and 6.5%, respectively, for cortisol, and 4.5% and 7.0%, respectively, for cortisone.

l-3,3’,5-triiodothyronine (T3) and thyroxine (T4) were analysed on an Elecsys 2010 (Hitachi High-Technologies Corporation, Tokyo, Japan). Mean inter- and intra-assay CVs were 1.1% and 1.3%, respectively, for T3, 3.1% and 1.7%, respectively, for T4.

Insulin was measured by radioimmunoassay (RIA) with ovine insulin (Sigma-Aldrich, St. Louis, Missouri, USA) as the standard (Oliver, Harding, Breier, Evans, & Gluckman, 1993). The minimum level of detection was 0.03 ng·mL⁻¹. Mean inter- and intra-assay CVs were 8.6% and 11.7%, respectively. IGF1 was measured by an IGFBP-blocked RIA (Blum & Breier, 1994). The minimum level of detection was 0.03 ng·mL⁻¹. Mean inter- and intra-assay CVs were 3.6 and 11.4%, respectively.

For adult physiological tests, adrenocorticotropic hormone (ACTH), cortisone, cortisol and 11-deoxycortisol, samples were analysed on an Elecsys 2010 (Hitachi High-Technologies Corporation, Tokyo, Japan). Mean inter- and intra-assay CVs were 3.1% and 2.2%, respectively, for ACTH, 3.6% and 6.9%, respectively, for cortisone, 5.9% and 6.5%, respectively, for cortisol, and 5.1% and 10.7%, respectively, for 11-deoxycortisol.

Plasma analyses of deuterium oxide dilution were performed using nuclear magnetic resonance spectroscopy by Massey University (Jaquiery, Oliver, Bloomfield, & Harding, 2011).
2.13. Body Composition

Dual X-ray absorptiometry (DXA) scans are a precise and accurate measure of body composition in the sheep (Pouilles et al., 2000). Following an overnight fast, DXA scans were conducted with a Norland, XR-800 (Cooper Surgical Ltd, Fort Atkinson, WI, USA) under jugular intravenous sedation on sheep at 1 week, 4, 12, and 17 months of age.

2.13.1. Jugular Intravenous Catheterisation and Sedation

In 1 week and in 4 and 12 month old sheep, restraint appropriate for age was used (e.g. padded cradle for 1 week old lambs and gentle manual stabilisation as needed). The neck was shaved and sprayed liberally with ethanolised hibitaine. An appropriately-sized (14-20 gauge 1.25-2.0 inch) Surflo™ intravenous catheter (Terumo, New South Wales, AU) was inserted into the jugular vein and secured to the skin with masking tape and superglue. In 1 week, 4 and 12 month old sheep sedation was induced with 5.0 mg·kg⁻¹ 100 mg·mL⁻¹ ketamine and 0.25 mg·kg⁻¹ 5.0 mg·mL⁻¹ diazepam (Parnell Co. Ltd. and Ceva Animal Health Ltd., respectively, Auckland, NZ) with additional anaesthetic administered as needed for induction. Anaesthesia was maintained throughout procedures with 10.0 mg·kg⁻¹ ketamine and 0.5 mg·kg⁻¹ diazepam. Palpebral reflex and arousal state were monitored and sedation titrated as needed. Sheep were closely monitored during rousing from anaesthesia for adverse reactions to the sedation until able to be returned to its ewe or pen.

In 17 month old sheep, both jugular veins were catheterised with indwelling catheters to enable use for multiple investigations. Intravenous sedation was induced in the animal’s pen via infusion of 5.0 mg·kg⁻¹ ketamine and 0.25 mg·kg⁻¹ diazepam, or more as needed, with an 18-gauge butterfly needle (BD Bioscience, Auckland, NZ) and maintained throughout the
procedures with 10.0 mg·kg\(^{-1}\) ketamine and 0.5 mg·kg\(^{-1}\) diazepam titrated to palpebral reflex and arousal state.

Sheep were placed in a supine position on a surgical trolley with forelimbs secured. 2% lignocaine hydrochloride (AstraZeneca, Auckland, NZ) was infiltrated subcutaneously directly over the vein and a 0.5 cm incision made perpendicular to the vein orientation. The vein was blunt-dissected from the connective tissue and a sterile 12-gauge stainless steel hypodermic needle (Perfektum, Popper & Sons, New Hyde Park, NY, USA) was introduced into the vein. An 80 cm length of 040 catheter tubing (SteriHealth, Victoria, AU) was inserted through the needle and advanced 10 cm into the vein. The catheter was secured with masking tape and Superglue™ at the point of insertion and again directly below the ear.

2.13.2. DXA Scanning

Sedated sheep were transferred to the scanning platform and rolled onto the side with the neck supported in line with the spine. DXA scans were undertaken at a spatial resolution of 3.0 x 3.0 mm in 1-week old lambs, and 6.0 x 6.0 mm in 4-, 12- and 18-month sheep. In 12- and 17-month sheep, the mouth was fixed open to allow for adequate drainage of saliva and mucus.

In the 1-week and 4-month lambs the area analysed was defined proximo-distally as the area of the trunk from the thoracic inlet along the curve of the spine through the base of the tail and around the rump through the pelvic limb just distal to the tibial plateau, along the curve of the abdomen and the anterior margin of the breast (Figure 2.2, A).

In 12- and 17-month old sheep the area analysed was divided into three distinct areas: the chest, abdomen and rump. The chest area was bounded by lines connecting the first cervical vertebra, the first lumbar vertebra, the diaphragm, the sternum and along the breast to
include the heads of the radius and ulna. The abdominal area was bounded by the chest area, the lumbosacral spine at the anterior line of the pelvis, along the anterior margin of the pelvic limb and around the ventral portion of the abdomen. The rump area was bounded by

Figure 2.2 Representative illustration of defined areas for dual X-ray absorptiometry analyses

*Defined areas for DXA analyses of A) one week and four month old sheep, and B) 12 and 18 month old sheep.*

the abdominal area, and included the base of the tail and the femorotibial joint (Figure 2.2, B). Fat and lean mass and bone mineral content were calculated using Norland software (Cooper Surgical Ltd, Fort Atkinson, WI, USA) and expressed in grams. Fat and lean mass of each area were calculated relative to both overall compartment mass and total bodyweight.

### 2.14. Neonatal Percutaneous Liver and Muscle Biopsies

Following the DXA procedure in sedated 1-week lambs, a percutaneous biopsy of the right lobe of the liver and a *vastus lateralis* biopsy were collected using aseptic techniques. The skin over the biopsy areas was scrubbed with an iodine surgical prep solution (Provet, Auckland, NZ) and sprayed thoroughly with ethanolic Hibitaine and iodine. Once the biopsies were obtained, the incision was closed with Superglue™ and sprayed with TetraVet (Bomac
Laboratories Ltd., Auckland, NZ). The biopsy was dabbed with gauze to remove blood, immediately frozen in dry ice and stored at -80°C until analysed. Sedation was maintained with the ketamine/diazepam mixture throughout the procedure by monitoring palpebral reflex and arousal state. The lamb was given 1.0 mL intramuscular Duplocillin in the semitendinosus muscle belly as a precautionary measure and closely monitored during recovery from anaesthesia for adverse reactions to either sedation or the biopsy procedures. The lamb was returned to the ewe once able to ambulate with minimal loss of balance, and with return of feed-seeking behaviour.

2.14.1. Percutaneous Liver Biopsy

With the lamb in left lateral recumbency over a bolster, which was placed immediately posterior to the margin of the ribs, and the neck supported in line with the spine, the point of the sternum was marked with indelible ink and a 10 cm area of wool centred between the sternum and spine shaved. With the thumb placed in the space between the last rib and the spine, moving cranially, the 4th rib was palpated and marked with a surgical pen.

The site was infiltrated with 2% lignocaine hydrochloride and a stab incision was made through the skin and intercostal muscles. A 10 gauge pencil point trocar in a razor-edged cannula was introduced into the thorax and the pleura punctured. The biopsy needle was advanced approximately 1.5 cm into the thorax and slowly oriented ventrocaudally to a 30° angle from the spine (pointing towards the umbilicus) and 20° from the external plane of the trunk. The needle was advanced into the thorax until the diaphragm was punctured. The needle was then rotated towards the point of the sternum and advanced approximately 2.0 cm into the liver at a 45-60° angle from the spine and 30° from the external plane of the trunk until a granular-textured tissue was identified. The trocar was removed and a 5.0 mL
sterile syringe was fitted to the cannula. The syringe was withdrawn approximately 2.5 mL and the cannula advanced 0.5-1.0 cm, depending on lamb size. The negative pressure was maintained while the cannula/syringe apparatus was withdrawn from the lamb. The incised skin was immediately closed over the wound avoid pneumothorax and pressure applied with sterile gauze to ensure haemostasis. If the biopsy was unsuccessful the procedure was repeated no more than twice depending on the level of sedation and general health of the lamb.

2.14.2. Skeletal Muscle Biopsy

The vastus lateralis was identified and an area 2 cm wide around the biopsy site was shaved. The site was infiltrated with 2% lignocaine hydrochloride and a stab incision was made through the skin and subcutaneous tissue. The epimysium was blunt-dissected with small mosquito forceps and a portion of the muscle belly grasped perpendicular to the orientation of the fibres with small Allis clamps. A 20-100 mg piece of muscle was dissected with small Vanna’s Scissors.

2.15. Intravenous Deuterium Oxide Assessment of Neonatal Milk Intake

Lambs are functionally monogastric (Wardrop & Coombe, 1961) and dependent exclusively upon milk to provide adequate nutrients during the first three weeks of life (Walker & Walker, 1961). Thus, water intake during this time is primarily a result of milk intake from the mother. Therefore, measuring total body water turnover in the lamb via deuterium oxide (D₂O) dilution over time can estimate milk intake. Milk intake was measured in the second week of life.
2.15.1. Preparation of 60% Deuterium Oxide

0.9 g NaCl (Scharlau Chemie, Barcelona, Spain) was aliquoted into a sterile glass bottle and 40 mL autoclaved MilliQ™ water was filtered through a 2.0 µm Supor polyethersulfone membrane Acrodisc® (PALL Life Sciences, New York, US) was added. 60 mL 99.9% D₂O (Sigma-Aldrich) was filtered through an Acrodisc® into the pottle and the solution was thoroughly mixed. The 60% D₂O was aliquoted into sterile, pyrogen-free 15 mL Falcon® tubes (BD Bioscience, Auckland, NZ), sealed with Parafilm™ and stored at 4°C until required.

2.15.2. Intravenous Deuterium Oxide Injection

Between 0800 and 0900 hours, the lamb was weighed and the 0.5 mL·kg⁻¹ 60% D₂O dose was calculated. The D₂O dose was drawn into an appropriately-sized syringe and the weight of the pre-injection syringe was recorded. A baseline blood sample was taken from the jugular vein with a 10 mL syringe with a 20 gauge butterfly needle. Immediately following the baseline sample the D₂O was infused and flushed through with a 5.0 mL 0.9% saline flush. The post-injection syringe weight (mg) was recorded and actual 60% D₂O dose administered was calculated:

\[
\text{[Final syringe weight (mg) - Start syringe weight (mg)]} \times 0.6
\]

Jugular venous blood samples were taken with a vacutainer in heparinised 10 mL tubes 2 and 6 hours post-bolus on day 1, and 1, 2, 3, 4, 5 and 8 days post-bolus. Sample time was recorded and plasma was collected as previously described. Lamb weight was recorded following the final sample.

2.15.3. Plasma Deuterium Oxide Analysis

Samples were analysed as previously described (Alsweiler, Harding, & Bloomfield, 2013).
2.15.4. Calculation of Water Turnover

A D\textsubscript{2}O calibration curve was generated for each lamb. The constant, \( k \), calculated from plasma samples, is the D\textsubscript{2}O exponential decay over time \( (e^{-kt}) \). A graph of plasma D\textsubscript{2}O concentrations over the second week of life was plotted. At each time point, the square roots of differences between measured and fitted data were summed and minimised to find the curve of best fit. Calculation of D\textsubscript{2}O concentration \( (C_{td}) \) took into account the changing volume of D\textsubscript{2}O distribution from time 0 \( (D_0) \) and the changing volume of the lamb from time 0 \( (V_0) \) due to growth over the experiment \( (\Delta V_t) \). This was calculated from the exponential growth velocity of the lamb and the exponential decay of D\textsubscript{2}O in plasma:

\[
C_{td} = \frac{D_0 \times e^{-kt}}{V_0 + \Delta V_t}
\]

Total milk intake \( (\text{mL/hr}^{-1}) \) was calculated from the disappearance of D\textsubscript{2}O over the sampling period from time 0 \( (T_0) \):

\[
\frac{\text{D}_2\text{O dose (mg)} \times k}{\text{D}_2\text{O at } T_0}
\]

Estimated total milk intake was calculated from the disappearance curve of D\textsubscript{2}O in plasma for each lamb. Milk intake \( (\text{mL/hr}^{-1}) \) was divided by the average weight \( (\text{kg}) \) across the testing period to give average intake \( (\text{mL/hr}^{-1} \cdot \text{kg}^{-1}) \):

\[
\frac{\text{D}_2\text{O dose (mg)} \times 1000}{\text{Net extrapolated D}_2\text{O at } T_0 \left(\text{mg}\cdot\text{mL}^{-1}\right) \times \text{weight at } T_0 \left(\text{kg}\right)}
\]

2.16. Animal Management and Paddock Care

Immediately following completion of the D\textsubscript{2}O series, ewe-lamb pairs were transferred to a group pen to allow lambs to acclimatise to a mob environment. At three weeks of age,
following collection of weight, linear measures and a blood sample, lambs were inoculated in the groin with active Orf parapox virus (Scabigard™ vaccine, Pfizer, Auckland, NZ), injected with 0.5 mL Prolaject 2000 (2000 µg hydroxocobalamin, 4.0 mg selenium·mL⁻¹, Bomac Laboratories Ltd., Auckland, NZ) and a rubber ring was applied to dock the tail just below the anus in males and the vulva in females. The mob was transferred outdoors to an appropriate paddock and monitored for complications. The ewe/lamb mob was brought into the feedlot for weight and linear measures at 1, 2 and 3 months. At 3 months lambs were weaned and sex-separated: ewe lambs were kept with un-weaned ewe-lamb pairs, and ram lambs were drafted into a separate mob. Males were not castrated. Sheep were vaccinated and drenched per farm protocol until the completion of the study. Animals were maintained to 18 months of age, defined here as “adulthood”, to enable completion of the project within the time allotted to conclude doctoral research.

2.17. Veterinary Care

2.17.1. Infections

Respiratory infection, pneumonia, or purulent hoof infection were treated with 2.2 mg·kg⁻¹ intravenous flunixin meglumine (Norbrook, Northamptonshire, UK) daily for 3 days and 1.0 mg·kg⁻¹ intramuscular ceftiofur hydrochloride (Pfizer, Auckland, NZ) daily for 5-7 days.

Uterine infections were initially treated with dilute iodine flush. If the infection persisted, ewes were given 2.0 mg·kg⁻¹ intramuscular ceftiofur hydrochloride for 7-10 days.

Joint infection in adults were treated with either 15 mg·kg⁻¹ amoxicillin and 2.5 mg·kg⁻¹ enorofloxacin (Bayer Animal Health, Manukau City, NZ) intramuscular injection or 2.0 mg·kg⁻¹ marbofloxacin (Vétoquinol, New South Wales, AU) and 6.0 mg·kg⁻¹ procaine penicillin/4.6
mg·kg$^{-1}$ benzathine penicillin (Intervet, Auckland, NZ) intramuscular injection twice-daily for 2 days.

All ram lambs were exposed to *Brucella ovis*-positive breeding rams. Therefore, ram lambs were injected with 10 mg·kg$^{-1}$ streptomycin sulphate and 10 mg·kg$^{-1}$ dihydrostreptomycin sulphate (Stockguard, Hamilton, NZ) intramuscularly daily for three days and blood-tested for *Brucella* monthly over a 90-day period.

Conjunctivitis was treated externally with a 2% tylan tartrate solution (Elance, Auckland, NZ) daily for 3 days. If the infection persisted, the eye was treated with a single application of 166 mg·mL$^{-1}$ cloxacillin (Pfizer, New South Wales, AU).

### 2.17.2. Facial Eczema

When pasture fungal spore counts increased in the autumn, sheep were given an oral 43 g zinc oxide bolus (Agri-feeds, Mount Maunganui, NZ) to aid in the prevention of facial eczema. Acute facial eczema was treated with 2.0 mg·kg$^{-1}$ intramuscular acetyl-dl-methionine (Ethical Agents, Manukau City, NZ) diluted 1:1 with sterile water, a 0.5 mL Prolaject 2000 injection (2000 µg hydroxocobalamin, 4.0 mg selenium·mL$^{-1}$, Bomac Laboratories Ltd., Auckland, NZ), 2.2 mg·kg$^{-1}$ intravenous flunixin meglumine (Norbrook, Northamptonshire, UK) daily for 3 days and 1.0 mg·kg$^{-1}$ intramuscular ceftiofur hydrochloride (Pfizer, Auckland, NZ) daily for 5 days. Weight, feed intake, temperature, skin condition and oedema were monitored throughout the treatment period. Eschar was removed and wounds debrided as necessary. Affected areas were covered with a cream containing 14% titanium dioxide, 8% zinc oxide, 0.3% cetrimide and 0.08% benzalkonium chloride (FiltaBac, Robin Pharmaceuticals Ltd., Rotorua, NZ) to protect the skin until sufficient wool regrowth was attained. Blood samples were analysed to assess GGT and liver function during the treatment period.
2.17.3. Gastrointestinal Conditions

Leptosporosis and gastroenteritis were treated with 2.2 mg·kg\(^{-1}\) intravenous flunixin meglumine (Norbrook, Northamptonshire, UK) twice daily for 1 day, and 13.3 mg·kg\(^{-1}\) sulphadimethyl pyrimidine 2.7 mg·kg\(^{-1}\) trimethoprim (Virbac, Manukau City, NZ) intravenously daily for 5 days. Urinalyses were conducted daily during the treatment period.

Coccidiosis was treated with 2.2 mg·kg\(^{-1}\) intravenous flunixin meglumine (Norbrook, Northamptonshire, UK) twice daily for 5 days and a single 20 mg·kg\(^{-1}\) oral toltrazuril suspension (Bayer Animal Health, Manukau City, NZ).

Rhododendron poisoning and cyanide intoxication were treated with oral 100 mg·kg\(^{-1}\) sodium thiosulphate and 1 g·kg\(^{-1}\) activated charcoal. In the instance of rhododendron poisoning, sheep were monitored until vomiting ceased. If appetite did not return after cessation of vomiting, sheep were given 0.5 mL·kg\(^{-1}\) oral Headstart Gold® (22.5 mg calcium, 4.6 mg magnesium in a stabilised thixotropic propionate solution, Virbac, Manukau City, NZ) and feed intake was monitored.

Scour was treated with 1.0 mL·kg\(^{-1}\) oral Scourban® (21.3 mg·mL\(^{-1}\) sulphadimine, 21.3 mg·mL\(^{-1}\) sulphaguanidine, 28.4 mg·mL\(^{-1}\) sulphadiazine, 103 mg·mL\(^{-1}\) kaolin, 0.02 mg·mL\(^{-1}\) hyoscine hydrobromide (Bomac, Manukau City, NZ) and 1.0 mL·kg\(^{-1}\) Electrolytes A/F® (Vétoquinol, New South Wales, AU) daily for 2-3 days.

Colic was treated with 200 mL oral liquid paraffin and 200 mL electrolytes. Gentle manual manipulation of the gut was conducted as tolerated by the animal to aid in the release of gas and to encourage gut motility.
Faecal egg counts were conducted to monitor parasite infestation in the cohorts. Administration of anthelminthics in addition to the standard worming protocol in sheep with increased susceptibility was conducted as needed.

2.17.4. Additional Veterinary Interventions

Anaemia was treated with a single 318 mg iron polymaltose (Sigma, Victoria, AU) intramuscular injection and a 0.5 mL Prolaject 2000 injection (2000 µg hydroxocobalamin, 4.0 mg selenium·mL⁻¹, Bomac Laboratories Ltd., Auckland, NZ).

Ewe lambs which were exposed to sexually mature rams were treated with a single 125 µg cloprostenol sodium (Bayer Animal Health, Manukau City, NZ) intramuscular injection of seven days post-exposure and ultrasound scans were conducted 30 days post-exposure.

To minimize the chance of hoof infection, sheep were run through a copper sulphate (Ravensdown, Christchurch, New Zealand) solution as needed. Minor infection was treated with TetraVet®.

2.18. Physiological Tests

Physiological tests were undertaken at 17-18 months of age. Sheep were brought into the feedlot and weighed. To synchronise estrous, a CIDR was inserted into ewes 3 days prior to the start of the testing series, and testing conducted with the CIDR in situ. In the rare instance of intrauterine infection prior to, or following insertion of the CIDR, the infection was treated and physiological tests conducted with the subsequent breeding group. Sheep were returned to individual pens following jugular venous catheterisation and DXA scans as previously described. Sheep which were not randomised to receive physiological tests were housed alongside the tested sheep throughout the testing series.
Three days prior to testing, feed intake was titrated to allow 10% by weight feed refusal per day, catheterisation wounds were tended and sheep were allowed to acclimatise prior to testing. Sheep underwent a glucose tolerance test (GTT), adrenaline stimulation test (ADR), hyperglycaemic clamp (HGC), Metrapone® test (MET), arginine vasopressin/corticotropin-releasing hormone stimulation test (AVPC), and growth hormone stimulation test (GHT). Dosing for all physiological tests were determined based on weight of the sheep when brought into the feedlot.

Catheters were flushed with heparinised saline following each test. If catheters became blocked or were irreparably damaged prior to the HGC, sheep were re-catheterised. If catheters became blocked or were irreparably damaged after the HGC, blood samples were collected with a vacutainer as previously described. All blood samples were collected in heparinised syringes and processed as previously described. Animals were free-moving in all tests except for the HGC, during which restraint was necessary due to the nature and duration of the test.

2.19. Physiological Testing Schedule

Testing was conducted as follows:

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<td>ADR</td>
<td>HGC</td>
<td>MET</td>
<td>AVPC</td>
<td>Free for Repeat Testing</td>
<td>Free for Repeat Testing</td>
<td>GHT</td>
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<td>GHT + biopsy</td>
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Table 2.2 Physiological testing series for adult sheep
In the instance of a failed test in the first week, the test was repeated the Saturday following the AVPC test.

2.20. Area Under the Curve Calculations

Area under the curve (AUC) with respect to the increase from baseline (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003) was calculated as appropriate for all tests.

2.20.1. Glucose Tolerance Test

Glucose tolerance tests were conducted at 0900 following an overnight (16-hour) fast with *ad libitum* access to water throughout the test.

After collecting a baseline blood sample, 0.5 g·kg⁻¹ 50% dextrose was infused in ≤60 seconds and followed by a 10 mL 0.9% saline flush. Blood samples were collected 2, 5, 10, 15, 20, 30, 40, 50, 60, 120 and 180 minutes after infusion.

2.20.1.1. Sample Analysis

Samples were analysed for glucose and insulin as previously described.

2.20.1.2. Data Analysis

Glucose (G) areas under the curve first 15 minutes following glucose infusion and 180 minutes following glucose infusion (mmol·L⁻¹; GTT-AUC₁₅ and GTT-AUC₉₀, respectively) were calculated. The GTT-AUC₁₅ was defined as the early phase insulin response in the first 15 minutes following the bolus (AIR₁₅) was calculated (Long et al., 2010; J. A. Owens, et al., 2007). The change in insulin (I) secretion from baseline in the first 15 minutes following glucose infusion (ng·mL⁻¹; ΔI₁₅, ng·mL⁻¹), absolute (ng·mL⁻¹; GTT-AUC₁₅) and relative insulin secretion (I₅, ng·mL⁻¹·mmol·L⁻¹; GTT-AUC₁₅/GTT-AUC₉₀) over the duration of the test were calculated (Gatford et al., 2004).
2.20.2. Adrenaline Stimulation Test

Adrenaline tests were conducted at 0900 in a fed state with *ad libitum* access to water throughout the test.

Following collection of a baseline blood sample, 1.0 µg·kg⁻¹ adrenaline (Mayne Pharma, Salisbury South, South Australia, AU) was infused and followed by a 10 mL 0.9% saline flush. Blood samples were collected 2.5, 5, 7.5, 10, 15, 20, 30, 45 and 60 minutes after infusion.

2.20.2.1. Sample Analysis

Samples were analysed for glucose, non-esterified fatty acids and β-hydroxybutyric acid as previously described.

2.20.2.2. Data Analysis

Glucose (G), insulin (I), free fatty acids (FFA) and β-hydroxybutyric acid (BHBA) areas under the curve (ADR-AUC) were calculated using the trapezoid rule from baseline over two periods: the first 15 minutes following ADR infusion (ADR-AUC₁₅) and 60 minutes following ADR infusion (ADR-AUC₆₀). Relative glucose (ADR-AUC₁₅ and ADR-AUC₆₀), FFA (ADR-AUC₁₅ and ADR-AUC₆₀) and β-hydroxybutyric acid (ADR-AUC₁₅ and ADR-AUC₆₀) secretion were calculated.

2.20.3. Hyperglycaemic Clamp

Hyperglycaemic clamps (HGCs) were conducted following an overnight fast. HGCs were performed in females at 0900 and in males at 1300.

2.20.3.1. L-Arginine Preparation

A 100 mg·mL⁻¹ L-Arginine (Acros Organics, Geel, Belgium) solution was prepared with 0.9% saline. The pH was adjusted to 8.0 with 2N hydrochloric acid. The solution was filtered
through a 0.2 µm Supor polyethersulfone membrane Acrodisc® immediately prior to the HGC and kept on ice until required.

2.20.3.2. Sample Collection

Glucose concentration in all blood samples collected throughout the HGC was immediately analysed with a YSI 2300. 3.0 mL baseline blood samples collected at -20 and -10 minutes were preserved as previously described for insulin assay. Additional 0.2 mL samples were collected at -15, -5 and 0 minutes to determine baseline glucose concentration (mmol·L⁻¹). A 7.7 mL·min⁻¹·(m²)⁻¹ body surface area (kg⁰.⁶⁷ x 0.09 (Mitchell, 1928)) 25% dextrose (Baxter Healthcare, Auckland, NZ) bolus was infused from 0-5 minutes to increase blood glucose concentration to 10 mmol·L⁻¹. The rate of glucose infusion thereafter was titrated to blood glucose using a computer algorithm (DeFronzo, Tobin, & Andres, 1979) to maintain blood glucose concentration at a 10 mmol·L⁻¹ steady state. Blood samples were collected at 5-minute intervals through 135 minutes to determine blood glucose concentration; samples collected at 15-minute intervals were preserved to assay plasma insulin concentration.

To maximize insulin secretion (Larsson & Ahrén, 1998), a 100 mg·kg⁻¹ l-Arginine bolus was given immediately following the 135 minute blood sample. Blood samples were collected at 5-minute intervals following the arginine bolus to determine blood glucose; samples collected at 5, 10, 20 and 30 minutes post-bolus were preserved for insulin assay.

2.20.3.3. Sample Analysis

Samples were analysed for glucose and insulin as previously described.
2.20.3.4. Data Analysis

Hyperglycaemic clamps were analysed if during steady state (75-135 minutes) the blood glucose concentration coefficient of variance was ≤10% throughout.

Insulin (I) area under the curve (HGC-AUC) was calculated using the trapezoid rule over two periods: from baseline to the end of steady state (HGC-AUC\textsubscript{ISS}) and following the L-Arginine bolus from the end of the steady state period to the completion of the clamp (HGC-AUC\textsubscript{ARG}). The mean glucose infusion during steady state (IG\textsubscript{SS}: mmol·kg\textsuperscript{-1}·min\textsuperscript{-1}) and following the L-Arginine bolus (IG\textsubscript{ARG}: mmol·kg\textsuperscript{-1}·min\textsuperscript{-1}) were calculated. Mean plasma insulin concentration during steady state (I\textsubscript{SS}: ng·mL\textsuperscript{-1}), peak plasma insulin concentration following the L-Arginine bolus (I\textsubscript{ARG}: ng·mL\textsuperscript{-1}), and the change in plasma insulin concentration from steady state in response to the L-Arginine bolus (ΔI\textsubscript{SS:ARG}) were calculated. Insulin sensitivity (S\textsubscript{i}) during steady state was calculated (IG\textsubscript{SS}/I\textsubscript{SS} (Mitrakou et al., 1992)). The insulin-dependent component of whole body glucose tolerance, glucose disposition index (DI\textsubscript{G}= S\textsubscript{i} x AIR\textsubscript{15} (Long, et al., 2010)), and glucose-stimulated insulin secretion (Mean I\textsubscript{SS}/baseline plasma insulin) (Carver, Anderson, Aldoretta, & Hay, 1996; Limesand & Hay, 2003) were calculated.

2.20.4. Metyrapone\textregistered Test

Metyrapone\textregistered tests were conducted at 0900 in a fed state with ad libitum access to water throughout the test.

2.20.4.1. Metyrapone\textregistered Preparation

A 100 mg·mL\textsuperscript{-1} Metyrapone\textregistered (kindly donated by Novartis, Auckland, NZ) solution was prepared with 0.9% saline acidified to pH 4.0 with 2N hydrochloric acid to ensure complete Metyrapone\textregistered suspension. The solution was filtered through a 0.2 µm Supor polyethersulfone membrane Acrodisc\textregistered prior to the test and kept on ice until required.
2.20.4.2. Sample Collection

Baseline blood samples were collected at -10 and -5 minutes. At time 0, 40 mg·kg⁻¹ Metyrapone was infused intravenously over a 2 minute period followed by a 10 mL 0.9% saline flush. Sheep were observed for 10 minutes post-bolus and any adverse events noted. Blood samples were collected at 30, 60 and 120 minutes post-bolus.

2.20.4.3. Sample Analysis

Samples were analysed for adrenocorticotropic hormone, cortisol, cortisone, and 11-deoxycortisol as previously described.

2.20.4.4. Data Analysis

Adrenocorticotropic hormone (ACTH), cortisol (F), cortisone (E) and 11-deoxycortisol (S) areas under the curve were calculated (MET-AUCₐᵢₙₜ, MET-AUCₓ, MET-AUCₛ). Absolute (ng·mL⁻¹) and relative (%) cortisol suppression from baseline to 30 minutes (MET-SUPPₓ), the cortisol nadir, peak 11-deoxycortisol and ACTH were calculated.

2.20.5. Arginine Vasopressin/Corticotropin-Releasing Hormone Stimulation Test

Arginine vasopressin-corticotropin-releasing hormone tests (AVPC) were conducted at 0900 in a fed state with ad libitum access to water throughout the test.

After baseline blood sample collection, equimolar doses of 0.1 µg·kg⁻¹ bovine arginine vasopressin and 0.5 µg·kg⁻¹ bovine corticotropin-releasing hormone (Sigma-Aldrich, St. Louis, Missouri, USA) were infused intravenously and followed by a 10 mL 0.9% saline flush. Blood samples were collected 15, 30, 45, 60, 120 and 240 minutes following infusion.
2.20.5.1. Sample Analysis

Samples were analysed for adrenocorticotropic hormone and cortisol as previously described.

2.20.5.2. Data Analysis

Adrenocorticotropic hormone (ACTH) and cortisol (F) areas under the curve (AVPC-AUC\text{ACTH}, AVPC-AUC\text{F}) and maximal ACTH and cortisol secretion calculated.

2.20.6. Growth Hormone Stimulation Test

Growth hormone stimulation tests (GHT) were conducted in a fed state with ad libitum access to water over a 3 day period. Feed intake was recorded beginning 3 days prior, and throughout the test. Ad libitum feed sufficient to allow 10% refusal by weight was maintained throughout the test.

2.20.6.1. Growth Hormone Preparation

Immediately prior to use, lyophilised bovine growth hormone (bGH, batch 7368-69Qm American Cyanamid, New Jersey, US) was re-suspended at 5.0 mg·mL\textsuperscript{-1} in carbonate-buffered saline (8.5 mg·mL\textsuperscript{-1} NaCl, 2.65 mg·mL\textsuperscript{-1} NaHCO\textsubscript{3}, 1.135 mg·mL\textsuperscript{-1} (Na)\textsubscript{2}CO\textsubscript{3} in sterile water, pH 9.4, Sigma-Aldrich, St. Louis, Missouri, USA) in an autoclaved glass scintillation vial and kept at 4\degree C until required.

2.20.6.2. Sample Collection

A baseline blood sample was collected at time 0; additional blood samples were collected at 4, 8, 12, 24, 28 and 48 hours post-baseline. Immediately following the baseline, 8 and 24 hour samples, 0.15 mg·kg\textsuperscript{-1} growth hormone was injected intramuscularly. A biceps femoris...
biopsy was collected immediately following the 48 hour blood sample and processed as previously described.

2.20.6.3. Sample Analysis
Samples were analysed for glucose, insulin, and non-esterified fatty acids as previously described.

2.20.6.4. Data Analysis
Glucose (G), insulin (I), IGF1 and FFA areas under the curve (GHT-AUC\textsubscript{G}, GHT-AUC\textsubscript{I}, GHT-AUC\textsubscript{IGF1}, GHT-AUC\textsubscript{FFA}) were calculated.

2.21. Adult Post Mortem Examination
Post mortem examination of the untested adult sheep was conducted 1-2 days following completion of the testing series. Post mortem examination of sheep randomised to receive physiological tests were conducted 5-6 days after the completion of the testing series to allow adequate time for drug elimination and recovery from testing procedures. All post mortems were conducted following an overnight fast. Live weight was collected in a crush and 100-120 mg·kg\textsuperscript{-1} sodium pentobarbitone was injected intravenously. Post mortem examination and tissue preservation were timed beginning at the point the animal lost consciousness. Following pentobarbitone injection sheep were immediately exsanguinated and decapitated.

2.21.1. Tissue Collection
Irregularities and abnormalities of the carcass and organs were noted. Organs were resected, weighed and select tissues were preserved.
2.21.1.1. Preservation Techniques

Tissues collected for molecular biology were snap-frozen in liquid nitrogen (BOC, Rotorua, NZ), crushed and stored at -80°C. Tissues collected for immunohistochemistry (IHC) were either sectioned into either Macrosettes™ (Simport, Quebec, Canada) or Fisherbrand SURE-TEK™ cassettes (Fisher Scientific, Auckland, NZ) and fixed in a 4% paraformaldehyde solution (PFA; extra pure paraformaldehyde, Scharlau, Barcelona, Sparin) mixed in phosphate-buffered saline, or mounted on a dot of optimal cutting temperature (OCT) compound (Andwin Scientific, Illinois, US) and frozen on a liquid nitrogen super-cooled steel block. PFA-preserved IHC tissues were stored at 4°C and PFA was changed after 24 hours. Frozen IHC tissues were wrapped in aluminium foil and stored at -80°C until analysis. Anterior coronal brain sections were preserved in PFA for 48 hours, cryoprotected in a 30% sucrose (NZ Sugar, Ltd., Auckland, NZ) solution mixed in MilliQ water, frozen and stored at -80°C.

2.21.2. Organ Resection and Tissue Sectioning

The pancreas was removed and an approximately 2 cm medial strip of the primary portion of the organ was dissected. The strip was bisected and sectioned further into the “head”, “body” and “tail” as determined by the tissue processor. One set of tissues was preserved for molecular biology and the other in PFA. The lymph node at the head of the pancreas was preserved for molecular biology.

A 2.0 cm³ section of both the deep medial portion of the left and right lobes of the liver were removed and preserved for molecular biology. The left adrenal and a portion of perirenal fat were preserved for molecular biology. Two approximately 1.0 cm³ midline sections including both the medulla and cortex immediate to the renal pelvis of the kidney were collected; one section was preserved in PFA and the other preserved for molecular biology.
The left gonad was collected. Female gonads were taken whole while an approximately 0.5 cm midline section of the male gonad was dissected and preserved for frozen IHC. The thyroid and right adrenal were preserved for frozen IHC.

The heart was bisected transmurally below the chordae tendinae. Septal, and left and right ventricular thicknesses were measured. An approximately 1.0 cm³ section of the lateral aspect of the left ventricle was preserved for frozen IHC. An approximately 1.0 cm length of descending aorta immediately inferior to the diaphragm was excised and preserved in PFA.

On the left side of the carcass, fat depth at the 12th rib (Clements, Thompson, Harris, & Lane, 1981) and the midline of the latissimus dorsi, as well as the latissimus width and depth (Kempster, Avis, Cuthbertson, & Harrington, 1976) were recorded. An approximately 1.0 cm³ section of subcutaneous fat immediately dorsal to the latissimus, and a portion of the latissimus muscle belly were preserved for molecular biology. An approximately 1.0 cm³ section of omental fat, and skeletal muscle from the muscle belly of the vastus lateralis, and semitendinosus were collected and preserved for molecular biology.

The brain was removed from skull. The pituitary, which cleaved off at the level of median eminence, was excised and preserved for frozen IHC.

The spinal cord immediately distal to the inferior margin of the medulla was excised and discarded. The medulla at the posterior margin of the pons and the cerebellum was removed and weighed individually. Cerebral anteroposterior and coronal widths were measured and an anterior coronal section of the frontal lobe approximately 4 mm thick was taken and preserved in PFA. The hypothalamic block was obtained by slicing through the lateral ventricles from the superior margin of the pons to the anterior margin made by the coronal
section and preserved for frozen IHC. The hippocampal lobes were obtained by bisecting the fornix and carefully dissecting. Hippocampi were preserved for frozen IHC.

The weight of the neck thymus, spleen, right kidney and associated perirenal fat, left and right lung and right gonad were recorded and the tissue was disposed of. Empty carcass weight was recorded.

2.22. Molecular Analyses

Molecular analyses were conducted on liver and muscle biopsies from 1-week old lambs, and post-growth hormone test biopsies from adult sheep.

2.22.1. Tissue Extractions

A TRIzol®-based parallel extraction protocol was developed to isolate mRNA, gDNA (Kimura et al., 2004; Triant & Whitehead, 2009) and protein (Likhite & Warardekar, 2011) from a 10-50 mg sample.

2.22.1.1. mRNA Extraction

10-50 mg snap-frozen tissue was ground with a liquid nitrogen pre-cooled stainless steel mortar and pestle and scooped into a 1.5 mL Eppendorf tube. 1.0 mL TRIzol® (Life Technologies, Auckland, NZ) was added and the solution vortexed to mix. Samples were sonicated at 35%, 2-cycle for 15 seconds and stored on ice. Sonication was repeated until sample was fully solubilised. Samples were centrifuged at 4°C, 10,621 g for 10 minutes; fatty layers were discarded.

200 μL chloroform (Sigma-Aldrich, St. Louis, Missouri, USA) per mL TRIzol® was added and the solution vortexed to mix. Samples were incubated for 5 minutes at room temperature, and centrifuged at 4°C, 17,949 g for 15 minutes. The aqueous phase was aspirated and
transferred to a fresh nuclease-free Eppendorf tube; the interphase and organic phase were stored at 4°C until gDNA and protein isolation were conducted.

500 μL cold isopropanol (Sigma-Aldrich, St. Louis, Missouri, USA) was added to the aqueous phase and the solution was incubated 30-40 minutes at -20°C. The precipitate was centrifuged at 4°C, 17,949 g for 15 minutes and the supernatant discarded. The pellet was washed with 1.0 mL 75% ethanol (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at 4°C, 17,949 g for 5 minutes. The wash step was repeated and the pellet semi-dried for 10 minutes at room temperature. The pellet was dissolved on ice in 10-20 μL nuclease-free water (Life Technologies, Auckland, NZ) and stored overnight at -80°C.

2.22.1.2. mRNA Quantification

The day following extraction, mRNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer (3.1.2 NanoDrop Software, BioLab Ltd., Auckland, NZ). Samples were of acceptable purity if 260/280 absorbance was >1.9 and 260/230 absorbance was >1.6. During protocol optimisation, mRNA integrity was visually confirmed with a 1.5% agarose (Life Technologies, Auckland, NZ) RNA denaturation gel.

2.22.1.3. gDNA Extraction

300 μL TNES-6U (10 mmol Tris HCl pH 7.5, 125 mmol NaCl, 10 mmol EDTA, 1.0% SDS, 6 M urea; all, Sigma-Aldrich, St. Louis, Missouri, USA) was added to the interphase and organic phase from the mRNA extraction. The tube was vortexed and incubated for 30 minutes at 60°C. Samples were centrifuged at 4°C, 17,949 g for 15 minutes. The supernatant was transferred to a fresh tube, and the interphase and organic phase was stored at 4°C until protein isolation was conducted.
An equal volume of cold isopropanol was added to the supernatant. The tube was gently mixed by inversion and the solution was incubated for 2 hours at -80°C. The precipitate was centrifuged at 4°C, 17,949 g for 15 minutes and the supernatant discarded. The pellet was washed in 1.0 mL 75% ethanol (Sigma-Aldrich, Auckland, NZ) and centrifuged at 4°C, 17,949 g for 5 minutes. The wash was repeated three times. The supernatant was discarded and the pellet was dried 10 minutes at room temperature. The pellet was gently dissolved in 10-20 μL nuclease-free water on ice and stored overnight at -80°C.

2.22.1.4. gDNA Quantification

The following day, gDNA concentration was analysed with a NanoDrop. Samples were of acceptable purity if 260/280 absorbance was approximately 1.8 and 260/230 absorbance was 2.0-2.2. During protocol optimisation, gDNA integrity was visually confirmed with a 1.0% agarose Tris-acetate-EDTA (TAE) gel.

2.22.1.5. Protein Extraction

The organic phase and interphase from the gDNA extraction was vortexed to mix and 3 volumes of acetone was added to the solution. The tube was vortexed and incubated 10 minutes at room temperature and centrifuged at 4°C, 20,817 g for 10 minutes. The supernatant was discarded and 500 μL of a 1:1 solution of 0.3 M guanidine HCl in 95% ethanol and 2.5% glycerol (Sigma-Aldrich, St. Louis, Missouri, USA) in MilliQ water was added. Samples were sonicated at 60%, 2-cycle for 15 seconds and iced for 2 minutes. Sonication was repeated until samples were fully homogenised. An additional 500 μL of the guanidine solution was added; the samples were vortexed and incubated for 10 minutes at room temperature. Samples were then centrifuged at 10,621 g for 5 minutes at room temperature. The supernatant was discarded, and the pellet was washed with 1.0 mL 1:1
guanidine/glycerol solution. 1.0 m 2.5% glycerol in ethanol was added and the pellet was re-
suspended and incubated for 10 minutes at room temperature. Samples were centrifuged at
10,621 g for 5 minutes at room temperature and the supernatant discarded. Tubes were
inverted and pellets semi-dried for 10 minutes at room temperature. Pellets were solubilised
in 100-200 μL 1.0% Triton, pH 5.3 (Sigma-Aldrich, St. Louis, Missouri, USA) and stored at -80°C
until quantification.

2.22.1.6. Protein Quantification
Protein was quantified using a modified Folin-Lowry method (Bio-Rad DC protein assay kit;
Bio-Rad Laboratories, Hercules, California, USA). Briefly, 25 μL working reagent (20 μL
Reagent S·Reagent A mL⁻¹) was added to each well of a flat-bottom 96-well plate. Bovine
serum albumin (BS; Sigma-Aldrich, St. Louis, Missouri, USA) standards were diluted in 0.01%
Triton from 5.0 to 0.0625 mg·mL⁻¹. 5.0 μL lysates, diluted 1:10 in MilliQ water to reduce
Triton interference, and BS standards were aliquoted in triplicate and 500 μL Reagent B was
added to the wells. The plate was incubated on an agitator for 15 minutes at room
temperature and absorbance at 750 nm was measured on a microplate reader (BioTek
Instruments, Inc., Winooski, Vermont, US). Total protein concentration was calculated from
the mean absorbance of the triplicate wells with the linear equation of the standard curve.
The result was corrected for the dilution factor.

2.22.2. Quantitative Polymerase Chain Reaction Analysis of mRNA Concentration
mRNA transcript levels of target genes were measured with quantitative polymerase chain
reaction (qPCR).
2.22.2.1. Synthesis of Complementary DNA

RNA template concentration was optimised for each tissue. 1.5 µg total RNA was incubated with 4 U RNase-free DNase I (Life Technologies, Auckland, NZ) for 15 minutes at room temperature to eliminate potential genomic DNA contamination. To halt DNA digestion, 1.0 µL 25 mmol EDTA was added and samples were incubated at 65°C for 10 minutes. Complementary DNA (cDNA) was synthesised in 20 µL reaction volumes with SuperScript® VILO™ cDNA synthesis kits (Life Technology, Auckland, NZ). 1x VILO™ reaction mix, 1x SuperScript® III enzyme mix containing SuperScript® III reverse transcriptase and RNaseOUT™ recombinant ribonuclease inhibitor were added to 1.5 µg DNase-treated RNA. Reverse transcription PCR (RT-PCR) was run on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) under the following conditions: 25°C for 10 minutes, 42°C for 60 minutes, 85°C for 5 minutes and cooled to 4°C. Complementary DNA was stored at -80°C until qPCR analysis.

2.22.2.2. Quantitative Polymerase Chain Reaction

Transcript abundance was determined by singleplex amplification in 384-well plates in triplicate with qPCR. 10.0 µL reaction volumes with 5.0 µL Taqman Master Mix (Applied Biosystems, Foster City, California, USA), 2.0 µL cDNA template, 900 nmol forward and reverse primers and 200 nmol probe (Applied Biosystems) were assayed on an ABI 7900HT sequence detector (Applied Biosystems) under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, 40 cycle-repeats of 95°C for 15 seconds and 60°C for 1 minute. FAM fluorescent reporter dye was bound to all Taqman probes except 18S (VIC reporter) at the 3’ end, and a molecular-groove binding non-fluorescence quencher (MGBNFQ) was bound at the 5’ end.

Ovine target genes analysed were insulin-like growth factor binding protein acid labile subunit (IGFALS), IGF1, IGF2, IGF1 receptor (IGF1R), growth hormone receptor (GHR), IGF
binding protein-1 (IGFBP1) and -3 (IGFBP3), glucose transporters SLC2A2 and SLC2A4, mammalian target of rapamycin (MTOR) and glucocorticoid receptor (NR3C1) (Table 2.3). The stability of seven potential housekeeping genes were determined for each tissue: 18S ribosomal protein 1 (18S), beta actin (ACTB), peptidylprolyl isomerase A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L19 (RPL19), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) (Table 2.4).
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<td>P</td>
<td>6FAM-AGT TCT GCC ATT TCT-MGBNFQ</td>
<td>69</td>
<td>552-547</td>
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</table>

Table 2.3 Quantitative polymerase chain reaction target genes

Primer and probe (P&P) sequences for forward (F) and reverse (R) primers and fluorescent probe, melting temperature (TM), location of the sequence (bp). Tissues analysed were liver and muscle biopsies at one week of age, and following the growth hormone (Post-GH) test.
Table 2.4 Quantitative polymerase chain reaction housekeeping genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession ID</th>
<th>P&amp;P</th>
<th>Sequences (5’-3’&lt;sup&gt;*&lt;/sup&gt;)</th>
<th>Location (bp)</th>
<th>cDNA Dilutions for qPCR</th>
<th>1 Wk Liver</th>
<th>1 Wk Muscle</th>
<th>Post-GH Muscle</th>
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<tr>
<td>RNA1851</td>
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<td>F</td>
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<td>49-65</td>
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</table>

*Primer and probe (P&P) sequences for forward (F) and reverse (R) primers and fluorescent probe, melting temperature (TM), location of the sequence (bp). Liver and skeletal muscle biopsies at one week of age, and skeletal muscle following the growth hormone (Post-GH) test.*
2.22.2.3. Analysis of Quantitative Polymerase Chain Reaction Data

The three most stable housekeeping genes for each tissue were amplified. Amplification curves for all housekeeping and target genes (visualised in Figure 2.3), and qPCR amplification efficiencies for all housekeeping and target genes were calculated from the slopes of the standard curves (Figure 2.4) which were run on cDNA from a representative sample for each tissue.

Figure 2.3 Representative quantitative polymerase chain reaction amplification curves

A 10-fold serial dilution curve and samples run in triplicate for IGFALS in 1-week old lamb liver biopsies. Cycle number (X-axis) of samples run in triplicate. The Y-axis ($\Delta R_n$) represents the log magnitude of the signal generated. The green horizontal line represents the threshold, which is set in the exponential phase of the amplification curve.
The Y-axis represents cycle threshold (Ct) for IGF1 over a 10-fold serial dilution, in 1-week old lamb liver. $R^2$ represents the line of the best fit ($y=mx + b$). Amplification efficiencies ($E$) were calculated from the slope, $m$: $E=10^{-1/m}$.

The geometric mean (GEO) of the three most stable housekeeping genes across the experimental groups were calculated for each tissue (Pfaffl, Tichopad, Prögomet, & Neuvians, 2004). The threshold cycle (Ct) for target gene (TG) mRNA expression levels were calculated relative to the housekeeping geometric mean with a mathematical model which accounts for variation in amplification efficiency amongst transcripts (Pfaffl, 2001). Standard error of the mean (SEM) was calculated for each group and a 99% confidence interval (CI) was used for statistical analysis of qPCR results, where $b$ is the experimental group compared with either Control or FGR-Saline ($a$):

$$99\% \text{ CI} = \frac{(E_{TG})^{\Delta \text{Ct}_{TG} (b-a)} \pm 2.58 \times \text{SEM}}{(E_{GEO})^{\Delta \text{Ct}_{TG} (b-a)} \pm 2.58 \times \text{SEM}}$$

Results are reported as fold-change with 99% CI relative to either Control (FGR-Saline and FGR-IGF1) or FGR-Saline (FGR-IGF1) groups.
2.23. Statistical Analyses

The number of animals required for the current study was determined by a power calculation based on growth trajectories from previous research with 80% power and an alpha of 0.05.

Data were recorded manually and an electronic database created in Excel (Microsoft, Seattle, Washington, USA). Calculations and analyses were conducted in JMP 10 (SAS Institute Inc., Cary, North Carolina, USA). Distribution was verified with the Shapiro-Wilk test. Non-parametric data were natural log transformed (ln) to approximate a normal distribution where necessary. Even distribution of key nominal variables between experimental groups was verified with $\chi^2$ tests.

The effects of FGR and intra-amniotic treatment of FGR with IGF1 were analysed by t-test, factorial, or repeated measures analysis of variance (ANOVA and RM ANOVA, respectively), and were conducted separately for each sex with breeding year as a random effect in ANOVA analyses. Breeding year was included in the RM ANOVA model to adjust for the effect on outcomes. Significance was set at $p<0.05$ unless otherwise stated. Tukey’s post hoc testing was conducted where appropriate. Results are presented as the least square means ± SEM, fold change (CI), or percentage (%) as appropriate.
Chapter 3. Generation of Experimental Groups

3.1. Introduction

This chapter reports the total number, characteristics and fate of animals produced for the purposes of this experiment.

FGR was induced via maternal uterine placental embolisation. Growth-restricted lambs were randomised to receive either intra-amniotic saline or IGF1 (FGR-Saline and FGR-IGF1, respectively, Chapter 2). Control animals were kept alongside experimental animals throughout.

The aims of this project were to determine whether intra-amniotic IGF1 treatment of the growth-restricted fetus improved fetal and perinatal survival and postnatal physiological and metabolic indices of disease risk in the growth-restricted offspring. We examined early postnatal growth and feed intake, body composition through 18 months of age, postnatal somatotrophic axis function, hypothalamic-pituitary-adrenal axis function, glucose tolerance and insulin sensitivity at 18 months of age.

3.2. Methods

Weight and body condition score were recorded for all ewes upon entry into the feedlot. Weight and linear measures were recorded for all experimental animals from birth through 18 months of age. Liver and muscle biopsies were collected at 1 week of age. If animals were deemed too weak and feeble to tolerate the biopsies, procedures were not conducted. Intravenous deuterium oxide (D₂O) dilution assessment of milk intake was conducted in the second week of life. DXA scans were performed at 1 week, 4, 12 and 18 months of age.
Adult animals were randomised to receive adult physiological tests at 17-18 months of age. If, after randomisation, animals died or were euthanised, the next animal in the randomisation list underwent physiological testing.

Animals with infections diagnosed in the two weeks prior to their scheduled testing period were treated per veterinary protocol (Chapter 2) and tested with the following breeding group. Failure of experimental procedures in some animals reduced the numbers of animals with complete data; precise numbers of animals with data for each test will be described in the relevant chapters.

3.3. Results

3.3.1. Animal Numbers and Experimental Groups

Maternal weight following feedlot acclimatisation was not significantly different between the 2010 and 2011 breeding years \((p=0.5, 67.5\pm0.5\text{ and } 66.9\pm0.7\text{ kg})\), whilst maternal body condition score (BCS) was significantly less \((p<0.0001)\) in 2010, median 3.5 (3.0-3.5) compared with 2011, median 3.5 (3.5-4.0) ewes.

One hundred and two singleton sheep were generated for the purposes of this experiment (Figure 3.1). Control, FGR-Saline and FGR-IGF1 animals produced for this experiment were evenly distributed across the January-July mating season in 2010 and 2011 \((\chi^2 p=0.10)\).
Animals utilised during the project according to experimental group and sex. Dashed boxes report exclusions, deaths and euthanasia. Split boxes reporting postnatal deaths indicate the number of females (top left) and males (bottom right) for each experimental group.

Circumstances constituting exclusion from the project were: *pregnancy toxaemia, †mammary abscess n=5, aspiration of rumen contents n=1, twin pregnancy n=2 and intrauterine infection n=1; ‡twin lambs n=2, intestinal atresia n=1; °preterm birth n=1.
3.3.2. Experimental Losses

Animals from all experimental groups died in both the prenatal (Table 3.1) and postnatal (Table 3.2) periods. Post mortem examinations were conducted and tissue was collected for histopathology where appropriate. The on-call veterinarian conducted post mortem examinations if zoonotic diseases were suspected or if pathology was uncertain.

Post mortem examination of abortive ewes found that 4 ewes displayed signs of intrauterine infection: purulence along the catheter tract associated with granular tissue, abscesses and inflammation at uterine catheterisation sites and creamy white or yellow discharge. Petechial placental haemorrhage and fibrotic adhesions of the uterus, intestine and omentum were also noted.

Eight aborted fetuses in breeding groups 1-4 were noted to have brachycephaly: maxillary hypoplasia and mandibular hyperplasia.

<table>
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<th>Losses during experimentation</th>
<th>Control</th>
<th>Surgical Ewes</th>
<th>FGR</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
</tr>
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<td>Pregnancy toxaemia</td>
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<td></td>
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<td></td>
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<tr>
<td>Exclusion during surgery</td>
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<td>11*</td>
<td></td>
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<tr>
<td>Experimentally-induced</td>
<td></td>
<td>12</td>
<td>26</td>
<td>21</td>
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<tr>
<td>Catheter tangles</td>
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<td>3</td>
<td></td>
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<tr>
<td>Congenital defect</td>
<td></td>
<td>3†</td>
<td>2†</td>
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</tr>
<tr>
<td>Intrauterine infection</td>
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<td>2</td>
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<tr>
<td>Stillbirth</td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
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</tr>
</tbody>
</table>

Table 3.1 Deaths during the prenatal period

*Circumstances constituting exclusion of surgical ewes include mammary abscess n=5, aspiration of rumen contents prior to intubation n=1, twin pregnancy n=2, intrauterine infection n=1, and fetal death during surgical procedures n=2. †Brachycephaly n=8. Stillbirth is defined as a ewe which delivers a dead lamb at term. Gestational age at death is defined as the date of abortion. Mean gestational age at death in embolised (FGR) fetuses was 108±2 days. Gestational age at fetal death was not different in either FGR-Saline (121±2) or FGR-IGF1 (122±3).
Table 3.2 Postnatal exclusions, deaths and euthanasia

Lambs excluded, euthanised, or found dead postnatally due to *intestinal atresia n=1, †respiratory distress/congenital heart failure secondary to pulmonary aneurysm n=2, ‡liver biopsy-induced injury n=2, ¥complication of anaesthesia n=1, "infection-related deaths: listeriosis n=2, pneumonia n=4, coccidiosis n=2, lymphocytic meningoencephalitis n=1, eosinophilic gastroenteritis and hepatocellular atrophy n=1, umbilical infection n=1, rhododendron poisoning n=1, °non-specific pulmonary oedema n=1, ●fight wounds n=1.

3.3.3. Morbidity

Postnatal illnesses, including pneumonia, gastrointestinal and foot rot were treated with antibiotics as previously described (Control, n=27; FGR-Saline, n=29; FGR-IGF1, n=30).

3.4. Discussion

Maternal body condition score, which is positively correlated with fat mass (Russel, et al., 1969), was greater upon entry into the feedlot in 2011 compared with 2010 ewes. We acknowledge the influence maternal body condition could have on experimental outcomes; thus, breeding year is included as an effect in statistical analyses.

Experimental procedures resulted in an average fetal mortality rate of 53%. Intra-amniotic IGF1 treatment of the growth-restricted fetus did not lead to increased fetal mortality. Postnatal mortality from birth to the completion of the experiment was 11% in Control animals, and 23% in both FGR-Saline and FGR-IGF1 groups. Whilst postnatal mortality was greater in growth-restricted groups compared with Control, weekly intra-amniotic IGF1
treatment of fetal growth restriction did not affect postnatal mortality compared with saline-treated sheep. Postnatal morbidity was not different amongst groups.

3.5. Conclusions

Intra-amniotic treatment of fetal growth restriction with weekly injection of 360 µg·mL$^{-1}$ of IGF1 did not affect fetal mortality or postnatal morbidity or mortality compared with the saline-treated growth-restricted lamb.
Chapter 4. Plasma Hormone and Metabolite Concentrations, Growth and Body Composition in the Perinatal Period

4.1. Introduction

Fetal growth restriction increases morbidity and mortality, and necessitates precise antenatal surveillance and specialist management in order to optimise the timing of birth relative to fetal survival (Alberry & Soothill, 2007). The fetal growth-restricted neonate is smaller and thinner than a normally-grown baby, with reduced adiposity (Verkauskiene, et al., 2007) and lean mass (Padoan et al., 2004). Inadequate energy stores, a higher brain to body weight ratio and the propensity for both increased peripheral insulin sensitivity (Diderholm, 2009; Ibáñez, et al., 2008) and glucose disposal (Bazaes, et al., 2003) predispose the growth-restricted neonate to hypoglycaemia (Diderholm, 2009). Although increased insulin sensitivity and glucose disposal is appropriate for a deprived environment, it is associated with accelerated growth and the rapid restoration of body weight after birth in the majority of growth-restricted infants (Beltrand, et al., 2009). Additionally, the growth-restricted neonate is at increased risk of adverse perinatal outcomes (Bernstein, Horbar, Badger, Ohlsson, & Golan, 2000; Unterscheider et al., 2013) and long-term complications (Alberry & Soothill, 2007). Currently, prenatal management of the growth-restricted fetus is limited to optimising delivery timing; however, improving fetal growth could provide a viable clinical intervention with which to improve adverse postnatal outcomes.

FGR in the sheep results in adaptations comparable to that of human placental insufficiency including reduced fetal growth velocity (Morrison, 2008), increased postnatal insulin sensitivity (De Blasio, Gatford, McMillen, Robinson, & Owens, 2007) and accelerated postnatal growth in early life (Louey, et al., 2005). Intra-amniotic IGF1 treatment of the
growth-restricted ovine fetus has been shown to increase fetal growth rate in late gestation (Eremia, et al., 2007) and partially abrogate the growth restriction-mediated reduction in fetal weight at 131 days gestational age (Wali, et al., 2012). However, the postnatal effects of IGF1 treatment of fetal growth restriction on growth, body composition, and plasma hormone metabolite concentrations have not been investigated. Thus, the aim of this experiment was to investigate the effect of intra-amniotic IGF1-treatment of the growth-restricted ovine fetus on size at birth, early postnatal growth velocity, body composition at one week of age, milk intake in the second week after birth, and plasma hormone and metabolite concentrations in the first two weeks after birth.

4.2. Methods

Fetal surgeries were conducted in fetal growth restriction groups, but not Controls; therefore, fetal data presented are for growth-restricted groups only. Fetal blood gas, plasma hormone and metabolite concentrations in chronically-catheterised fetal Control sheep have been published previously from similar preparations (Bloomfield, Bauer, et al., 2002; de Boo, et al., 2008; Wali, et al., 2012). All lambs in the current study were born spontaneously, and vaginally. Statistical analyses were conducted as previously described (Section 2.23).

4.3. Results

4.3.1. Fetal Characteristics

4.3.1.1. Fetal Biometry

Fetal hock-to-toe at 102 days gestational age (dGA) was not different between FGR-Saline and FGR-IGF1 groups (both 9.9±0.1 cm).
4.3.1.2. Fetal Whole Blood gases and Metabolite Concentrations

During the embolisation period the partial pressure of oxygen (PaO₂) and pH decreased (p=0.04 and p=0.004, respectively). Arterial blood lactate concentration increased throughout the embolization period (p=0.002). The partial pressure of carbon dioxide (PaCO₂) and blood glucose concentration did not change (p=0.2 and p=0.4, respectively). Although blood lactate was variable in FGR-Saline fetuses, blood gas and metabolite concentrations did not differ between experimental groups (Figure 4.1: A-E).

4.3.1.3. Experimental Losses and Post Mortem Examination

Fetal mortality did not differ amongst breeding groups (p=0.2, Figure 4.1: F). Gestational age at death did not differ between FGR-Saline and FGR-IGF1 fetuses (122±2 and 123±2 days), and there was no effect of fetal sex (p=0.3).

Eight fetuses sired by a single ram underwent surgery in breeding groups 1-4 and subsequently aborted. All were noted to have brachycephaly, maxillary hypoplasia and mandibular hyperplasia. The ram was removed from the breeding program. Refer to Table 3.1 for gestational losses.

4.3.2. Lambing of Chronically Catheterised Ewes

Dystocia, defined as 30 minutes of unproductive labour with the thoracic limbs of the lamb becoming lodged in the pelvic canal with nose and bilateral front hooves protruding, and exhaustion were noted in Control ewes. Lack of amniotic fluid at delivery, malpresentation and dystocia including posterior longitudinal with bilateral hip flexion (breech), unilateral carpal flexion, deviation of the head and neck, and ewe exhaustion were noted in ewes carrying FGR-Saline and FGR-IGF1 fetuses. Frequency of assisted lambing was not significantly
different amongst groups (Control, 33%; FGR-Saline, 39%; FGR-IGF1, 43%). Postnatal infections in experimental ewes were noted and treated but not analysed.

4.3.3. Birth Characteristics

4.3.3.1. Developmental Abnormalities

Lack of wool cover on the face (FGR-Saline, \( n = 1 \); FGR-IGF1 \( n = 1 \)), fused, inverted or malformed eyelids (FGR-Saline, \( n = 5 \); FGR-IGF1 \( n = 3 \)), prognathia (mandibular protrusion; FGR-Saline, \( n = 1 \); FGR-IGF1 \( n = 1 \)), stifle (knee) deformation with lateral bone spur (FGR-IGF1, \( n = 1 \)), and increased metacarpophalangeal and metatarsophalangeal joint laxity (as diagnosed by the on-call veterinarian) resulting in abnormal gait (\( n = 18 \)) were noted in all groups and were more prevalent in the 2011 (\( n = 15 \)) compared with the 2010 (\( n = 3 \)) breeding season. Joint laxity was noted in male Control (\( n = 2 \)), FGR-Saline (\( n = 5 \)) and FGR-IGF1 (\( n = 3 \)) lambs and female FGR-Saline (\( n = 5 \)) and FGR-IGF1 (\( n = 3 \)) lambs, but not in female Control lambs. Respiratory distress in the first two days after birth was noted in Control (\( n = 1 \)), FGR-Saline (\( n = 1 \)) and FGR-IGF1 (\( n = 4 \)) lambs.

4.3.4. Perinatal Characteristics

4.3.4.1. Biometry at Birth

At birth, female FGR-Saline lambs were smaller and lighter with reduced abdominal circumference and birthweight (BW) Z-score, body mass index (BMI) and G-Index (GI, refer to section 2.11.1 for calculations) compared with Controls whilst female FGR-IGF1 lambs had shorter hindlimb length than Controls (Table 4.1). There were no significant differences in weight or linear measures between FGR-Saline and FGR-IGF1 groups.
BW and BW Z-score in male FGR-Saline and FGR-IGF1 lambs were reduced compared with Controls. Whilst male FGR-Saline lambs had shorter hock-to-toe and forelimb length than Controls, male FGR-IGF1 lambs also had lesser chest circumference, hindlimb length, BMI and G-index than Controls (Table 4.1). There was no significant difference in ponderal index (PI) amongst groups in either sex.

4.3.4.2. Biometry in the First Two Weeks after Birth

Female FGR-Saline lambs were lighter than Controls with shorter hindlimb and hock-to-toe length from birth to seven days after birth (Figure 4.2: A-H). At two weeks after birth, male FGR-IGF1 lambs had reduced crown-rump length hindlimb length, chest circumference and biparietal diameter and were lighter than Controls (Figure 4.3: A-H).

4.3.4.3. Early Postnatal Growth Velocity

Weight and forelimb length growth velocity were greater in FGR-Saline lambs than Controls in the first week after birth, whilst in FGR-IGF1 lambs growth velocity of weight was greater than controls in the second week after birth (Table 4.2).

In males, growth velocity of weight in FGR-Saline lambs was greater than Controls in the first week after birth, whilst there were no differences in growth velocity of weight in FGR-IGF1 compared with either FGR-Saline or Control groups in the second week (Table 4.2).

In females, growth velocity of crown-rump length in FGR-Saline lambs was greater than Controls in the second week after, but growth velocities for all other measurements were similar amongst groups in females (Table 4.2).

In the second week after birth, growth velocity of weight of Control and FGR-Saline lambs decreased by 25% and 32%, respectively, compared with the first week after birth, whilst
growth velocity of weight of FGR-IGF1 lambs decreased by only 17% and was significantly
greater than Control lambs.

4.3.4.4. Body Composition One Week after Birth

Bone mineral content were reduced in FGR-Saline females compared with Controls, which
bone mineral density was reduced in both FGR-Saline and FGR-IGF1 lambs compared with
Controls. Bone mineral composition and bone mineral density were reduced in FGR-Saline
lambs compared with Controls whilst bone mineral density was reduced in both FGR-Saline
and FGR-IGF1 lambs compared with Controls (Table 4.3). Fat mass at one week of age was
below the limit of detection.
Figure 4.1 Fetal whole blood measures and fetal mortality

Whole blood measures of A) partial pressure of carbon dioxide (PaCO₂, mmHg); B) partial pressure of oxygen (PaO₂, mmHg); C) pH; D) glucose concentration (mmol·L⁻¹); and E) Lactate concentration (mmol·L⁻¹) in FGR-Saline (blue, n=20-34) and FGR-IGF1 (red, n=20-32) fetuses; data are least square means ± SEM. F) Fetal mortality in fetal sheep randomised to FGR-Saline (blue, n=64) and FGR-IGF1 (red, n=56) experimental groups from 2010 (breeding groups 1-9) and 2011 (breeding groups 10-14).
### Table 4.1 Characteristics at birth according to sex and experimental group

Gestational age (GA), birthweight (BW), crown rump length (CRL), chest circumference (Chest), abdominal circumference, hock-to-toe length, hindlimb length, forelimb length, biparietal diameter (BPD), body mass index (BMI, kg·m\(^{-2}\)), g index (GI, kg·m\(^{-1.5}\)), and ponderal index (PI, kg·m\(^{-3}\)) at birth. Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females, **p<0.01, females; †p<0.05, males, ††p<0.01, males.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control, n=15</td>
<td>FGR-Saline, n=18</td>
</tr>
<tr>
<td>GA at Birth (days)</td>
<td>147±1</td>
<td>147±1</td>
</tr>
<tr>
<td>BW (kg) * †</td>
<td>5.7±0.3(^a)</td>
<td>4.6±0.3(^b)</td>
</tr>
<tr>
<td>BW Z-Score * ††</td>
<td>0.3±0.2(^a)</td>
<td>-0.4±0.2(^b)</td>
</tr>
<tr>
<td>CRL (cm)</td>
<td>51.0±0.8</td>
<td>48.5±0.7</td>
</tr>
<tr>
<td>Chest (cm) ††</td>
<td>39.5±0.6</td>
<td>37.4±0.5</td>
</tr>
<tr>
<td>Abdomen (cm) *</td>
<td>40.7±1.0(^a)</td>
<td>36.9±0.9(^b)</td>
</tr>
<tr>
<td>Hock to Toe (cm) †</td>
<td>21.1±0.3</td>
<td>19.9±0.3</td>
</tr>
<tr>
<td>Hindlimb (cm) * ††</td>
<td>39.5±0.6(^a)</td>
<td>37.4±0.5(^b)</td>
</tr>
<tr>
<td>Forelimb (cm) †</td>
<td>34.1±0.5</td>
<td>32.5±0.5</td>
</tr>
<tr>
<td>BPD (cm)</td>
<td>6.4±0.1</td>
<td>6.2±0.1</td>
</tr>
<tr>
<td>BMI (kg·CRL(^{-2})) *</td>
<td>22.0±0.9(^a)</td>
<td>19.5±0.9(^b)</td>
</tr>
<tr>
<td>GI (kg·CRL(^{-1.5})) *</td>
<td>15.7±0.7(^a)</td>
<td>13.6±0.6(^b)</td>
</tr>
<tr>
<td>PI (kg·CRL(^{-3}))</td>
<td>43.6±1.9</td>
<td>40.1±1.8</td>
</tr>
</tbody>
</table>

|                       | Control, n=22           | FGR-Saline, n=13        | FGR-IGF1, n=19  |
| BW (kg) * †           | 6.4±0.2\(^a\)          | 5.4±0.2\(^b\)          | 5.4±0.2\(^b\) |
| BW Z-Score *          | 0.8±0.1\(^a\)          | 0.2±0.2\(^b\)          | 0.1±0.2\(^b\) |
| CRL (cm)              | 52.3±0.6                | 50.0±0.7                | 49.0±1.0      |
| Chest (cm)            | 41.2±0.4\(^a\)         | 39.2±0.6\(^a,b\)       | 38.3±0.6\(^b\) |
| Hock to Toe (cm) †     | 41.8±0.6                | 40.3±0.8                | 39.2±0.8      |
| Hindlimb (cm) *       | 41.4±0.3\(^a\)         | 39.3±0.5\(^a,b\)       | 38.5±0.2\(^b\) |
| Forelimb (cm) †       | 35.5±0.4\(^a\)         | 33.7±0.5\(^b\)         | 33.9±0.5\(^a,b\) |
| BPD (cm)              | 6.6±0.04                | 6.5±0.06                | 6.4±0.03      |
| BMI (kg·CRL\(^{-2}\)) * | 23.6±0.8                | 21.6±0.9                | 22.01±0.9     |
| GI (kg·CRL\(^{-1.5}\)) * | 17.0±0.5                | 15.3±0.6                | 15.5±0.6      |
| PI (kg·CRL\(^{-3}\))  | 45.6±1.8                | 43.1±2.2                | 44.6±2.0      |
Figure 4.2 Effect of FGR and intra-amniotic IGF1 treatment on early postnatal growth in females

A) Bodyweight (WT); B) crown rump length (CRL); C) chest circumference; D) abdominal circumference (Abdo); E) hock-to-toe (HT); F) hindlimb length (HL); G) forelimb length (FL); H) biparietal diameter (BPD). Control (green, n=15), FGR-Saline (blue, n=16-18), FGR-IGF1 (red, n=13). Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, p<0.05; ii, p<0.01; iii, time*experimental group interaction p<0.05), letters refer to p-values for differences between experimental groups at each time point (factorial ANOVA: Control vs. Saline: a, p<0.05 and A, p<0.01; Control vs. IGF1: b, p<0.05; B, p<0.01).
**Figure 4.3** Effect of FGR and intra-amniotic IGF1 treatment on early postnatal growth in males

A) Bodyweight (WT); B) crown rump length (CRL); C) chest circumference; D) abdominal circumference (Abdo); E) hock-to-toe (HT); F) hindlimb (HL); G) forelimb (FL); H) biparietal diameter (BPD). Control (green, n=21-22), FGR-Saline (blue, n=13), FGR-IGF1 (red, n=18-19). Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, p<0.05; ii, p<0.01; letters refer to p-values for differences between experimental groups at each time point (factorial ANOVA: Control vs. Saline: a, p<0.05 and A, p<0.01; Control vs. IGF1: b, p<0.05; B, p<0.01).
### Table 4.2 Growth velocity in the first two weeks after birth

Growth velocity (GV) in the first (GV\(_{0-1}\)) and second (GV\(_{1-2}\)) week after birth for body weight (WT; g·kg\(^{-1}\)·d\(^{-1}\)), crown rump length (CRL; cm·m\(^{-1}\)·d\(^{-1}\)), chest circumference (cm·m\(^{-1}\)·d\(^{-1}\)), abdominal circumference (Abdo; cm·m\(^{-1}\)·d\(^{-1}\)), hock-to-toe (HT; cm·m\(^{-1}\)·d\(^{-1}\)), hindlimb (HL; cm·m\(^{-1}\)·d\(^{-1}\)), forelimb (FL; cm·m\(^{-1}\)·d\(^{-1}\)) and biparietal diameter (BPD; mm·cm\(^{-1}\)·d\(^{-1}\)). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, female; †p<0.05, male; ¥p<0.05, female and male combined; ¥¥p<0.01, female and male combined.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
<th>Control</th>
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<th>FGR-IGF1</th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
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<tbody>
<tr>
<td><strong>GV(_{0-1}) WT</strong> ¥</td>
<td>53.6±3.4</td>
<td>62.1±3.2</td>
<td>55.6±4.0</td>
<td>52.3±3.3</td>
<td>63.9±4.2</td>
<td>56.4±3.8</td>
<td>53.0±2.9</td>
<td>62.8±3.1</td>
<td>56.1±3.0</td>
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<tr>
<td><strong>GV(_{0-1}) CRL</strong></td>
<td>2.0±0.2</td>
<td>1.6±0.2</td>
<td>1.8±0.3</td>
<td>1.7±0.2</td>
<td>1.6±0.2</td>
<td>1.7±0.2</td>
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<tr>
<td><strong>GV(_{0-1}) Chest</strong></td>
<td>2.3±0.1</td>
<td>2.4±0.1</td>
<td>2.4±0.1</td>
<td>2.2±0.2</td>
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<tr>
<td><strong>GV(_{0-1}) Abdo</strong></td>
<td>2.7±0.2</td>
<td>3.2±0.2</td>
<td>2.8±0.3</td>
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<tr>
<td><strong>GV(_{0-1}) HT</strong></td>
<td>0.9±0.1</td>
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<td>0.8±0.1</td>
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<tr>
<td><strong>GV(_{0-1}) Abdo</strong></td>
<td>0.6±0.2</td>
<td>1.2±0.2</td>
<td>1.2±0.2</td>
<td>0.6±0.2</td>
<td>1.2±0.2</td>
<td>1.2±0.2</td>
<td>0.7±0.1</td>
<td>1.1±0.1</td>
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<tr>
<td><strong>GV(_{0-1}) FL</strong></td>
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<td>1.0±0.2</td>
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<td>0.8±0.2</td>
<td>1.0±0.2</td>
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<td>1.0±0.1</td>
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<tr>
<td><strong>GV(_{0-1}) BPD</strong></td>
<td>0.8±0.1</td>
<td>0.9±0.1</td>
<td>0.7±0.5</td>
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<tr>
<th></th>
<th>Control</th>
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<th>FGR-IGF1</th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
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<tr>
<td><strong>GV(_{1-2}) WT</strong> ¥</td>
<td>41.2±3.4</td>
<td>46.4±3.2</td>
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<td><strong>GV(_{1-2}) CRL</strong> *</td>
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<td>1.9±0.2</td>
<td>1.6±0.2</td>
<td>1.6±0.1</td>
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<td>1.6±0.1</td>
<td>1.4±0.1</td>
<td>1.8±0.1</td>
<td>1.6±0.1</td>
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<td><strong>GV(_{1-2}) Chest</strong></td>
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<td>0.7±0.1</td>
<td>0.7±0.1</td>
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<td>0.7±0.1</td>
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<tr>
<td><strong>GV(_{1-2}) Abdo</strong></td>
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<tr>
<td><strong>GV(_{1-2}) FL</strong></td>
<td>0.8±0.2</td>
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<tr>
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<tr>
<td></td>
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<td>( n=15 )</td>
<td>( n=13 )</td>
<td>( n=20 )</td>
<td>( n=11 )</td>
<td>( n=14 )</td>
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<tr>
<td>BMC (g) * ¥¥</td>
<td>96±5(^a)</td>
<td>78±4(^b)</td>
<td>83±4(^a,b)</td>
<td>104±4</td>
<td>91±5</td>
<td>91±6</td>
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<tr>
<td>BMD (g·cm(^{-2})) * ¥¥</td>
<td>0.53±0.02(^a)</td>
<td>0.45±0.01(^b)</td>
<td>0.46±0.01(^b)</td>
<td>0.53±0.01</td>
<td>0.49±0.02</td>
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<td></td>
</tr>
<tr>
<td>LM (kg) ¥¥</td>
<td>6.3±0.3</td>
<td>5.6±0.2</td>
<td>5.7±0.3</td>
<td>6.9±0.3</td>
<td>6.1±0.4</td>
<td>6.2±0.3</td>
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<tr>
<td>LM:WT (%)</td>
<td>75.8±0.5</td>
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<td>75.7±0.5</td>
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<td>76.3±1.1</td>
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</table>

Table 4.3 Effect of FGR and intra-amniotic IGF1 treatment on body composition at one week of age

Bone mineral content (BMC), bone mineral density (BMD), lean mass (LM) and lean mass relative to bodyweight (LM:WT). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: ¥¥p<0.01, female and male combined.
4.3.5. Plasma Hormone and Metabolite Concentrations

4.3.5.1. Plasma Insulin and Metabolite Concentrations

FGR-Saline lambs had lower plasma insulin concentration at birth than Control lambs, but these rose to comparable levels by day seven (time*experimental group interaction, \( p=0.05 \), Figure 4.4: B). Female FGR-Saline lambs had higher plasma lactate concentration at birth than both Control and FGR-IGF1 groups, but these fell to similar levels of other groups by day seven (time*experimental group interaction, \( p=0.05 \), Figure 4.4: F). Plasma free fatty acid and urea concentrations and the natural log transformation of the glucose to insulin ratio were not different amongst groups (Figure 4.4: A-E).

Male FGR-Saline lambs had higher plasma free fatty acid concentration at birth than both Control and FGR-IGF1 groups, but these fell to similar levels of other groups by day seven (time*experimental group interaction, \( p=0.05 \), Figure 4.5: D). Male FGR-Saline and FGR-IGF1 lambs had higher plasma lactate concentrations at birth than Control lambs, but these fell to levels similar to that of Controls by day seven (time*experimental group interaction, \( p=0.05 \), Figure 4.5: F). Plasma glucose, insulin, urea, and the glucose to insulin ratio were not different amongst groups (Figure 4.5: A-C, E).

4.3.5.2. Plasma IGF1 Concentrations

FGR-Saline female lambs had lower plasma IGF1 concentration at birth than Control lambs, but these rose to comparable levels by day seven (time*experimental group interaction, \( p=0.05 \), Figure 4.6: A). There were no differences in plasma IGF1 concentration amongst groups in males (Figure 4.6).
4.3.5.3. Plasma Corticosteroid Concentrations
Plasma concentrations of cortisol, cortisone and the 11-deoxycortisol, the cortisol to cortisone ratio, the cortisol:11-deoxycortisol ratio, and the cortisol to weight ratio were all not different amongst groups in either sex (Table 4.4).

4.3.5.4. Plasma Thyroid Hormone Concentrations
Plasma T3 concentration at birth was lower in male FGR-IGF1 lambs than in Controls. Plasma T4 concentration at birth was lower in both female FGR-Saline and FGR-IGF1 lambs than in Controls. In FGR-IGF1 females, plasma T4 concentration remained lower than Controls at seven days after birth. There were no differences in either sex in the T3:T4 ratio (Table 4.4).

4.3.6. Milk Intake
Milk intake in the second week after birth was greater in FGR-IGF1 lambs (p=0.02) compared with Controls but not different from FGR-Saline (162±5, 142±5 and 147±5 ml·kg⁻¹·d⁻¹, respectively), and not different amongst groups in males (Control, 148±5; FGR-Saline, 148±5; FGR-IGF1, 151±5 ml·kg⁻¹·d⁻¹).
<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
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<tr>
<td></td>
<td>n=11-15</td>
<td>n=10-13</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
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<tr>
<td>Cortisol (ng·mL⁻¹)</td>
<td>70.1±9.9</td>
<td>59.5±9.5</td>
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<tr>
<td>Cortisone (ng·mL⁻¹)</td>
<td>20.0±5.6</td>
<td>22.2±5.8</td>
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<tr>
<td>Ln 11-Deoxycortisol</td>
<td>-0.8±0.2</td>
<td>-1.2±0.2</td>
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<tr>
<td>Ln Cortisol:Cortisone Ratio</td>
<td>1.4±0.2</td>
<td>1.5±0.2</td>
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<tr>
<td>Cortisol:11-Deoxycortisol Ratio</td>
<td>155±42</td>
<td>183±45</td>
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<tr>
<td>Ln Cortisol:WT (ng·mL⁻¹·kg⁻¹)</td>
<td>2.4±0.2</td>
<td>2.6±0.2</td>
</tr>
<tr>
<td>T3 (pg·mL⁻¹) †</td>
<td>17.5±1.7</td>
<td>14.0±1.9</td>
</tr>
<tr>
<td>T4 (pmol·mL⁻¹) **</td>
<td>73.0±4.5⁴</td>
<td>49.7±5.8³</td>
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<td>Ln T3:T4 Ratio</td>
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<tr>
<td>Day 7</td>
<td></td>
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</tr>
<tr>
<td>Cortisol (ng·mL⁻¹)</td>
<td>31.2±8.8</td>
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<tr>
<td>Cortisone (ng·mL⁻¹)</td>
<td>8.3±1.3</td>
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<tr>
<td>Ln 11-Deoxycortisol</td>
<td>-1.3±0.5</td>
<td>-0.9±1.2</td>
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<td>Ln Cortisol:Cortisone Ratio</td>
<td>1.6±0.2</td>
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<tr>
<td>Cortisol:11-Deoxycortisol Ratio</td>
<td>98.5±16.9</td>
<td>76.0±19.4</td>
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<td>Ln Cortisol:WT (ng·mL⁻¹·kg⁻¹)</td>
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<tr>
<td>T3 (pg·mL⁻¹)</td>
<td>16.0±0.6</td>
<td>16.7±0.7</td>
</tr>
<tr>
<td>T4 (pmol·mL⁻¹) *</td>
<td>45.2±1.1⁴</td>
<td>44.0±1.3³</td>
</tr>
<tr>
<td>Ln T3:T4 Ratio</td>
<td>-1.5±0.1</td>
<td>-1.1±0.1</td>
</tr>
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</table>

Table 4.4 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormones in the early perinatal period

Plasma concentrations of cortisol and cortisone, the natural log transformation of plasma 11-deoxycortisol, the natural log transformation of the cortisol to cortisone ratio, cortisol to 11-deoxycortisol ratio, the natural log transformation of the cortisol to bodyweight ratio, -3,3',5-triiodothyronine (T3), thyroxine (T4) and the natural log transformation of the T3:T4 ratio. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females, **p<0.01, females; †p<0.05, males.
Figure 4.4 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations in the early postnatal period in female lambs

Plasma concentrations of A) glucose, B) insulin, D) free fatty acids (FFA), E) urea and F) lactate, and the natural log transformation of the glucose to insulin (G:I) ratio in the first two weeks after birth in female Control (green, n=14), FGR-Saline (blue, n=11-14) and FGR-IGF1 (red, n=13) lambs. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, p<0.05; iii, time*experimental group interaction p<0.05), letters refer to p-values for differences between experimental groups at each time point (factorial ANOVA: Control vs. Saline: A, p<0.01; Saline vs. IGF1: C, p<0.01).
Figure 4.5 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations in the early postnatal period in male lambs

Plasma concentrations of A) glucose, B) insulin, D) free fatty acids (FFA), E) urea and F) lactate, and the natural log transformation of the glucose to insulin (G:I) ratio in the first two weeks after birth in male Control (green, n=16), FGR-Saline (blue, n=11) and FGR-IGF1 (red, n=10-11) lambs. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Roman numerals denote the significant difference between experimental groups (RM ANOVA: iii, time*experimental group interaction p<0.05).
Figure 4.6 Effect of FGR and intra-amniotic IGF1 treatment on plasma insulin-like growth factor-1 concentrations in the early postnatal period in lambs

Plasma concentrations of insulin-like growth factor 1 (IGF1) in the first two weeks after birth in A) female Control (green, n=13-15), FGR-Saline (blue, n=10-13) and FGR-IGF1 (red, n=12), and B) male Control (green, n=15-16), FGR-Saline (blue, n=11) and FGR-IGF1 (red, n=9-10) lambs. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: iii, time*experimental group interaction p<0.05), letters refer to p-values for differences between experimental groups at each time point (factorial ANOVA: Control vs. Saline: A, p<0.01).

4.4. Discussion

4.4.1. Effect of IGF1 Treatment on the Growth-Restricted Singleton Ovine Fetus

This is the first study which shows that intra-amniotic IGF1 treatment of the growth-restricted ovine fetus results in a birthweight and size not significantly different from non-instrumented Control lambs. This effect was strongest in female lambs. Previous research suggests that intra-amniotic IGF1 treatment of the growth-restricted sheep increases fetal weight and linear measures at 128 days gestational age (Eremia, et al., 2007), which could be attributed to up-regulation of placental amino acid transporters and thus increased nutrient delivery to the fetus (Wali, et al., 2012). Despite a non-significant 10% reduction in growth velocity of weight in the first week after birth compared to FGR-Saline lambs, lean mass at one week of age was not significantly less in IGF1-treated lambs than in Control lambs, which could indicate increased skeletal muscle protein synthesis prior to birth (Shen, et al., 2003) of a magnitude not great enough to be manifest by a significantly increased birthweight.
Compared to the first week after birth, in the second week, growth velocity of weight of Control and FGR-Saline lambs decreased by 25% and 32%, respectively, whilst growth velocity of weight of FGR-IGF1 lambs decreased by only 17% and was significantly greater than Control lambs during this period suggesting sustained growth during the early postnatal rapid growth phase compared with both Control and saline-treated lambs. Growth velocity of weight in FGR-IGF1 lambs may indicate a sustained increase in postnatal insulin sensitivity. Increased systemic (De Blasio, Gatford, McMillen, et al., 2007) insulin sensitivity following fetal deprivation has previously been reported to potentiate accelerated postnatal growth velocity. Investigating insulin and nutrient signalling in skeletal muscle and liver biopsy tissues collected at one week of age could help clarify tissue-specific mechanisms which could alter early postnatal growth patterns.

Fetal growth restriction in sheep reduces gut growth, maturation and absorptive capacity (Avila, Harding, Rees, & Robinson, 1989). Intra-amniotic IGF1 treatment of fetal growth restriction in the ovine fetus thickens the gut wall and increases fetal-type enterocytes and endocytic complexes, which suggests reduced maturation and increased absorptive capacity (Bloomfield, Bauer, et al., 2002). Abnormal gut development alters nutrient absorption in fetal and postnatal life (Sangild, Fowden, & Trahair, 2000), potentially influencing both feed intake and growth patterns. Whilst we report greater milk intake in FGR-IGF1 females compared with FGR-Saline, structural and functional characteristics of the gut have not been investigated. Data show greater milk intake concurrent with greater growth velocity in the second week of life in FGR-IGF1 females. This could be due to a direct relationship between nutrient intake and somatic growth in the neonatal period in females; however, if gut development is delayed in growth-restricted lambs as reported previously, this may indicate increased efficiency.
4.4.2. Effect of FGR

Although blood lactate concentrations were variable during the embolisation period in fetuses later randomised to the FGR-Saline group, these differences were not statistically significant. Reduced weight and linear measures at birth in FGR-Saline lambs compared with Controls in both males and females suggests that the level of placental embolisation in the current study was sufficient to reduce fetal growth velocity in the ovine fetus. Whilst in humans, lower ponderal index (PI) is indicative of reduced soft tissue growth in the growth-restricted neonate (Landmann, Reiss, Misselwitz, & Gortner, 2006), in the current study the 10% reduction in PI at birth in FGR-Saline lambs was not statistically significant. Previous research suggests that fat deposition in lambs is greatest during rapid postnatal growth (Ricordeau, Boccard, Damiani & van Willigen, 1961) due to activation of adipogenic and lipogenic pathways (Graugnard et al., 2009; Isganaitis et al., 2009) and increased peripheral insulin sensitivity (Gardner et al., 2005). Low birth weight lambs have increased fat accretion in early life compared to high birth weight counterparts (Greenwood, Hunt, Hermanson, & Bell, 1998), whilst fetal growth restriction induced by carunclectomy increases early postnatal accumulation of visceral adipose (De Blasio, Gatford, Robinson, et al., 2007). Despite the increased growth velocity in the first week after birth in FGR-Saline lambs compared to Controls, fat mass at one week of age was below the level of detection by DXA. Interestingly, the accelerated growth velocity in saline-treated lambs was not associated with increased milk intake, which could suggest greater feed efficiency, rather than greater feeding activity (De Blasio, Gatford, McMillen, et al., 2007). In addition to dynamic testing of tissue accretion using isotopic metabolite studies, future investigations into molecular adipogenic pathways would be useful in elucidating postnatal tissue-specific metabolic pathways of nutrient uptake and responsiveness following fetal growth restriction.
4.4.3. Hormone Ontogeny in the Growth-Restricted Ovine Fetus

4.4.3.1. HPA Axis Activation

Although previous research in intra-amniotic IGF1-treatment of the placental insufficiency-induced growth restricted ovine fetus reported decreased fetal plasma cortisol concentrations in the late gestation growth-restricted fetus compared to saline-treated fetuses (Wali, et al., 2012), there was not a significant difference amongst groups in plasma cortisol concentration at birth. Interestingly, these data are not consistent with cortisol profiles at birth in either the growth-restricted human (Verkausiene, et al., 2007) or sheep (Morrison, 2008). High variability in plasma cortisol after birth in the current study could be due to dramatic changes in plasma cortisol from birth to the time at which the first blood sample was collected. Although, these data could also suggest that the embolisation procedure was not sufficient to up-regulate HPA axis activity. This may also indicate that up-regulated HPA axis activity prior to birth in response to a stressful intrauterine environment has not resulted in a fundamental change in the HPA axis homeostatic set-point in the first two weeks after birth. This is consistent with human literature of cortisol concentrations in growth-restricted newborns after birth (Bazaes, et al., 2003). Indeed, cortisol concentrations tended to be decreased (20%) in FGR-Saline lambs, perhaps suggesting a degree of HPA suppression after birth (Osterholm, Hostinar, & Gunnar, 2012; Schaffer et al., 2009); however, as samples were taken within two hours after birth, dramatic changes in plasma cortisol concentration following birth (Chantal Wrutniak & Cabello, 1987) could contribute to the high level of variability we report, especially in male lambs.

4.4.3.2. Thyroid Hormone Concentration

Plasma T3 has been suggested to promote accelerated postnatal growth velocity and adipose deposition (De Blasio, et al., 2006). Although postnatal growth velocity of weight was
accelerated in FGR-Saline and FGR-IGF1 lambs, plasma T3 concentration was decreased in FGR-IGF1 males at birth. Concurrent with a 20% (non-significant) decrease in plasma T4 concentration and the natural log transformation of the plasma T3:T4 ratio in IGF1-treated males compared with Controls, this could suggest compromised thyroid hormone secretion. The pre-partum rise in plasma T3 is associated with hepatic upregulation of phosphoenolpyruvate kinase (PEPCK) and glucose-6-phosphatase (G6P) expression (Forhead, Poore, Mapstone, & Fowden, 2003) and increased hepatic glyconeogenesis (Alison J. Forhead, et al., 2009); thus, reduced plasma T3 in IGF1-treated growth-restricted males could suggest compromised gluconeogenic capacity at birth. Although not significant, a 2.0 mmol·L⁻¹ decrease in plasma glucose concentration in FGR-IGF1 males compared with Control and FGR-Saline groups 1-2 hours after birth could support this. Assessment of hepatic molecular gluconeogenic genes in liver biopsies taken at this time could help determine whether reduced hepatic glucose transporter SLC2A2 mRNA expression in IGF1 males (Chapter 5) is associated with compromised hepatic glucose metabolism in the early postnatal period.

Growth restriction in female lambs reduced plasma T4 concentrations compared with Controls at birth. Whilst plasma T4 concentration recovered in saline-treated females by day seven, it remained suppressed in IGF1-treated females. Reduced relative thyroid mass in adulthood (Chapter 6) could suggest lesser capacity for T4 secretion. Although, as previous fetal research using the current paradigm does not suggest that intra-amniotic IGF1 treatment alters relative thyroid mass (Wali, et al., 2012), reduced plasma T4 concentrations may be due to reduced activity of the hypothalamic-pituitary-thyroid axis. Indeed, in the FGR-IGF1 female, HPA axis testing at 18 months of age suggests reduced pituitary activity (Chapter 8), which could, thus, result in reduced thyroid activity.
4.4.3.3. Somatotrophic Axis Activity

Neonatal plasma IGF1 concentrations in the perinatal period (Figure 4.6) were higher than fetal concentrations in previous research using the same embolisation methodology (Wali, et al., 2012). This difference is consistent with observations in postnatal sheep, which report greater plasma IGF1 concentrations in the first week following birth than during fetal life (Gluckman & Butler, 1983). Plasma IGF1 concentration was decreased in FGR-Saline females compared with Controls at birth. This is consistent with the large body of literature reporting an association between IGF1 concentration at birth and fetal weight (Bennett et al., 1983; Ong, Kratzsch, Kiess, ALSPAC Study Team, et al., 2000; Ong, Kratzsch, Kiess, Costello, et al., 2000; Verhaeghe et al., 1993). In previous fetal studies of IGF1-treatment of the growth-restricted ovine fetus, a decrease in plasma IGF1 concentrations was observed (Bloomfield, Bauer, et al., 2002). In the current study, plasma IGF1 concentrations were not different from Controls at birth. Despite suppression of fetal hepatic IGF1 mRNA expression during intra-amniotic IGF1 treatment (Darp, et al., 2010), the data in this chapter suggest that following cessation of the intervention, circulating plasma IGF1 concentrations after birth are not compromised in either sex.

4.5. Limitations

Ewe milk composition varies seasonally (Sevi, Albenzio, Marino, Santillo, & Muscio, 2004). We did not assess milk composition in the current study and thus assume comparable macro- and micro-nutrient concentrations amongst groups.

4.6. Conclusions

Weekly intra-amniotic IGF1 treatment of fetal growth restriction does not affect fetal or perinatal mortality, early perinatal morbidity, or gestational length. However, intra-amniotic IGF1 treatment abrogates the growth restriction-mediated reduction in birthweight and body
size in females, and reduces the accelerated postnatal growth velocity of weight in the first week of life evident in saline-treated lambs. Whilst early postnatal growth velocity is consistent between sexes, in males IGF1 treatment did not appreciably alter weight or body size at birth compared with FGR-Saline. Future studies should assess sex-specific fetal adaptations to determine whether innate mechanistic differences perpetuate sexually dimorphic somatic growth in response to intra-amniotic IGF1 treatment. This sexually dimorphic response could suggest that intrinsic differences to developmental stimuli persist postnatally.

Both fetal growth restriction and accelerated postnatal growth velocity are implicated in chronic disease development. Accelerated postnatal growth velocity of weight in the second week of life in FGR-IGF1 lambs compared with Control could suggest that the period during which accelerated early postnatal growth contributes to the susceptibility of chronic disease development later in life is prolonged in IGF1-treated growth-restricted lambs. Although preliminary investigations of somatotrophic and metabolic adaptation at one week of age in skeletal muscle and liver biopsies have been conducted (Chapter 5), thorough elucidation of molecular mechanisms of adaptation to intra-amniotic IGF1 treatment of fetal growth restriction in these lambs is necessary to determine whether the intervention results in beneficial effects at the tissue level, which may not be apparent at the whole-body level.
Chapter 5. Hepatic and Skeletal Muscle mRNA Expression at One Week of Age

5.1. Introduction

The somatotrophic axis, which promotes fetal growth throughout gestation in autocrine and paracrine fashion (Murray & Clayton, 2013), is regulated predominantly through nutritional supply (Bloomfield, et al., 2013). Maturation of the somatotrophic axis and endocrine activation occurs around parturition (Gluckman & Butler, 1983). Cortisol-mediated activation of the adult growth hormone receptor in liver (Li, Gilmour, Saunders, Dauncey, & Fowden, 1999) and reduction of paracrine IGF1 synthesis in skeletal muscle (Forhead, et al., 2002; Li, Forhead, Dauncey, Gilmour, & Fowden, 2002) facilitates the shift from fetal to adult somatotrophic regulation (Li et al., 1996). Circulating IGF1 concentration is positively correlated with fetal growth in the sheep (Owens, Kind, Carbone, Robinson, & Owens, 1994), and with in utero growth potential (Verhaeghe et al., 2003) and birth weight (Verhaeghe, et al., 1993) in the human. Reduced cord blood IGF1 and IGFBP3 concentrations at birth in both small for gestational age and appropriate for gestational age babies with fetal growth restriction, defined as a reduction by more than 20 percentiles from 22 weeks gestation to birth, compared to those with stable intrauterine growth (Verkauskiene, et al., 2007) could suggest reduced somatotrophic sensitivity due to fetal malnutrition (Leger, Noel, Limal, & Czernichow, 1996; Ohkawa et al., 2010).

Postnatally, increased plasma IGF1, IGF2 and IGFBP3 concentrations in FGR children when compared to height, weight and puberty-matched controls (Cutfield, et al., 2002) may suggest later somatotrophic insensitivity. This could decrease capacity for growth through childhood, resulting in the failure to attain age-appropriate body size (Chakraborty, Joseph,
Bankart, Petersen, & Wailoo, 2007). Growth hormone resistance in the low birth weight child (Cutfield et al., 2004) and altered skeletal muscle growth patterns in the fetal growth-restricted child following one year of growth hormone treatment (Lee, Blizzard, Cheek, & Holt, 1974) could suggest that an early-life intervention, prior to the development of somatotrophic insensitivity, is more appropriate. Antenatal intervention to modulate the fetal growth restriction-mediated in utero somatotrophic down-regulation could provide a potential therapeutic avenue to regulate growth in the growth-restricted offspring postnatally.

Although previous in vivo ovine studies have investigated alterations in fetal expression of select somatotrophic axis genes in response to FGR and IGF1-treatment of FGR, the postnatal effects of IGF1 treatment on tissue-specific somatotrophic axis genes have not been investigated. Thus, the aim of this experiment was to investigate the effect of intra-amniotic IGF1-treatment of the growth-restricted ovine fetus on skeletal muscle and hepatic mRNA expression of key genes at one week of age.

5.2. Methods

To determine the tissue-specific effects of FGR and intra-amniotic IGF1 treatment of FGR as close to birth as possible without potentially compromising lamb survival, mRNA expression at one week of age was analysed in tissue obtained via percutaneous biopsy, as previously described (Section 2.22). Quantitative real-time polymerase chain reaction was conducted on skeletal muscle (Female: Control, n=8; FGR-Saline, n=6; FGR-IGF1, n=4 and Male: Control, n=8; FGR-Saline, n=7; FGR-IGF1, n=8), and liver (Female: Control, n=7; FGR-Saline, n=8; FGR-IGF1, n=8 and Male: Control, n=8; FGR-Saline, n=8; FGR-IGF1, n=8) biopsies.
5.3. Results

5.3.1. Effects of FGR and Intra-amniotic IGF1 Treatment of FGR on Skeletal Muscle mRNA Expression at One Week of Age

In females, FGR decreased GHR expression to 75% and increased IGF2 and IGF1R to 129% and 114%, respectively, of Control values (Figure 5.1: A). IGF1-treatment of FGR increased GHR, IGF1 and IGFBP3 and decreased IGF1R expression to 109%, 153%, 116% and 89%, respectively, of Control, and to 144%, 173%, 126% and 78%, respectively, of FGR-Saline values whilst IGF2 expression was increased to 116% of Control values and decreased to 90% of FGR-Saline values (Figure 5.1: B and C).

FGR decreased glucose transporter SLC2A4 and MTOR, and increased glucocorticoid receptor NR3C1 to 82%, 74% and 105%, respectively, of Control values (Figure 5.2: A). IGF1-treatment of FGR increased MTOR to 128% and 174%, respectively, of Control and FGR-Saline values, NR3C1 expression to 104% of Control values and SLC2A4 expression to 121% of FGR-Saline values (Figure 5.2: B and C).

In males, FGR decreased IGF1 to 87% and increased IGF2, IGF1R and IGFBP3 to 160%, 112% and 114%, respectively, of Control values (Figure 5.2: A). IGF1-treatment of FGR decreased GHR and increased IGF1R to 74% and 133%, respectively, of Control and to 69% and 119%, respectively, of FGR-Saline values, whilst IGF2 expression increased to 203% of Control values and IGF1 increased 121% of FGR-Saline values (Figure 5.3: B and C).

FGR increased SLC2A4 to 161% of Control values (Figure 5.4: A). IGF1-treatment of FGR increased SLC2A4, MTOR, and NR3C1 to 294%, 246% and 213%, respectively, of Control and to 183%, 182% and 173%, respectively, of FGR-Saline values (Figure 5.4: B and C).
Results are reported as fold-change with a 99% confidence interval relative to Control (n=8): (A) FGR-Saline (n=6) and (B) FGR-IGF1 (n=4); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes beta actin (ACTB), peptidylprolyl isomerase A (PPIA) and hypoxanthine phosphoribosyltransferase 1 (HRPT1). Data points with error bars not intersecting the x-axis are significantly different.
Figure 5.2 Skeletal muscle mRNA expression at one week of age in females

Results are reported as fold-change with a 99% confidence interval relative to Control (n=8): (A) FGR-Saline (n=6) and (B) FGR-IGF1 (n=4); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes beta actin (ACTB), peptidylprolyl isomerase A (PPIA) and hypoxanthine phosphoribosyltransferase 1 (HRPT1). Data points with error bars not intersecting the x-axis are significantly different.
Figure 5.3 Skeletal muscle somatotrophic mRNA expression at one week of age in males

Results are reported as fold-change with a 99% confidence interval relative to Control (n=8): (A) FGR-Saline (n=7 and (B) FGR-IGF1 (n=8); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes beta actin (ACTB), peptidylprolyl isomerase A (PPIA) and hypoxanthine phosphoribosyltransferase 1 (HRPT1). Data points with error bars not intersecting the x-axis are significantly different.
Figure 5.4 Skeletal muscle mRNA expression at one week of age in males

Results are reported as fold-change with a 99% confidence interval relative to Control (n=8): (A) FGR-Saline (n=7) and (B) FGR-IGF1 (n=8); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes beta actin (ACTB), peptidylprolyl isomerase A (PPIA) and hypoxanthine phosphoribosyltransferase 1 (HRPT1). Data points with error bars not intersecting the x-axis are significantly different.
5.3.2. Effects of FGR and Intra-amniotic IGF1 Treatment of FGR on Hepatic mRNA Expression at One Week of Age

In contrast to skeletal muscle, FGR and IGF1 treatment had no effect on hepatic mRNA expression of GHR in either females or males (Figures 5.5 and 5.7).

In females, FGR decreased IGF1 to 63% and increased IGF2, IGF1R and IGFALS to 117%, 338% and 122%, respectively, of Control values without a change in IGFBP3 (Figure 5.5: A). IGF1 treatment of FGR increased IGF1R and IGFBP1 to 249% and 256%, respectively, of Control values and decreased IGFBP3 to 83% of Control values without a change in IGFALS (Figure 5.5: B). Compared to FGR-Saline values, IGF1 treatment increased IGF1 to 126% but decreased IGF1R and IGFBP3 expression to 74% and 84%, respectively (Figure 5.5: C).

FGR decreased SLC2A2 to 50% and increased MTOR and NR3C1 to 199% and 207%, respectively, of Control values (Figure 5.6: A). IGF1-treatment of FGR decreased SLC2A2 to 50% and increased MTOR and NR3C1 to 161% and 160%, respectively, of Control values, and decreased MTOR and NR3C1 to 81% and 77%, respectively, of FGR-Saline values (Figure 5.6: B and C).

In males, FGR decreased IGF1, IGFBP1 and IGFBP3 to 81%, 22% and 53%, respectively, and increased IGFALS to 146% of Control values (Figure 5.7: A). IGF1-treatment of FGR decreased IGF2, IGF1R, IGFALS, IGFBP1 and IGFBP3 to 62%, 83%, 80%, 35% and 52%, respectively, of Control values (Figure 5.7: B), decreased IGF2, IGF1R and IGFALS to 60%, 77% and 55%, respectively, and increased IGF1 and IGFBP1 to 181% and 159%, respectively, of FGR-Saline values (Figure 5.7: C).

FGR increased NR3C1 to 140% of Control values (Figure 5.8: A), but had no effect on SLC2A2 or MTOR. IGF1-treatment of FGR decreased SLC2A2 to 68% of Control and 66% of FGR-Saline values, and decreased NR3C1 to 76% of FGR-Saline values (Figure 5.8: B and C).
Figure 5.5 Hepatic somatotrophic mRNA expression at one week of age in females

Results are reported as fold-change with a 99% confidence interval relative to Control (n=7): (A) FGR-Saline (n=8) and (B) FGR-IGF1 (n=8); or relative to FGR-Saline: (C) FGR-IGF1. Data are normalised to the geometric (GEO) mean of housekeeping genes GAPDH, hypoxanthine phosphoribosyltransferase 1 (HRPT1) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ). Data points with error bars not intersecting the x-axis are significantly different.
Figure 5.6 Hepatic mRNA expression at one week of age in females

Results are reported as fold-change with a 99% confidence interval relative to Control (n=7): (A) FGR-Saline (n=8) and (B) FGR-IGF1 (n=8); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes GAPDH, hypoxanthine phosphoribosyltransferase 1 (HRPT1) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ). Data points with error bars not intersecting the x-axis are significantly different.
Figure 5.7 Hepatic somatotrophic mRNA expression at one week of age in males

Results are reported as fold-change with a 99% confidence interval relative to Control (n=8): (A) FGR-Saline (n=8) and (B) FGR-IGF1 (n=8); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes GAPDH, hypoxanthine phosphoribosyltransferase 1 (HRPT1) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ). Data points with error bars not intersecting the x-axis are significantly different.
Figure 5.8 Hepatic mRNA expression at one week of age in males

Results are reported as fold-change with a 99% confidence interval relative to Control (n=8): (A) FGR-Saline (n=8) and (B) FGR-IGF1 (n=8); or relative to FGR-Saline: (C) FGR-IGF. Data are normalised to the geometric (GEO) mean of housekeeping genes GAPDH, hypoxanthine phosphoribosyltransferase 1 (HRPT1) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ). Data points with error bars not intersecting the x-axis are significantly different.
5.4. Discussion

These data show that brief intra-amniotic IGF1 treatment of fetal growth restriction in the sheep alters hepatic and skeletal muscle mRNA expression of key genes in the offspring one week after birth compared with both Control and growth-restricted saline-treated lambs. The somatotrophic axis is sexually dimorphic: males, which are larger at birth than females, have lower mean cord plasma IGF1 and IGFBP3 than females but higher plasma growth hormone, suggesting greater somatotrophic sensitivity (Geary, et al., 2003). Sexually divergent somatotrophic axis maturation and innate differences in fetal growth hormone sensitivity are apparent during fetal life in the sheep (Bassett & Gluckman, 1986) and persist postnatally. The sexually dimorphic somatotrophic response to intra-amniotic IGF1 treatment of fetal growth restriction in the current study was apparent in differential hepatic and skeletal muscle mRNA expression at one week of age. This could be associated with altered perinatal metabolic processes and, ultimately, growth patterns.

5.4.1. Effects of FGR and Intra-Amniotic IGF1 Treatment on mRNA Expression at One Week of Age

Greater skeletal muscle IGF2 expression at seven days of age in FGR-Saline and FGR-IGF1 male lambs compared with Controls is concurrent with rapid somatic growth (Chapter 4) and, thus, suggests delayed postnatal deceleration of growth velocity (Finkielstain et al., 2009; Lui, Finkielstain, Barnes, & Baron, 2008). Previous research reports that circulating IGF2 concentrations in sheep fall to adult levels by 12 hours post-birth (Gluckman & Butler, 1983). However, at six months of age hepatic abundance of IGF2 receptor protein is constitutively up-regulated in nutrient-mediated late gestation fetal growth-restricted offspring with no difference in growth hormone receptor (Hyatt, Walker, Stephenson, & Symonds, 2004) and could suggest an IGF2-mediated mechanism for “catch-up growth” in the growth-restricted sheep during the postnatal period. A concurrent increase in skeletal muscle SLC2A4 and
MTOR could also suggest that increased peripheral nutrient sensitivity and uptake contributes to accelerated growth velocity of weight in the second week of life in FGR-IGF1 males (Chapter 4).

Increased skeletal muscle IGF2 in FGR-Saline females and decreased GHR could suggest an IGF2-mediated suppression of somatotrophic maturation in this tissue, whilst increased GHR and IGF1 expression in FGR-IGF1 females could suggest treatment-mediated recovery of somatotrophic function. Interestingly, this was concurrent with increased MTOR expression and greater milk intake in the second week of life compared with Controls (Chapter 4); although, hepatic IGF2 was not different amongst groups. Tissue samples were taken during a rapid growth phase (Chapters 4 and 6). Thus, we cannot discount the coordinate up-regulation of growth-promoting pathways contributing to accelerated organ growth during this period (Finkielstain, et al., 2009; Lui, et al., 2008; Lui et al., 2010).

In males a growth restriction-mediated reduction in hepatic IGFBP1 and IGFBP3 could suggest reduced endocrine IGFBP secretion and, thus increased capacity for somatotrophic growth. An increase in IGF1 in FGR-IGF1 males could suggest an intra-amniotic IGF1-mediated increase in IGF1 bioavailability which could further promote growth in the early postnatal period (Chapter 4). Together, these data could provide a mechanism for accelerated growth velocity in FGR-IGF1 males in the second week after birth. However, marked variability in hepatic GHR expression suggests that inherent differences in somatotrophic response to intra-amniotic IGF1 treatment could exist in males and should, thus, be interpreted with care. In females, fetal growth restriction resulted in a dramatic up-regulation of hepatic IGF1R expression, which was not altered by intra-amniotic treatment. This was concurrent with increased MTOR and NR3C1, which could suggest that increased nutrient-sensitivity at one week of age and accelerated growth velocity in the early postnatal
period (Chapter 4) may occur despite hepatic glucocorticoid insensitivity. Marked (~2.5-6.0-fold) variance in IGFBP1 expression in FGR-Saline females, which is amplified with intra-amniotic IGF1 treatment, suggests that innate differences in the adaptation to both fetal growth-restriction and IGF1 treatment should be considered when interpreting these data.

Fetal growth restriction can result in abnormal growth hormone responsiveness and IGF1 synthesis in the ovine fetus (Kind et al., 1995), which could suggest disruption of somatotrophic axis maturation. Previous fetal research using the current paradigm shows comparable right hepatic lobe IGF1 and IGF1R mRNA expression compared with Controls and decreased compared with FGR-Saline (Darp, et al., 2010) with no difference amongst groups in relative or absolute liver weight (Wali, et al., 2012). Together, these data could suggest that following discontinuation of intra-amniotic IGF1 treatment, somatotrophic suppression is terminated, resulting in postnatal up-regulation concurrent with greater nutrient-mediated pathways in the periphery. However, as there is no protein or epigenetic data available, these data require more thorough investigation in order to elucidate significant mechanistic alterations. We acknowledge that discrete and limited tissue-specific measurements should be interpreted with care when inferring somatotrophic axis alteration in the perinatal period. Future time-course studies from birth throughout the early postnatal period could help determine the timing and magnitude of tissue-specific somatotrophic alterations in response to intra-amniotic IGF1 treatment.

5.5. Limitations

Due to the small number of samples analysed in the female FGR-IGF1 group, the accuracy of the results is debateable. Additional samples must be analysed to increase the robustness of the data.
Expression of IGF system genes differ amongst skeletal muscle types in the sheep (Nattrass et al., 2006). Although every effort has been made to obtain skeletal muscle tissue solely from the vastus lateralis, procedural error is a possibility.

IGF system mRNA expression differs between the two hepatic lobes (Darp, et al., 2010). Although every effort has been made to obtain tissue solely from the right lobe of the liver, procedural error is a possibility.

5.6. Conclusions

Weekly intra-amniotic IGF1 treatment of the growth-restricted fetus results in postnatal alteration in the mRNA expression of somatotrophic and nutrient sensitive genes at one week of age. Whether apparent changes in mRNA expression in IGF1-treated lambs are advantageous in abrogating growth restriction-mediated alterations in somatotrophic sensitivity remains unclear. Thorough investigation of fetal hypothalamic, pituitary and adrenal tissues from previous studies could help determine whether this intervention alters regulation of the HPA axis in utero and affects change in somatotrophic axis maturation near term. Although sexually dimorphic responses could suggest that a sex-specific intervention for the treatment of the growth-restricted fetus should be considered, comprehensive elucidation of tissue-specific molecular adaptation to intra-amniotic IGF1 intervention would help to determine if these mechanisms endure. From the limited data available in this study and previous fetal work, it is not feasible to conclude whether these changes reported in the perinatal period are of importance.
Chapter 6. Postnatal Plasma Hormone and Metabolites, Growth, Body Composition, and Post Mortem Carcass and Organ Characteristics

6.1. Introduction

Whilst early embryonic and fetal development establish a framework for progressive somatic growth throughout life, fetoplacental integration of environmental cues influences fetal growth, tissue maturation and ultimately postnatal survival (Sandovici, et al., 2012). Aberrant metabolic (Danielsen, Moeller, & Rewitz, 2013) and endocrine (Fowden & Forhead, 2004) signals in the environmental milieu, such as those present during placental insufficiency, can result in in utero physiological adaptation to deprivation. Although postnatal organ growth is determined in early life (Lui, et al., 2010), fetal growth restriction results in a phenotypic adaptation apparent in reduced organ and somatic growth of the organism. Circulatory adaptation to fetal growth restriction resulting in disproportionate growth and “brain-sparing” is commonly reported (Ebbing, Rasmussen, Godfrey, Hanson, & Kiserud, 2009; Godfrey, et al., 2012). The preservation of adequate blood supply during fetal life to the developing brain occurs at the expense of peripheral tissues, such as the liver. Interestingly, recent research suggests that maternal undernutrition which does not reduce brain or body weight in the mid-gestation non-human primate fetus nonetheless compromises cerebral development (Antonow-Schlorke, et al., 2011). This could suggest that despite appropriate gross fetal somatic and brain growth, growth restriction-mediated alterations in development occur and could, thus compromise postnatal function.

In the ovine fetus, compromised growth, such as that seen in the brain (Rees, et al., 1988), pancreas (Limesand et al., 2013) and liver (Cheung, et al., 2004), and compromised
development, such as that seen in the gut (Bloomfield, Bauer, et al., 2002), skeletal muscle
(Costello, et al., 2008) and heart (Bubb, et al., 2007) can alter postnatal functional capacity
and thus developmental trajectory. Accelerated postnatal growth velocity, which is an
upward crossing of centiles, can be a compensatory adaptation to fetal growth restriction
(Claris, Beltrand, & Levy-Marchal, 2010). The growth-restricted neonate has reduced lean
mass and subcutaneous lipid stores at birth (Thorn, et al., 2011), and is prone to accelerated
neonatal growth, which is associated with increased visceral adiposity in childhood (Ong,
Ahmed, Emmett, Preece, & Dunger, 2000) and early adulthood (Leunissen, Kerkhof, Stijnen,
& Hokken-Koelega, 2009). Fetal metabolic adaptations to an adverse intrauterine
environment which promote survival may perpetuate adverse alterations in postnatal body
composition, which could compromise later health of the offspring.

Consistent with observations in humans (Diderholm, et al., 2007; Enzi, Zanardo, Caretta,
Inelmen, & Rubaltelli, 1981; Perucchin et al., 2011), fetal growth restriction in sheep reduces
soft tissue accretion and size at birth, and is associated with accelerated postnatal growth
velocity and increased insulin action via enhanced insulin sensitivity (De Blasio, Gatford,
McMillen, et al., 2007). In the placental insufficiency-induced growth-restricted fetus,
upregulation of insulin-signalling, gluconeogenic and glucose storage pathways concurrent
with reduced activation of nutrient sensors provides both a mechanism for in utero survival
and postnatal metabolic adaptation resulting in accelerated growth (Morrison, 2008; Thorn,
et al., 2009). Indeed, in fetal growth-restricted male sheep, increased early postnatal growth
velocity is associated with impaired early glucose clearance during a glucose challenge at one
year of age, whilst in females growth restriction resulting in thinness at birth is associated
with increased insulin sensitivity and size in adulthood (Owens, et al., 2007). Intra-amniotic
IGF1-treatment of fetal growth restriction increases fetal growth in the sheep (Wali, et al.,
2012), but the effects of IGF1 treatment on postnatal growth, body composition, and plasma hormones and metabolite concentrations have not been investigated. Thus, the aim of this experiment was to investigate the effect of intra-amniotic IGF1 treatment of the growth-restricted ovine fetus on postnatal growth, body composition and plasma hormone and metabolite concentrations through to 18 months of postnatal age.

6.2. Methods

The progression of animals through the time-course of the experiment is shown in Chapter 3, Figure 3.1. Phenotypic investigations (Section 2.11, 2.13, and 2.21) and metabolite and hormone analyses (Section 2.12) were performed at specified time points. Statistical analyses were conducted as previously described (Section 2.23).

6.3. Results

6.3.1. Postnatal Growth Through 18 Months of Age

FGR-Saline and FGR-IGF1 females had a shorter crown-rump length and reduced chest circumference, without comparable weight through 18 months of age compared with Controls. Both FGR-Saline and FGR-IGF1 males had a smaller chest circumference and hock-to-toe length compared with Controls, whilst FGR-IGF1 males were lighter than Controls at 18 months of age (Figure 6.1). In females, growth velocity of the biparietal diameter from 4-8 months of age was greater in FGR-Saline compared with Control females (Table 6.1).

Growth velocities of weight and a variety of linear measures were not different amongst groups in females over any period beyond the early postnatal period (Table 6.1). In males, growth velocity of the hindlimb from 0.5-4 months of age and growth velocity of the biparietal diameter from 12-18 months of age were greater in FGR-Saline compared with Control (Table 6.2).
6.3.2. Body Composition

Body composition assessed by DXA (see Chapter 2) was not different between the groups of males at 4, 12 or 18 months (Tables 6.3-6.5). Whilst FGR-IGF1 females had lesser absolute lean mass at 4 months than Controls, there were no differences amongst groups at 12 months. At 18 months in females, chest lean mass was less compared with Controls, total lean mass relative to bodyweight was greater and relative abdominal fat mass less than Controls (Tables 6.3-6.5).
Figure 6.1 Effect of FGR and intra-amniotic IGF1 treatment on growth through to 18 months of age

In the above graphs females are on the left panel, males on the right panel. Bodyweight (WT), crown rump length (CRL), chest circumference, and hock-to-toe (HT) in Control (green: females, n=11; males, n=20), FGR-Saline (blue: females, n=13; males, n=10) and FGR-IGF1 (red: females, n=12-13; males, n=12-14) sheep. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (i, experimental group, p<0.05; ii, experimental group, p<0.01; iii, experimental group*time interaction, p<0.05).
Table 6.1 Effect of FGR and intra-amniotic IGF1 treatment on growth in females

Growth velocity from 0.5-4 (GV0.5-4), 4-8 (GV4-8), 8-12 (GV8-12) and 12-18 (GV12-18) months of age for body weight (WT; g·kg⁻¹·d⁻¹), crown rump length (CRL; cm·m⁻¹·d⁻¹), chest circumference (cm·m⁻¹·d⁻¹), abdominal circumference (Abdo; cm·m⁻¹·d⁻¹), hock-to-toe (HT; cm·m⁻¹·d⁻¹), hindlimb (HL; cm·m⁻¹·d⁻¹), forelimb (FL; cm·m⁻¹·d⁻¹) and biparietal diameter (BPD; mm·cm⁻¹·d⁻¹). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=11</td>
<td>n=13</td>
<td>n=13</td>
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<tr>
<td>GV0.5-4 WT</td>
<td>11.9±0.7</td>
<td>12.3±0.7</td>
<td>11.6±0.7</td>
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<td>4.2±0.4</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>GV0.5-4 Chest</td>
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<td>4.2±0.3</td>
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<td>5.6±0.2</td>
<td>5.1±0.3</td>
</tr>
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<td>2.0±0.2</td>
</tr>
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<td>GV 4-8 Abdo</td>
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<td>0.5±0.1</td>
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<tr>
<td>GV 4-8 HT</td>
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<td>0.4±0.1</td>
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<td>n=12</td>
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<td>0.01±0.01</td>
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<td>0.04±0.02</td>
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<tr>
<td>GV 12-18 BPD</td>
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<td>0.04±0.02</td>
<td>0.03±0.02</td>
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<tr>
<td></td>
<td>Control</td>
<td>FGR-Saline</td>
<td>FGR-IGF1</td>
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<td>---------------</td>
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</tr>
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<td>n=20</td>
<td>n=10</td>
<td>n=14</td>
</tr>
<tr>
<td>GV0.5-4 WT</td>
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<td>11.7±0.4</td>
<td>12.3±0.4</td>
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</table>

Table 6.2 Effect of FGR and intra-amniotic IGF1 treatment on growth in males

Data are least square means ± SEM. Growth velocity from 0.5-4 (GV0.5-4), 4-8 (GV4-8), 8-12 (GV8-12) and 12-18 (GV12-18) months of age for body weight (WT; g·kg⁻¹·d⁻¹), crown rump length (CRL; cm·m⁻¹·d⁻¹), chest circumference (cm·m⁻¹·d⁻¹), abdominal circumference (Abdo; cm·m⁻¹·d⁻¹), hock-to-toe (HT; cm·m⁻¹·d⁻¹), hindlimb (HL; cm·m⁻¹·d⁻¹), forelimb (FL; cm·m⁻¹·d⁻¹) and biparietal diameter (BPD; mm·cm⁻¹·d⁻¹). Values with different superscripts report significant differences amongst experimental groups on post hoc testing: †p<0.05.
Table 6.3 Effect of FGR and intra-amniotic IGF1 treatment on body composition at 4 months of age

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<th>Male</th>
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<tr>
<td>BMC (g)</td>
<td>390±44</td>
<td>347±43</td>
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<tr>
<td>BMD (g·cm⁻²)</td>
<td>0.99±0.05</td>
<td>0.93±0.05</td>
</tr>
<tr>
<td>LM (kg)</td>
<td>28.2±2.2ᵃ</td>
<td>25.7±2.2ᵃᵇ</td>
</tr>
<tr>
<td>LM:WT (%)</td>
<td>80.1±3.0</td>
<td>78.5±3.0</td>
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</tbody>
</table>

*Bone mineral content (BMC), bone mineral density (BMD), lean mass (LM) and lean mass relative to bodyweight (LM:WT). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: ᵃp<0.05, female.
<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th></th>
<th>Male</th>
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<tbody>
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<td><strong>BMC (g)</strong></td>
<td>745±45</td>
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<td><strong>BMD (g·cm⁻²)</strong></td>
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<td><strong>Chest LM (kg)</strong></td>
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<td><strong>Chest FM (kg)</strong></td>
<td>2.5±0.6</td>
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<td><strong>Abdo LM (kg)</strong></td>
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<td><strong>Abdo FM (kg)</strong></td>
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<td><strong>Rump LM (kg)</strong></td>
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<td><strong>Rump FM (kg)</strong></td>
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<td><strong>Total LM (%)</strong></td>
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<td><strong>Total FM (%)</strong></td>
<td>8.4±2.0</td>
<td>7.0±1.9</td>
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<td><strong>LM:WT (%)</strong></td>
<td>70.0±1.5</td>
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<td><strong>FM:WT (%)</strong></td>
<td>14.1±2.7</td>
<td>12.4±2.6</td>
<td>12.1±2.6</td>
</tr>
<tr>
<td><strong>Chest FM:WT (%)</strong></td>
<td>4.2±0.8</td>
<td>3.7±0.8</td>
<td>3.6±0.8</td>
</tr>
<tr>
<td><strong>Abdo FM:WT (%)</strong></td>
<td>6.8±1.3</td>
<td>6.0±1.2</td>
<td>5.8±1.3</td>
</tr>
<tr>
<td><strong>Rump FM:WT (%)</strong></td>
<td>3.0±0.6</td>
<td>2.7±0.5</td>
<td>2.6±0.6</td>
</tr>
<tr>
<td><strong>Abdo FM:Total FM (%)</strong></td>
<td>48.8±0.6</td>
<td>48.5±0.5</td>
<td>47.3±0.7</td>
</tr>
</tbody>
</table>

Table 6.4 Effect of FGR and intra-amniotic IGF1 treatment on body composition at 12 months of age

Bone mineral content (BMC), bone mineral density (BMD), lean mass (LM), fat mass (FM) measures of the chest, abdomen (Abdo) and rump, and LM and FM relative to bodyweight (WT). Data are least square means ± SEM.
<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FGR-Saline</td>
</tr>
<tr>
<td></td>
<td>n=11</td>
<td>n=13</td>
</tr>
<tr>
<td>BMC (kg)</td>
<td>1.3±0.3</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>BMD (g·cm⁻²)</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Chest LM (kg) *</td>
<td>15.1±0.4 a</td>
<td>15.0±0.3 a</td>
</tr>
<tr>
<td>Chest FM (kg)</td>
<td>6.1±2.0</td>
<td>5.3±2.0</td>
</tr>
<tr>
<td>Abdo LM (kg)</td>
<td>24.6±0.5</td>
<td>24.1±0.5</td>
</tr>
<tr>
<td>Abdo FM (kg)</td>
<td>9.9±3.3</td>
<td>8.5±3.3</td>
</tr>
<tr>
<td>Rump LM (kg)</td>
<td>10.4±2.1</td>
<td>12.5±2.0</td>
</tr>
<tr>
<td>Rump FM (kg)</td>
<td>4.2±1.0</td>
<td>4.4±1.0</td>
</tr>
<tr>
<td>Total LM (%)</td>
<td>50.2±2.0</td>
<td>51.5±1.8</td>
</tr>
<tr>
<td>Total FM (%)</td>
<td>20.2±6.3</td>
<td>18.1±6.2</td>
</tr>
<tr>
<td>LM:WT (%) *</td>
<td>61.4±8.7 b</td>
<td>67.6±8.7 a,b</td>
</tr>
<tr>
<td>FM:WT (%)</td>
<td>24.0±5.3</td>
<td>23.1±5.3</td>
</tr>
<tr>
<td>Chest FM:WT (%)</td>
<td>7.2±1.8</td>
<td>6.7±1.8</td>
</tr>
<tr>
<td>Abdo FM:WT (%)</td>
<td>11.8±2.9</td>
<td>10.8±2.9</td>
</tr>
<tr>
<td>Rump FM:WT (%)</td>
<td>4.9±0.8</td>
<td>5.7±0.8</td>
</tr>
<tr>
<td>Abdo FM:Total FM (%) *</td>
<td>49.2±1.6 a</td>
<td>46.9±1.6 a,b</td>
</tr>
</tbody>
</table>

Table 6.5 Effect of FGR and intra-amniotic IGF1 treatment on body composition at 18 months of age

Bone mineral content (BMC), bone mineral density (BMD), lean mass (LM), fat mass (FM) measures of the chest, abdomen (Abdo) and rump, and LM and FM relative to bodyweight (WT). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, female.
6.3.3. Plasma Hormone and Metabolite Concentrations Through 18 Months of Age

6.3.3.1. Plasma IGF1 Concentrations

At 5 months of age, plasma IGF1 concentration was higher in FGR-Saline females compared with Controls, whilst there were no differences amongst males (Figure 6.2).

6.3.4.2 Plasma Insulin and Metabolite Concentrations

There were no differences amongst groups in plasma concentrations of glucose, urea, IGF1, free fatty acid, the natural transformation of insulin and the natural log transformation of the G:I ratio, from 2-12 weeks of age in females (Figure 6.3).

Whilst increased plasma glucose concentration at 4 weeks of age was observed in FGR-IGF1 males compared with Controls, there were no differences amongst males in concentrations of urea, IGF1, free fatty acids, the natural log transformation of insulin and the natural log transformation of the G:I ratio, from 2-12 weeks of age (Figure 6.4).

Figure 6.2 Effect of FGR and intra-amniotic IGF1 treatment on plasma IGF1 from weaning to 18 months of age

Insulin-like growth factor-1 (IGF1) plasma concentrations in A) female Control (green, n=9-12), FGR-Saline (blue, n=9-12) and FGR-IGF1 (red, n=10-13), and B) male Control (green, n=8-17), FGR-Saline (blue, n=8-11) and FGR-IGF1 (red, n=8-13) sheep. Data are least square means ± SEM, letters refer to p-values for differences between experimental groups at each time point (factorial ANOVA: Control vs. Saline: a, p<0.05).
Figure 6.3 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations from the perinatal period to weaning in females

Plasma concentrations of A) glucose, B) insulin, C) the natural log transformation of the glucose:insulin ratio, D) free fatty acids (FFA), E) urea and F) IGF1 from the perinatal period to weaning in female Control (green, n=11-13), FGR-Saline (blue, n=8-10) and FGR-IGF1 (red, n=10-12) lambs. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation.
Figure 6.4 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations from the perinatal period to weaning in males

Plasma concentrations of A) glucose, B) insulin, C) the natural log transformation of the glucose:insulin ratio, D) free fatty acids (FFA), E) urea and F) IGF1 from the perinatal period to weaning in male Control (green, n=12-15), FGR-Saline (blue, n=10-11) and FGR-IGF1 (red, n=8-11) lambs. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Letters refer to experimental groups at each time point (factorial ANOVA: Control vs. IGF1: b, p<0.05).
6.3.4. Post Mortem Analysis of Carcass Characteristics and Organ Size

Live weight, carcass weight and relative brain weight were reduced in FGR-Saline males compared with Controls (Table 6.6). There were no significant differences amongst groups in either sex in the absolute weight of the majority of organs. FGR-IGF1 females had lighter hearts and shorter anteroposterior cerebral length (Table 6.6), and decreased thyroid weight compared with Controls, whilst relative cerebellar weight and relative liver weight were increased (Table 6.6).
### Female

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>n=11</td>
<td>n=13</td>
<td>n=12</td>
</tr>
<tr>
<td>Live Weight (kg) †</td>
<td>83.6±7.0</td>
<td>78.8±6.9</td>
<td>76.7±7.0</td>
</tr>
<tr>
<td>Carcass Weight (kg) †</td>
<td>54.8±4.0</td>
<td>52.9±4.0</td>
<td>49.8±4.0</td>
</tr>
<tr>
<td>Fat Depth at 12th Rib (mm)</td>
<td>23.1±3.3</td>
<td>21.0±3.3</td>
<td>18.9±3.3</td>
</tr>
<tr>
<td>Fat Depth at LD (mm)</td>
<td>11.3±1.6</td>
<td>10.8±1.5</td>
<td>10.5±1.6</td>
</tr>
<tr>
<td>LD Width (mm)</td>
<td>63.6±1.7</td>
<td>62.8±1.4</td>
<td>64.0±1.0</td>
</tr>
<tr>
<td>LD Depth (mm)</td>
<td>52.3±4.2</td>
<td>47.9±4.1</td>
<td>48.3±4.1</td>
</tr>
<tr>
<td>Adrenal, Total (g)</td>
<td>5.3±0.3</td>
<td>4.8±0.3</td>
<td>5.7±0.2</td>
</tr>
<tr>
<td>Brain, Total (g)</td>
<td>105±4</td>
<td>102±4</td>
<td>100±4</td>
</tr>
<tr>
<td>Brain Stem (g)</td>
<td>6.9±0.4</td>
<td>6.4±0.3</td>
<td>6.1±0.7</td>
</tr>
<tr>
<td>Cerebrum (g)</td>
<td>87.4±4.4</td>
<td>85.0±4.2</td>
<td>78.2±4.4</td>
</tr>
<tr>
<td>Cerebral AP Length (mm) *</td>
<td>69.6±0.9a</td>
<td>68.4±0.8ab</td>
<td>66.4±1.0b</td>
</tr>
<tr>
<td>Cerebral Width (mm)</td>
<td>66.3±1.0</td>
<td>66.0±0.8</td>
<td>64.1±1.4</td>
</tr>
<tr>
<td>Cerebellum (g)</td>
<td>10.8±0.5</td>
<td>10.9±0.5</td>
<td>11.6±0.5</td>
</tr>
<tr>
<td>Gonads, Total (g)</td>
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<td>2.3±0.1</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Heart (g) *</td>
<td>335±22a</td>
<td>311±21ab</td>
<td>302±22b</td>
</tr>
<tr>
<td>LV Wall (mm)</td>
<td>13.9±2.2</td>
<td>15.6±2.2</td>
<td>14.5±2.2</td>
</tr>
<tr>
<td>RV Wall (mm)</td>
<td>6.9±1.0</td>
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<td>6.8±1.0</td>
</tr>
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<td>Septal Wall (mm)</td>
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<td>15.3±1.6</td>
<td>14.4±1.6</td>
</tr>
<tr>
<td>Kidney, Total (g)</td>
<td>233±12</td>
<td>215±12</td>
<td>220±12</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1,365±78</td>
<td>1,310±76</td>
<td>1,365±78</td>
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<tr>
<td>Lungs, Total (g)</td>
<td>621±51</td>
<td>617±50</td>
<td>618±51</td>
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<td>Pancreas (g)</td>
<td>115±14</td>
<td>81±13</td>
<td>89±14</td>
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<td>Perirenal Fat (g)</td>
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<td>1,071±155</td>
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<td>Spleen (g)</td>
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<td>353±56</td>
<td>334±59</td>
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<tr>
<td>Thymus, Neck (g)</td>
<td>38.2±7.1</td>
<td>30.6±6.9</td>
<td>35.5±7.2</td>
</tr>
<tr>
<td>Thyroid (g)</td>
<td>10.7±1.8</td>
<td>9.1±1.8</td>
<td>8.5±1.8</td>
</tr>
</tbody>
</table>

### Male

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>n=20</td>
<td>n=10</td>
<td>n=12</td>
</tr>
<tr>
<td>Live Weight (kg) †</td>
<td>93.7±6.5a</td>
<td>86.8±6.7b</td>
<td>89.4±6.7a,b</td>
</tr>
<tr>
<td>Carcass Weight (kg) †</td>
<td>60.4±2.9a</td>
<td>54.8±3.2b</td>
<td>58.3±3.1a,b</td>
</tr>
<tr>
<td>Fat Depth at 12th Rib (mm)</td>
<td>16.2±3.0</td>
<td>14.5±3.1</td>
<td>12.8±3.1</td>
</tr>
<tr>
<td>Fat Depth at LD (mm)</td>
<td>7.1±1.1</td>
<td>5.7±1.2</td>
<td>5.7±1.2</td>
</tr>
<tr>
<td>LD Width (mm)</td>
<td>68.1±2.1</td>
<td>68.2±2.6</td>
<td>65.4±2.5</td>
</tr>
<tr>
<td>LD Depth (mm)</td>
<td>52.4±3.0</td>
<td>45.6±3.4</td>
<td>50.4±3.3</td>
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<tr>
<td>Adrenal, Total (g)</td>
<td>5.6±0.2</td>
<td>5.2±0.3</td>
<td>5.2±0.3</td>
</tr>
<tr>
<td>Brain, Total (g)</td>
<td>110±2</td>
<td>112±2</td>
<td>109±2</td>
</tr>
<tr>
<td>Brain Stem (g)</td>
<td>7.5±0.7</td>
<td>7.3±0.8</td>
<td>6.9±0.8</td>
</tr>
<tr>
<td>Cerebrum (g)</td>
<td>89.7±2.1</td>
<td>92.1±2.8</td>
<td>85.1±2.7</td>
</tr>
<tr>
<td>Cerebral AP Length (mm) *</td>
<td>72.3±0.8</td>
<td>72.3±1.1</td>
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</tr>
<tr>
<td>Cerebral Width (mm)</td>
<td>66.4±1.2</td>
<td>67.4±1.4</td>
<td>66.3±1.3</td>
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<tr>
<td>Cerebellum (g)</td>
<td>12.9±0.3</td>
<td>13.2±0.4</td>
<td>13.2±0.4</td>
</tr>
<tr>
<td>Gonads, Total (g)</td>
<td>743±27</td>
<td>750±40</td>
<td>766±40</td>
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<tr>
<td>Heart (g) *</td>
<td>378±16</td>
<td>368±18</td>
<td>354±18</td>
</tr>
<tr>
<td>LV Wall (mm)</td>
<td>16.4±1.3</td>
<td>15.9±1.6</td>
<td>14.8±1.5</td>
</tr>
<tr>
<td>RV Wall (mm)</td>
<td>7.5±0.7</td>
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<td>7.0±0.8</td>
</tr>
<tr>
<td>Septal Wall (mm)</td>
<td>16.0±1.7</td>
<td>15.2±1.9</td>
<td>15.6±1.8</td>
</tr>
<tr>
<td>Kidney, Total (g)</td>
<td>277±12</td>
<td>280±14</td>
<td>292±14</td>
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<tr>
<td>Liver (g)</td>
<td>1,631±119</td>
<td>1,620±126</td>
<td>1,599±124</td>
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<tr>
<td>Lungs, Total (g)</td>
<td>754±21</td>
<td>688±28</td>
<td>746±26</td>
</tr>
<tr>
<td>Pancreas (g)</td>
<td>103±4</td>
<td>101±5</td>
<td>113±5</td>
</tr>
<tr>
<td>Perirenal Fat (g)</td>
<td>510±79</td>
<td>441±85</td>
<td>451±84</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>386±31</td>
<td>363±40</td>
<td>428±38</td>
</tr>
<tr>
<td>Thymus, Neck (g)</td>
<td>22.7±5.9</td>
<td>23.0±6.7</td>
<td>23.0±6.5</td>
</tr>
<tr>
<td>Thyroid (g)</td>
<td>10.8±2.5</td>
<td>9.0±2.7</td>
<td>7.5±2.6</td>
</tr>
</tbody>
</table>
Table 6.6 Effect of FGR and intra-amniotic IGF1 treatment on carcass characteristics and organ size at 18 months of age

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=11</td>
<td>n=13</td>
<td>n=12</td>
</tr>
<tr>
<td>Carcass:Live Weight (%)</td>
<td>65.8±1.7</td>
<td>67.0±1.6</td>
<td>65.6±1.9</td>
</tr>
<tr>
<td>Adrenal:Live Weight (%)</td>
<td>0.006±0.001</td>
<td>0.006±0.001</td>
<td>0.008±0.001</td>
</tr>
<tr>
<td>Brain:Live Weight (%)</td>
<td>0.13±0.01a</td>
<td>0.13±0.01a</td>
<td>0.13±0.01a</td>
</tr>
<tr>
<td>Brain:Liver Weight (%)</td>
<td>8.0±0.3</td>
<td>8.0±0.3</td>
<td>7.5±0.4</td>
</tr>
<tr>
<td>Cerebellum:Live Weight (%)</td>
<td>0.013±0.001b</td>
<td>0.014±0.001ab</td>
<td>0.015±0.001a</td>
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<tr>
<td>Heart:Live Weight (%)</td>
<td>0.40±0.01</td>
<td>0.40±0.01</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td>LV Wall Depth:Live Weight (mm·kg⁻¹)</td>
<td>0.17±0.04</td>
<td>0.20±0.04</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td>RV Wall Depth:Live Weight (mm·kg⁻¹)</td>
<td>0.08±0.02</td>
<td>0.10±0.02</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>Septum:Live Weight (mm·kg⁻¹)</td>
<td>0.17±0.04</td>
<td>0.20±0.04</td>
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<tr>
<td>Heart Weight:BSA (g·m⁻²)</td>
<td>2,767±164a</td>
<td>2,580±161ab</td>
<td>2,505±164b</td>
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<td>Kidney:Live Weight (%)</td>
<td>0.28±0.01</td>
<td>0.27±0.01</td>
<td>0.29±0.01</td>
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<td>Liver:Live Weight (%)</td>
<td>1.63±0.07a</td>
<td>1.67±0.07ab</td>
<td>1.80±0.07a</td>
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<td>Pancreas:Live Weight (%)</td>
<td>0.14±0.01</td>
<td>0.10±0.01</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>Perirenal Fat:Live Weight (%)</td>
<td>1.25±0.1</td>
<td>1.36±0.2</td>
<td>1.18±0.1</td>
</tr>
<tr>
<td>Perirenal:Abdo Fat (%)</td>
<td>11.9±2.5</td>
<td>13.3±2.5</td>
<td>12.0±2.5</td>
</tr>
<tr>
<td>Thyroid:Live Weight (g·kg⁻¹)</td>
<td>0.013±0.001a</td>
<td>0.012±0.001ab</td>
<td>0.009±0.001b</td>
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<td>BMI (kg·m⁻²)</td>
<td>63.1±6.6</td>
<td>61.8±6.5</td>
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<tr>
<td>GI (kg·m⁻¹·s⁻¹)</td>
<td>67.3±7.0</td>
<td>65.4±7.0</td>
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<tr>
<td>PI (kg·m⁻³)</td>
<td>55.6±5.7</td>
<td>55.4±5.7</td>
<td>56.5±5.7</td>
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<tr>
<td>Body Surface Area (m²)</td>
<td>0.121±0.001</td>
<td>0.121±0.001</td>
<td>0.120±0.001</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FGR-Saline</th>
<th>FGR-IGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=20</td>
<td>n=10</td>
<td>n=12</td>
</tr>
<tr>
<td>Carcass:Live Weight (%)</td>
<td>64.6±1.6</td>
<td>63.2±1.7</td>
<td>65.0±1.7</td>
</tr>
<tr>
<td>Adrenal:Live Weight (%)</td>
<td>0.006±0.001</td>
<td>0.006±0.001</td>
<td>0.006±0.001</td>
</tr>
<tr>
<td>Brain:Live Weight (%)</td>
<td>0.12±0.01a</td>
<td>0.13±0.01ab</td>
<td>0.12±0.01ab</td>
</tr>
<tr>
<td>Brain:Liver Weight (%)</td>
<td>7.0±0.6</td>
<td>7.1±0.6</td>
<td>7.0±0.6</td>
</tr>
<tr>
<td>Cerebellum:Live Weight (%)</td>
<td>0.014±0.001</td>
<td>0.015±0.001</td>
<td>0.015±0.001</td>
</tr>
<tr>
<td>Heart:Live Weight (%)</td>
<td>0.40±0.01</td>
<td>0.40±0.02</td>
<td>0.42±0.02</td>
</tr>
<tr>
<td>LV Wall Depth:Live Weight (mm·kg⁻¹)</td>
<td>0.18±0.03</td>
<td>0.19±0.03</td>
<td>0.17±0.03</td>
</tr>
<tr>
<td>RV Wall Depth:Live Weight (mm·kg⁻¹)</td>
<td>0.08±0.01</td>
<td>0.08±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>Septum:Live Weight (mm·kg⁻¹)</td>
<td>0.17±0.03</td>
<td>0.18±0.03</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>Heart Weight:BSA (g·m⁻²)</td>
<td>3,099±115</td>
<td>3,029±137</td>
<td>2,910±132</td>
</tr>
<tr>
<td>Kidney:Live Weight (%)</td>
<td>0.30±0.01</td>
<td>0.32±0.02</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>Liver:Live Weight (%)</td>
<td>1.75±0.02</td>
<td>1.86±0.05</td>
<td>1.78±0.05</td>
</tr>
<tr>
<td>Pancreas:Live Weight (%)</td>
<td>0.11±0.01</td>
<td>0.12±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Perirenal Fat:Live Weight (%)</td>
<td>0.54±0.05</td>
<td>0.50±0.06</td>
<td>0.51±0.06</td>
</tr>
<tr>
<td>Perirenal:Abdo Fat (%)</td>
<td>10.8±2.5</td>
<td>12.8±2.7</td>
<td>11.5±2.6</td>
</tr>
<tr>
<td>Thyroid:Live Weight (g·kg⁻¹)</td>
<td>0.010±0.001</td>
<td>0.010±0.001</td>
<td>0.009±0.001</td>
</tr>
<tr>
<td>BMI (kg·m⁻²)</td>
<td>65.7±6.7</td>
<td>65.1±6.7</td>
<td>64.2±6.7</td>
</tr>
<tr>
<td>GI (kg·m⁻¹·s⁻¹)</td>
<td>71.7±7.1</td>
<td>70.1±7.1</td>
<td>69.6±7.1</td>
</tr>
<tr>
<td>PI (kg·m⁻³)</td>
<td>55.2±5.9</td>
<td>56.3±6.0</td>
<td>54.7±6.0</td>
</tr>
<tr>
<td>Body Surface Area (m²)</td>
<td>0.122±0.001</td>
<td>0.121±0.001</td>
<td>0.122±0.001</td>
</tr>
</tbody>
</table>

Absolute body and organ weight, and organ weight relative to body weight. Latissimus dorsi (LD), anteroposterior (AP), left ventricle (LV), right ventricle (RV), body mass index (BMI), g index (GI), ponderal index (PI), indices of weight (kg) relative to crown-rump-length (m), and body surface area (BSA). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females; **p<0.01, females; †p<0.05, males; ††p<0.01, males.
6.4. Discussion

It was hypothesised that intra-amniotic IGF1-treatment of the growth-restricted fetus would improve in utero growth, abrogate accelerated postnatal growth velocity and improve adult body composition. These findings suggest that intra-amniotic IGF1 treatment of placental insufficiency-mediated fetal growth restriction has long-term sex-specific effects on weight and body proportions through 18 months of age, body composition, and organ size at 18 months of age. However, given the number of comparisons, a type 1 error cannot be excluded.

6.4.1. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Growth

Previous pilot data from our research group suggest that a 15% reduction in birth weight is adequate to induce early postnatal changes in growth velocity (Berry, 2012). In the current study, birthweight in growth-restricted lambs was reduced by ~20% in combined groups (Chapter 4). The lack of appreciable differences amongst groups in adult body size, and few differences in physiological capacity (Chapter 8) could suggest that in the current paradigm, 18 months may be too early for any long-term phenotypic consequences of FGR to be manifest. However, as the current study suggests potential alteration in postnatal organ growth and visceral adipose deposition at 18 months of age in IGF1-treated sheep, epigenetic pathways involved in postnatal organ growth and adult body size (Lui, et al., 2008) should also be investigated.

Although there were no differences in growth velocity during the defined periods amongst groups in females, Control sheep had the greatest fat mass at 18 months. Whilst differences in growth velocity in males were not appreciable, FGR-Saline males remained significantly smaller and lighter than Controls at 18 month, suggesting a failure to catch-up. This is
consistent with a recent meta-analysis in humans which demonstrates that low birth weight individuals have a decreased risk of being overweight (Schellong, et al., 2012), but contrary to postnatal sequelae in other mammalian paradigms of fetal growth restriction such as uteroplacental insufficiency in the rat (Nusken, Dotsch, Rauh, Rascher, & Schneider, 2008), maternal undernutrition in the mouse (Isganaitis, et al., 2009) and naturally-occurring low birth weight in pigs (Poore & Fowden, 2004). These data suggest that ovine placental embolisation-induced fetal growth restriction is a more appropriate paradigm for comparison to postnatal sequelae in the growth-restricted human.

There were no differences in measures of body composition amongst groups in either sex at 4 or 12 months of age. At 18 months of age, total lean mass relative to body weight is increased in female FGR-IGF1 sheep compared with Controls. Additionally, a significant reduction (~10%) in relative abdominal fat mass of FGR-IGF1 females compared with Controls suggests a persistent treatment-effect of intra-amniotic IGF1 on both skeletal muscle and fat deposition in adulthood. Reduced fat deposition as determined by the DXA scan, defined in the current study as visceral fat, is concurrent with a 20% reduction in subcutaneous fat depth superficial to the 12th rib, and a 10% reduction in perirenal fat mass at post-mortem examination. Although not significant, IGF1-treated females also had a 42% increase in rump lean mass at 18 months of age. Interestingly, at 4 months of age (post-weaning), there is a decrease in absolute lean mass in IGF1-treated females compared with Controls, with no difference in relative lean mass. This indicates that at 4 months of age, FGR-IGF1 females have size-appropriate musculature, which could suggest that changes in body composition in females do not arise until the post-pubertal period. Additional physiological and molecular investigations could indicate that augmented musculature in adult FGR-IGF1 females is concurrent with increased skeletal muscle somatotrophic sensitivity (Chapter 7). Although no
statistically significant differences in body composition existed amongst males as determined by DXA at 4, 12 or 18 months of age, at post-mortem IGF1-treated sheep had a 20% reduction in subcutaneous fat depth superficial to the 12th rib as well as that covering the latissimus dorsi and a 10% reduction in perirenal fat mass compared with Controls. Although these findings are not significant, they could indicate the potential for reduced fat mass in IGF1-treated males later in life as total body mass increases. Future studies should investigate feed efficiency to determine if changes in metabolic rate are associated with differential postnatal growth patterns and body composition.

6.4.2. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Hepatic Growth

In the current study, IGF1 treatment of the growth-restricted female ovine fetus increased relative liver weight at post mortem compared with Controls. This effect was not observed in males. Although not significant, FGR-Saline and FGR-IGF1 females tended to be lighter at post mortem, but without evidence of brain-sparing. In contrast, increased relative liver weight at post mortem in FGR-IGF1 females and decreased relative visceral adipose compared with Controls could suggest better maintained abdominal visceral nutrition in FGR-IGF1 females. Acute hypoxia in the fetal sheep increases ductus venosus shunting, decreasing perfusion to the right lobe of the liver (Jensen, Roman, & Rudolph, 1991; Sørensen, et al., 2011). In the growth-restricted human fetus ductus venosus shunting prioritizes left lobe blood flow (Bellotti et al., 2004; Kessler, et al., 2009) whilst total hepatic blood flow is decreased (Kiserud, et al., 2006). Interestingly, greater liver hepatic blood flow in human fetuses is associated with greater fat mass at birth and at 4 years of age (Godfrey, et al., 2012). Although IGF1 treatment of the chronically-catheterised ovine fetus has been shown to stimulate hepatic mitogenesis (Lok, et al., 1996), previous fetal studies using the current paradigm and intervention reveal no difference in absolute or relative liver weight, or brain...
to liver ratio at 131 days gestational age (Darp, et al., 2010; Wali, et al., 2012). Additionally, portal delivery of intra-amniotically-delivered IGF1, which decreases hepatic IGF1 mRNA expression in both the left and right lobes of the growth-restricted liver (Darp, et al., 2010), and increased postnatal hepatic somatotrophic and nutrient sensitivity (Chapter 5) in the FGR-IGF1 females could suggest that differences in hepatic growth are dependent upon postnatally-mediated mechanisms. Increased hepatic somatotrophic sensitivity could mediate postnatal mitogenesis and hyperplastic response, providing a mechanism for increased hepatic growth. However, as fetal growth restriction is associated with increased hepatic lipid content (Alisi, Panera, Agostoni, & Nobili, 2011), and lipolytic response is altered in IGF1-treated females (Chapter 8), assessment of the ultrastructure of the liver is necessary to determine the mechanisms of hepatic growth. Immunohistochemical studies of hepatic morphology and metabolic capacity could help elucidate whether increased liver mass is due to appropriate cellularity and growth, or hepatic lipid accretion.

6.4.3. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Brain Growth

Brain-sparing in FGR-Saline males, but not females, could suggest that placental insufficiency results in more severe haemodynamic compromise in males. Interestingly, the human male fetus has reduced middle cerebral artery pulsatility index and peak velocity, and umbilical venous flow at term compared with females (Prior, Wild, Mullins, Bennett, & Kumar, 2013). Lower middle cerebral artery pulsatility could suggest reduced capacity for adaptive response. Additionally, placental adaptation following fetal growth restriction may not be adequate to maintain appropriate growth in the male (Clifton, 2010), thus, requiring prioritisation of brain growth at the expense of other organ systems (Eriksson, Kajantie, Osmond, Thornburg, & Barker, 2010). Interestingly, whilst brain-sparing was not apparent in
growth-restricted females, there was a significant increase in relative cerebellar weight in IGF1-treated females.

6.4.4. Effect of Intra-Amniotic IGF1 Treatment of FGR on Thyroid Growth

Relative thyroid weight was reduced in IGF1 females at 18 months of age. At birth, both FGR-Saline and FGR-IGF1 females had reduced plasma T4 concentrations compared with Controls (Chapter 4); however, plasma T4 remained depressed at seven days of age in FGR-IGF1 females. This could suggest reduced capacity for T4 secretion. IGF1 has previously been observed to localise to the fetal ovine thyroid in vivo following intra-amniotic injection (Bloomfield, Breier, et al., 2002). In porcine thyroid monolayers, IGF1 acts synergistically with thyroid stimulating hormone (TSH) (Ericson & Nilsson, 1996), whilst in transgenic mice over-expressing IGF1 and IGF1R in the thyroid gland, thyroid function is potentiated concurrent with reduced TSH secretion (Clément, Refetoff, Robaye, Dumont, & Schurmans, 2001). Interestingly, whilst TSH participates in thyrocyte specification in mouse cells in vitro, insulin and IGF1 induce terminal differentiation and maturation of thyrocytes (Arufe, Lu, & Lin, 2009). In the fetal sheep, maturation of the fetal thyroid begins around 100 days gestational age (Fisher, 1991). Reduced relative thyroid weight at 18 months of age could suggest that localisation of intra-amniotic IGF1 to the fetal thyroid stimulates terminal differentiation and premature functional maturation of thyrocytes in utero. Whether this contributes to thyroid dysregulation at 18 months of age in females is unknown.

6.4.5. Effect of Intra-Amniotic IGF1 Treatment of FGR on Postnatal Cardiac Growth

Ventricular mass relative to body surface area increases with age in children (Rowland & Gutgesell, 1995), whilst heart weight relative to body surface area increases with age in adults (Gaitskell, Perera, & Soilleux, 2011). In the current study heart weight relative to body surface area was reduced in FGR-IGF1 females compared with Controls, which could suggest
that age-appropriate cardiac hypertrophy is reduced and, thus, myocardial capacity compromised. Immunohistochemical investigations of the cardiac ultrastructure in these sheep could help elucidate whether intra-amniotic IGF1 treatment detrimentally impacts cardiac development at 18 months of age.

6.5. Limitations

The current study was conducted in a homogenous breeding group which were produced during extended breeding seasons over a two year period. Ewe body condition around the time of conception and pastoral conditions during postnatal life could influence postnatal growth. Additionally, as sheep were sexually intact, seasonal variations in reproductive capacity could influence body condition and metabolic characteristics leading up to the experimental period.

Plasma IGF1 concentrations change throughout the oestrous cycle in sheep (Spicer, et al., 1993). We did not synchronise oestrous in females for routine plasma sampling procedures, which could result in sufficient variability to mask any significant changes in plasma IGF1 concentrations.

6.6. Conclusions

Sexually dimorphic adaptations to fetal growth restriction and intra-amniotic IGF1 intervention persist through to 18 months of age in the sheep. Whilst differences amongst groups in males are primarily limited to somatic growth patterns and body size at 18 months of age, significant alterations in body composition and the growth of key organs is concerning. A significant post-pubertal increase in relative lean mass concurrent with reduced relative visceral adipose in females could suggest that sex-specific growth patterns are disrupted. Both greater postnatal hepatic growth and reduced thyroid growth could alter
metabolic capacity in these animals. Additionally, phenotypic data are limited to discrete points within a relatively brief period of time and to an age comparable to very young adulthood in the human. It is difficult to interpret whether treatment-induced alterations are in any way beneficial in preventing the long-term sequelae of restricted fetal growth. Future studies using this paradigm should endeavour to maintain sheep to a more advanced age, whilst molecular research is needed to elucidate the sex-specific mechanisms perpetuating sex-specific adaptations postnatally.
Chapter 7. The Somatotrophic Axis at 18 Months of Age

7.1. Introduction

Growth hormone is synthesised and secreted by anterior pituitary somatotrophic cells in a pulsatile, sexually dimorphic pattern: male mammals display distinct peaks, whilst females display a relatively constant low-level of secretion (Murray & Clayton, 2013). Somatic growth and metabolic processes are promoted via numerous proteins involved in the insulin and AKT/MTOR pathways (Ray et al., 2012) resulting in varied and divergent cellular responses to growth hormone stimulation. Hepatic endocrine IGF1, the secretion of which is stimulated by growth hormone and mediated through hepatic STAT5 (Udy et al., 1997), promotes cellular proliferation and differentiation and provides a negative feedback mechanism to inhibit pituitary growth hormone secretion and expression of the hepatic growth hormone receptor (Fletcher, Thomas, Dunshea, Moore, & Clarke, 1995; S H Min, MacKenzie, Breier, McCutcheon, & Gluckman, 1996). In sheep, activation of growth hormone-dependent growth around parturition is associated with endocrine maturation (Li, et al., 1999). Altered somatotrophic axis sensitivity in response to fetal deprivation (Gallaher, Breier, Keven, Harding, & Gluckman, 1998) could, thus, result in life-long “reprogramming” of the somatotrophic axis, altering the postnatal response to growth hormone. Indeed, in children born SGA, relative somatotrophic axis insensitivity is apparent (Cabrol et al., 2011).

Despite improved growth in IGF1-treated compared with saline-treated growth-restricted fetuses (Wali, et al., 2012), plasma IGF1 concentrations are reduced in both saline- and IGF1-treated FGR fetuses (Wali, et al., 2012). Reduced hepatic IGF1 and IGF1R mRNA expression (Darp, et al., 2010) and increased hepatic IGFBP1 and IGFBP3 mRNA (Shaikh, et al., 2005) in
IGF1-treated compared with saline-treated FGR fetuses could suggest delayed somatotrophic axis maturation and endocrine activation.

Although previous studies have investigated somatotrophic hormones and genes at discrete time points in response to IGF1-treatment of the growth-restricted fetus, postnatal molecular and physiological consequence of intra-amniotic IGF1 treatment on somatotrophic axis sensitivity have not been investigated. Thus, the aim of this experiment was to investigate the effect of intra-amniotic IGF1 treatment of the growth-restricted ovine fetus on physiological and molecular skeletal muscle response to a growth hormone test at 18 months of age.

7.2. Methods

A growth hormone test was conducted as described previously (Section 2.20.6). Briefly, blood was collected at 0, 4, 8, 12, 24, 28 and 48 hours. Growth hormone (0.15 mg·kg⁻¹) was injected intramuscularly immediately following the baseline, 8 and 24 hour blood samples. A percutaneous biceps femoris skeletal muscle biopsy was collected following the 48 hour blood sample in conscious animals. Plasma glucose (G), insulin (I), FFA and IGF1 concentrations were analysed. Messenger ribonucleic acid (mRNA) expression was analysed by qPCR in biceps femoris biopsies (Female: Control, n=8; FGR-Saline, n=7; FGR-IGF1, n=9 and Male: Control, n=8; FGR-Saline, n=7; FGR-IGF1, n=8) as described previously (Section 2.22). Statistical analyses of the physiological response to a growth hormone test were conducted as described previously (Section 2.23). The liver was not biopsied following the growth hormone test as general anaesthesia could compromise hepatic function prior to the subsequent post-mortem examination, and influence further tissue analyses.
7.3. Results

7.3.1. Plasma Glucose Concentration

Plasma glucose concentrations increased significantly throughout the test (p<0.0001) in both female (Figure 7.1: A) and male (Figure 7.2: A) sheep. There was a significant effect of breeding year in males, with plasma glucose concentrations greater in 2011 than 2010 sheep. Female FGR-Saline sheep had significantly lower peak plasma glucose concentration compared with both Control and FGR-IGF1. There was a trend towards decreased plasma glucose AUC in FGR-Saline females, but no difference amongst groups in males (Table 7.1). Females had significantly greater glucose area under the curve compared with males (p=0.01).

7.3.2. Plasma Insulin Concentration

Plasma insulin concentrations increased significantly throughout the test (p<0.0001) in both female (Figure 7.1: B) and male (Figure 7.2: B) sheep. There was a significant effect of experimental group with plasma insulin concentration greater in FGR-IGF1 females compared with Control and FGR-Saline. Plasma insulin concentrations were greater in 2011 sheep compared with 2010, with the greatest plasma concentrations in the 2011 FGR-IGF1 group (experimental group*breeding year interaction, p<0.05). There was no difference in plasma insulin AUC in either sex amongst groups (Table 7.1); however, insulin area under the curve tended to be greater in females compared with males (p=0.05).

7.3.3. Plasma Glucose to Insulin Ratio

Plasma glucose to insulin ratio (G:I) decreased significantly throughout the test (p<0.0001) in both female (Figure 7.1: C) and male (Figure 7.2: C) sheep. Plasma G:I was greater in 2011 than 2010 males (experimental group*breeding year interaction, p<0.05)
7.3.4. Plasma Free Fatty Acid Concentration

Plasma FFA concentrations increased significantly throughout the test (p<0.0001) in both female (Figure 7.1: D) and male (Figure 7.2: D) sheep.

Control males had higher plasma free fatty acid concentrations than both FGR-Saline and FGR-IGF1 (p=0.05), and 2011 males had greater plasma free fatty acid concentrations than 2010 (p<0.01). There was no difference in plasma FFA AUC in either sex amongst groups (Table 7.1); however, females had significantly greater FFA area under the curve compared with males (p=0.002).

Figure 7.1 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations during a growth hormone stimulation test in female sheep at 18 months of age

Plasma concentrations of A) glucose, B) insulin, C) the glucose to insulin (G:I) ratio and D) free fatty acids (FFA) in female Control (green, n=8-10), FGR-Saline (blue, n=9-10) and FGR-IGF1 (red, n=8-10) sheep during a growth hormone test. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, experimental group p<0.05; ii, breeding year p<0.05; iii, experimental group*breeding year interaction p<0.05.)
Figure 7.2 Effect of FGR and intra-amniotic IGF1 treatment on plasma hormone and metabolite concentrations during a growth hormone stimulation test in male sheep at 18 months of age

Plasma concentrations of A) glucose, B) insulin, C) the glucose to insulin (G:I) ratio and D) free fatty acids (FFA) in male Control (green, n=7-8), FGR-Saline (blue, n=6-8) and FGR-IGF1 (red, n=7-8) sheep during a growth hormone test. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, experimental group p<0.05; ii, breeding year p<0.05).

7.3.5. Plasma Insulin-Like Growth Factor-1 Concentration

Plasma IGF1 increased significantly throughout the test (p<0.0001) in both female (Figure 7.3: A) and male (Figure 7.3: B) sheep, whilst plasma IGF1 was greater in 2011 compared with 2010 females (p<0.01). There was no difference in plasma IGF1 AUC amongst groups in either sex (Table 7.1).

There was a significant positive correlation between basal plasma IGF1 concentration and bodyweight (kg) in Control ($R^2=0.44$, $p<0.05$) and FGR-IGF1 ($R^2=0.41$, $p<0.05$), but not FGR-Saline ($R^2=0.01$, $p=0.77$) females (Figure 7.4: A). In males there was a significant positive
correlation between basal plasma IGF1 concentration and bodyweight in Control ($R^2=0.53$, $p<0.05$) and a trend towards a positive correlation in FGR-Saline ($R^2=0.44$, $p=0.07$), but not in FGR-IGF1 ($R^2=8.5 \times 10^{-6}$, $p=0.99$) sheep (Figure 7.4: B). There was no difference between sexes in plasma IGF1 area under the curve ($p=0.20$).

7.3.6. Feed Intake

Although pre-test feed intake relative to bodyweight tended to be greater in FGR-IGF1 females ($p=0.06$) compared with Control, there were no significant differences amongst groups in either sex (Table 7.1).

![Figure 7.3 Effect of FGR and intra-amniotic IGF1 treatment on plasma IGF1 and during a growth hormone test in adult sheep](image)

*Plasma IGF1 concentrations in A) female, and B) male Control (green: female, n=7-9; male, n=7-8), FGR-Saline (blue: female, n=8-9; male, n=7-8) and FGR-IGF1 (red: female, n=10; male, n=7-8) sheep during a growth hormone test. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, breeding year $p<0.05$).*
Figure 7.4 Effect of FGR and intra-amniotic IGF1 treatment on the correlation between weight and basal plasma IGF1 concentration in adult sheep

The correlation between basal plasma IGF1 concentration and weight at 18 months in A) female, and B) male Control (green: female, n=9; male, n=8), FGR-Saline (blue: female, n=9; male, n=8) and FGR-IGF1 (red: female, n=10; male, n=8) sheep.
### Table 7.1 Effect of FGR and intra-amniotic IGF1 treatment on FGR on plasma hormone and metabolite concentrations, areas under the curve and feed intake during a growth hormone stimulation test

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th></th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FGR-Saline</td>
<td>FGR-IGF1</td>
</tr>
<tr>
<td></td>
<td>n=7-9</td>
<td>n=8-10</td>
<td>n=9-10</td>
</tr>
<tr>
<td><strong>GHT-AUC₆ (mmol·L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*</td>
<td>277±27</td>
<td>200±25</td>
<td>268±31</td>
</tr>
<tr>
<td>Ln Peak Glucose (mmol·L⁻¹)</td>
<td>2.55±0.05³</td>
<td>2.35±0.05ᵇ</td>
<td>2.59±0.09ᵃ</td>
</tr>
<tr>
<td>Ln GHT-AUC₁ (ng·mL⁻¹)</td>
<td>5.4±0.2</td>
<td>5.4±0.2</td>
<td>5.5±0.2</td>
</tr>
<tr>
<td>Ln Peak Insulin (ng·mL⁻¹)</td>
<td>2.2±0.2</td>
<td>2.1±0.2</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>Ln GHT-AUC₇α (mmol·L⁻¹)</td>
<td>2.8±0.2</td>
<td>2.6±0.2</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Ln Plasma FFA 48h (mmol·L⁻¹)</td>
<td>-0.8±0.2</td>
<td>-1.1±0.2</td>
<td>-1.4±0.2</td>
</tr>
<tr>
<td>Ln GHT-AUCIGF₁ (ng·mL⁻¹)</td>
<td>9.38±0.05</td>
<td>9.43±0.03</td>
<td>9.41±0.07</td>
</tr>
<tr>
<td>Ln Peak IGF1 (ng·mL⁻¹)</td>
<td>5.99±0.07</td>
<td>5.95±0.07</td>
<td>6.02±0.07</td>
</tr>
<tr>
<td>Pre-Test Feed Intake (g·kg·d⁻¹)</td>
<td>36.9±8.3</td>
<td>37.6±8.2</td>
<td>44.5±8.3</td>
</tr>
<tr>
<td>Test Feed Intake (g·kg·d⁻¹)</td>
<td>44.2±6.7</td>
<td>41.2±6.6</td>
<td>49.2±6.7</td>
</tr>
<tr>
<td>Maximal Test Feed Intake (kg·d⁻¹)</td>
<td>4.0±0.1</td>
<td>3.7±0.1</td>
<td>4.0±0.1</td>
</tr>
</tbody>
</table>

*Table 7.1 Effect of FGR and intra-amniotic IGF1 treatment on FGR on plasma hormone and metabolite concentrations, areas under the curve and feed intake during a growth hormone stimulation test.

Area under the curve (AUC) of glucose (G) and the natural logarithmic transformation of the peak plasma glucose concentration (mmol·L⁻¹), the natural logarithmic transformation of the AUC of insulin (I) and peak plasma insulin concentration (ng·mL⁻¹), the natural logarithmic transformation of the area under the curve of free fatty acid (FFA) and plasma FFA concentration at 48 hours (48h, mmol·L⁻¹), the natural logarithmic transformation of the IGF1 area under the curve and peak plasma IGF1 concentration (ng·mL⁻¹), relative feed intake (g·kg·d⁻¹) prior to and during a growth hormone test, and maximal feed intake (kg·d⁻¹). Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females; *p=0.06, females; *p=0.07, females; †p<0.05, males.
7.3.7. Effects of FGR and Intra-Amniotic IGF1 Treatment of FGR on Skeletal Muscle mRNA Expression Following a Growth Hormone Test in adult sheep

In FGR-Saline females, growth hormone GH increased *IGF1*, *IGF1R*, *IGFBP3*, and *NR3C1* (50%, 41%, 54% and 57%, respectively) compared with Control sheep. In FGR-IGF1 females, growth hormone increased *GHR*, *IGF1*, and *IGFBP3* (17%, 99% and 29%, respectively) and decreased *IGF1R* and *MTOR* (26% and 34%) compared with Control, but further increased *IGF1* 32% and decreased *IGF1R*, *IGFBP3*, *MTOR*, and *NR3C1* (47%, 16%, 39% and 49%, respectively) compared with FGR-Saline females (Figure 7.5).

In FGR-Saline males, GH decreased *GHR* 10% and increased *IGF1R*, *IGFBP3*, *MTOR* and *NR3C1* (90%, 46%, 38% and 135%, respectively) compared with Control sheep. Compared with both Control and FGR-Saline, in FGR-IGF1, GH increased *IGF1* (48% and 69%, respectively), *IGFBP3* (84% and 25%, respectively) and *MTOR* (59% and 15% respectively), whilst *IGF1R* was increased 86% compared with Control sheep, but not different from FGR-Saline (Figure 7.6).

Compared with females, GH increased *GHR* and *IGF1* (25% and 34%) and decreased *IGF1R*, *MTOR* and *NR3C1* (27%, 39% and 34%, respectively) in male Controls, whilst in FGR-Saline males, both IGFBP3 and MTOR were reduced (12% and 21%), and in FGR-IGF1 males *IGF1R*, *IGFBP3*, *MTOR* and *NR3C1* were increased (82%, 32%, 48% and 100%, respectively) (Table 7.2).

*IGF1:IGFBP3* ratio was significantly greater (*p*=0.04) in FGR-IGF1 females compared with Controls, whilst there were no differences amongst groups in males (*p*=0.22).
Figure 7.5 Effect of FGR and intra-amniotic IGF1 treatment of FGR on skeletal muscle mRNA expression following a growth hormone test in females

Results are reported as fold-change (99%CI) relative to Control (n=8): (A) FGR-Saline (n=7) and (B) FGR-IGF1 (n=9); or relative to FGR-Saline: (C) FGR-IGF, with a 99% confidence interval. Data are normalised to the geometric (GEO) mean of housekeeping genes GAPDH, hypoxanthine phosphoribosyltransferase 1 (HRPT1) and ribosomal protein L 19 (RPL19). Data points with error bars not intersecting the x-axis are significantly different.
Figure 7.6 Effect of FGR and intra-amniotic IGF1 treatment of FGR on skeletal muscle mRNA expression following a growth hormone test in males

Results are reported as fold-change (99%CI) relative to Control (n=8): (A) FGR-Saline (n=6) and (B) FGR-IGF1 (n=5); or relative to FGR-Saline: (C) FGR-IGF, with a 99% confidence interval. Data are normalised to the geometric (GEO) mean of housekeeping genes GAPDH, hypoxanthine phosphoribosyltransferase 1 (HRPT1) and ribosomal protein L 19 (RPL19). Data points with error bars not intersecting the x-axis are significantly different.
Control | FGR-Saline | FGR-IGF1
---|---|---
GHR | 1.25 (1.15-1.36)* | 1.02 (0.92-1.13) | 1.07 (0.99-1.16)
IGF1 | 1.34 (1.29-1.40)* | 0.78 (0.58-1.04) | 1.00 (0.97-1.02)
IGF1R | 0.73 (0.57-0.92)* | 0.98 (0.96-1.00) | 1.82 (1.52-2.17)*
IGFBP3 | 0.92 (0.77-1.12) | 0.88 (0.87-0.89)* | 1.32 (1.11-1.56)*
NR3C1 | 0.61 (0.48-0.78)* | 0.79 (0.67-0.93)* | 1.48 (1.26-1.75)*
MTOR | 0.66 (0.52-0.83)* | 0.98 (0.94-1.03) | 2.0 (1.63-2.57)*

Table 7.2 Effect of sex on skeletal muscle mRNA expression following a growth hormone stimulation test

Data are fold-change (99%CI) in Control, FGR-Saline and FGR-IGF1 male sheep relative to female. Where 99% confidence intervals do not cross 1.0, mRNA expression is significantly different (shown by *).

7.4. Discussion

These data show that brief intra-amniotic IGF1 treatment of fetal growth restriction in the sheep alters plasma hormone and metabolite concentrations and skeletal muscle mRNA expression of key somatotrophic genes compared with both Control and saline-treated growth-restricted sheep following a growth hormone test at 18 months of age. The long-term effect of intra-amniotic IGF1 treatment of FGR could reflect sexually dimorphic somatotrophic axis dysregulation which persists to 18 months of age.

7.4.1. Effect of FGR and Intra-Amniotic IGF1 Treatment of FGR on Plasma Hormone and Metabolite Response to a Growth Hormone Stimulation Test

Sheep became hyperlipidaemic, hyperglycaemic, and hyperinsulinaemic with a reduced glucose to insulin ratio throughout the test. Greater maternal body condition score in 2011 compared with 2010 breeding ewes (Chapter 3) altered plasma hormone and metabolite response to a growth hormone test. If maternal body condition is increased in the periconceptional period in sheep, fetal pancreatic development (Zhang et al., 2011) and, thus postnatal metabolic capacity (Miller, Blache, Jackson, Downie, & Roche, 2010) is compromised. Additionally, increased skeletal muscle glycogen storage protein expression and decreased insulin signalling molecules (Nicholas et al., 2013) could increase growth...
hormone-induced glucose uptake, which may be reflected in reducing plasma glucose concentration in FGR-Saline females near the completion of the test. Interestingly, breeding year effects on plasma hormone and metabolite concentrations were more prevalent in males than females, which may reflect reduced robustness and adaptability in the male fetus (Eriksson, et al., 2010). However, as glucose and insulin signalling in skeletal muscle following a growth hormone test was not within the scope of the study, the relevance of physiological response to tissue-specific alterations following a growth hormone test is unknown.

A single intravenous 0.15 mg·kg⁻¹ bovine growth hormone injection in sheep increases free fatty acid concentrations within two hours, which persist for five hours, returning to baseline by nine hours (Hart et al., 1984). In the current study, growth hormone was injected intramuscularly at 0, 8 and 24 hours; distinct plasma FFA peaks in males and Control females suggest delayed response to growth hormone, possibly due to mode of delivery (Figure 7.2: C). FGR-IGF1 males display three distinct peaks four hours following each dose, which may be indicative of increased growth hormone sensitivity in adipose tissue in response to IGF1 treatment, but overall lesser area under the curve compared with females, which may be due to lesser visceral adipose in males (Chapter 6). In females, both FGR-Saline and FGR-IGF1 sheep had a delayed rise in plasma FFA concentration compared with Control following growth hormone injection. Whilst FGR-Saline females displayed a 2-fold increase in plasma FFA concentration by eight hours, there was no appreciable change in FGR-IGF1 females until 12 hours (Figure 7.1: C).

Although growth hormone promotes hepatic glucose production via glycogenolysis (Ghanaat & Tayek, 2005; Höybye et al., 2008), in the current study elevated plasma free fatty acid concentration precedes both elevated plasma glucose and insulin concentrations (Figure 7.1 and 7.2). Excess free fatty acid inhibits glucose disposal by inducing insulin resistance in
skeletal muscle (Schmitz-Peiffer, 2000), inhibiting pancreatic β-cell function (Boden & Shulman, 2002; Lupi et al., 2002) and antagonising hepatic insulin sensitivity, thus perpetuating hyperglycaemia and hyperinsulinaemia. Although testing was conducted in an induced follicular phase, females had greater growth hormone-mediated free fatty acid response compared with males, which is in agreement with the literature (Lichanska & Waters, 2008). A significant reduction in plasma free fatty acid concentration in FGR-IGF1 males, and a trend towards reduced plasma concentration at the completion of the test in FGR-IGF1 females could reflect compromised fatty acid mobilisation. Growth hormone has been shown to initiate lipolysis in visceral adipose (Gravholt et al., 1999). Although FGR-IGF1 females have reduced visceral adiposity (Chapter 6), the dose-dependent effects of growth hormone are modulated via circulating growth hormone binding protein, which is derived from the growth hormone receptor. As we did not assess growth hormone receptor protein content in this study, tissue-specific growth hormone sensitivity is unknown; however, the delayed rise in plasma free fatty acid concentration following growth hormone injection could suggest reduced adipose growth hormone sensitivity. The relevance of skeletal muscle lipolysis and metabolic substrate utilisation on the physiological response to growth hormone injection is unknown.

Growth hormone-mediated inhibition of glucose disposal is apparent in the progressive hyperglycaemia (Figures 7.1 and 7.2: A) and reduced glucose to insulin ratio throughout the test (Figures 7.1 and 7.2: D). Female FGR-Saline sheep had significantly reduced peak plasma glucose concentration compared with both Control and FGR-Saline, and a trend towards decreased plasma glucose area under the curve (Table 7.1). This could reflect reduced glycogenolytic capacity, or a measure of growth hormone resistance in FGR-Saline females. As a group, females had significantly greater glucose area under the curve and a trend
towards greater insulin area under the curve compared with males. These data support the sexually dimorphic nature of the growth hormone response (Lichanska & Waters, 2008).

### 7.4.2. Effect of FGR and Intra-Amniotic IGF1 Treatment of FGR on the Somatotrophic Response to a Growth Hormone Stimulation Test

Growth hormone, which binds the GHR, activates JAK and promotes phosphorylation of STAT5 which stimulates transcription of IGF1 (Rotwein, 2012; Woelfle & Rotwein, 2004). Growth hormone recruits STAT5B to multiple redundant sites within the igf1 promoter, which amplifies growth hormone-stimulated activity (Chia et al., 2006) via epigenetic modifications (Chia, Varco-Merth, & Rotwein, 2010). STAT5B, which is necessary for skeletal muscle IGF1 production and appropriate postnatal growth (Klover & Hennighausen, 2007), is implicated in sexual dimorphism of the somatotrophic axis (Holloway et al., 2007; Udy, et al., 1997): activating male-predominant and repressing female-predominant gene expression (Clodfelter et al., 2006). Interestingly, there was little difference in mRNA expression between the sexes in FGR-Saline sheep, which could suggest a growth restriction-mediated alteration of sexual dimorphism. Both FGR-Saline and FGR-IGF1 males had GHR and IGF1 skeletal muscle mRNA expression comparable to that of females (Table 7.2), which could suggest a growth restriction-mediated alteration in skeletal muscle growth hormone sensitivity. As growth hormone promotes hepatic secretion of IGF1, a significant positive correlation between weight and basal plasma IGF1 concentrations in Control and FGR-IGF1 females and Control males, which approached significance in FGR-Saline males (Figure 7.4: A and B, respectively) could suggest recovery of growth restriction-induced IGF1 insensitivity in females. Paired with mRNA expression data at one week of age (Chapter 5), the lack of an association in basal plasma IGF1 concentration and weight at 18 months of age in both FGR-
Saline females and FGR-IGF1 males may also suggest a persistent decrease in somatotrophic sensitivity.

Differential alteration at the level STAT5B, which mediates growth hormone action after binding of growth hormone to its receptor, regulates post-receptor IGF1 action. In rodents, STAT5B is necessary for growth hormone-mediated IGF1, IGFALS, IGFBP3 (Woelfle & Rotwein, 2004), skeletal muscle androgen receptor (Klover, Chen, Zhu, & Hennighausen, 2009) expression, and the sexually dimorphic accretion of skeletal muscle mass (Klover & Hennighausen, 2007). Greater IGF1 and IGFBP3, but reduced MTOR expression in FGR-IGF1 female skeletal muscle compared with both Control and FGR-Saline could support altered STAT5B, rather than MTOR-mediated mechanisms. Interestingly, reduced absolute lean mass at 12 months of age, yet greater relative lean mass at 18 months of age in FGR-IGF1 females compared with controls (Chapter 6) could reflect differences in puberty-associated mechanisms regulating skeletal muscle accretion. This could suggest the maintenance of greater somatotrophic sensitivity and, thus, skeletal muscle mass, both of which are known to diminish during aging (Bartke, 2009).

As it was not possible to directly assess functional protein accretion in the current study via isotopic metabolite methods, analysing plasma urea throughout the growth hormone test could be useful in determining whether differences in nitrogen retention exist in response to intervention. In yearling lambs, 0.1 μg·kg⁻¹ of growth hormone for 5 days increases plasma IGF1 and decreases plasma urea concentration (Min, Mackenzie, Breier, McCutcheon, & Gluckman, 1999), whilst in pigs, 7-10 days of 150 μg·kg⁻¹·d⁻¹ increases plasma IGF1 concentrations and reduces urea nitrogen (Wilson et al., 2008) suggesting increased protein synthesis in response to growth hormone treatment. If plasma urea is decreased in FGR-IGF1 females compared with Controls despite comparable plasma IGF1 concentrations, this could
reflect greater peripheral somatotrophic sensitivity. Additionally, analysis of post mortem
tissues could help to determine if there exists an underlying post-growth hormone receptor
STAT5B-mediated adaptation which persists in unstimulated skeletal muscle.

*In vitro*, growth hormone has been shown to rapidly phosphorylate MAPK/ERK (Hayashi &
Proud, 2007) and multiple proteins in the PI3K/MTOR pathway (Ray, et al., 2012), which are
critical for myofibre differentiation and development (Gardner, Anguiano, & Rotwein, 2012).
However, research in neonatal pigs suggests that MTORC1 signalling in muscle is stimulated
within 30 minutes of feeding (Gazzaneo et al., 2011) and translation initiation and
subsequent protein synthesis is enhanced for 120 minutes, not returning to baseline until
240 minutes (Wilson et al., 2009). Sheep were fed 30-60 minutes prior to skeletal muscle
biopsies. In females, MTOR expression was decreased in FGR-IGF1 compared with both
Control and FGR-Saline sheep, whilst in males MTOR expression was increased in both FGR-
Saline and FGR-IGF1 compared with Control sheep (Figure 7.5). This sexually-dimorphic
change in MTOR in response to FGR and IGF1 treatment requires further investigation. As we
did not assess genes of interest at the protein level, the relevance of altered gene expression
relative to physiological alterations remains is unclear. Although fetal somatotrophic axis
regulation in response to growth restriction in mammalian paradigms is well-established
(Fowden, Giussani, & Forhead, 2005), the effects of fetal deprivation on long-term postnatal
somatotrophic axis function focuses on early maternal nutrient deprivation (Hyatt, Budge,
Walker, Stephenson, & Symonds, 2007; Hyatt, et al., 2004). Although various ovine
paradigms may produce similar postnatal phenotypic characteristics, mechanistic
adaptations are distinct (Morrison, 2008; Wallace, Milne, Adam, & Aitken, 2011) and, thus
should be compared with care.
7.5. Limitations

Experimental procedures such as blood sampling, restraint and conscious skeletal muscle tissue biopsy could induce a stress response. We acknowledge that prenatal deprivation increases the sheep’s response to restraint stress (Erhard, Boissy, Rae, & Rhind, 2004), whilst increased adiposity is also associated with greater restraint stress, adrenergic and corticosteroid secretion (Tilbrook, Rivalland, Turner, Lambert, & Clarke, 2008).

The skeletal muscle biopsy was conducted following the final blood sample and feed distribution. Feeding increases skeletal muscle and liver MTOR mRNA expression and phosphorylation (Gazzaneo, et al., 2011) and protein synthesis from 30-120 minutes after feeding in piglets (Wilson, et al., 2009). As such, the effect of feeding on skeletal muscle gene transcription cannot be overlooked.

Dietary ratio of feed concentrates relative to roughage alters growth hormone and IGF1 secretion in sheep (Hagino et al., 2005). As such, care should be taken when comparing responses to a growth hormone test in the 100% concentrate-fed sheep in the current study to the response to growth hormone in other studies.

7.6. Conclusions

Whilst intra-amniotic IGF1 treatment of the FGR female fetus restored the relationship between bodyweight and basal plasma IGF1 concentration, the relationship in males was dysregulated; reduced robustness of the growth-restricted male fetus could necessitate sex-specific optimisation of the intervention. Nonetheless, we have shown that intra-amniotic IGF1 treatment of ovine fetal growth restriction results in sexually dimorphic alteration of somatotrophic gene transcription and physiological parameters in response to a growth hormone test. The persistent effects of this brief in utero intervention on the somatotrophic
axis could affect metabolic and mitogenic capacity throughout life. As we did not investigate glucose or insulin signalling, or molecular characteristics of lipid metabolism in skeletal muscle, the relevance of these changes to plasma hormone and metabolite concentrations is unknown. Whether somatotrophic axis alteration in IGF1-treated animals is associated with compromised metabolic capacity requires further investigation as reduced metabolic flexibility could affect growth and have implications for long-term health in the treated offspring. Additionally, sexually dimorphic actions of intra-amniotic IGF1 treatment on postnatal molecular and physiological response to a growth hormone test could suggest that sex-specific treatment protocols be investigated in order to optimise the effectiveness of the intervention.
Chapter 8. Physiological Tests

8.1. Introduction

The developmental origins of health and disease hypothesis suggests that deprivation during critical periods of fetal growth can predispose individuals to the development of chronic disease as life progresses (Barker, 2007; Gluckman & Hanson, 2004; Hanson, Godfrey, Lillycrop, Burdge, & Gluckman, 2011). Placental insufficiency, which limits nutrient and oxygen availability to the fetus, reduces pancreatic β-cell mass and insulin secretion (Limesand, et al., 2013; Limesand, Rozance, Zerbe, Hutton, & Hay, 2006) and restricts fetal growth (Owens, 1991). Whilst fetal plasticity is advantageous for in utero survival, compromised metabolism established during fetal development may be perpetuated postnatally. Impaired pancreatic β-cell insulin secretory capacity (Gatford et al., 2008), and increased insulin sensitivity persist at one month of age and are associated with greater accretion of visceral adiposity (De Blasio, Gatford, McMillen, et al., 2007).

Lasting alterations in metabolic capacity throughout life, including impaired pancreatic β-cell secretory capacity, reduced skeletal muscle insulin sensitivity and reduced peripheral insulin signalling (Gatford, Simmons, Blatio, Robinson, & Owens, 2010) could be due to activation of the hypothalamic-pituitary-adrenal axis in response to deprivation (Gardner, Fletcher, Bloomfield, Fowden, & Giussani, 2002; Gardner, Fletcher, Fowden, & Giussani, 2001). Reduced fetal glucose supply is associated with increased insulin receptor mRNA expression in the hypothalamic arcuate nucleus and fetal growth restriction at 130 days gestational age in over-nourished pregnant adolescent ewes (Adam, et al., 2011). Whilst precocious activation of the neuroendocrine HPA axis in the growth-restricted fetal sheep is well-documented (Morrison, 2008), data on long-term HPA sensitivity are focused on
nutritionally-mediated fetal growth restriction (Bloomfield et al., 2003; Wallace, Milne, Green, & Aitken, 2011) and are confounded by fetal number (Rumball, et al., 2008) and postnatal age at testing (Oliver et al., 2012). Additionally, there are currently no studies investigating long-term adaptation following intervention in the growth-restricted ovine fetus. Thus, the purpose of the current study is to investigate persistent HPA axis and metabolic adaptation in adult offspring which had fetal growth restriction secondary to placental embolisation and which were treated with intra-amniotic IGF1.

8.2. Methods

Males and females from each experimental group were electronically randomised to receive physiological testing. Refer to table 8.2 for the number of sheep tested. Physiological testing included a glucose tolerance test (GTT), adrenaline stimulation test (ADR), hyperglycaemic clamp (HGC), Metyrapone® test (MET), arginine vasopressin/corticotropin-releasing hormone stimulation test (AVPC) at 18 months of age:

<table>
<thead>
<tr>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
<th>Sunday</th>
</tr>
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<tr>
<td>GTT</td>
<td>ADR</td>
<td>HGC</td>
<td>MET</td>
<td>AVPC</td>
<td>Free For Repeat Test</td>
<td>Free For Repeat Test</td>
</tr>
</tbody>
</table>

Table 8.1 Physiological testing series at 18 months of age

If tests failed, they were repeated on the Saturday or Sunday following completion of the remainder of the physiological tests. Areas under the curve (AUC) were calculated as previously described (Section 2.20). Statistical analyses were conducted as previously described (Section 2.23).
8.2.1. Glucose Tolerance Test

After collecting a baseline blood sample, 0.5 g·kg⁻¹ 50% dextrose was infused in ≤60 seconds and followed by a 10 mL 0.9% saline flush. Blood samples were collected 2, 5, 10, 15, 20, 30, 40, 50, 60, 120 and 180 minutes after glucose infusion. Plasma was processed and glucose (G, mmol·L⁻¹) and insulin (I, ng·mL⁻¹) concentrations were analysed as previously described (Section 2.20.1). Glucose (G) areas under the curve first 15 minutes following glucose infusion and 180 minutes following glucose infusion (mmol·L⁻¹; GTT-AUC_G15 and GTT-AUC_GT, respectively) were calculated. The GTT-AUC_G15 was defined as the early phase insulin response in the first 15 minutes following the bolus (AIR₁₅) was calculated (Long, et al., 2010; Owens, et al., 2007). The change in insulin (I) secretion from baseline in the first 15 minutes following glucose infusion (ng·mL⁻¹; ΔI₁₅, ng·mL⁻¹), absolute (ng·mL⁻¹; GTT-AUC_I) and relative insulin secretion (Iₛ, ng·mL⁻¹·mmol·L⁻¹; GTT-AUC_I/GTT-AUC_GT) over the duration of the test were calculated (Gatford, et al., 2004). Statistical analyses were conducted as previously described (Section 2.23).

8.2.2. Hyperglycaemic Clamp

After collecting baseline blood samples at -20, -15, -10 and 0 minutes to determine basal whole blood glucose concentration, a 7.7 mL·min⁻¹·m⁻² body surface area (kg⁰.⁶₇ x 0.09 (Mitchell, 1928)) 25% dextrose bolus was infused from 0-5 minutes to increase blood glucose concentration to 10 mmol·L⁻¹. The rate of glucose infusion thereafter was titrated using a computer algorithm to maintain blood glucose concentration at a 10 mmol·L⁻¹ steady state (SS). Blood samples were collected at 5-minute intervals through 135 minutes to determine whole blood glucose concentration; samples collected at 15-minute intervals were preserved for plasma insulin assay. To maximize insulin secretion, a 100 mg·kg⁻¹ l-Arginine bolus was given immediately following the 135 minute blood sample. Blood samples were collected at
5-minute intervals following the \( \text{L-Arginine} \) bolus to determine whole blood glucose concentration; samples collected at 5, 10, 20 and 30 minutes post-bolus were preserved for plasma insulin assay as previously described (Section 2.20.3). Hyperglycaemic clamps were analysed if during steady state (75-135 minutes) the blood glucose concentration coefficient of variance was \( \leq 10\% \) throughout. Insulin (I) area under the curve (HGC-AUC\(_{I}\)) was calculated using the trapezoid rule over two periods: from baseline to the end of steady state (HGC-AUC\(_{S\,SS}\)) and following the \( \text{L-Arginine} \) bolus from the end of the steady state period to the completion of the clamp (HGC-AUC\(_{I\,ARG}\)). The mean glucose infusion during steady state (IG\(_{S\,SS}\): mmol·kg\(^{-1}\)·min\(^{-1}\)) and following the \( \text{L-Arginine} \) bolus (IG\(_{I\,ARG}\): mmol·kg\(^{-1}\)·min\(^{-1}\)) were calculated. Mean plasma insulin concentration during steady state (I\(_{S\,SS}\): ng·mL\(^{-1}\)), peak plasma insulin concentration following the \( \text{L-Arginine} \) bolus (I\(_{I\,ARG}\): ng·mL\(^{-1}\)), and the change in insulin secretion from steady state in response to the \( \text{L-Arginine} \) bolus (\( \Delta I_{S\,SS:ARG} \)) were calculated. Insulin sensitivity during steady state \( (S = IG_{S\,SS}/I_{S\,SS}) \) was calculated (Mitrakou, et al., 1992). The insulin-dependent component of whole body glucose tolerance during steady state, glucose disposition index \( (DI_{G} = S_{I} \times AIR_{15} \text{ (Long, et al., 2010)}) \), and glucose-stimulated insulin secretion \( (\text{Mean } I_{S\,SS}/\text{basal plasma insulin} \text{ (Carver, et al., 1996; Limesand & Hay, 2003)}) \) were calculated. Statistical analyses were conducted as previously described (Section 2.23).

### 8.2.3. Adrenaline Stimulation Test

After collecting a baseline blood sample, 1.0 \( \mu \text{g} \cdot \text{kg}^{-1} \) adrenaline was intravenously injected and followed by a 10 mL 0.9% saline flush. Blood samples were collected 2.5, 5, 7.5, 10, 15, 20, 30, 45 and 60 minutes after infusion. Plasma was processed and glucose (G), FFA and BHBA concentrations were analysed as previously described (Section 2.20.2). The AUC for glucose, FFA and BHBA in the first 15 minutes \( (ADR-\text{AUC}_{G15}, ADR-\text{AUC}_{FFA15}, \text{ and } ADR-\text{AUC}_{BHBA15}) \) following glucose infusion and at the completion of the test \( (ADR-\text{AUC}_{GT}, ADR-\text{AUC}_{FT}, \text{ and } ADR-\text{AUC}_{BHBA15}) \) were calculated.
AUC_{FFAT}, and ADR-AUC_{BHBAT}), peak glucose, FFA and BHBA were calculated. Statistical analyses were conducted as previously described (Section 2.23).

8.2.4. Metyrapone® Test

After collecting a baseline blood sample, 40 mg·kg^{-1} Metyrapone® was infused intravenously over a 2 minute period. Blood samples were collected at 30, 60 and 120 minutes following the bolus. Blood samples were processed and plasma ACTH cortisol (F), cortisone (E), 11-deoxycortisol (S) and were analysed as previously described (Section 2.20.4) and the logarithmic area under the curve (MET-AUC_{F}, MET-AUC_{E}, MET-AUC_{S} and MET-AUC_{ACTH}), absolute (ng·mL^{-1}) and relative (%) cortisol suppression (MET-SUPP_{F}) from baseline to 30 minutes, the cortisol nadir, peak 11-deoxycortisol, basal and peak ACTH, basal S:F and E:F, and peak:basal ACTH ratios were calculated. Statistical analyses were conducted as previously described (Section 2.23).

8.2.5. Arginine Vasopressin/Corticotropin-Releasing Hormone Stimulation Test

After collecting a baseline blood sample, equimolar doses of 0.1 µg·kg^{-1} bovine arginine vasopressin and 0.5 µg·kg^{-1} bovine corticotropin-releasing hormone were injected intravenously. Blood samples were collected 15, 30, 45, 60, 120 and 240 minutes following infusion and plasma ACTH cortisol (F) and were analysed as previously described (Section 2.20.5). Area under the curve for cortisol and (AVPC-AUC_{F} and AVPC-AUC_{ACTH}), basal and peak cortisol, and peak:basal ACTH ratio were calculated. Statistical analyses were conducted as previously described (Section 2.23).
8.3. Results

8.3.1. Glucose Tolerance Test

Plasma glucose and insulin concentrations changed significantly over the first 15 minutes, and over the total duration of the test in female (all, $p<0.0001$) and male (G15, GT and I15, $p<0.0001$; IT, $p<0.005$) sheep (Figure 8.1: A-D). There was a significant time* experimental group interaction ($p<0.05$) in female plasma insulin concentrations over the duration of the test; FGR-Saline and FGR-IGF1 sheep had a lower plasma insulin response compared with Controls (Figure 8.1: C).

The areas under the curve of glucose and insulin for the first 15 minutes and the total duration of the test were not different amongst groups of either sex (Table 8.3). Whilst first-phase insulin secretion ($AIR_{15}$), relative insulin secretion ($I_{5}$) and peak insulin secretion were not different amongst groups in either sex, peak glucose concentration was greater ($p<0.05$) in FGR-Saline compared with Control males and the change in plasma insulin concentration from basal levels in the first 15 minutes of the test was greater ($p<0.05$) in FGR-Saline compared with both Control and FGR-IGF1 males (Table 8.3).

The area under the curve of glucose (GTT-AUC<sub>GT</sub>) was significantly positively correlated with glucose to insulin ratio (G:I) at birth in female Control and FGR-IGF1 sheep, and tended towards significance ($p=0.07$) in FGR-Saline females (Figure 8.2: A). In males, the area under the curve was significantly negatively correlated with G:I ratio at birth in Control and FGR-IGF1 sheep, whilst in FGR-Saline males tended towards ($p=0.09$) a significant positive correlation (Figure 8.2: B).

Acute insulin response ($AIR_{15}$) was not correlated with birth weight, glucose to insulin ratio at birth, or growth velocity in the first or second weeks of life amongst groups in either sex.
Figure 8.1 Plasma glucose and insulin during a glucose tolerance test at 18 months of age

Left panels: females (Control, green, n=7-10; FGR-Saline, blue, n=8-10; FGR-IGF1, red, n=8-9); right panels: males (Control, green, n=7-9; FGR-Saline, blue, n=6-8; FGR-IGF1, red, n=6-8). Concentrations of plasma glucose (A and B) and insulin (C and D) during an intravenous glucose tolerance test with the first 15 minutes shown by a box. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, effect of time, p<0.0001; ii, effect of time, p<0.01; iii, time*experimental group interaction, p<0.05).

Figure 8.2 Area under the curve of glucose following a glucose tolerance test at 18 months of age relative to glucose to insulin ratio at birth

The correlation between the area under the curve during a glucose tolerance test (GTT-AUCGT) at 18 months of age and glucose to insulin (G:I) ratio at birth in female (A: Control, green, n=6; FGR-Saline, blue, n=5; FGR-IGF1, red, n=6) and male (B: Control, green, n=6; FGR-Saline, blue, n=5; FGR-IGF1, red, n=6) sheep.
### Table 8.2 Plasma glucose and insulin response to a glucose tolerance test at 18 months of age

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control n=7-10</td>
<td>FGR-Saline n=8-10</td>
</tr>
<tr>
<td></td>
<td>Control n=7-9</td>
<td>FGR-Saline n=6-8</td>
</tr>
<tr>
<td>GTT-AUC&lt;sub&gt;G15&lt;/sub&gt; (mmol·L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>192±10</td>
<td>191±10</td>
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<tr>
<td>GTT-AUC&lt;sub&gt;G&lt;/sub&gt; (mmol·L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1,048±81</td>
<td>1,074±80</td>
</tr>
<tr>
<td>Ln GTT-AUC&lt;sub&gt;I15&lt;/sub&gt; (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>2.8±0.2</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>Ln GTT-AUC&lt;sub&gt;I&lt;/sub&gt; (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>5.3±0.4</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>Peak Glucose (mmol·L&lt;sup&gt;-1&lt;/sup&gt;) †</td>
<td>28.1±2.2</td>
<td>26.5±2.2</td>
</tr>
<tr>
<td>Peak Insulin (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.0±0.9</td>
<td>2.7±0.9</td>
</tr>
<tr>
<td>AIR&lt;sub&gt;I15&lt;/sub&gt; (ng·mL&lt;sup&gt;-1&lt;/sup&gt;·mmol·L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.21±0.03</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Ln Relative I&lt;sub&gt;S&lt;/sub&gt; (ng·mmol&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-1.7±0.4</td>
<td>-1.8±0.4</td>
</tr>
<tr>
<td>Δ I&lt;sub&gt;S15&lt;/sub&gt; (ng·mL&lt;sup&gt;-1&lt;/sup&gt;) †</td>
<td>2.5±0.4</td>
<td>1.3±0.4</td>
</tr>
</tbody>
</table>

Area under the curve (AUC) for plasma glucose (G) and insulin (I) for the first 15 minutes (GTT-AUC<sub>G15</sub>, GTT-AUC<sub>I15</sub>) and 180 minutes (GTT-AUC<sub>G</sub>, GTT-AUC<sub>I</sub>), peak glucose and insulin concentrations, first-phase insulin secretion (AIR<sub>I15</sub>), the natural logarithmic transformation of the relative insulin secretion (I<sub>S</sub>), and the change in insulin secretion in the first 15 (ΔI<sub>S15</sub>) minutes of a glucose tolerance test. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: †p<0.05, males.
8.3.2. Hyperglycaemic Clamp

Plasma insulin concentration increased throughout the hyperglycaemic clamp \((p<0.05)\) and following the \(\text{L-Arginine}\) bolus in females, and was greater during steady state in FGR-IGF1 than Control (group effect, \(p<0.01\)). Plasma insulin concentration increased significantly following the \(\text{L-Arginine}\) bolus in both sexes (female, \(p<0.05\); males, \(p<0.001\)) and there was a time*experimental group interaction in females with greater concentrations in FGR-IGF1 than in Controls (Figure 8.3).

The rate of glucose infusion to maintain a 10 mmol·L\(^{-1}\) whole blood glucose concentration during steady state glucose concentration was significantly greater in FGR-IGF1 females compared with FGR-Saline \((p<0.05, \text{Table 8.4})\), whilst the rate of glucose infusion to maintain 10 mmol·L\(^{-1}\) following the \(\text{L-Arginine}\) bolus was significantly greater in both Control and FGR-IGF1 females compared with FGR-Saline \((p<0.001, \text{Table 8.4})\); there were no differences in glucose infusion rate amongst groups in males. The change in plasma insulin concentration from steady state following the \(\text{L-Arginine}\) bolus was greater \((p<0.05)\) in FGR-IGF1 males compared with FGR-Saline, and approached significance \((p<0.07)\) in females with 12% greater plasma insulin concentration in FGR-Saline females compared with FGR-IGF1 and Control. Although not significant, mean insulin secretion during steady state was increased 43% in FGR-IGF1 compared with Control females, and decreased 20% in FGR-IGF1 compared with both Control and FGR-Saline males. Although not significant, insulin sensitivity was decreased 23% in FGR-Saline females compared with Control, but increased 40% in FGR-IGF1 compared with FGR-Saline females, whilst in males insulin sensitivity was increased 67% in FGR-IGF1 compared with FGR-Saline groups. Glucose disposition index approached significance \((p=0.06)\) in females, with FGR-Saline 50% less than Control and FGR-IGF1 79% greater than FGR-Saline. Although not significant, peak plasma insulin concentration following the \(\text{L-}\)
Arginine bolus was decreased 20% in FGR-Saline females compared with Control, but increased 47% in FGR-IGF1 females compared with Control. There were no differences amongst groups in either sex in or glucose-stimulated insulin secretion.

In female sheep, glucose disposition index (D\textsubscript{IG}) was negatively correlated with visceral adipose in Controls ($R^2=0.73$, β-coefficient=-0.05±0.01, $p=0.01$), but not in FGR-Saline ($R^2=0.01$, β-coefficient=-0.002±0.01, $p=0.85$), and tended towards positive correlation in FGR-IGF1 ($R^2=0.64$, β-coefficient=0.10±0.04, $p=0.06$).

In male sheep, D\textsubscript{IG} was positively correlated with visceral adipose in Controls ($R^2=0.74$, β-coefficient=0.03±0.01, $p=0.03$), but tended towards negative correlation in FGR-Saline ($R^2=0.65$, β-coefficient=-0.01±0.01, $p=0.05$) and FGR-IGF1 ($R^2=0.56$, β-coefficient=-0.04±0.02, $p=0.05$).

Insulin sensitivity and glucose-stimulated insulin secretion were not correlated with birth weight or growth velocity of weight in the first and second weeks after birth amongst groups in either sex.
Figure 8.3 Whole blood glucose and plasma insulin responses during a hyperglycaemic clamp at 18 months of age

Left panels: females (Control, green, n=6-9; FGR-Saline, blue, n=9-10; FGR-IGF1, red, n=8-10); right panels: males (Control, green, n=6-9; FGR-Saline, blue, n=6-8; FGR-IGF1, red, n=6-8). Concentrations of whole blood glucose (A and B) and plasma insulin (C and D) with period of steady state hyperglycaemia (SS) and the hour following an L-arginine bolus (ARG) shown. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, main effect of experimental group, p<0.05; ii, main effect of experimental group, p<0.01; iii, effect of time, p<0.05; iv, effect of time, p<0.001; v, time*experimental group interaction, p<0.05).
Table 8.3 Plasma glucose and insulin responses during a hyperglycaemic clamp at 18 months of age

Area under the curve (AUC) for plasma insulin (I) during steady state (HGC-AUC\text{ISS}) and in response to \textit{l}-Arginine bolus (HGC-AUC\text{ARG}), glucose infusion rate (mmol·kg·min\textsuperscript{-1}) during steady state and in response to an \textit{l}-Arginine bolus, change in insulin secretion from steady state to the peak following the \textit{l}-Arginine bolus (ΔI\textsubscript{S}), insulin sensitivity (S\textsubscript{i}), glucose disposition index (D\textsubscript{IG}), and glucose-stimulated insulin secretion (GSIS). Data are least square means ± SEM. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females; **p<0.001, females; †p<0.05, males; •p=0.06, females; °p=0.07, females.
8.3.3. Adrenaline Stimulation Test

Plasma glucose, FFA and BHBA concentrations changed significantly over the duration of the test in females and males (glucose and FFA: \( p < 0.0001 \), BHBA: \( p < 0.01 \)). In females, there was a time*experimental group interaction for plasma glucose concentration (\( p < 0.05 \)) and FFA (\( p < 0.01 \)), with plasma glucose concentration plateauing from 5-15 minutes in Control, whilst FGR-Saline and FGR-IGF1 females had a peak plasma concentration at 5 minutes, and FFA showing a peak at 5 minutes followed by a gradual decrease in both Control and FGR-Saline sheep, whilst FGR-IGF1 females showed a rapid post-peak decrease. In males there was a time*experimental group interaction for plasma FFA and BHBA (both: \( p < 0.05 \)), with FFA in FGR-IGF1 males sustained at a higher plasma concentration after the initial peak response, and higher plasma BHBA concentration in FGR-Saline compared with Control males from 10 minutes for the duration of the test (8.4: A-F).

Basal plasma BHBA concentration tended to be greater (\( p = 0.06 \)) in FGR-IGF1 females compared with FGR-Saline. The logarithmic transformation of the area under the curve for BHBA for the duration of the test (ADR-AUC_{BHBA}) tended to be greater in FGR-IGF1 females compared with FGR-Saline (\( p = 0.07 \)). Refer to Table 8.5. Peak plasma glucose concentration tended to be greater in Control and FGR-IGF1 males compared with FGR-Saline (\( p = 0.08 \)). The area under the curve for glucose in the first 15 minutes of the adrenaline stimulation test (ADR-AUC_{G15}) tended to be greater (\( p = 0.10 \)) in Control compared with FGR-IGF1 males (Table 8.5).

There was a significant positive correlation between peak plasma FFA concentration during the adrenaline stimulation test and absolute abdominal fat mass as determine by dual X-ray absorptiometry (DXA) scan (reported in Chapter 6) in FGR-IGF1 sheep (female: \( p < 0.0001 \),
male: \( p < 0.008 \), and a trend towards a significant positive correlation in Control female \( p = 0.10 \) and FGR-Saline male \( p = 0.08 \) sheep (Figure 8.5: A, B).

**Figure 8.4** Plasma glucose, free fatty acids and \( \beta \)-hydroxybutyric acid during an adrenaline stimulation test at 18 months of age

*Left panels: females (Control, green, \( n = 7-9 \); FGR-Saline, blue, \( n = 7-10 \); FGR-IGF1, red, \( n = 7-10 \)); right panels: males (Control, green, \( n = 7-9 \); FGR-Saline, blue, \( n = 6-9 \); FGR-IGF1, red, \( n = 6-8 \)). Concentrations of plasma glucose (A and B), free fatty acids (FFA, C and D) and \( \beta \)-hydroxybutyric acid (BHBA, E and F) during an intravenous adrenaline stimulation test. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, effect of time, \( p < 0.0001 \); ii, effect of time, \( p < 0.01 \); iii, time*experimental group interaction, \( p < 0.01 \); iv, time*experimental group interaction, \( p < 0.05 \)).
<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control  n=7-9</td>
<td>FGR-Saline n=7-10</td>
</tr>
<tr>
<td>ADR-AUCG₁₅ (mmol·L⁻¹) **</td>
<td>14.1±1.2</td>
<td>13.5±1.2</td>
</tr>
<tr>
<td>ADR-AUCGT (mmol·L⁻¹)</td>
<td>44.5±5.6</td>
<td>45.5±4.9</td>
</tr>
<tr>
<td>Ln ADR-AUCFFA₁₅ (mmol·L⁻¹)</td>
<td>0.4±0.4</td>
<td>0.5±0.3</td>
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<tr>
<td>Ln ADR-AUCFFAT (mmol·L⁻¹)</td>
<td>0.7±0.8</td>
<td>1.2±0.8</td>
</tr>
<tr>
<td>Ln ADR-ACBHBA₁₅ (mmol·L⁻¹)</td>
<td>-0.5±0.5</td>
<td>-0.8±0.5</td>
</tr>
<tr>
<td>Ln ADR-ACBHBAT (mmol·L⁻¹)</td>
<td>0.8±0.4</td>
<td>0.4±0.3</td>
</tr>
<tr>
<td>Peak Glucose (mmol·L⁻¹) *</td>
<td>5.2±0.1</td>
<td>5.0±0.1</td>
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<tr>
<td>Ln Peak FFA (mmol·L⁻¹)</td>
<td>-1.0±0.5</td>
<td>-1.1±0.5</td>
</tr>
<tr>
<td>Basal BHBA (mmol·L⁻¹) *</td>
<td>0.37±0.05</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td></td>
<td>Control  n=7-9</td>
<td>FGR-Saline n=6-9</td>
</tr>
<tr>
<td>ADR-AUCG₁₅ (mmol·L⁻¹) **</td>
<td>13.4±2.7</td>
<td>12.8±2.7</td>
</tr>
<tr>
<td>ADR-AUCGT (mmol·L⁻¹)</td>
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<td>40.0±5.2</td>
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<tr>
<td>Ln ADR-AUCFFA₁₅ (mmol·L⁻¹)</td>
<td>0.6±0.3</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>Ln ADR-AUCFFAT (mmol·L⁻¹)</td>
<td>1.6±0.5</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>Ln ADR-ACBHBA₁₅ (mmol·L⁻¹)</td>
<td>-0.8±0.5</td>
<td>-0.9±0.5</td>
</tr>
<tr>
<td>Ln ADR-ACBHBAT (mmol·L⁻¹)</td>
<td>0.8±0.3</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>Peak Glucose (mmol·L⁻¹) *</td>
<td>5.1±0.2</td>
<td>5.1±0.2</td>
</tr>
<tr>
<td>Ln Peak FFA (mmol·L⁻¹)</td>
<td>-1.3±0.4</td>
<td>-1.4±0.4</td>
</tr>
<tr>
<td>Basal BHBA (mmol·L⁻¹) *</td>
<td>0.37±0.03</td>
<td>0.39±0.04</td>
</tr>
</tbody>
</table>

Table 8.4 Plasma glucose, free fatty acids and β-hydroxybutyric acid response to an adrenaline stimulation test at 18 months of age

Area under the curve (AUC) for plasma glucose (G), free fatty acids (FFA) and β-hydroxybutyric acid (BHBA) in the early phase (first 15 minutes: ADR-AUCG₁₅, ADR-AUCFFA₁₅ and ADR-ACBHBA₁₅) and 60 minutes (ADR-AUC₁₅, ADR-AUCFFA₁₅, and ADR-ACBHBA₁₅), and peak glucose, FFA and BHBA during an adrenaline stimulation test. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females, **p=0.06, females; °p=0.07, females; °°p=0.08, males, °°°p=0.10, males.
Figure 8.5 Peak plasma free fatty acid concentration during an adrenaline stimulation test relative to abdominal fat tissue at 18 months of age

The correlation between peak concentration of plasma free fatty acids (FFA) and weight at 18 months of age in female (A: Control, green, n=9; FGR-Saline, blue, n=11; FGR-IGF1, red, n=10) and male (B: Control, green, n=9; FGR-Saline, blue, n=7; FGR-IGF1, red, n=8) sheep.

8.3.4. Metyrapone® test

Plasma ACTH, cortisol, 11-deoxycortisol, cortisol to cortisone ratio and changed significantly over the duration of the test in females and males (Figure 8.6: A-H). There was a trend (p=0.1) towards reduced basal plasma cortisol concentration in FGR-Saline females compared with Control, and reduced (p<0.05) basal plasma 11-deoxycortisol concentration in FGR-Saline compared with Control. In females, there was a main effect of experimental group (p<0.05) and a time*experimental group interaction for plasma cortisol concentration (p<0.01) with reduced cortisol in FGR-Saline females from baseline to the 30 minute sample (Figure 8.6: A). Plasma corticosteroid and ACTH concentrations did not return to baseline within the duration of the test (Figure 8.6 A-H). Plasma cortisol concentration did not return to basal concentration by 24 hours post-Metyrapone® infusion (Figure 8.6: A and B).

The logarithmic area under the curve of plasma 11-deoxycortisol (MET-AUC₃) was significantly less (p<0.05) in FGR-IGF1 compared with Control females, whilst plasma area under the curve for cortisol (MET-AUC₇), cortisone (MET-AUC₉), and ACTH (MET-AUC₉)
were not different amongst groups in either sex (Table 8.6). Peak plasma 11-deoxycortisol concentration ($p<0.01$) and peak ACTH ($p<0.05$) were significantly lower in FGR-IGF1 females compared with Controls. There was a trend towards a significant decrease in basal plasma 11-deoxycortisol to cortisol in females ($p=0.1$), and the logarithmic transformation of the peak plasma ACTH concentration ($p=0.05$) in males. The logarithmic transformation of absolute cortisol suppression ($\text{Ln MET-SUPP}_r$), relative cortisol suppression ($\%$, MET-SUPP$_r$), cortisol nadir, basal cortisone to cortisol ratio, basal ACTH and peak to basal ACTH ratio were not different amongst groups in either sex.
Figure 8.6 Plasma cortisol, 11-deoxycortisol, cortisone and adrenocorticotropic hormone during a Metyrapone® test at 18 months of age

Left panels: females (Control, green, n=6-7; FGR-Saline, blue, n=7; FGR-IGF1, red, n=8-10); right panels: males (Control, green, n=6-7; FGR-Saline, blue, n=8-9; FGR-IGF1, red, n=8). Concentrations of plasma cortisol (A and B), 11-deoxycortisol (C and D), the cortisol to cortisone ratio (E and F) and concentration of plasma adrenocorticotropic hormone (ACTH, G and H) during an intravenous Metyrapone® test. Data are least square means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, main effect of experimental group, p<0.05; ii, effect of time, p<0.0001; iii, effect of time, p<0.05; iv, time*experimental group interaction, p<0.01.)
Table 8.5 Plasma cortisol, cortisone, 11-deoxycortisol, and adrenocorticotropic hormone in response to a Metyrapone® test at 18 months of age

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th></th>
<th>Male</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FGR-Saline</td>
<td>FGR-IGF1</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>n=6-8</td>
<td>n=7</td>
<td>n=8-10</td>
<td>n=6-7</td>
</tr>
<tr>
<td>Ln MET-AUC_F (ng·mL⁻¹)</td>
<td>5.1±0.3</td>
<td>4.8±0.3</td>
<td>5.1±0.3</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>MET-AUC_F (ng·mL⁻¹)</td>
<td>122±22</td>
<td>102±28</td>
<td>117±22</td>
<td>65.5±8.9</td>
</tr>
<tr>
<td>MET-AUC_E (ng·mL⁻¹) *</td>
<td>2,855±316a</td>
<td>2,159±342b,a</td>
<td>1,607±279b</td>
<td>1,948±176</td>
</tr>
<tr>
<td>Ln MET-AUC_ACTH (pg·mL⁻¹)</td>
<td>10.1±0.3</td>
<td>9.3±0.3</td>
<td>9.2±0.2</td>
<td>9.4±0.1</td>
</tr>
<tr>
<td>Ln MET-SUPP_F (ng·mL⁻¹)</td>
<td>2.3±0.5</td>
<td>1.4±0.6</td>
<td>1.8±0.5</td>
<td>1.2±0.6</td>
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<td>MET-SUPP_F (%)</td>
<td>-95.6±3.0</td>
<td>-94.3±3.6</td>
<td>-91.1±3.0</td>
<td>-93.2±0.9</td>
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<td>Basal Cortisol (ng·mL⁻¹)</td>
<td>8.7±1.3</td>
<td>3.8±1.3</td>
<td>6.0±1.0</td>
<td>7.1±1.5</td>
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<tr>
<td>Ln Cortisol Nadir (ng·mL⁻¹)</td>
<td>-0.8±0.2</td>
<td>-1.3±0.2</td>
<td>-1.0±0.1</td>
<td>-1.2±0.2</td>
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<tr>
<td>Basal S (ng·mL⁻¹) *</td>
<td>0.088±0.012a</td>
<td>0.067±0.012b</td>
<td>0.072±0.012a,b</td>
<td>0.107±0.047</td>
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<td>Peak S (ng·mL⁻¹) **</td>
<td>38.2±3.2a</td>
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<td>21.2±1.4</td>
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<td>Ln Basal S:F</td>
<td>-3.1±0.2</td>
<td>-2.5±0.2</td>
<td>-2.6±0.1</td>
<td>-2.2±0.2</td>
</tr>
<tr>
<td>Ln Basal E:F</td>
<td>-1.6±0.1</td>
<td>-1.4±0.1</td>
<td>-1.5±0.1</td>
<td>-1.3±0.1</td>
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<tr>
<td>Basal ACTH (pg·mL⁻¹)</td>
<td>2.4±0.5</td>
<td>1.9±0.6</td>
<td>2.3±0.5</td>
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<tr>
<td>Ln Peak ACTH (pg·mL⁻¹) * **</td>
<td>5.8±0.2a</td>
<td>5.1±0.2a,b</td>
<td>4.8±0.2b</td>
<td>5.2±0.1</td>
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<td>Ln Peak:Basal ACTH</td>
<td>3.4±0.5</td>
<td>3.3±0.6</td>
<td>2.8±0.5</td>
<td>2.6±0.1</td>
</tr>
</tbody>
</table>

Area under the curve (AUC) for plasma cortisol (F), cortisone (E), 11-deoxycortisol (S), adrenocorticotropic hormone (ACTH) (MET-AUC_F, MET-AUC_E, MET-AUC_S, MET-AUC_ACTH), the natural logarithmic transformation of the absolute (Ln MET-SUPP_F), and relative cortisol suppression (MET-SUPP_F, %) from baseline to the nadir, the natural logarithmic transformation of the cortisol nadir, peak 11-deoxycortisol (S), basal 11-deoxycortisol to cortisol ratio (S:F), basal cortisone to cortisol ratio (E:F), basal ACTH, the natural logarithmic transformation of peak ACTH, and the natural logarithmic transformation of the peak to basal ACTH ratio in response to a Metyrapone® test. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: *p<0.05, females; **p<0.01, females; *p=0.1, females; ** p=0.05 males.
8.3.5. Arginine Vasopressin-Corticotropin-Releasing Hormone Stimulation Test

Plasma ACTH and cortisol changed significantly over the duration of the test in females and males (Figure 8.7: A-D). In females, there was a time*experimental group interaction for plasma ACTH concentration ($p<0.05$, Figure 8.7: A) with FGR-IGF1 concentrations less than Control during the initial peak response.

The logarithmic transformation of the peak to basal ACTH concentration difference was significantly greater in FGR-IGF1 males compared with FGR-Saline ($p<0.05$, Table 8.7), whilst there were no differences amongst groups in females. The area under the curve of plasma cortisol (AVPC-AUC$_{c}$), ACTH (AVPC-AUC$_{ACTH}$), the logarithmic transformation of basal and peak cortisol concentrations, basal and peak ACTH concentrations were not different amongst groups in either sex.

Basal plasma cortisol prior to the arginine vasopressin-corticotropin-releasing hormone stimulation test did not return to a concentration comparable to the pre- Metyrapone® test basal sample.
Figure 8.7 Plasma cortisol and adrenocorticotropic hormone during an arginine vasopressin corticotropin-releasing hormone stimulation test at 18 months of age

Left panels: females (Control, green, n=8-9; FGR-Saline, blue, n=8; FGR-IGF1, red, n=9-10); right panels: males (Control, green, n=6-8; FGR-Saline, blue, n=7-9; FGR-IGF1, red, n=7-8). Concentrations of plasma cortisol (A and B) and ACTH (C and D) during an intravenous arginine vasopressin corticotropin-releasing hormone test. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Roman numerals denote the significant difference between experimental groups (RM ANOVA: i, effect of time, p<0.001; ii, time*experimental group interaction, p<0.05).
<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th></th>
<th></th>
<th>Male</th>
<th></th>
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<tr>
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<td>Control</td>
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<td>n=9-10</td>
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<td>n=7-8</td>
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<tr>
<td>AVPC-AUC&lt;sub&gt;F&lt;/sub&gt; (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>7,344±933</td>
<td>7,418±1,194</td>
<td>8,262±1,192</td>
<td>5,592±646</td>
<td>5,228±747</td>
<td>5,518±747</td>
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<tr>
<td>AVPC-AUC&lt;sub&gt;A&lt;sub&gt;ACTH&lt;/sub&gt;&lt;/sub&gt; (pg·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>19,986±4,561</td>
<td>19,225±4,775</td>
<td>18,313±4,711</td>
<td>14,580±1,546</td>
<td>10,011±1,978</td>
<td>17,081±2,142</td>
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<tr>
<td>Ln Basal Cortisol (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.9±0.2</td>
<td>2.0±0.2</td>
<td>2.1±0.2</td>
<td>1.9±0.1</td>
<td>2.0±0.2</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>Ln Peak Cortisol (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.6±0.1</td>
<td>4.5±0.1</td>
<td>4.7±0.1</td>
<td>4.3±0.1</td>
<td>4.3±0.1</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>Basal ACTH (pg·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>11.3±2.0</td>
<td>8.8±2.5</td>
<td>11.0±2.4</td>
<td>12.7±3.1</td>
<td>14.5±2.3</td>
<td>9.8±2.3</td>
</tr>
<tr>
<td>Peak ACTH (pg·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>741±140</td>
<td>568±152</td>
<td>495±148</td>
<td>359±86</td>
<td>285±74</td>
<td>394±74</td>
</tr>
<tr>
<td>Ln Peak:Basal ACTH †</td>
<td>4.6±0.1</td>
<td>4.5±0.1</td>
<td>4.7±0.1</td>
<td>3.5±0.4&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 8.6 Plasma cortisol and adrenocorticotropic hormone during an arginine vasopressin/corticotropin-releasing hormone test at 18 months of age**

Area under the curve (AUC) for plasma cortisol (F) and adrenocorticotropic hormone (ACTH) (AVPC-AUC<sub>F</sub>, AVPC-AUC<sub>A<sub>ACTH</sub></sub>), the natural logarithmic transformation of the basal and peak cortisol and ACTH, and the natural logarithmic transformation of the peak to basal plasma ACTH ratio in response to an arginine vasopressin/corticotropin-releasing hormone test. Data are least square means ± SEM of either the raw data or natural logarithmic (Ln) transformation. Values with different superscripts report significant differences amongst experimental groups on post hoc testing: †p<0.05, males.
8.4. Discussion

8.4.1. Glucose and Insulin Axis Response at 18 Months of Age in Females

Although not statistically significant, insulin sensitivity during the steady state period of a hyperglycaemic clamp was decreased 23% in FGR-Saline females compared with Control, but increased 40% in FGR-IGF1 females. FGR-IGF1 females also required a significantly greater rate of glucose infusion to maintain blood glucose during this period compared with FGR-Saline whilst, interestingly, mean insulin secretion was increased 43% in FGR-IGF1 compared with Control females. Following an L-Arginine bolus, both Control and FGR-IGF1 females required greater glucose infusion than FGR-Saline to maintain blood glucose, whilst the change in plasma insulin concentration from steady state following the L-Arginine infusion tended to be greater in FGR-Saline females compared with FGR-IGF1 and Control. Interestingly, glucose disposition index approached significance in females, with FGR-Saline 50% less than Control and FGR-IGF1 79% greater than FGR-Saline, whilst the change in plasma insulin concentration in the first 15 minutes of a GTT tended to be less (p=0.06) in FGR-Saline females compared with Control, but was 77% greater in FGR-IGF1 females compared with FGR-Saline. These data could suggest reduced peripheral glucose uptake in FGR-Saline females despite increased insulin secretion.

A negative correlation between insulin sensitivity at birth, as estimated by glucose to insulin ratio (Chapter 4), and plasma glucose area under the curve following a GTT is consistent within groups in females. This could suggest that fetal growth restriction resulted in compromised glucose handling, which could promote the development of metabolic dysfunction at a more advanced age.
In female Control sheep, increased visceral adipose was correlated with decreased glucose disposition, or peripheral sensitivity to glucose uptake (Gatford, et al., 2008), suggesting a relationship between reduced pancreatic function and greater visceral adiposity; interestingly, in FGR-IGF1 females, the opposite was true: increased glucose disposition was correlated with increased visceral adiposity. The reduction in relative visceral adipose of FGR-IGF1 females compared with Controls at 18 months of age (Chapter 6) could suggest that, as glucose disposition was not different between these groups other mechanisms, such as disturbed lipogenesis or lipid metabolism, may have contributed to reduced adiposity in IGF1 females. A 32% reduction in glucose disposition in FGR-Saline females is consistent with data in small-for-gestational age children (Mericq et al., 2005). Mericq and colleagues suggest that reduced pancreatic β-cell compensation is associated with increased propensity for visceral adipose deposition; however, in the current study, FGR-Saline females did not have increased visceral adipose compared with Controls at 18 months of age. Recent research reports that hepatic and skeletal muscle insulin resistance is independent of organ-specific lipid deposition in low birth weight adults when matched for body weight and body mass index (Dufour & Falk Petersen, 2011). However in obese adolescents reduced glucose disposition (Bacha, Saad, Gungor, & Arslanian, 2006) and hepatic lipid deposition (D'Adamo et al., 2010) are thought to contribute to insulin resistance. Future investigations in post mortem tissues in these animals should assess tissue-specific insulin signalling pathways and intramyocellular and hepatic lipid content to elucidate whether organ-specific lipid deposition precedes alteration of visceral adiposity in this paradigm, and whether intra-amniotic IGF1 disturbs this relationship in females.
8.4.2. Adrenergic Response at 18 Months of Age in Females

A significant increase in basal plasma BHBA in FGR-IGF1 females compared to FGR-Saline, and a trend towards increased BHBA response could suggest increased capacity for ketotic response in IGF1 females. Whilst abdominal fat mass at 18 months is positively correlated with greater peak plasma free fatty acid concentration in response to adrenaline infusion in FGR-IGF1 females and approaches significance in Control females, this relationship is not present in FGR-Saline females. Fetal growth restriction desensitises fetal adipose \( \beta_2 \)-adrenergic receptors and impairs lipolysis (Chen et al., 2010) and free fatty acid mobilisation and oxidation postnatally (Yates et al., 2012) in the sheep. As FGR-Saline females had comparable visceral adipose compared with Controls at 18 months (Chapter 6), reduced fatty acid mobilisation could be due to adrenergic desensitisation. Greater basal plasma BHBA concentration and a trend towards increased BHBA area under the curve despite comparable plasma free fatty acid concentration in FGR-IGF1 females compared with Control could suggest increased adrenergic-stimulated fat mobilisation, or, perhaps an underlying disruption of hepatic ketone body metabolism. This could contribute to reduced visceral adipose in FGR-IGF1 females compared with Control. To identify whether adrenergic sensitivity is increased in response to intra-amniotic IGF1 treatment of fetal growth restriction in the female adipose tissue adrenergic receptor content and molecular mechanisms associated with hepatic BHBA production and hepatic mitochondrial oxidative capacity should be investigated. Future studies should investigate de novo lipogenesis and lipolysis as well to identify potential underlying disruption.
8.4.3. Hypothalamic-Pituitary-Adrenal Axis Response at 18 Months of Age in Females

The lack of major differences amongst groups in females in response to HPA axis stimulation with intravenous AVP/CRH suggests that homeostatic regulation at 18 months of age in the fetal growth-restricted female sheep is sufficient to account for discrete dysregulation. Our data are in agreement with a previous study investigating the effects of placental restriction and chronic maternal undernutrition on postnatal response to AVP/CRH (Wallace, et al., 2011). However, compensatory modulation of the HPA axis may maintain homeostatic regulation, only becoming apparent with more specific investigations. Indeed, testing pituitary feedback response to a decrease in circulating cortisol and the adrenal response to ACTH stimulation with an intravenous Metyrapone® bolus suggests abnormal responses in FGR-IGF1 females compared with Controls.

There was a significant 25% reduction in basal plasma 11-deoxycortisol concentration in FGR-Saline females compared with Controls prior to the Metyrapone® test. A Metyrapone® bolus resulted in reduced peak plasma ACTH concentration in FGR-IGF1 sheep compared to Controls. A 20% reduction in peak plasma ACTH could suggest reduced pituitary ACTH secretion (Leisti, 1977), rather than increased negative feedback (Conte-Devolx et al., 1992). Additionally, a non-significant ~40% decrease in both peak plasma 11-deoxycortisol and area under the curve could suggest reduced adrenal response. 11-deoxycortisol is synthesised from 17-hydroxyprogesterone by cytochrome P450 21-hydroxylase (CYP21a1) in the adrenal gland; substrate and enzyme inadequacies in this steroidogenic pathway can result in adrenal insufficiency (Arlt & Allolio, 2003). However, as work in rats reports activation in the paraventricular nucleus of the hypothalamus two hours following a 50 mg·kg⁻¹ Metyrapone® injection in rats (Rotllant, Ons, Carrasco, & Armario, 2002), and a three-hour 100 mg·kg⁻¹
infusion in rams mediates hypothalamic arginine vasopressin secretion, a confounding effect on hypothalamic centres cannot be discounted. Interestingly, although previous fetal research using the current paradigm reports relative adrenal hyperplasia in FGR-Saline fetuses compared to Controls at 130 days gestational age, there are no differences in adrenal weight in FGR-IGF1 fetuses (Wali, et al., 2012), whilst in the current study there is a trend towards increased relative adrenal weight at post mortem (Chapter 6). This could suggest compensatory adrenal hyperplasia. Assessing molecular capacity for steroidogenesis in post mortem tissues could help determine whether HPA axis dysfunction and adrenal hyperplasia in adult female FGR-IGF1 sheep is associated with postnatal steroidogenic dysregulation.

8.4.4. Glucose and Insulin Axis Response at 18 Months of Age in Males

Greater peak glucose concentration and change in insulin secretion in the first 15 minutes of the glucose tolerance test in FGR-Saline males compared to both Control and FGR-IGF1 could suggest reduced peripheral sensitivity of glucose uptake to insulin, possibly due to reduced skeletal muscle insulin sensitivity (De Blasio, et al., 2012). Although a 30% increase in insulin sensitivity in FGR-IGF1 males compared with FGR-Saline as determined by hyperglycaemic clamp was not significant at this relatively young age, future studies should elucidate whether manifest dysfunction in homeostatic control of the glucose-insulin access due to FGR is corrected by intra-amniotic IGF1 treatment in males.

Interestingly, FGR-IGF1 males had a greater change in plasma insulin concentration following an L-Arginine bolus compared with FGR-Saline, which could suggest greater pancreatic insulin stores (Limesand, et al., 2006), possibly due to pancreatic β-cell compensation (Gatford, et al., 2008). Whilst in Control males insulin secretion appropriate for glucose uptake (glucose disposition index) was positively correlated with visceral adiposity, the opposite was true for
FGR-Saline and FGR-IGF1 males. This could suggest impaired balance between glucose sensitivity and insulin secretion, which could affect peripheral glucose uptake. However, increased insulin sensitivity at birth in males, as estimated by glucose to insulin ratio (Chapter 4), is correlated with improved glucose tolerance in both Control and FGR-IGF1 males at 18 months of age, whilst in FGR-Saline males there is a trend towards an association with reduced glucose tolerance.

Reduced glucose tolerance in the early phase of a GTT in FGR-Saline males could be due to reduced glucose sensitivity and reduced capacity for glucose-stimulated insulin secretion. These data could suggest that intra-amniotic treatment of fetal growth restriction with IGF1 results in normalisation of peripheral insulin sensitivity, which persists postnatally. Admittedly, as glucose regulation changes with age (Clarke et al., 2000) and developmental plasticity persists through adolescence (Gatford, et al., 2004; Poore et al., 2007), discrete metabolic testing at 18 months may be inadequate to assess long-term programming effects.

Accelerated early postnatal growth velocity, which was apparent in both FGR-Saline and FGR-IGF1 sheep (Chapter 4), is associated with increased insulin sensitivity following placental restriction-mediated fetal growth restriction (De Blasio, Gatford, McMillen, et al., 2007), but peripheral insulin resistance at 16 months of age (Sebert, et al., 2011). Accelerated postnatal growth velocity could indicate susceptibility to reduced peripheral insulin sensitivity in adulthood. As there were no differences in adult visceral adipose at 18 months of age amongst males (Chapter 6), this could suggest that growth-restricted males are predisposed to ageing-associated increase in visceral adipose accretion as insulin sensitivity decreases, regardless of intra-amniotic IGF1 treatment. As such, 18 months of age might be too early to identify abject physiological dysfunction. Post mortem assessment of peripheral insulin
signalling and pancreatic β-cell islet function could help determine if tissue-specific adaptations occur prior to whole-body physiological level.

8.4.5. Adrenergic Response at 18 Months of Age in Males

A trend towards a reduced glucose response at 15 minutes and reduced peak plasma glucose concentration in response to an adrenaline stimulation test in FGR-IGF1 sheep compared with Controls could suggest adrenergic insensitivity. In the growth-restricted fetal and neonatal sheep, adrenergic insensitivity is associated with reduced glucose oxidation (Yates, et al., 2012); reduced glucose response to adrenaline in FGR-IGF1 males could suggest reduced hepatic glucose production and a “thrifty” metabolic state resulting from intra-amniotic treatment. Interestingly, increased abdominal fat mass at 18 months is associated with greater peak plasma free fatty acid concentration in response to intravenous adrenaline injection in FGR-IGF1 males, whilst the correlation approaches significance in FGR-Saline males. These data could suggest that fetal growth restriction promotes metabolic flexibility in males by improving lipid mobilisation; assaying plasma free fatty acid concentrations during the glucose tolerance test could help determine if there are differences in insulin-induced inhibition of lipolysis in response to fetal growth restriction in males (Poore et al., 2014). Post mortem analyses in visceral and perirenal adipose should assess tissue-specific lipolytic capacity and adrenergic receptor content to investigate whether lipolytic and oxidative capacity is increased in growth-restricted males in adulthood.

8.4.6. Hypothalamic-Pituitary-Adrenal Axis Response at 18 Months of Age in Males

Whilst neither FGR-Saline nor FGR-IGF1 were different from Control males in response to either the Metyrapone® or AVP/CRH tests in the current study, FGR-Saline rams had reduced
AVP/CRH-stimulated relative ACTH response, and a non-significant 40% reduction in the ACTH area under the curve compared to FGR-IGF1 males. This could suggest a lack of overt pituitary dysregulation at the whole-body level in the current paradigm in response to fetal growth restriction. Functional development of the ovine fetal hypothalamus throughout gestation (Mühlhäusler, et al., 2004) suggests that in the current paradigm both fetal growth restriction and the intra-amniotic IGF1 intervention are conducted during a time period which could produce a persistent programming-effect. A significantly greater change in ACTH secretion following AVP/CRH infusion in FGR-IGF1 compared to FGR-Saline males could suggest increased hypothalamic responsiveness and greater corticotrophic response in the pituitary of FGR-IGF1 males. Both AVP and CRH increase ACTH secretion in adult ovine pituitary cells (van de Pavert, Clarke, Rao, Vrana, & Schwartz, 1997); greater corticotrophic response in IGF1-treated males could suggest increased hypothalamic sensitivity, which persists postnatally. Although there was a trend towards reduced peak ACTH and ~40% decrease in the area under the curve of plasma 11-deoxycortisol following the Metyrapone® bolus in FGR-IGF1 males compared with both Control and FGR-Saline, these were not significant.

8.5. Limitations

Isolation and restraint was utilised during hyperglycaemic clamps. We acknowledge the effect of adiposity on response to restraint stress (Tilbrook, et al., 2008).

Although the hyperglycaemic clamp yields an estimate of insulin sensitivity comparable to that of the hyperinsulinaemic-euglycaemic clamp (Mitrakou, et al., 1992), we are unable to
elucidate differences in hepatic versus peripheral insulin sensitivity with the hyperglycaemic clamp (Færch, Brøns, Alibegovic, & Vaag, 2010).

8.6. Conclusions

Data suggest that intra-amniotic IGF1 treatment of the growth restricted fetus results in persistent, sex-specific alteration of hypothalamic-pituitary-adrenal axis regulation and metabolic capacity at 18 months of age. Postnatal sequelae of intra-amniotic IGF1 treatment in the growth-restricted female sheep may suggest compromised hepatic ketone metabolism and greater insulin sensitivity concurrent with reduced pituitary and adrenal responsiveness, which may result in compensatory adrenal hyperplasia. Intra-amniotic IGF1-treatment may increase pancreatic insulin stores in males, reduce adrenergic sensitivity and increase corticotrophic responsiveness. However, the failure of plasma ACTH and cortisol to return to basal concentration in sheep following Metyrapone® administration could affect the subsequent AVP/CRH test on the following day. Interestingly, there were no significant differences in basal plasma ACTH or cortisol response between FGR-Saline and FGR-IGF1 sheep compared to Control, which could suggest that HPA axis stimulation was conducted from a suppressed, rather than a basal, state. Altered physiological responsiveness in response to discrete testing at 18 months of age does not seem to result in overt dysfunction in intra-amniotically treated sheep. Tissue-specific adaptations occur prior to the development of physiological compromise; thus, molecular studies are needed to elucidate whether detrimental adaptations exist prior to the development of overt systemic dysfunction. Additionally, 18 months of age might be too young to observe detrimental long-term effects of intra-amniotic IGF1 intervention of the growth-restricted fetus. As such, further research should be conducted in aged animals to confirm long-term effects.
Chapter 9. Conclusions

There are currently no interventions which improve clinical outcomes in fetal growth-restricted offspring. Adverse events are common. Increased risk of perinatal morbidity and mortality, and a predisposition to the development of metabolic disease in later life suggest that the most appropriate time to intervene in order to improve the health status of the in utero growth-restricted individual is prior to the development of postnatal complications.

The purpose of this thesis was to examine the postnatal outcomes of an experimental intervention for the treatment of fetal growth restriction in utero. Sheep were utilised in the experimental paradigm as singleton pregnancies, allowing the investigation of late-gestation placental insufficiency without the confounding effect of multiple pregnancies (Bloomfield, Oliver, & Harding, 2007; Rumball, et al., 2008). Additionally, the size of the ovine conceptus enabled direct fetal assessment during the experimentation period, and precocial maturation allowed investigation of a fetal intervention during developmental stages comparable to that of humans. Several ovine paradigms inducing FGR exist, including carunclectomy (Owens, Falconer, & Robinson, 1986), maternal hyperthermia (Bell, Wilkening, & Meschia, 1987), umbilical artery ligation (Oh, Omori, Hobel, Erenberg, & Emmanouilides et al., 1975), over-nourished adolescent pregnancy established by embryo transfer (Wallace, Aitken, & Cheyne, 1996), and placental embolisation (Creasy, Barrett, De Swiet, Kahanpää, & Rudolph, 1972). Whilst some physiological adaptations associated with FGR are common amongst various paradigms, placental embolisation-induced placental insufficiency enabled simulation of human late gestation onset FGR (Morrison, 2008). Although physiological studies and tissue assays in previous fetal investigations utilising IGF1 intervention of placental embolisation-
induced FGR in sheep do not provide evidence of a sexually dimorphic response (Darp, et al., 2010; Eremia, et al., 2007; Wali, et al., 2012), this could be due to experimental groups of insufficient numbers to demonstrate a sex-specific effect. As the placenta and, thus, the fetus, responds to adverse intrauterine events in an intrinsically sex-specific manner (Clifton, 2010), a differential response in male and female offspring to intra-amniotic IGF1 intervention was expected.

The experiments described in this thesis were designed to investigate the effects of intra-amniotic IGF1 treatment of the growth-restricted ovine fetus on perinatal morbidity and mortality, phenotypic and endocrine characteristics in early postnatal life, tissue-specific somatotrophic and metabolic gene expression, postnatal growth patterns, and later indices of somatotrophic, hypothalamic-pituitary-adrenal axis and metabolic function. Importantly, these experiments allowed the effects of sexual dimorphism to become manifest as physiological testing was conducted on post-pubertal sheep with gonads intact. These experiments demonstrate that the effects of an intra-amniotic intervention for the treatment of fetal growth restriction persist well into adulthood and have distinct sex-specific outcomes.

Weekly intra-amniotic administration of 360 μg IGF1 to the growth-restricted fetus did not increase the incidence of adverse perinatal events, nor did it improve morbidity or mortality in the first two weeks after birth. This could suggest that increased fetal growth trajectory (Wali, et al., 2012) and, thus, metabolic demand do not alter susceptibility to adverse perinatal events. Conversely, intra-amniotic IGF1-treatment did not decrease mortality, which could suggest that increased growth does not improve survival in the growth-restricted neonatal lamb during the perinatal period.
The treatment-mediated alterations in neonatal characteristics include a modest increase in size at birth in IGF1-treated females and a prolonged period of accelerated growth velocity of weight during the early postnatal “rapid growth phase” in both sexes. The growth restriction-mediated neonatal acceleration of growth velocity of weight, possibly through IGF2-mediated mechanisms (Finkielstain, et al., 2009), could prolong the period of susceptibility to the development of later metabolic disease. Accelerated weight gain during infancy, from 0-3 months of age, is positively associated with both greater visceral adiposity and lean mass in adolescent humans (Chomtho, et al., 2008) and inversely associated with insulin sensitivity in young adulthood (Leunissen, et al., 2009). This could suggest that despite recovery of body size, underlying metabolic homeostatic disturbance persists. Whilst in the current study both FGR-Saline and FGR-IGF1 groups displayed accelerated postnatal growth velocity of weight compared with Controls, only FGR-IGF1 females exhibited lean mass measures different from Controls after the perinatal period. Pubertal acceleration of lean mass accretion in FGR-IGF1 females compared with Controls could suggest alteration of puberty-associated endocrine control of growth (Veldhuis et al., 2005).

Increased growth rates associated with increased insulin-signalling in the growth-restricted neonate (Morrison, Duffield, Muhlhausler, Gentili, & McMillen, 2010) could predispose to the development of cardio-metabolic risk factors (Bruce & Hanson, 2010; Varga, Harangi, Olsson, & Hansen, 2010). Although accelerated postnatal growth velocity following carunclectomy-mediated placentally-restricted fetal growth is associated with the development of visceral obesity in the sheep (De Blasio, Gatford, McMillen, et al., 2007), in the current study, placental insufficiency-induced fetal growth-restriction followed by accelerated postnatal growth velocity of weight did not significantly increase visceral adiposity. Interestingly, IGF1
treatment of the growth-restricted fetus decreased visceral adiposity in females compared with Controls at 18 months of age. This novel and unexpected finding could have implications for the prevention of excess visceral adipose in the growth-restricted offspring.

Previous work assessing the pharmacokinetics of intra-amniotically-administered IGF1 reported direct uptake into fetal circulation and localisation to fetal tissues (Bloomfield, Breier, et al., 2002), and tissue-specific alteration of key somatotrophic genes (Shaikh, et al., 2005), which could alter somatotrophic sensitivity postnatally. The lack of appreciable differences in size at birth amongst growth-restricted groups in males in the current studies could be due to a sex-specific reduction in somatotrophic sensitivity (Geary, et al., 2003). Interestingly, data suggest that intra-amniotic IGF1 increased GHR and IGF1 skeletal muscle and hepatic IGF1R mRNA at one week of age in females, and restored the growth restriction-mediated disturbance of the correlation between plasma IGF1 concentration and body weight, which could suggest improved somatotrophic sensitivity. Whilst in FGR-IGF1 males, increased hepatic IGF1 expression compared with FGR-Saline at one week of age and increased hepatic and skeletal muscle IGF1 expression could suggest greater bioavailability, but a perturbed IGF to weight relationship in adulthood could suggest reduced somatotrophic sensitivity. These data could suggest that elucidating sexually dimorphic somatotrophic sensitivity may be of importance when designing interventions using IGF1; therefore, optimising a sex-specific treatment protocol, which addresses differential somatotrophic maturation, may augment the efficacy of the intra-amniotic IGF1 intervention in males. A multi-centre randomised controlled trial (NCT01096784), which is currently underway to investigate the efficacy of binary recombinant human IGF1/IGFBP3 administration in preterm infants to prevent complications of preterm birth, including
retinopathy of prematurity, bronchopulmonary dysplasia, and growth failure, should help answer the question of whether sexually dimorphic somatotrophic sensitivity contributes to differential response to treatment with IGF1 in premature babies in early life.

Postnatal sequelae of intra-amniotic IGF1 treatment on somatic and organ growth, and body composition through 18 months of age are largely confined to females. Reduced relative lean mass at 4 months of age and greater relative lean mass at 18 months of age compared with Controls could suggest that puberty-associated changes in body composition, which are due to complex gonadotrophic and somatotrophic interactions (Veldhuis, Roemmich, Richmond, & Bowers, 2006), may be altered. These data suggest that greater gross lean tissue accretion could be due to increased skeletal muscle somatotrophic sensitivity, which may be augmented by peripheral nutrient signalling pathways and glucose utilisation. Interestingly, divergences in measures of body composition in FGR-IGF1 females do not become apparent until after the pubertal period. These data suggest dysregulation of the hypothalamic-pituitary axis in growth-restricted females postnatally, which could impact pubertal maturation-associated mechanisms and, thus, influence subsequent phenotypic development. The lack of appreciable differences amongst groups in males could suggest that the relatively young age of 18 months may be too early for the long-term consequences of both placental embolisation-induced FGR and intra-amniotic IGF1 intervention to manifest in males.

Reduced heart weight relative to body surface area at 18 months of age in IGF1-treated females could suggest a treatment-mediated cardiovascular change, which persists into young adulthood. Although FGR has been shown to reduce cardiac efficiency in children (Crispi, et al., 2010), we did not investigate cardiovascular outcomes in these sheep and are
unable to elucidate whether physiological compromise is evident. However, molecular studies at the tissue level could help inform as to whether gross cardiac microstructural abnormalities occur in response to treatment in females. Of concern to the cardiovascular health in these females is the apparent “liver-sparing” effect, reflected in a greater relative liver size in adulthood compared with Controls, which is associated with greater disease risk in humans (Haugen et al., 2005). As we did not directly assess the hepatic contribution to metabolic homeostasis in this study, we are unable comment on the cardio-metabolic health in these animals. Long-term glucose and insulin sensitivity has not previously been investigated in this paradigm. Whilst there was no overt dysfunction in the maintenance of physiological homeostasis in either the saline-treated or IGF1-treated growth-restricted groups, discrete changes in the glucose-insulin and hypothalamic-pituitary-adrenal axes in response to physiological testing were found.

FGR compromises insulin sensitivity postnatally in both humans (Veening, van Weissenbruch, & Delemarre-van de Waal, 2002; Veening, van Weissenbruch, Heine, & Delemarre-van de Waal, 2003) and in the sheep (Gatford, et al., 2008), and can contribute to impaired metabolic homeostasis in later life. Reduced insulin-stimulated peripheral glucose uptake and \(\text{l-Arginine-stimulated} \) insulin secretion in growth-restricted males, which is consistent with the literature (Gatford, et al., 2008), could suggest reduced pancreatic \(\beta\)-cell secretory capacity. Although not statistically significant, in IGF1-treated males there was a strong trend with a large effect size toward increased \(\text{l-Arginine-stimulated} \) insulin secretion 40%, whilst in females IGF1 treatment abrogated the growth restriction-mediated reduction (50%) in insulin sensitivity and glucose disposition (24%). Investigation of pancreatic cellularity and immunohistochemical assessment of \(\beta\)-cell mass, islet size and functional capacity could help
determine whether intra-amniotic IGF1 treatment potentiates increased insulin secretion and improves glucose disposition. Assessing genes involved in glucose signalling and the maintenance of metabolic homeostasis could help elucidate the mechanisms associated with systemic and tissue-specific glucose-insulin axis regulation and peripheral insulin signalling in adulthood. For future studies, \textit{in vitro} analysis of \( \beta \)-cell secretory capacity throughout life would be invaluable in determining whether apparent changes at the tissue level precede later systemic changes.

Data suggest that IGF1 treatment in growth-restricted females normalises the growth restriction-mediated reduction in glucose disposition despite increased capacity for relative \( \lambda \)-Arginine-stimulated insulin secretion compared with Controls. Restoration of the capacity for glucose disposition and, possibly, insulin sensitivity, could prevent a growth restriction-mediated exacerbation of age-related metabolic decline (Gatford, et al., 2004). Future studies in this paradigm should include time-course postnatal tissue analyses to determine whether glucose and insulin axis alterations in intra-amniotic IGF1-treated sheep are preceded by alterations in peripheral tissue insulin sensitivity. Additionally, following animals to a later age could help determine whether metabolic homeostasis may be dysregulated in later life.

Precocious HPA activation in the growth-restricted fetus \textit{in utero} occurs in the majority of ovine growth restriction paradigms (Morrison, 2008). Morrison and colleagues report a lack of precocious fetal HPA axis activation in the adolescent over-nourished ewe model of placental restriction. Indeed, in offspring of adolescent over-nourished ewes, HPA axis function is age- and sex-appropriate (Wallace, et al., 2011). Contrary to the adolescent ewe paradigm of FGR utilised by Wallace and colleagues, we report dysregulated hypothalamic-
pituitary-adrenal axis function in growth-restricted females postnatally. Although there were no differences in plasma glucocorticoid concentrations at birth, interestingly, intra-amniotic IGF1 treatment of the growth-restricted ovine fetus reduced pituitary and adrenal steroidogenesis in females, possibly resulting in compensatory adrenal hyperplasia to maintain homeostasis, and increased corticotrophic responsiveness in males at 18 months of age. Persistent dysregulation of the HPA axis can promote chronic disease development. As such, although overt homeostatic HPA axis dysregulation is not apparent at 18 months of age, the changes reported in the current studies are of concern for the future health of the offspring should they precede physiological dysfunction. Thus, further investigations are needed to determine if these physiological changes solely reflect peripheral mechanistic alterations, or if central alterations in hypothalamic regulation exist.

Conclusion

Intra-amniotic IGF1 tended to increase birth weight in female lambs, but did not improve fetal or perinatal morbidity or mortality. Sustained acceleration of growth velocity of weight in the perinatal period led to decreased relative visceral adiposity and increased relative lean mass in females in adulthood. IGF1 treatment increased insulin sensitivity in both sexes which, although not different from Controls, was 50% greater than FGR-Saline sheep. In contrast, changes in HPA axis modulation in IGF1-treated females in young adulthood could indicate a disturbance in glucocorticoid sensitivity and neuroendocrine regulation, which could have implications as age progresses. The long-term effects of intra-amniotic IGF1 treatment are sex-specific and persist to young adulthood; however, the life-long outcomes are not known and would require the maintenance of experimental animals to an older age, with longitudinal molecular analyses to ascertain mechanistic differences across the lifespan.
Improved placental nutrient transport in the IGF1-treated growth-restricted fetus could abrogate FGR-mediated hypermethylation of the IGF1 promoter, and influence growth and metabolism postnatally (Oberbauer, 2013). Additionally, altering the fetoplacental hormonal milieu via intra-amniotically injected IGF1 could modify the environmental cues associated with the fetal programming of deprivation (Fowden & Forhead, 2008), which could influence lifelong metabolic and physiological capacity. Investigating epigenetic mechanisms underlying postnatal alterations in phenotypic and physiologic adaptation to intra-amniotic IGF1 intervention of the growth-restricted ovine fetus could help elucidate whether this treatment induces persistent alteration of fetal programming of deprivation. Assessment of epigenetic control of pathways associated with postnatal organ growth, body size, and physiological responses would be invaluable in determining whether this intervention is of long-term benefit.
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272


