HYPOPHYSIAL AND LOCAL MEDIATORS OF ADRENOCORTICAL GROWTH AND FUNCTION BEFORE BIRTH

Jacob T. Ross B.Sc. (Hons)

Department of Physiology

The University of Adelaide

South Australia

A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

February 2000

For Robert, June and Terry Ross, and my wife Kerri - my family.

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SUMMARY

In the sheep fetus, adrenocortical growth and increases in steroidogenesis are essential for the maturation of a range of organ systems including the lungs, gut and brain, vital to successful adaptation to extra-uterine life; for the appropriate hormonal responses to acute and chronic stress *in utero* and for the timing and process of parturition. The precise mechanisms that coordinate growth and functional development of the ovine fetal adrenal gland during the last two weeks of gestation (term≈150 days of gestation) are unknown. This thesis examines the relative roles of pituitary hormones, glucocorticoids, tissue growth factors and fetal stress, in modulating the increase in adrenocortical growth and steroid synthesis in late-gestation. Chapter 1 reviews the literature regarding adrenal growth and the synthesis of steroid hormones within the adrenal gland of the sheep fetus. The relative roles of the fetal pituitary gland and cortisol in modulating the growth and functional activation of the adrenal cortex are also reviewed. The potential role of peptides arising from the amino (N)-terminal region of the adrenocorticotrophin (ACTH) precursor, pro-opiomelanocortin (POMC), is specifically discussed. Finally, the role of the insulin-like growth factors (IGFs) and fetal stress are each considered in the regulation of the growth and functional development of the ovine fetal adrenal gland prior to birth.

Chapter 2 examines the changes in adrenal growth throughout gestation, and the messenger ribonucleic acid (mRNA) abundance of the adrenal steroid-synthesising enzymes during the two weeks prior to birth in the fetal sheep. The input of the hypothalamus in the regulation of fetal adrenal growth and function is also studied, by surgical disconnection of the fetal hypothalamus and pituitary (hypothalamo-pituitary disconnection; HPD) at approximately 110 days (d) of gestation. Maintenance of fetal cortisol from 135-140 d of gestation, following fetal HPD, is also included to compare the separate effects of HPD and cortisol on adrenal development in late-gestation. This study demonstrates that there is a differential temporal regulation of mRNA expression of the steroid-synthesising enzymes within the fetal adrenal during the two weeks prior to delivery. While there is no change in the adrenal mRNA abundance of IGF-II during the two weeks prior to delivery, the adrenal expression of mRNA for IGF binding protein-2 (IGFBP-2), the major fetal IGF-II binding protein, decreases during late-gestation, suggesting that bioactivity of the IGF axis may be modulated through altered abundance and activity of the IGF binding proteins during this

time. Fetal HPD at 110 d of gestation abolishes the late-gestation increase in adrenal growth and steroidogenic enzyme mRNA abundance. Replacement of cortisol following fetal HPD, however, stimulates adrenal growth in the absence of any effects on adrenal levels of IGF-II or steroidogenic enzyme mRNA. Cortisol may, therefore, act directly at the fetal adrenal to modulate the activity of locally produced growth factors, in part through altered abundance of the IGF binding proteins, or indirectly at the surgically disconnected pituitary to stimulate adrenal growth.

Chapter 3 examines the effects of a premature elevation of fetal plasma cortisol levels on adrenal growth and steroidogenesis in fetuses with an intact hypothalamo-pituitary-adrenal (HPA) axis, prior to the onset of the ontogenic rise in fetal circulating levels of adrenocorticotrophin (ACTH) and endogenous cortisol. While exposure to elevated fetal circulating levels of cortisol during the period 109-116 d of gestation does not stimulate adrenal growth or steroidogenic enzyme gene expression, the adrenal expression of mRNA for IGFBP-2 and the steroid-metabolising enzyme 11β hydroxysteroid dehydrogenase (11βHSD-2) is decreased. It is possible that a decrease in adrenal IGFBP-2 and 11βHSD-2 expression and increased intra-adrenal glucocorticoids may enhance adrenal growth and steroidogenic responsiveness only when the fetal adrenal is simultaneously exposed to the high ACTH concentrations which prevail in the week before birth, or during chronic intra-uterine stress.

Studies in the fetal sheep have demonstrated that pro-ACTH and *N*-terminal POMC(1-77) are present in 20-50 fold higher concentrations than is ACTH(1-39) in fetal sheep circulation. While the *N*-POMC peptides have potent mitogenic and steroidogenic effects on adult rat adrenocortical cells *in vivo* and *in vitro*, no studies have examined their effect on fetal adrenal development *in utero*. Chapter 4 examines the role of peptides derived from the *N*-terminal region of POMC in the regulation of fetal adrenal growth and development. *N*-POMC(1-77) and *N*-POMC(1-49) were extracted and purified from adult bovine pituitaries and infused into the circulation of fetal sheep during late-gestation. Intra-fetal infusion of *N*-POMC(1-77), but not *N*-POMC(1-49), results in an increase in adrenal weight and in the expression of mRNA for the adrenal steroid-synthesising enzyme P450c17 (CYP17). These data indicate a possible novel role for the *N*-terminal POMC peptide in adrenal growth and steroidogenesis before birth.

IGFs are potent mitogenic factors and are important in the regulation of many aspects of fetal growth. Previous studies have demonstrated the presence of IGF-I and IGF-II mRNA

and peptide and IGF receptors in the adrenal glands of the developing fetus. Thus, IGFs potentially play an important role in the growth and development of the fetal adrenal. In Chapter 5, the effect of intra-fetal infusion of IGF-I on adrenal maturation has been investigated. Consistent with *in vitro* studies, a 10 d intra-fetal infusion of recombinant human IGF-I results in a marked increase in adrenal growth, in the absence of an effect on the expression of mRNA for the steroidogenic enzymes. These results demonstrate for the first time that IGF-I has a substantial growth promoting effect on the adrenal gland of the ovine fetus *in vivo*.

Finally, in Chapter 6, the response of the fetal adrenal gland to chronic fetal growth restriction has been examined in an experimental model wherein restriction of placental growth is secondary to the surgical removal of most of the sites of placental development (the maternal caruncles) prior to mating. A quantitative relationship has been demonstrated between adrenal growth and the degree of fetal growth restriction, regardless of the cause of growth restriction. Thus, there is a continuum among fetuses in which growth restriction is induced experimentally by carunclectomy and in fetuses where growth restriction occurs spontaneously, *i.e.* those control singleton and twin fetuses which were <3.5 kg. Placentally restricted fetal sheep have a higher ratio of adrenal : fetal body weight, and an enhanced adrenal expression of mRNA for the steroid-synthesising enzyme P450scc (CYP11A1), the rate-limiting enzyme in steroid synthesis, in addition to higher fetal circulating levels of cortisol. While adrenal growth is promoted in the placentally restricted group, the adrenal expression of mRNA for IGF-II is suppressed, in the absence of any changes in IGFBP-2 mRNA expression.

In conclusion, this dissertation describes the interactions among pituitary-derived peptides, intra-adrenal exposure to glucocorticoids and the local adrenal and endocrine IGF axes in the growth and functional activation of the ovine fetal adrenal gland before birth. The involvement of these systems in the fetal response to chronic stress and intra-uterine growth restriction is also considered. Throughout this thesis, several conceptual models of the control of adrenal growth and function in late-gestation are proposed and developed.

DECLARATION OF ORIGINALITY

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, it contains no material that has been published previously by any other person except where due reference is made. The author consents to the thesis being available for photocopying and loan.

Jacob Ross

29 February 2000

ACKNOWLEDGMENTS

This thesis was carried out in the Department of Physiology at The University of Adelaide. I would like to thank all of the people working in and associated with the Department for providing such a vibrant and interesting environment in which to conduct research.

I am indebted to my supervisors, Professor Caroline McMillen and Dr. Julie Owens, for their supervision, guidance, support and encouragement. I would like to express my sincere gratitude for their inexhaustible patience, endless enthusiasm and priceless humour, which often provided light relief from the impending seriousness of life. Thanks to Dr. Michael Roberts for his initial encouragement and advice to move to Adelaide to continue my studies, and for his irrepressible spirit. Thanks also to Associate Professor Tim Miles for his encouragement and enthusiasm in his role as postgraduate coordinator and all-round good role model. Financial support for the research presented in this thesis was provided by the University of Adelaide's postgraduate scholarship scheme, and generously supplemented by the Department, Professor McMillen and Dr. Owens.

I would particularly like to thank Dr. Giuseppe Simonetta and Ms. Linda Mundy for their advice and constant support, provided throughout many years of friendship and professional interaction. I also thank Dr. Simonetta for sharing his expertise in fetal surgery. Joe's surgical skill and natural flair for teaching has been an essential part of the success of these studies. Learning fetal surgery from this exceptional teacher was as fun as it was challenging.

Thanks go to Mr. Dan McHolm for many hours of technical advice and instruction in the numerous software applications used in the production of this thesis. Thanks also for many years of laughter and friendship, and for the occasional beer after work. I'm not sure which of these elements were more important in producing this thesis, however, I think that all were essential ingredients in its completion. Many thanks to all the members of the Department, the general staff, teaching and research staff and co-workers in Professor McMillen's and Dr. Owens' laboratories for providing a relaxed and fun working environment.

My thanks also go to Dr. Ian Phillips for his patience and perseverance in training a 'non molecular biologist' in the mystic art of genes and bugs. Thanks to Mr. Frank Carbone for

his invaluable assistance during sheep surgeries. The daily care and feeding of the sheep used in these studies was carried out by the friendly and helpful animal house staff, whose assistance I am very grateful for. I also thank Dr. Ross Young for performing the fetal hypothalamo-pituitary disconnection surgery. I would also like to thank Mark Salkeld and Andrew Bryce for their expert assistance in proof reading and knowledge of all matters 'molecular'. Their expertise in molecular biology theory and practice, and their willingness and ability to teach these arcane arts to others has been an invaluable resource.

I am indebted to several people for their advice and assistance in proof-reading and editing this manuscript. Dr. Cathie Coulter, Dr. Jeff Schwartz, Kerri Supple, Prof. McMillen, Dr. Owens and Dr. Simonetta are among the many people to whom I am grateful for helpful comments and constructive criticism. I have benefited from the assistance of numerous critical reviewers, and I would like to thank them all for the many hours spent corrrctign my speling mistaakes and grammatical fawlts. Any mistakes which remain are entirely my own.

I would particularly like to acknowledge several people whose cheerful demeanour, sense of fun and positive outlook (not to mention wicked sense of humour) have often provided a refreshing change of perspective: Joe Simonetta, Carolyn 'Smiles' Gadd, Damian 'Ginger' Adams, Dane Horton, Jeff Schwartz, Daniel Houghton, Dan McHolm, Marie Senn, Pat Buckley, Chris Wallace, Julia Pitcher, Michael Roberts, Cathie Coulter and Tom Foster, Jen Washington, Chris Wong, Garry Kerry, Chris Daly, Kerri Diener, Caroline McMillen and Julie Owens. Thank you all for sharing your sparkle. The power of laughter is an amazing thing.

Thanks also go to a very special group of people who helped to sustain me through a critical period during my candidature. To Joe and Michael Cullen; Andrew Linn; Glen Borlace; Paul and Rachel Feuerherdt and Alex Blood. You know what you did and I thank you all. Last but not least, I am very grateful to the most important people in my life, who have helped me throughout my PhD and over the many years before. To my Mum and Dad (June and Robert Ross), my sister Terry and my wife Kerri, thanks for all the love and support you have given me over the years. I couldn't have done it without you. P.S. don't stop!

ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis, in addition to those abbreviations commonly accepted. In addition, the reader is referred to Figures 1.1, 1.3 and 1.4 for the structures and relationships among some of the steroids and peptides listed below.

A .... absorbance

A_4 .... androstenedione

ACAT .... acyl coenzyme A-cholesterol acyl transferase

ACTH .... adrenocorticotrophin

ad libitum .... (Latin, 'without preparation')

AII .... angiotensin II

Amp .... ampicillin

α-MSH .... α-melanocyte-stimulating hormone

ANOVA .... analysis of variance

$17\alpha P_4$ .... 17α hydroxy progesterone

$17\alpha P_5$ .... 17α hydroxy pregnenolone

AT-1 .... angiotensin II type-1 receptor

AVP .... arginine vasopressin

b .... bioactive

b .... bovine

B .... corticosterone

3βHSD .... 3β hydroxysteroid dehydrogenase / $\Delta^5\Delta^4$ isomerase (EC1.1.1.145) enzyme, mRNA, cDNA and gene

Bt_2cAMP .... dibutyryl cyclic adenosine monophosphate

C .... Carbon atom in a steroid molecule

$[^{14}C]$ .... ^{14}Carbon

° C .... degrees Celsius

$CaCl_2$ .... calcium chloride

cAMP .... cyclic adenosine monophosphate

cDNA .... complementary deoxyribonucleic acid

CEH .... cholesterol ester hydrolase

Ci .... Curie(s)

CLIP .... corticotrophin-like intermediate lobe peptide

cpm .... counts per minute

CRH .... corticotrophin releasing hormone

CRIF .... corticotrophin-release inhibiting factor

CsCl .... cesium chloride

CYP11A1 .... P-450 cholesterol side-chain cleavage enzyme, mRNA and cDNA

CYP11A1 .... P-450 cholesterol side-chain cleavage gene

CYP11B1 .... P-450 11β hydroxylase enzyme, mRNA and cDNA

CYP11B1 .... P-450 11β hydroxylase gene

CYP17 .... P-450 17α hydroxylase enzyme, mRNA and cDNA

CYP17 .... P-450 17α hydroxylase gene

CYP21A1 .... P-450 21 hydroxylase enzyme, mRNA and cDNA

CYP21A1 .... P-450 21 hydroxylase gene

d .... day(s)

Da .... Dalton (1 atomic mass unit)

ddH_2O .... double distilled water

DHEA .... dehydroepiandrosterone

DNA ....deoxyribonucleic acid

DOC...........................11 deoxycorticosterone

Δ^4 steroids .... steroids with a $\Delta^4\Delta^3$ double bond in ring A (*i.e.* post 3βHSD activity)

Δ^5 steroids .... steroids with a $\Delta^5\Delta^4$ double bond in ring B (*i.e.* pre 3βHSD activity)

E .... embryonic day

E. coli ....*Escherichia coli*

ED_{50} ....half maximal dose (effective dose to stimulate 50 % of the maximal response)

EDTA ....ethylenediaminetetraacetic acid di-sodium salt

e.g. ...... *exempli gratia* (Latin, 'for example')

eq. ....Equation

et al. ....*et alii* (Latin, 'and others')

EtBr ....ethidium bromide

F...cortisol

FSH................... follicle-stimulating hormone

$F_w : P_w$ ....fetal weight : placental weight

g...gram(s)

g.......................................acceleration due to Earth's gravity (9.8 m·s^{-2})

GH ....growth hormone

γ_3-MSH .... γ_3-melanocyte-stimulating hormone [*N*-POMC(51-77)]

GR .... glucocorticoid receptor

GTP .... guanosine triphosphate

h... hour(s)

h... human

[^{3}H] .... ^{3}Hydrogen (Tritium)

Hb .... haemoglobin content

*h*CG .... human chorionic gonadotrophin

HDL.......................high density lipoprotein

Hg ....Mercury

HMG-CoA reductase................... 3-hydroxy-methylglutaryl coenzyme A reductase

HMW......................... high molecular weight

HP.............................hypothalamo-pituitary

HPA.......................... hypothalamo-pituitary-adrenal

HPD..................................hypothalamo-pituitary disconnection

HPLC............................. high pressure liquid chromatography

Hx .... hypophysectomised

[^{125}I] .... ^{125}Iodine

i.e. .... *id est* (Latin, 'that is')

IGF-I....................insulin-like growth factor-I

IGF-II................insulin-like growth factor-II

ir .... immunoreactive

IRMA ....immunoradiometric assay

IU.......................... international units

IUGR .... intra-uterine growth restriction

J ....Joules

kb..kilobase(s)

KIU...................... kallikrein inhibitory units

l...litre(s)

LB broth .... Luria-Bertani broth

LDL .... low density lipoprotein

LH .... luteinising hormone

lys-γ_3-MSH .... lys-γ_3- melanocyte-stimulating hormone [*N*-POMC(50-77)]

m...metre(s)

M .... moles·l^{-1}

MAb .... monoclonal antibody

mer.............................. single unit of a multi-unit structure (*i.e.* 1 base of a DNA polymer)

min...minute(s)

mol..moles

M6P mannose-6-phosphate

mRNA ....messenger ribonucleic acid

n....................... number of animals

n.......................number of samples

NaCl .... sodium chloride

N-linked......................Nitrogen atom-linked

N-terminal............................ amino-terminal

o .... ovine

O_2.. molecular oxygen

OD ....optical density

O-linked........................ Oxygen atom-linked

P_4 .... progesterone

P_5 .... pregnenolone

$[^{32}P]$ ....^{32}Phosphorus

p_aCO_2............................... partial pressure of carbon dioxide in arterial blood

p_aO_2.................................... partial pressure of oxygen in arterial blood

PC .... pro-hormone convertase

pg...page

PGE_2 ....prostaglandin E_2

POMC......................... pro-opiomelanocortin

pp. .... pages

pro-γ-MSH........................pro-γ-melanocyte-stimulating hormone 16 kDa fragment of POMC [*N*-POMC(1-103)]

PVN...........................paraventricular nucleus

RE......................................restriction enzyme

rh .... recombinant human

RIA .... radioimmunoassay

RNA...................................... ribonucleic acid

rpm............................revolutions per minute

RT..room temperature

RT-PCR........................ reverse transcriptase-polymerase chain reaction

S............................... 11 deoxycortisol

SCP-2......................... sterol carrier protein-2

SD.................standard deviation of the mean

SDS.......................... sodium dodecylsulphate

sec ....second(s)

SEM....................standard error of the mean

SER.............. smooth endoplasmic reticulum

SSC.............sodium chloride, sodium citrate

StAR protein.................... steroidogenic acute regulatory protein

T_3 ....tri-iodothyronine

T_4 ....thyroxine

TBE .... tris borate electrophoresis buffer

Tet ....tetracycline

T4PNK ....T4 polynucleotide kinase

tris HCl...............tris[hydroxymethyl]-amino methane hydrochloride

TSH .... thyroid-stimulating hormone

U .... Pharmacia enzyme activity units

For Klenow fragment: one unit catalyses the incorporation of 10 nM of total deoxynucleotide into acid-insoluble product in 30 min at 37 °C using poly(dA-dT) as substrate.

For T4PNK: one unit catalyses the transfer of 1 nM of phosphate from ATP to the polynucleotide in 30 min at 37 °C (Richardson units).

UBF ....uterine blood flow

UV ....ultra violet

V .... Volt(s)

vs. .... versus

$v \cdot v^{-1}$ .... volume·volume^{-1} (ml·ml^{-1})

$w \cdot v^{-1}$ .... weight·volume^{-1} (g·ml^{-1})

$w \cdot w^{-1}$ ....weight·weight^{-1} (g·g^{-1})

Mathematical prefixes

M ....mega (10^6)

k...kilo (10^3)

c...centi (10^{-1})

m...milli (10^{-3})

μ...micro (10^{-6})

n...nano (10^{-9})

p... pico (10^{-12})

PUBLICATIONS ARISING FROM THIS THESIS

1. **Ross JT, Bennett HPJ, James S, McMillen IC**. Infusion of N-proopiomelanocortin (1-77) increases adrenal weight and mRNA levels of cytochrome P450 17α hydroxylase in the sheep fetus during late gestation. *Endocrinology*. In press, 2000.

2. **Ross JT, McMillen IC, Adams MB, Coulter CL**. A premature increase in circulating cortisol suppresses expression of 11β hydroxysteroid dehydrogenase type 2 messenger ribonucleic acid in the adrenal of the fetal sheep. *Biology of Reproduction*. In press, 2000.

3. **Ross JT, Phillips ID, Simonetta G, Owens JA, Robinson JS, McMillen IC**. Differential effects of placental restriction on IGF-II, ACTH receptor and steroidogenic enzyme mRNA levels in the foetal sheep adrenal. *Journal of Neuroendocrinology*. 12(1): 79-86, 2000.

4. **Ross JT, Phillips ID, Owens JA, Young IR, McMillen IC**. Cortisol differentially regulates pituitary-adrenal function in the sheep fetus after disconnection of the hypothalamus and pituitary. *Journal of Neuroendocrinology*. 9: 663-668, 1997.

5. **Phillips ID, Ross JT, Young IR, McMillen IC**. The peptide ACTH(1-39), adrenal growth and steroidogenesis in the sheep fetus after disconnection of the hypothalamus and pituitary. *Journal of Physiology*. 491(3): 871-879, 1996.

CHAPTER 1.   LITERATURE REVIEW

1.1   THE PHYSIOLOGICAL ROLE OF ADRENAL CORTICOSTEROIDS

1.1.1   *Historical perspective*

The mammalian adrenal gland consists of two morphologically and functionally separate parts, the cortex and medulla. The cortical parenchymal cells originate from the embryonic mesoderm, whereas the medullary, or chromaffin, cells are derived from the embryonic neural crest, as are the closely related neurons within the sympathetic branch of the autonomic nervous system ([310]Norman and Litwack, 1997).

The first detailed description of the adrenal gland dates back to 1552, when Bartholomeus Eustachius (1520-1574) published a number of illustrations of human anatomy, including one depicting the adrenal gland. In 1866, the currently accepted histological description of adrenal anatomy established the nomenclature of the three concentric zones within the mammalian adrenal cortex. These include the outer: *zona glomerulosa*, middle: *zona fasciculata* and inner: *zona reticularis* layers of the adrenal cortex, which produce steroids with primarily mineralocorticoid, glucocorticoid and adrenal androgenic activity, respectively. In some species the chromaffin cells of the adrenal medulla can also be divided into subtypes based on their endocrine characteristics. One population of chromaffin cells produces adrenaline, whereas the remaining parenchymal cells synthesise noradrenaline.

Many observations have followed these early descriptions of the adrenal gland. The discovery by Oliver and Schafer that extracts of the adrenal medulla increase blood pressure and accelerate heart rate led to further investigations to identify and characterise the chemical compound responsible for these effects. In 1901, Takamine and Aldrich independently succeeded in purifying and elucidating the chemical structure of this compound, naming it 'adrenaline'. Later in the same year it was shown that adrenaline, in addition to its effect on blood pressure and heart rate, also increases the level of blood glucose. Thus, adrenaline became the first chemically defined compound with a hormonal function, a finding that also established the adrenal gland as an endocrine organ.

During the mid-1930's a second type of hormone produced by the adrenal gland was discovered. These hormones, known as steroids, are produced by the different layers of

the adrenal cortex and have been classified based on their main physiological effects: (*1*) glucocorticoids, *e.g.* cortisol and corticosterone, regulate carbohydrate mobilisation and protein and fat metabolism; (*2*) mineralocorticoids, *e.g.* aldosterone, regulate sodium reabsorption in the renal tubules, salivary glands and gut; and (*3*) adrenal androgens (C_{19} steroids, containing only 19 Carbon atoms) exert effects on the reproductive system and can serve as precursors to sex hormones in extra-adrenal tissues ([310]Norman and Litwack, 1997).

Glucocorticoids act by binding to specific steroid receptors in the cytoplasm. Two 'glucocorticoid' receptors (GRs) have been identified: the type-1 GR, which paradoxically is associated with mineralocorticoid activity and therefore also called the mineralocorticoid receptor; and the type-2 GR or glucocorticoid receptor. The deoxyribonucleic acid (DNA) sequence of the GRs is highly conserved across species. The presence or absence of GRs determines whether or not a particular tissue is a glucocorticoid responsive target. Fetal systems which are important for the transition to the extra-uterine environment show enhanced tissue maturation after glucocorticoid administration, and it has been shown that GRs are abundantly present throughout fetal tissues.

Within the cell, most effects of glucocorticoids are genomic, being mediated by binding to GRs within the cytoplasm. When the glucocorticoid has bound to the GR, the steroid-receptor complex translocates to the nucleus where it binds to steroid response elements on DNA. The steroid-receptor complex is essentially a trans-activating factor that stimulates or inhibits the genes to which it binds. The proteins that are produced may themselves be trans-activating factors that stimulate RNA transcription from a number of genes. In this fashion glucocorticoids may initiate multiple effects in a single cell ([423]Walker and Williams, 1992).

Some effects of steroids may be independent of classical receptors and steroid response elements ([423]Walker and Williams, 1992). There is evidence that this so-called non-genomic effect specifically occurs in neural tissue, where a cell-surface GR has been identified ([316]Orchinik, Murray and Moore, 1991). By binding to receptors located on the cell membrane of neurons, glucocorticoids can rapidly modulate GABA-ergic inhibitory neurons, resulting in suppression of neural activity ([231]de Kloet, Reul and Sutanto, 1990). These non-genomic effects of glucocorticoids are different from the classical genomic effects. Further research is needed to illuminate the differences between the cell-surface and intra-cellular GRs. Finally, in terms of fetal development, it has become clear that the

dose and timing of exposure of the fetus to glucocorticoids may have important long-term sequelae, particularly on the fetal cardiovascular and nervous systems.

1.1.2   The physiological role of cortisol before birth

There is considerable evidence that glucocorticoids influence the maturation of various systems in the developing fetus in preparation for the transition to the extra-uterine environment. The lungs mature structurally and functionally, becoming distensible and capable of coping with high surface tension when air enters the alveoli with the first breath ([244]Liggins, 1976; [248]Liggins, Schellenberg *et al.*, 1985; [57]Boshier, Holloway *et al.*, 1989). In the liver, glycogen accumulates and gluconeogenesis is initiated to meet the demands for glucose until feeding begins ([154]Fowden, Coulson and Silver, 1990). There is an increase in the production of tri-iodothyronine (T$_3$), and catecholamines ([155]Fowden, Mundy and Silver, 1998) in preparation for the increase in metabolic rate and thermogenesis required post-natally ([89]Cheung, 1984). These maturational events are regulated by cortisol, preparing the fetus for the loss of the placenta at birth, when alternative sources of oxygen (O$_2$) and nutrients must be available immediately for the survival of the new born.

In mammalian species the fetal adrenal gland plays a key role during late-gestation since glucocorticoids trigger cellular differentiation, and are thereby responsible for a range of biochemical and morphological changes which occur in fetal tissues in preparation for extra-uterine life. Moreover, in domestic ruminants as well as in the pig, cortisol from the fetal adrenal gland provides the trigger for estrogen and prostaglandin synthesis within the placenta and fetal membranes, and for the timing of birth ([315]Olson, Lye and Challis, 1986; [349]Power and Challis, 1987; [80]Challis and Brooks, 1989; [81]Challis and Lye, 1994). Myometrial responses to contractile agonists such as prostaglandins are enhanced by these steroid changes which, accompanied with cervical softening, eventuate in birth ([189]Hillier and Wallis, 1981). Indeed, Liggins and colleagues demonstrated that fetal hypophysectomy ([247]Liggins, Kennedy and Holm, 1967; [246]Liggins and Kennedy, 1968), which results in adrenal hypoplasia, or bilateral fetal adrenalectomy ([128]Drost and Holm, 1968) lead to prolonged gestation (term≈150 days in the sheep). Conversely, administration of adrenocorticotrophin (ACTH) or of a glucocorticoid (cortisol or dexamethasone) into the fetus *in utero* leads to premature birth ([245]Liggins, Fairclough *et al.*, 1973). In these studies, the timing of parturition was unaltered by maternal hypophysectomy or by the administration of ACTH or glucocorticoids to the mother ([245]Liggins, Fairclough *et al.*, 1973).

Cortisol also acts to inhibit growth during late-gestation and during chronic fetal stress, such that in adverse conditions wherein the exogenous nutrient supply is restricted, elevated cortisol may contribute to the reduced rates of cell proliferation and fetal tissue accretion ([156]Fowden, Szemere *et al.*, 1996). The predominant effects of chronic stress on the hypothalamo-pituitary-adrenal (HPA) of the adult rat are an increase in adrenal weight and plasma corticosteroid concentrations, an increased sensitivity of the adrenal to ACTH, and a maintained or diminished pituitary sensitivity to corticotrophin releasing hormone (CRH) ([7]Akana, Dallman *et al.*, 1992; [8]Akana, Scribner *et al.*, 1992). Similarly, in the fetal sheep during late-gestation there is an increase in adrenal weight, in adrenocortical steroid output and in the adrenocortical responsiveness to ACTH ([80]Challis and Brooks, 1989). Fetal pituitary responsiveness to CRH and arginine vasopressin (AVP) also appears to diminish after 135 d of gestation ([80]Challis and Brooks, 1989). These changes are all consistent with chronic stimulation of the fetal HPA axis. The nature and source of the stimulus, however, remain unclear as there are many factors in the fetal environment which may act separately or in concert to stimulate the fetal HPA axis in late-gestation.

The ovine fetus is able to respond to several pathophysiological stimuli during the period preceding delivery, including hypoxaemia ([6]Akagi and Challis, 1990), hypotension ([370]Rose, Meis and Morris, 1981) and haemorrhage ([369]Rose, MacDonald *et al.*, 1978). The fetal HPA axis also responds to low blood glucose concentrations during late-gestation, resulting in increased fetal ACTH and cortisol levels ([327]Ozolins, Young and McMillen, 1992). In all species, the fetus is hypoglycaemic relative to the mother, thus maintaining the maternal-fetal glucose gradient essential to drive the placental delivery of glucose from mother to fetus ([181]Hay, 1991). In addition, while there is an inverse correlation between fetal plasma glucose and ACTH or cortisol during 24 h sampling periods between 135 d and 144 d of gestation, this relationship is absent prior to 134 d of gestation ([398]Simonetta, Walker and McMillen, 1991). Thus, there may be an increase in the sensitivity of the fetal HPA axis to low glucose concentrations during late-gestation. In this way, birth may represent an escape from an intra-uterine environment, characterised by chronic stress and hypercortisolism ([280]McMillen, Phillips *et al.*, 1995).

1.1.3 *Post-natal consequences of exposure to glucocorticoids in utero*

1.1.3.1 *Effects of glucocorticoids on cardiovascular development*

Barker and colleagues have established an inverse relationship between human birthweight and blood pressure in adulthood, such that small-at-term human babies with large placentae are at markedly higher risk for developing hypertension in adulthood

([26]Barker, Osmond *et al.,* 1989; [25]Barker, Bull *et al.,* 1990). One hypothetical reason for this relationship is the effect of *in utero* exposure to glucocorticoids. Data from studies of adult rats suggest that the efficiency of the placental barrier to maternal glucocorticoid varies considerably between individuals ([34]Benediktsson, Lindsay *et al.,* 1993). These authors observed a strong positive correlation between the activity of placental 11β hydroxysteroid dehydrogenase (11βHSD, which metabolises cortisol to the inactive cortisone) and fetal body weight at term, and an inverse correlation between placental 11βHSD activity and placental weight ([34]Benediktsson, Lindsay *et al.,* 1993). The lowest placental 11βHSD activity, and therefore presumably the highest fetal exposure to maternal glucocorticoids was seen in the smallest term fetuses with the largest placentae. Physiological consequences of exposure to glucocorticoids may be seen in a number of studies. Offspring of rats treated during pregnancy with the synthetic glucocorticoid, dexamethasone (which has a very low affinity for 11βHSD), have lower birth weights and higher blood pressure when mature, than offspring of control rats ([34]Benediktsson, Lindsay *et al.,* 1993; [139]Edwards, Benediktsson *et al.,* 1993). Whether humans exposed to therapeutic regimens of beta- or dexamethasone *in utero* will develop hypertension in adult life remains to be elucidated, since the children examined in the longest follow-up study ([383]Schmand, Neuvel *et al.,* 1990; [401]Smolders-de Haas, Neuvel *et al.,* 1990) are now only about to reach puberty.

The effects of cortisol infusion on cardiovascular development in fetal sheep have also been examined ([435]Wood, Cheung and Brace, 1987; [409]Tangalakis, Lumbers *et al.,* 1992). Both studies described an increase in fetal blood pressure during cortisol infusion, presumably due to enhanced vascular resistance. Although circulating concentrations of vasoconstrictor hormones such as renin, angiotensin II (AII) or noradrenaline were not increased during the study period ([435]Wood, Cheung and Brace, 1987), vascular responsiveness to AII was enhanced following cortisol treatment, suggesting an increased sensitivity of the AII type-1 receptor (AT-1) for AII ([409]Tangalakis, Lumbers *et al.,* 1992). Treatment of pregnant sheep with dexamethasone (0.28 mg·d^{-1}·kg^{-1} for 48 h) in early gestation (27 d of gestation) also leads to persistent elevation of blood pressure in the offspring, while treatment for 48 h at 64 d of gestation is without effect ([126]Dodic, May *et al.,* 1998).

1.1.3.2 *Effects of glucocorticoids on neuroendocrine development*

The effects of therapeutic regimens of steroids on fetal neuronal development are of major concern, as neurons undergo cell division for only a limited time span during development

and inhibition of cell mitosis by therapeutic doses of glucocorticoids is likely to have lasting effects on fetal brain development. Glucocorticoids also influence post-mitotic events in nervous system development, including cell differentiation, phenotypic expression of specific neurotransmitter characteristics, synaptogenesis and synaptic reactivity. High physiological levels of glucocorticoids, whether administered exogenously or produced endogenously, are able to cause marked hippocampal degeneration in newborn ([413]Uno, Lohmiller *et al.*, 1990) and adult monkeys ([414]Uno, Tarara *et al.*, 1989; [378]Sapolsky, Uno *et al.*, 1990).

A number of studies in rats and mice also described marked changes in brain development and motor behaviour after glucocorticoid exposure. One simple manipulation, such as handling in the neo-natal period, results in changes in the HPA axis that persist throughout the life of the animal. Rats handled during infancy have a permanent increase in the concentration of type-2 GRs in the hippocampus, a critical region of glucocorticoid negative feedback ([282]Meaney, Aitkin *et al.*, 1988). Significantly, rats that were not handled secrete more glucocorticoids in response to stress when compared with handled rats. The increased exposure to glucocorticoids throughout life in the non-handled group accelerated hippocampal neuron loss and cognitive impairment in aging ([282]Meaney, Aitkin *et al.*, 1988). Hippocampal cell loss and pronounced spatial memory deficits emerged with age in the non-handled rats, but were almost absent in handled rats. Thus, these authors suggested that relatively subtle individual differences in early experience can determine the stress response of the HPA axis for the rest of a lifetime, and alter profoundly the quality of aging years later ([282]Meaney, Aitkin *et al.*, 1988).

In summary, exposure to cortisol at specific times in fetal and neo-natal life can irreversibly trigger the programmed development of a range of fetal tissues including the brain ([282]Meaney, Aitkin *et al.*, 1988; [414]Uno, Tarara *et al.*, 1989; [378]Sapolsky, Uno *et al.*, 1990), lung ([244]Liggins, 1976), liver ([154]Fowden, Coulson and Silver, 1990), and cardiovascular system ([26]Barker, Osmond *et al.*, 1989; [25]Barker, Bull *et al.*, 1990; [34]Benediktsson, Lindsay *et al.*, 1993; [139]Edwards, Benediktsson *et al.*, 1993). Thus, it is critical that during fetal life, adrenal growth and steroidogenesis are tightly regulated to ensure the appropriate exposure of developing tissues to cortisol. While a series of elegant *in vitro* and *in vivo* studies have elucidated key regulatory factors which are involved in adrenal maturation in the late-gestation fetus, the precise role of these factors in the stimulation of adrenal growth and steroid output are unclear.

This thesis, therefore, examines the role of a number of key fetal factors that modulate the growth and development of the fetal sheep adrenal during gestation. This dissertation describes the interactions among pituitary-derived peptides, intra-adrenal exposure to glucocorticoids and the local adrenal and endocrine IGF axes in the growth and functional activation of the ovine fetal adrenal gland before birth. The involvement of these systems in the fetal response to chronic stress and intra-uterine growth restriction is also considered.

1.2 FETAL ADRENAL STEROID SYNTHESIS

1.2.1 Cholesterol structure

The principal tissues of synthesis of the five classical categories of steroid hormones (glucocorticoids, mineralocorticoids, estrogens, androgens and progestins) are the adrenal cortex, ovaries, testes and the placenta. The sites of synthesis of the sixth class of steroid hormones, the pro-hormone vitamin D_3 and its metabolites, are the skin, liver, and kidneys. The bile acids are also steroids, but have no known hormonal activity and are synthesised in the liver.

All steroids possess a phenanthrene ring structure (*Figure 1.1, structure 1*) to which a pentano ring has been attached to yield cyclopentanoperhydrophenanthrene, or the sterane ring structure (*Figure 1.1, structure 2*). This structure consists of four rings: three six-Carbon cyclohexane rings (labelled A, B and C) and one five-Carbon cyclopentane ring (labelled D). Cholestane, which has twenty-seven Carbon atoms, differs from sterane by the addition of an eight-Carbon side-chain on Carbon atom 17 (*C17*) of ring D, and the presence of two methyl groups at *C10* and *C13* (*Figure 1.1, structure 3*). The fully saturated cholestane molecule forms the parent ring structure for cholesterol, the biosynthetic precursor of adrenal corticosteroids. Also indicated is the standard numbering system for the Carbon atoms in the four rings (*Figure 1.1, structures 2 and 3*). This structure is fused in the *trans* configuration to give a roughly planar structure.

Within the cholesterol molecule there is one double bond in ring B (*C5-C6* ene) and one polar hydroxyl group in ring A (*C3*-β-hydroxyl). Conventionally, substituents in the four-ringed nucleus are classified by their orientation in relation to the plane of the molecule. Thus, groups projecting above this plane are β-substituents, while those projecting below it are α-substituents. The basic cholestane nucleus is common to all steroid hormones but the different categories are distinguished by the length of the side-chain, as well as the number, location and orientation of substituent groups, such as

(1) **Phenanthrene**
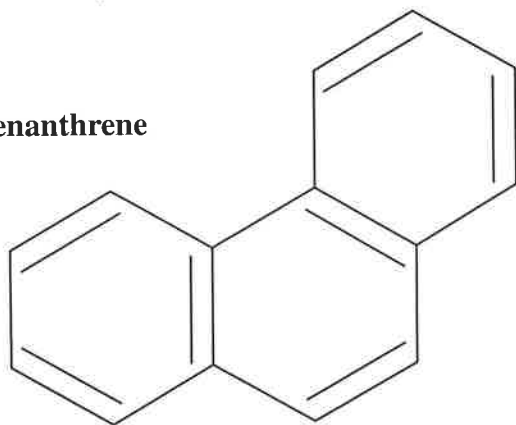
(2) **Cyclopentanoperhydrophenanthrene**

   (Sterane)
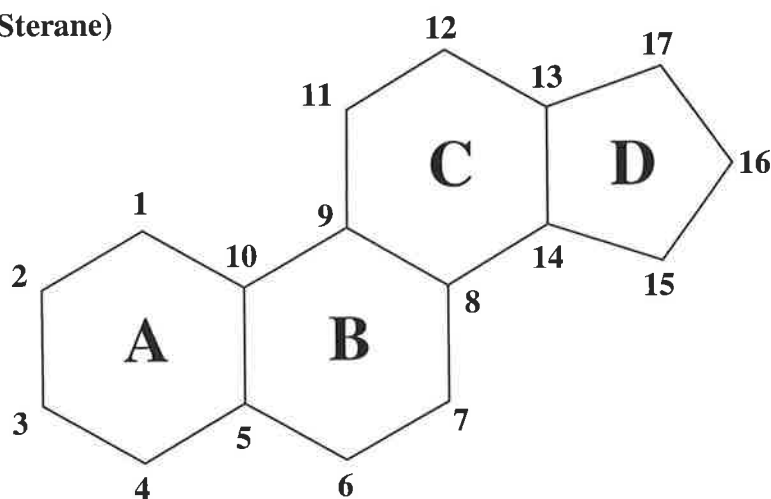
(3) **Cholestane**
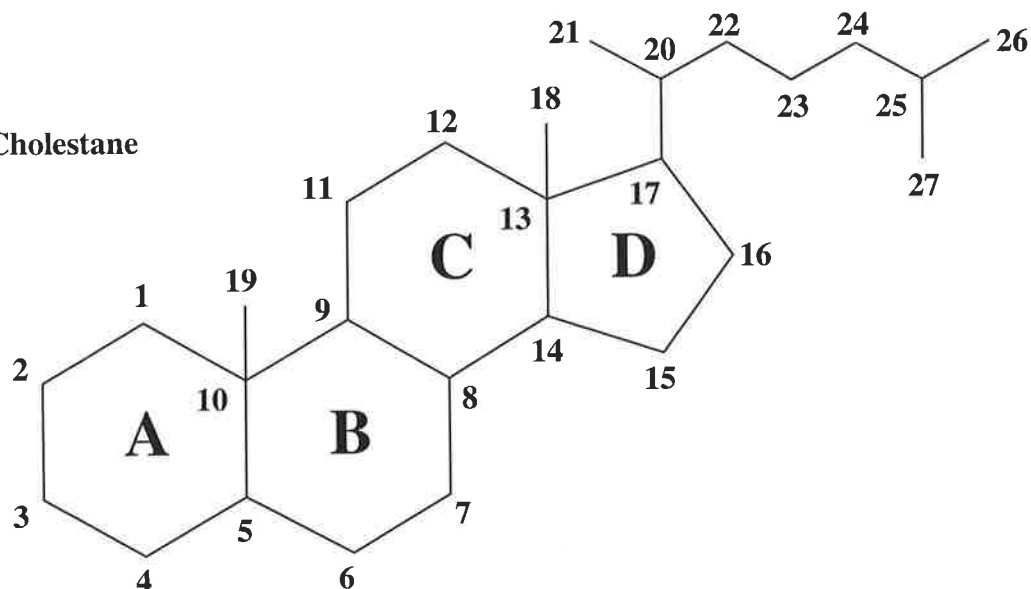
Figure 1.1   **Parent ring structure for cholesterol, the biosynthetic precursor of adrenal corticosteroids.**

Basic ring structure of *(1)* phenanthrene, *(2)* cyclopentanoperhydrophenanthrene and *(3)* cholestane.

hydroxyl groups, which affect polarity. Relatively small changes in these substituent groups have a profound effect on the potency and specificity of steroid biological activity ([310]Norman and Litwack, 1997).

1.2.2   Cholesterol uptake, synthesis and mobilisation

Steroidogenic tissues may synthesise cholesterol *de novo* from acetate. Ovine fetal adrenocortical cells, however, possess a low activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis) and contain lower amounts of cholesterol than adrenal cells of newborn lambs ([135]Durand, Cathiard *et al.*, 1988). Thus, the delivery of cholesterol via plasma low-density lipoproteins (LDLs) in the sheep ([134]Durand, Cathiard *et al.*, 1987) and human fetus ([69]Carr and Simpson, 1981), plays an important role providing cholesterol as substrate for steroid hormone biosynthesis in the fetal adrenal gland. The pathway of LDL metabolism in the adrenal cortex involves binding of the LDL to specific receptors on the cell surface, localised in clathrin coated pits. LDL-receptor complexes are internalised via coated vesicles and LDL cholesterol esters may then be stored in lipid droplets or converted to free cholesterol by cholesterol ester hydrolase (CEH) and used for steroid hormone synthesis. Alternatively, the free cholesterol can be re-esterified for storage inside the cell by acyl coenzyme A-cholesterol acyl transferase (ACAT), whose activity is stimulated by free cholesterol. Thus, intra-cellular cholesterol storage and release is controlled by the action of two opposing enzymes, ACAT and CEH.

Adrenocorticotrophic hormone (ACTH) and γ_3-melanocyte-stimulating hormone (γ_3-MSH), two adrenal trophic peptides from the pituitary multi-hormone precursor pro-opiomelanocortin (POMC), have complementary actions on adrenal steroid synthesis. In hypophysectomised (Hx) rats, infusion of γ_3-MSH potentiates the ACTH-induced increase in serum concentrations of corticosterone and aldosterone, and results in a dose-dependent rise in intra-mitochondrial free cholesterol via enhanced adrenal CEH enzyme activity ([332]Pedersen, Brownie and Ling, 1980b; [330]Pedersen and Brownie, 1987a). Pedersen and colleagues further demonstrated that the effects of γ_3-MSH also synergise with that of ACTH in rat adrenocortical cells *in vitro* ([329]Pedersen and Brownie, 1983), requiring the inclusion of high density lipoprotein (HDL, the primary source of steroid precursors in the rat) in the incubation medium ([331]Pedersen and Brownie, 1987b). In the absence of ACTH, however, there is no effect of γ_3-MSH on cholesterol esterification or on steroid synthesis *in vitro* ([331]Pedersen and Brownie, 1987b). Thus, these authors concluded that the full potentiation of adrenal steroidogenesis by γ_3-MSH is facilitated by a

transient pool of substrate whose source is plasma lipoproteins and is sustained in a manner which is acutely dependent on ACTH.

Pedersen and co-workers also demonstrated that ACTH(1-24), a fully bioactive synthetic form of ACTH diminishes ACAT enzyme activity without altering the rate of cholesterol hydrolysis ([330]Pedersen and Brownie, 1987a). Thus, γ_3-MSH and ACTH appear to coordinate CEH and ACAT enzyme activity, resulting in a substantial shift in the set-point of cholesterol ester ↔ free cholesterol cycling, in favour of free cholesterol. Since ACTH also stimulates steroid-synthesising enzyme activity ([267]Manchester and Challis, 1982) and gene expression ([408]Tangalakis, Coghlan *et al.*, 1990) in the fetal sheep adrenal, this coordinated control of cholesterol substrate flux results in a potentiation of steroidogenesis when these peptides act in concert.

Once sequestered within the steroidogenic cell, cholesterol transport to the steroid-synthesising enzymes in response to trophic hormone stimulation occurs in two phases. The first phase is the mobilisation of cholesterol from cellular stores, such as lipid droplets, to the outer mitochondrial membrane, while the second phase consists of translocation of cholesterol across the aqueous inter-membrane space of the mitochondria to the inner membrane. While both processes are necessary to ensure maximal rates of steroid production in response to hormone stimulation, the latter event is the key hormone-stimulated regulatory step (*for reviews see* [427]White, 1994; [402]Stocco and Clarke, 1996).

1.2.3 *Steroidogenic acute regulatory protein*

The first and rate-limiting step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone by the mitochondrial cytochrome P450 enzyme, cholesterol side-chain cleavage (CYP11A1). Trophic hormones such as ACTH and gonadotrophins induce steroidogenesis via stimulating intra-cellular cyclic adenosine monophosphate (cAMP) formation, which stimulates CYP11A1 activity in two distinct ways. Chronic stimulation (hours to days) occurs through the induction of *CYP*11A1 gene transcription, leading to increased CYP11A1 protein and consequent increased steroidogenic capacity. Acute regulation, over minutes, occurs via a rapid increase in the flow of cholesterol into mitochondria, thus regulating substrate availability to the existing pool of CYP11A1 enzyme ([402]Stocco and Clarke, 1996). Cholesterol translocation is blocked by inhibitors of protein synthesis (*i.e.* cycloheximide) indicating that the effect of trophic hormones, acting via cAMP, most likely involves the *de novo* synthesis of a protein that is rapidly inactivated ([225]Kallen, Arakane *et al.*, 1998). The recently identified steroidogenic acute

regulatory protein (StAR) appears to be the most likely candidate for the labile protein, as: (*1*) cAMP stimulates the phosphorylation of pre-existing StAR protein and rapid synthesis of new StAR protein, while StAR pre-protein disappears rapidly in the presence of inhibitors of protein synthesis ([287]Miller and Strauss, 1999); (*2*) StAR has an *N*-terminal 'targeting' sequence that directs the protein to the mitochondria ([402]Stocco and Clarke, 1996); and (*3*) StAR protein is expressed almost exclusively in steroid-producing cells, its presence is correlated with steroid hormone production, and lack of functional StAR causes the autosomal recessive disease congenital lipoid adrenal hyperplasia, characterised by markedly impaired gonadal and adrenal steroid hormone synthesis ([225]Kallen, Arakane *et al.*, 1998).

Under acute ACTH stimulation, the level of expression of mRNA for StAR increases within 0.5 h in both *zona glomerulosa* and *zonae fasciculata-reticularis* of the adult rat adrenal ([239]Lehoux, Fleury and Ducharme, 1998). Increases in the level of StAR protein within the *zona glomerulosa* and the *zonae fasciculata-reticularis* also occur, but are delayed compared with those of their mRNA ([239]Lehoux, Fleury and Ducharme, 1998). In contrast, hypophysectomy of adult rats produces a 3-5 fold decrease in adrenal StAR mRNA along with a loss of adrenal steroids ([21]Ariyoshi, Kim *et al.*, 1998). These authors suggested that post-translational modifications of the StAR precursor peptide occur during the early stimulatory phase and before the apparent translation of the newly formed mRNA. The addition of dibutyryl cAMP (Bt$_2$cAMP) and forskolin, or phorbol ester plus calcium ionophore 23187 to adult bovine adrenal fasciculata cells *in vitro* activates expression of the StAR protein as well as cortisol production ([306]Nishikawa, Sasano *et al.*, 1996). In addition, the stimulatory effects of ACTH on cAMP formation and on StAR protein and cortisol production are inhibited by pre-treatment with calphostin C (a protein kinase C inhibitor), suggesting that ACTH enhances the expression of StAR protein, possibly via two signal transduction systems, cAMP-dependent and protein kinase C-dependent processes ([306]Nishikawa, Sasano *et al.*, 1996).

It has been proposed that, in response to hormone stimulation, a StAR precursor protein is rapidly synthesised in the cytoplasm and is 'targeted' to the mitochondria ([229]King, Ronen-Fuhrmann *et al.*, 1996). As the transfer of this protein to the inner mitochondrial compartment begins, 'contact sites' between the inner and outer mitochondrial membranes form ([402]Stocco and Clarke, 1996). Both the 'signal' sequence and the 'targeting' sequence of the peptide are removed by proteolytic processing while the StAR peptide traverses these 'contact sites'. The mechanism of enhanced cholesterol transfer in response to StAR activation may result from physical contact between the membranes, or

from the formation of a protein 'pore' between the membranes ([209]Jefcoate, McNamara *et al.*, 1992). The hydrophobic steroid precursors could then pass through this 'pore', without traversing the aqueous inter-membrane space ([402]Stocco and Clarke, 1996). It has also been suggested that StAR facilitates cholesterol desorption from mitochondrial membranes, stimulating transfer from the outer mitochondrial (donor) membrane to the inner mitochondrial (acceptor) membrane ([287]Miller and Strauss, 1999). In addition, the processing of StAR within the mitochondria necessitates the continued synthesis of its precursor to allow the continued transport of cholesterol to the inner membrane. It is interesting that enzymes involved in steroid synthesis are associated with the mitochondrial 'contact sites' involved in the trans-location of StAR to the inner mitochondrial membrane ([88]Cherradi, Defaye and Chambaz, 1994). Thus, it has been speculated that the importing and processing of StAR through the mitochondrial 'contact sites', which house a complex of steroid-synthesising enzymes, allows cholesterol transfer and subsequent steroidogenesis ([402]Stocco and Clarke, 1996).

1.2.4   Adrenal steroidogenic enzymes

The synthesis of cortisol, aldosterone and adrenal androgens requires the sequential actions of only five enzymes, although several of these mediate more than one enzymatic reaction. Four of these enzymes are members of the ubiquitous cytochrome P450 family of haeme-containing mixed function oxidases. These steroid hydroxylases are membrane bound and are present in either the mitochondria or microsomal endoplasmic reticulum of the cell (*Figure 1.2*). The two mitochondrial P450 enzymes that mediate cholesterol side-chain cleavage (CYP11A1) and hydroxylation of *C*11 and *C*18 (CYP11B1) are the terminal components of an electron transport chain involving NADPH, a flavoprotein (adrenodoxin reductase) and an iron-sulfur protein (adrenodoxin) ([310]Norman and Litwack, 1997). The two microsomal P450 enzymes, one with 17α-hydroxylase and *C*17-*C*20 desmolase activities (CYP17) and the other with 21-hydroxylase activity (CYP21A1), utilise NADPH and an intermediary flavoprotein (NADPH-cytochrome P450 reductase) as co-enzymes. The fifth enzyme, having both 3β-hydroxysteroid dehydrogenase and $\Delta^5\Delta^4$-*C*3-ketosteroid isomerase activities (3βHSD), is also located in the smooth endoplasmic reticulum and utilises NAD as a co-factor ([310]Norman and Litwack, 1997) (*Figure 1.2*). In recent years, complementary DNA (cDNA) probes for all of these enzymes have been cloned, permitting sequencing and mapping of the relevant genes and investigation of their cell-specific expression ([95]Chung, Matteson *et al.*, 1986; [96]Chung, Picado-Leonard *et al.*, 1987; [274]Matteson, Phillips *et al.*, 1987; [254]Lorence, Murray *et al.*, 1990).
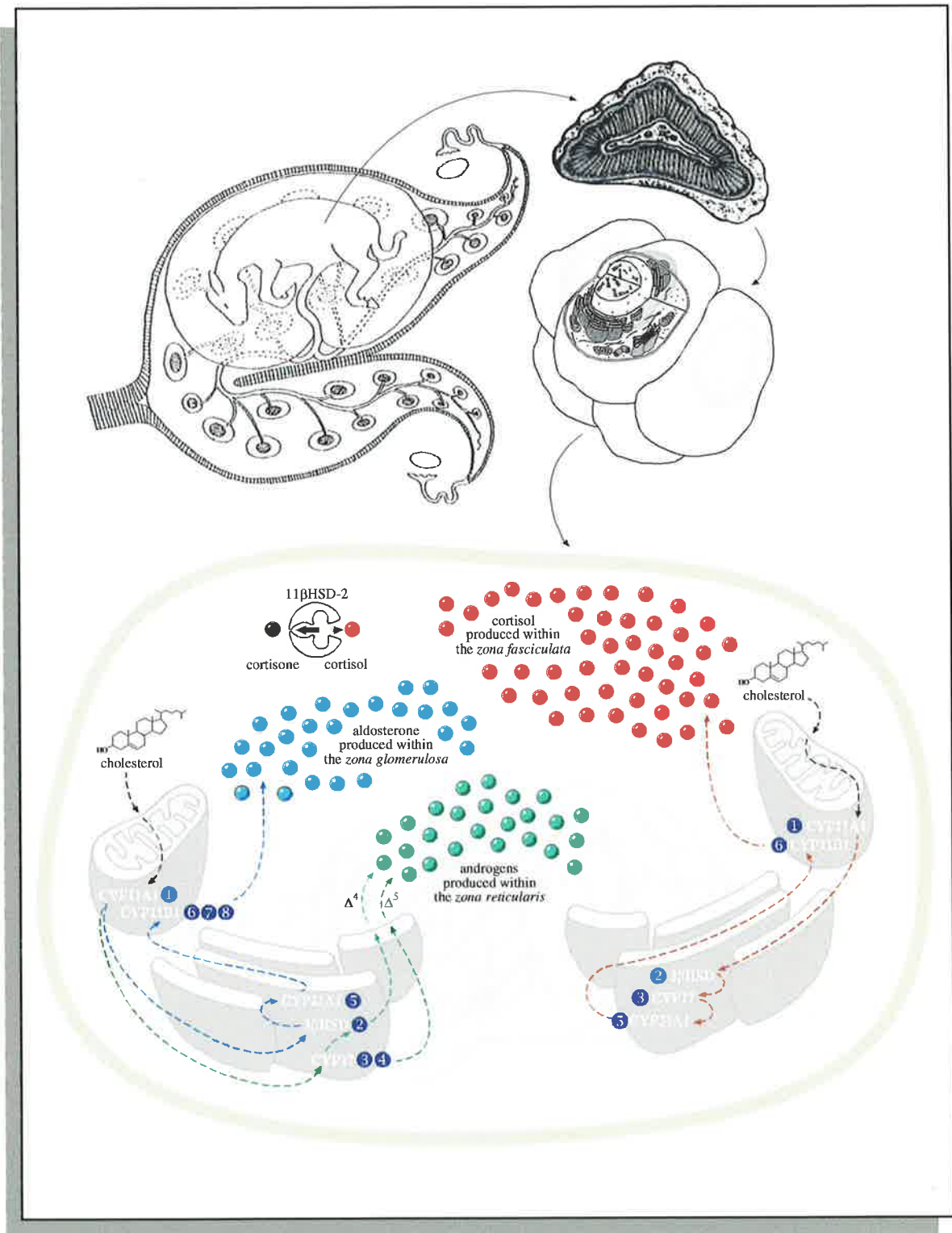
Figure 1.2   **Ovine fetal adrenal steroid synthesis.**

Intra-cellular pathways of glucocorticoid, mineralocorticoid and androgen synthesis in the *zonae* of the fetal sheep adrenal cortex. Biosynthetic intermediates are exchanged between mitochondria and microsomal endoplasmic reticulum. Descriptions of the enzymatic steps ❶ through ❽ are provided in *Figure 1.3* and in the text.

As the cytochrome P450s are approximately 500 amino acids in length, the mRNAs that encode these enzymes are somewhat larger than 1500 bases. Bovine and human *CYP*11A1 and *CYP*17 genes are encoded by single mRNA species, each approximately 2 kilobases (kb) in length. There are two mRNAs specific for the bovine *CYP*21A1 gene, one ~2.0 kb and one ~2.2 kb in length, although the human *CYP*21A1 gene is encoded by a single 2.0 kb mRNA transcript. Nelson and colleagues have described a unified nomenclature for the naming of genes, cDNAs, mRNAs and enzymes of the P450 super-family ([305]Nelson, Koymans *et al.*, 1996). Briefly, the recommendations for naming a P450 gene include the italicised root symbol '*CYP*' ('*Cyp*' for the mouse), denoting c̲y̲tochrome P̲450, an Arabic number designating the P450 family, a letter indicating the sub-family when two or more sub-families exist, and an Arabic number representing the individual gene. A non-italicised root symbol 'CYP' is used for the gene product (enzyme), mRNA and cDNA related to the gene.

Several laboratories have contributed to the characterisation of DNAs complementary to mRNA species encoding adrenocortical steroid hydroxylases, and identification of their genes. Characterisation of cDNAs specific for the steroid hydroxylases has allowed detailed investigation of the developmental expression and regulation of the steroidogenic enzymes. The first to be characterised were cDNAs for the bovine *CYP*11A1 gene ([289]Morohashi, Fujii-Kuriyama *et al.*, 1984; [273]Matteson, Chung *et al.*, 1986). Subsequently, cDNAs for the human *CYP*11A1 gene were characterised ([95]Chung, Matteson *et al.*, 1986; [290]Morohashi, Sogawa *et al.*, 1987), as were clones specific for the bovine ([291]Morohashi, Yoshioka *et al.*, 1987), rat ([309]Nonaka, Matsukawa *et al.*, 1989) and human ([94]Chua, Szabo *et al.*, 1987; [428]White, DuPont *et al.*, 1991) *CYP*11B1 gene. Likewise, cDNA clones specific for the microsomal components of the adrenocortical steroidogenic pathway have also been characterised, including the bovine ([447]Zuber, John *et al.*, 1986) and human ([96]Chung, Picado-Leonard *et al.*, 1987) *CYP*17 gene; the bovine ([429]White, New and DuPont, 1984; [445]Yoshioka, Morohashi *et al.*, 1986) and human ([430]White, New and DuPont, 1986) *CYP*21A1 gene, and the human 3βHSD gene ([234]Lachance, Luu-The *et al.*, 1990). These cDNAs have subsequently been utilised as probes to investigate the regulation of mRNAs encoding the steroid hydroxylase enzymes.

1.2.5 *Adrenal steroid synthesis*

Aldosterone is the main mineralocorticoid synthesised within the adrenal cortex, and is secreted from the *zona glomerulosa*, regulated primarily by AII and potassium. Aldosterone is present only in the outer layer of the gland. Failure to demonstrate CYP17

immunoreactivity in the human *zona glomerulosa* confirms the inability of this zone to produce cortisol and sex steroids ([380]Sasano, Mason and Sasano, 1989) (*Figure 1.3*). In contrast, the *zonae fasciculata* and *reticularis* are thought to act as a functional unit in the production of cortisol and androgens, under the influence of ACTH ([286]Miller, 1988; [427]White, 1994; [402]Stocco and Clarke, 1996). Both *zonae fasciculata* and *reticularis* contain the appropriate enzymes for the biosynthesis of glucocorticoids and androgens in most mammalian species, although a distinct *zona reticularis* layer is not evident in the fetal sheep adrenal ([380]Sasano, Mason and Sasano, 1989; [381]Sasano, Sasano and Okamoto, 1989; [407]Tangalakis, Coghlan *et al.*, 1989). The biochemical reactions leading to the three classes of adrenocortical steroids, the trivial and abbreviated names of the steroid intermediates and products, and their primary sites of synthesis are illustrated in *Figure 1.3*.

The modifications necessary to yield corticosteroid hormones may be summarised as follows:

(1) Cleavage of the hydrophobic side-chain of cholesterol between $C20$ and $C22$, to a two-carbon side-chain, forming pregnenolone.

(2) Isomerisation, in which the double bond in ring (B) is 'moved' to ring (A) and the $C3$-β-hydroxy group is dehydrogenated to an oxo group.

(3) A series of 'hydroxylation' reactions at positions $C17$, $C21$, $C11$ and $C18$.

Initially, cholesterol released by the action of CEH must be transported to the inner mitochondrial membrane. Although the side-chain cleavage reaction is known to be the rate-limiting step for adrenal steroid synthesis, it is not the catalytic process of the enzyme, rather the delivery of cholesterol from the cytoplasm, that is rate-limiting ([402]Stocco and Clarke, 1996). The side-chain cleavage reaction comprises hydroxylations at $C20$ and $C22$, followed by scission of the $C20$-$C22$ bond, to yield pregnenolone (P_5). All these reactions are catalysed by the same enzyme CYP11A1 (*Figure 1.3, step 1*). After pregnenolone is produced, it is translocated from the mitochondria to the endoplasmic reticulum where it may undergo one of two conversions. It may undergo $C17$-α-hydroxylation to 17α hydroxy pregnenolone ($17\alpha P_5$) (*Figure 1.3, step 3*), or it may be converted to progesterone (P_4) (*Figure 1.3, step 2*), the first biologically important steroid in the pathway. The enzyme catalysing the dehydrogenation of the $C3$-β-hydroxyl group and isomerisation of the double bond from the B ring (Δ^5 steroids) to the A ring (Δ^4 steroids) is 3βHSD (*Figure 1.3, step 2*).
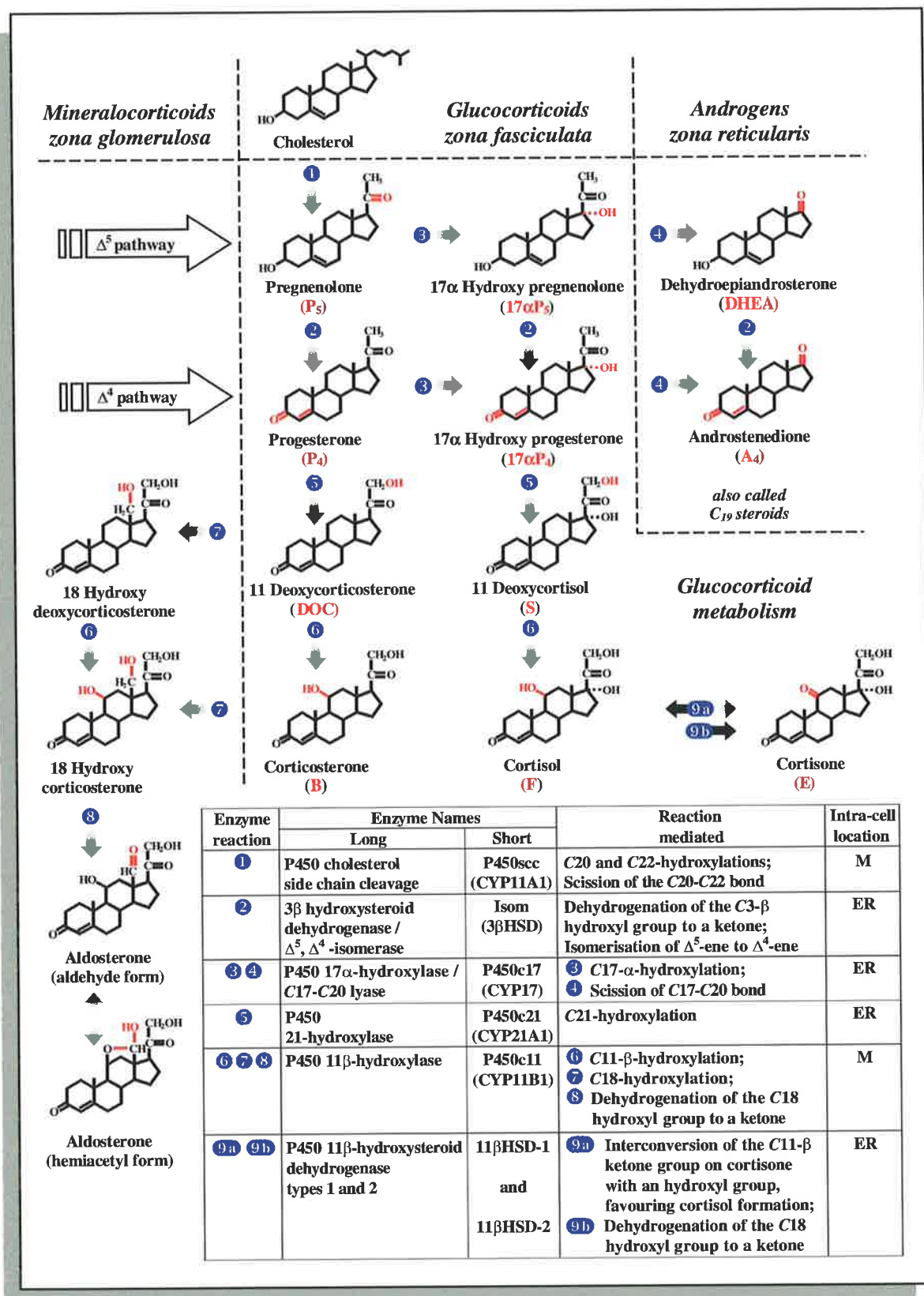
Figure 1.3   Biosynthetic pathway of adrenal corticosteroids.

Enzymatic steps are numbered (in blue) according to the reactions described in the table. Sub-cellular locations are coded as M, mitochondria and ER, endoplasmic reticulum. Trivial names of steroid products are given, including accepted abbreviations in red.

Pregnenolone and progesterone are both substrates for CYP17, forming 17α hydroxy pregnenolone and 17α hydroxy progesterone (17αP₄) respectively (*Figure 1.3, step 3*). It is noteworthy that there is no CYP17 activity in the rat or mouse adrenal cortex, rendering it incapable of synthesising cortisol (F). The principal glucocorticoid secreted by the rat and mouse adrenal cortex is corticosterone (B). In contrast, the CYP17 enzyme is pivotal in steroidogenesis in the fetal zone of the human and primate adrenal cortex, in which the C_{19} steroid dehydroepiandrosterone (DHEA) is synthesised by cleavage of the *C17-C20* side-chain of 17α hydroxy pregnenolone (*Figure 1.3, step 4*). DHEA is the primary steroid secreted from the fetal zone of the adrenal cortex in the human fetus and is the primary precursor for estradiol synthesis in the human placenta, which cannot synthesise the necessary precursors due to the absence of CYP17 enzyme activity ([80]Challis and Brooks, 1989). Thus, since CYP17 has both 17α-hydroxylase activity and *C17-C20* lyase activity, it is a key branch point in steroid hormone synthesis. If neither activity is exerted, pregnenolone is metabolised to mineralocorticoids; glucocorticoids result from 17-α-hydroxylation but not *C17-C20* lyase activity (*Figure 1.3, step 3 only*); and sex steroids result from both CYP17 enzyme activities (*Figure 1.3, steps 3 and 4*).

The fetal sheep *zona fasciculata* possesses abundant levels of CYP17 and 3βHSD enzyme activities. Thus, most of the 17α hydroxy pregnenolone formed by CYP17 is converted to 17α hydroxy progesterone by 3βHSD (*Figure 1.3, step 2*). The sheep placenta also differs from the primate and human placentae, operating autonomously in its production of androgenic precursors and estrogens, due to the presence of placental CYP17 enzyme activity ([80]Challis and Brooks, 1989). After the synthesis of progesterone and 17α hydroxy progesterone, these steroids are hydroxylated at *C21* to yield 11 deoxycorticosterone (DOC) and 11 deoxycortisol (S) respectively. This hydroxylation step is carried out by CYP21A1 within the endoplasmic reticulum and is the penultimate step in glucocorticoid synthesis (*Figure 1.3, step 5*). At the final stage of glucocorticoid synthesis in the fetal sheep, 11 deoxycortisol is returned to the mitochondria for the final *C11*-β-hydroxylation by CYP11B1 (*Figure 1.3, step 6*).

The final enzyme in the synthesis of adrenal mineralocorticoids within the *glomerulosa* of the fetal sheep adrenal is also CYP11B1 (although in humans and rats it is CYP11B2). 11 deoxycorticosterone is converted to aldosterone by the sequential actions of CYP11B. This enzyme mediates all three final steps in the synthesis of aldosterone. *C11*-β and *C18* hydroxylations result in the formation of 18 hydroxy corticosterone (*Figure 1.3, steps 6 and 7*). Finally, dehydrogenation of the *C18* hydroxyl group completes the formation of

aldosterone, which has two configurations, the aldehyde and hemiacetyl forms (*Figure 1.3, step 8*).

1.3 GROWTH AND FUNCTIONAL DEVELOPMENT OF THE FETAL ADRENAL

1.3.1 Adrenocortical cell proliferation and zonation

In the developing rat adrenal cortex, most of the proliferative activity occurs at the periphery of the cortex ([436]Wright and Voncina, 1977). Using DNA pulse labelling with tritiated thymidine it was shown that cells originally labelled at the *glomerulosa* or outer *fasciculata* boundary are displaced sequentially to the inner-most *zonae* of the cortex, where labelled cells finally disappear. Boshier and colleagues have compared the distribution of mitotic figures within the *zona fasciculata* of the fetal sheep adrenal cortex at 130 d and 144 d of gestation ([54]Boshier and Holloway, 1991). In sections of 130 d fetal adrenals, mitotic figures were distributed evenly throughout the *zona fasciculata*, however, at 144 d of gestation adrenocortical mitotic figures located in the 'outer' half of the *zona fasciculata* were double those of the 'inner' half. In the most mature glands, mitotic figures were confined predominantly to the outer one-third of the *zona fasciculata* ([54]Boshier and Holloway, 1991). Thus, cell replication proceeds randomly throughout the entire cortex until late-gestation in the sheep fetus, at which time mitosis is found predominantly at the *zona glomerulosa / fasciculata* boundary, the so-called 'zona intermedia'.

A mechanism has been proposed by Nussdorfer and colleagues to reconcile *zona intermedia* cell proliferation with adrenocortical growth *in utero* ([313]Nussdorfer, 1986). Nussdorfer has suggested that dividing 'stem cells' are located at the tips of cellular cords aligned centripetally towards the medulla. The stem cells originating in the *zona glomerulosa* form straight, radial cords, whereas cells originating within the *zona intermedia* first migrate upwards into the *zona glomerulosa*, then curl down towards the inner *zonae*. Cell migration from the outer cortex to the inner *zonae* has been demonstrated by nuclear labelling experiments in fetal and neo-natal guinea pigs ([60]Bransome, 1968), pre-pubertal rats ([437]Wright, Voncina and Morley, 1973), and adult rats ([446]Zajicek, Ariel and Arber, 1986). As stem cells divide and differentiate, they displace neighbouring cells along the radial cords, resulting in the centripetal migration pattern seen in DNA labelling experiments ([313]Nussdorfer, 1986; [446]Zajicek, Ariel and Arber, 1986).

The adrenal gland of the fetal sheep can first be recognised by 28 d of gestation ([433]Wintour, Brown *et al.*, 1975). Morphologically, however, zonation and maturation of the adrenal cortex is only apparent from 60 d of gestation ([367]Robinson, Rowe and Wintour, 1979; [425]Webb, 1980). Prior to 60 d of gestation an 'immature' cortical cell type, characterised by small mitochondria with irregular tubulo-lamellar cristae and little smooth endoplasmic reticulum (SER), predominates throughout the *zona fasciculata*. In contrast, by 53 d of gestation the *zona glomerulosa* is well defined and displays signs of histological maturity ([53]Boshier and Holloway, 1989).

A study of ovine fetal adrenal development described two distinct phases of rapid adrenocortical growth during gestation, separated by a period of 'quiescence' in mid-gestation ([53]Boshier and Holloway, 1989). Adrenal development from 53-100 d of gestation is characterised by the completion of sympatho-chromaffin cell migration and the histological re-organisation of the adrenal cortex. The volume of steroidogenic cells within the *zona fasciculata* increases rapidly, primarily due to cellular hyperplasia, while the parenchymal cells become organised into radial cords, supported and separated by anastomosing sinusoids and vascular capillaries ([367]Robinson, Rowe and Wintour, 1979; [53]Boshier and Holloway, 1989).

Two qualitative studies of adrenal development have described the cytological maturation of the *zonae glomerulosa* and *fasciculata* ([367]Robinson, Rowe and Wintour, 1979; [425]Webb, 1980). Between 60 d and 80 d of gestation the morphologically 'mature' glomerular cell clusters beneath the adrenal capsule were characterised by extensive networks of SER, and mitochondria with vesicular cristae, typical of organelles of adult steroid-secreting cells. A few cortical cells situated at the cortico-medullary border also showed signs of cytological maturity at this time, although the majority of the cells of the *zona fasciculata* remained histologically 'immature' ([367]Robinson, Rowe and Wintour, 1979; [425]Webb, 1980). Few changes are seen in adrenocortical size between 100 d and 120 d of gestation, marking the gland's lowest rate of organ growth and steroidogenic cell multiplication during gestation.

Subsequent to the period of quiescence from 100-120 d of gestation, the juxta-medullary cortical cells display signs of accelerated histological maturation ([367]Robinson, Rowe and Wintour, 1979), attaining characteristics of adult *zona fasciculata* cells. From 130 d of gestation until birth, the second phase of rapid adrenocortical growth is characterised by rapid cellular hypertrophy, predominantly within the outer *zona fasciculata*, followed by intense hyperplastic activity, continuing into the post-natal period

([53]Boshier and Holloway, 1989). Between 130 d and 136 d of gestation, the inner 25 % of the *zona fasciculata* consist of histologically 'mature' parenchymal cells, with this proportion increasing in a radial, centrifugal fashion towards the *zona glomerulosa* as gestation continues ([366]Robinson, Comline *et al.*, 1983).

In contrast to the qualitative observations of Robinson and Webb, Boshier and colleagues have provided quantitative ultra-structural evidence of cellular differentiation between 130 d and 144 d of gestation ([54]Boshier and Holloway, 1991), which suggests a centripetal pattern of maturation. These authors demonstrated that the cytoplasmic volume density of SER within the steroidogenic cells of the outer *zona fasciculata* is greater than that of the inner zone at both 130 d and 144 d of gestation ([54]Boshier and Holloway, 1991). Based on the relative volume density of SER within steroidogenic cells, these authors have suggested that cortical maturation originates within the outer region of the *zona fasciculata*, progressing centripetally towards the *zona reticularis*.

A cell migration theory has been proposed to describe adrenocortical cell division and zonation. Hornsby and co-workers have suggested that the steroid gradient occurring inward throughout the adrenocortical parenchyma, which results from the centripetal direction of blood flow, may induce functional and morphological differentiation as cells are displaced inwards from the *zonae glomerulosa* and *intermedia* ([197]Hornsby and Crivello, 1983; [196]Hornsby, 1985b). Thus, functional zonation of the cortex is thought to depend on a sequential switching of steroid-synthesising enzymes within the migrating parenchymal cells, stimulated by the steroid gradient and possibly other endocrine and paracrine factors operating together, and resulting in adrenal functional and histological zonation. Initially, cells differentiating into *glomerulosa* cells are displaced towards the cortico-medullary boundary where they differentiate into *fasciculata* cells. Upon reaching the inner cortex these cells become *reticularis* cells, where they are eliminated. Typically, many of the features indicative of cell senescence are found within the *zona reticularis* ([438]Wylie, Kerr and Currie, 1973). In addition, Wintour and colleagues have postulated an autocrine / paracrine effect of cortisol in the ontogenic maturation of fetal *zona fasciculata* cells such that low concentrations of ACTH have increasingly larger effects on the growth and steroid synthesis within the adrenal cortex ([432]Wintour, 1984).

The two most significant events that occur during the last two weeks of gestation are a dramatic increase in the volume of the *zona fasciculata*, and the functional differentiation of this zone for maximum steroidogenic capacity. During this period, fetal plasma ACTH concentrations more than double ([219]Jones, Boddy and Robinson, 1977) and, by the time of

parturition, adrenal corticosteroid production has increased nine fold ([245]Liggins, Fairclough *et al.*, 1973).

1.3.2 Ontogeny of fetal adrenal steroidogenic enzyme activity

Three distinct phases of adrenocortical steroidogenic potential can be identified in fetal sheep. During the first period, which lasts until approximately 90 d of gestation, the capacity of adrenocortical cells to secrete cortisol *in vitro* is greater, per gram of fetal body weight, than at any other time in gestation ([433]Wintour, Brown *et al.*, 1975), although the half-maximal dose (ED$_{50}$) of ACTH for cortisol output is considerably lower at term ([168]Glickman and Challis, 1980). The steroid synthetic potential at this stage of development is suprising, as key organelles which contain the steroidogenic enzymes (CYP17, 3βHSD and CYP21A1 within the SER and CYP11A1 and CYP11B1 within the mitochondria) appear undeveloped in the immature cortical cells prior to 100 d of gestation ([367]Robinson, Rowe and Wintour, 1979; [425]Webb, 1980).

The cortisol-synthetic rate of adrenocortical cells in response to either exogenous ACTH *in vitro*, or to physiological stressors that elicit the endogenous release of ACTH *in vivo*, reaches a nadir between 90-129 d of gestation ([433]Wintour, Brown *et al.*, 1975; [370]Rose, Meis and Morris, 1981). The diminished responsiveness of the fetal ovine adrenal has been attributed to both an attenuated cAMP response to activation of the ACTH receptor ([133]Durand, Cathiard *et al.*, 1981) and to a decline in the number of adrenocortical ACTH receptors ([129]Durand, 1979). Prior to 125 d of gestation, the transfer of maternal cortisol across the placenta is responsible for the level of cortisol in the fetal circulation, being indistinguishable from the level measured in bilaterally adrenalectomised fetuses ([183]Hennessy, Coghlan *et al.*, 1982). In addition to the mid-gestation decline in fetal adrenocortical ACTH receptor number, and association with adenylate cyclase, impaired steroid synthetic capacity is also associated with a functional block at the level of the steroidogenic enzymes.

1.3.2.1 CYP17 and 3βHSD enzyme activity

Dispersed adrenal cells isolated from ovine fetuses secrete large amounts of cortisol in response to ACTH or Bt$_2$cAMP at 50 d of gestation and at term, but not at 100 d or 130 d of gestation ([168]Glickman and Challis, 1980; [267]Manchester and Challis, 1982). In contrast, Bt$_2$cAMP stimulates progesterone secretion from fetal adrenal cells at 50 d, 100 d, 130 d of gestation and term, whereas ACTH is only effective at 50 d of gestation and at term ([267]Manchester and Challis, 1982). These results confirm the mid-gestation functional block between the ACTH receptor and the adenylate cyclase enzyme, which is overcome

by the addition of Bt_2cAMP into the incubation medium. In addition, the ability of fetal adrenal cells to secrete progesterone, but not cortisol, at 100 d and 130 d of gestation is indicative of a blockade in the steroid synthetic pathway downstream of the formation of progesterone.

Anderson and colleagues reported that minced adrenal tissue from fetuses at 122 d of gestation have little capacity to metabolise [^{3}H]pregnenolone (*see Figure 1.3, step 2 on pg. 16*), although there is formation of [^{3}H]-labelled corticosteroids in adrenal glands from fetuses at 133 d of gestation or later ([15]Anderson, Pierrepoint *et al.*, 1972). In addition, adrenal cells from 113 d fetuses produce predominantly 11 deoxycorticosterone and corticosterone, and lower amounts of the 17α-hydroxylated steroids (11 deoxycortisol and cortisol) *in vitro* ([78]Cathiard, Crozat *et al.*, 1985). These results indicate a blockade at the level of the CYP17 enzyme.

The ontogeny of this enzymatic step was demonstrated further by Manchester and colleagues, who showed that the ratio of cortisol : corticosterone (F : B) production from isolated fetal adrenal cells in response to ACTH increases from 3.3 at 50 d of gestation to 11.3 at term ([267]Manchester and Challis, 1982). The progressive increase in the F : B ratio between 100-145 d of gestation has also been demonstrated using fetal adrenal slices stimulated by ACTH ([264]Madill and Bassett, 1973; [433]Wintour, Brown *et al.*, 1975). Similarly, Durand and colleagues demonstrated that the F : B ratio observed in newborn lambs is higher than that in fetuses at 120 d of gestation ([131]Durand, Cathiard *et al.*, 1982). Finally, the gestational increase in the F : B ratio measured in adrenal venous blood ([12]Alexander, 1968) and in the fetal circulation ([65]Brown, Coghlan *et al.*, 1978) confirms these *in vitro* findings and is consistent with the suggestion that the increased responsiveness of the fetal adrenal near term is associated with increased activity of the CYP17 enzyme.

Manchester and colleagues have confirmed the association of decreased activity of CYP17 with the blockade to steroid synthesis in mid-gestation. These authors incubated isolated fetal adrenal cells with potential precursors of cortisol production: pregnenolone, 17α hydroxy pregnenolone, progesterone and 17α hydroxy progesterone ([267]Manchester and Challis, 1982). At 100 d of gestation, the conversion quotient of progesterone and 17α hydroxy progesterone to cortisol [(P_4 : F)/(17αP_4 : F)] is 0.09, indicating a blockade at the CYP17 enzyme step (*see Figure 1.3, step 3 on pg. 16*). At 130 d of gestation, however, this ratio is 0.36, while at term CYP17 enzyme activity is no longer rate-limiting, as the [(P_4 : F)/(17αP_4 : F)] conversion quotient rises to 0.8 ([267]Manchester and Challis, 1982).

These authors concluded that there is a marked increase in CYP17 activity through the second half of gestation such that, at term, CYP17 activity ceases to be a major rate-limiting step in cortisol production.

Manchester and colleagues have also shown that the conversion of the Δ^4 substrates (progesterone and 17α hydroxy progesterone) to cortisol is faster than the conversion of the Δ^5 substrates (pregnenolone and 17α hydroxy pregnenolone), suggesting a rate-limiting role of 3βHSD in fetal sheep adrenals at mid-gestation ([267]Manchester and Challis, 1982) (*see Figure 1.3, step 2 on pg. 16*). Indeed, the conversion of Δ^5 substrates to cortisol remains about 30 % of the corresponding Δ^4 substrates at term, suggesting a rate-limiting role of 3βHSD from mid-gestation until term ([267]Manchester and Challis, 1982) (*see Figure 1.3, step 2 on pg. 16*).

The administration of ACTH *in vitro* increases F biosynthesis dramatically in both younger and older fetuses ([433]Wintour, Brown *et al.*, 1975; [168]Glickman and Challis, 1980; [267]Manchester and Challis, 1982). Administration of ACTH to the ovine fetus *in vivo* markedly increases the capacity of the adrenal to synthesise pregnenolone ([131]Durand, Cathiard *et al.*, 1982). In 120 d fetal adrenal cells incubated with 5×10^{-5} M [^{14}C]pregnenolone, only 45 % of the pregnenolone had been metabolised by 2 h, of which 85 % was metabolised through the 17α-deoxy pathway (*i.e.* towards B) (*see Figure 1.3 on pg. 16*). In contrast, adrenal cells from fetuses pre-treated with a 5 d infusion of ACTH *in vivo* metabolised all of the [^{14}C]pregnenolone by 2 h, of which 75 % was metabolised through the 17α-hydroxy pathway (*i.e.* towards F) (*see Figure 1.3 on pg. 16*). Taken together, these data indicate that ACTH induces a marked increase in CYP17 activity in the ovine fetal adrenal ([131]Durand, Cathiard *et al.*, 1982).

Pulsatile ACTH administration to mid-gestation fetuses for 72 h *in vivo* increases the relative incorporation of progesterone : 17α hydroxy progesterone into F to 0.8 (*i.e.* similar to the ratio observed in term fetuses), and continued pulsatile ACTH treatment until labour results in equivalent rates of conversion of these two substrates into F ([268]Manchester, Lye and Challis, 1983). Pregnenolone and 17α hydroxy pregnenolone are also converted into F at equivalent rates following pulsatile ACTH administration, but the conversion rates of these Δ^5 steroids remain at 20-25 % of the corresponding Δ^4 steroids ([268]Manchester, Lye and Challis, 1983). It would appear, therefore, that pulsatile ACTH administration ameliorates the rate-limiting nature of CYP17, similar to the ontogenic increase in the activity of this enzyme. 3βHSD enzyme activity, however, remains rate-limiting in

late-gestation, and the activity of this enzyme is not affected by pulsatile ACTH administration ([268]Manchester, Lye and Challis, 1983) (*see Figure 1.3, step 2 on pg. 16*).

1.3.2.2   CYP11A1 enzyme activity

Durand and colleagues have suggested that CYP17 enzyme activity is not the only rate-limiting step to steroidogenesis during the mid-gestation hypo-responsive phase of fetal adrenal development. Adrenal cells from 120 d fetuses are able to synthesise cortisol and corticosterone spontaneously from exogenous [^{14}C]pregnenolone substrate *in vitro*, to a greater extent than that observed under maximal ACTH stimulation without [^{14}C]pregnenolone substrate ([131]Durand, Cathiard *et al.*, 1982). In addition, although the basal conversion quotient of cortisol and corticosterone from [^{14}C]pregnenolone substrate [([^{14}C]P$_5$: F)/([^{14}C]P$_5$: B)] significantly increases following a 5 d intra-fetal infusion of ACTH(1-24) prior to 120 d of gestation, the formation of steroids is still limited by the availability of exogenous [^{14}C]pregnenolone substrate. Taken together, these results are consistent with the availability of endogenous pregnenolone substrate being another important limiting step in the biosynthesis of corticosteroids in mid-gestation, and activity of the CYP11A1 enzyme is therefore suggested to influence adrenal corticosteroid secretion ([131]Durand, Cathiard *et al.*, 1982). Finally, adrenal cells from fetuses at 89-144 d of gestation, incubated in the presence of 25 µM 22R-hydroxy cholesterol (a synthetic substrate for CYP11A1), produce 75-80 % less pregnenolone than those of 3-10 d old lamb adrenal cells under similar conditions ([300]Naaman-Reperant, Cathiard and Durand, 1992). These results suggest that CYP11A1 enzyme activity is rate-limiting for steroidogenesis in the ovine fetal adrenal gland. The production of pregnenolone by isolated fetal adrenal mitochondria is also lower than that of neo-natal mitochondria when 22R-hydroxy cholesterol is added to the incubation medium, indicating that there is an increase either in CYP11A1 enzyme activity (*see Figure 1.3, step 1 on pg. 16*), or an increase in StAR synthesis between mid-gestation and early post-natal life. Interestingly, Coulter and colleagues have recently demonstrated a significant increase in StAR mRNA within the fetal adrenal between 90 d and 141 d of gestation (Dr. CL Coulter, personal communication).

In addition to the rate-limiting nature of pregnenolone production by CYP11A1, the accumulation of progesterone by 120 d fetal adrenal cells incubated with [^{14}C]pregnenolone suggests an additional defect in CYP21A1 enzyme activity ([131]Durand, Cathiard *et al.*, 1982). The slower metabolism of [^{14}C]pregnenolone and formation of 17α hydroxy progesterone and progesterone by mid-gestation, compared with newborn

adrenal cells, supports the finding of lower CYP17 and / or 3βHSD activity demonstrated by Manchester and colleagues ([267]Manchester and Challis, 1982).

1.3.2.3 Adrenocortical reactivation

In sheep, fetal adrenal reactivation occurs at approximately 125-130 d of gestation, and is characterised by an increasing sensitivity to exogenous ACTH or physiological stress ([370]Rose, Meis and Morris, 1981; [263]Lye, Sprague *et al.*, 1983) and the re-emergence of adrenocortical steroidogenic enzyme activity ([168]Glickman and Challis, 1980). Placental transfer accounts for only 37 % of the cortisol measured in fetal blood from 122-135 d of gestation, and for <12 % after 136 d of gestation ([183]Hennessy, Coghlan *et al.*, 1982). After approximately day 125 of gestation there is an increase in cortisol synthesis which is accompanied by an increase in the number of adrenal ACTH receptors ([129]Durand, 1979) and their coupling to adenylate cyclase ([133]Durand, Cathiard *et al.*, 1981). In addition, changes in the ratio of bioactive : immunoreactive (ir)-ACTH in the fetal circulation reflect an increase in trophic stimulation from the fetal pituitary throughout late-gestation ([62]Brieu and Durand, 1987; [63]Brieu and Durand, 1989). These changes in enzyme activity, and the increased abundance and function of ACTH receptors ensure the adrenal is maximally responsive to the increasing trophic stimulation of the fetal pituitary in late-gestation.

1.3.3 Ontogeny of fetal adrenal steroidogenic enzyme gene expression

Concomitant with the functional block to steroid synthesis in mid-gestation at the level of steroidogenic enzyme activity, several studies have demonstrated a decrease in the mRNA levels of a number of key steroid-synthesising enzymes ([267]Manchester and Challis, 1982; [407]Tangalakis, Coghlan *et al.*, 1989; [298]Myers, McDonald and Nathanielsz, 1992b). Several studies have measured CYP11A1, CYP17 and CYP21A1 mRNA levels in the fetal sheep adrenal between 50 d and 135 d of gestation ([216]John, Simpson *et al.*, 1987; [407]Tangalakis, Coghlan *et al.*, 1989; [298]Myers, McDonald and Nathanielsz, 1992b). *In situ* hybridisation of 40-50 d fetal adrenal sections revealed that *zona fasciculata* cells contain the three mRNAs, however, in small groups of sub-capsular cells, no signal for CYP17 mRNA is detected ([407]Tangalakis, Coghlan *et al.*, 1989). These data indicate that there is 'functional' differentiation of the *zona glomerulosa* at this gestational age range, as cells capable of synthesising cortisol should contain all three mRNAs, while cells synthesising aldosterone should lack mRNA for CYP17 (*see Figure 1.2 on pg. 13 and Figure 1.3, step 1 on pg. 16*).

Tangalakis and colleagues also demonstrated a decrease in the hybridisation signal for CYP17 mRNA between 40-50 d and 114 d of gestation in the *zona fasciculata*, where the

labelling for this enzyme is restricted ([407]Tangalakis, Coghlan *et al.*, 1989). Concurrently, a weaker signal for CYP11A1 mRNA is also observed in both the *zonae glomerulosa* and *fasciculata* at the later gestational age, when adrenal secretion of steroids and responsiveness to ACTH are low. Similarly, Myers and colleagues reported a significant decline in the expression of the mRNAs for these enzymes between 105 d and 120 d of gestation ([298]Myers, McDonald and Nathanielsz, 1992b). These investigators also noted that the expression of CYP21A1 mRNA is suppressed to a lesser extent than CYP11A1 and CYP17 at 120 d of gestation ([298]Myers, McDonald and Nathanielsz, 1992b). These findings are consistent with studies using dispersed ovine fetal adrenal cells, which have demonstrated a decrease in CYP11A1, CYP17 and 3βHSD and to a lesser extent CYP21A1 and CYP11B1 enzyme activities, during the adrenal quiescent period between 90 d and 125 d of gestation ([267]Manchester and Challis, 1982; [136]Durand, Cathiard and Saez, 1984a).

Adrenocortical steroidogenesis resumes at around 125 d of gestation, and reactivation is associated with an increased activity of the CYP11A1, CYP17 and 3βHSD enzymes ([267]Manchester and Challis, 1982; [136]Durand, Cathiard and Saez, 1984a). Tangalakis and co-workers also reported that there is a qualitative increase in the expression of CYP11A1, CYP17 and CYP21A1 mRNAs in the fetal adrenal between 114 d and 132 d of gestation ([407]Tangalakis, Coghlan *et al.*, 1989). These findings are supported by those of Myers and co-workers, who demonstrated 10-20 fold increases in CYP11A1 and CYP17 and a ~2 fold increase in CYP21A1 mRNA expression between 120 d of gestation and birth ([298]Myers, McDonald and Nathanielsz, 1992b). John and co-workers demonstrated, using steroid enzyme immunoblotting, that CYP17 undergoes a greater than 10 fold increase in protein concentration between 127-145 d of gestation ([216]John, Simpson *et al.*, 1987). These data indicate that increases in steroid hydroxylase mRNA and protein expression are tightly coupled. Indeed, immunocytochemical examination of the levels of CYP17 and 3βHSD in bovine fetal adrenal glands correlate with the results of Western and Northern analyses ([106]Conley, Head *et al.*, 1992).

Increases in transcription of the *CYP*11A1, *CYP*11B1, *CYP*17 and *CYP*21A1 genes have been measured in nuclear run-on transcription experiments of isolated nuclei from bovine adrenocortical cells incubated with ACTH ([214]John, John *et al.*, 1986). Utilising primary cultures of bovine adrenocortical cells, it has been established that ACTH treatment leads to accumulation of mRNA for CYP11A1 ([213]John, John *et al.*, 1984), CYP17 ([447]Zuber, John *et al.*, 1986) and CYP21A1 ([215]John, Okamura *et al.*, 1986). ACTH treatment has also been found to increase the half-life of bovine CYP11A1 mRNA, but to have no effect

on the stability of mRNAs encoding the other bovine steroid hydroxylases ([48]Boggaram, John *et al.*, 1989).

While basal expression of mRNA for CYP11A1, 3βHSD, CYP21A1 and CYP11B1 is observed in the absence of ACTH in cultured adrenocortical cells, maintenance of *CYP*17 gene expression ([260]Lund, Faucher *et al.*, 1988) and enzyme activity ([78]Cathiard, Crozat *et al.*, 1985) appear to be essentially dependent on ACTH. ACTH can restore CYP17 mRNA expression and, to a lesser extent, increase expression of the other steroid hydroxylases in dispersed bovine adrenocortical cells obtained from mid-gestation fetuses ([260]Lund, Faucher *et al.*, 1988). ACTH also restores the low CYP17 activity in adrenal cells from hypophysectomised ovine fetuses ([78]Cathiard, Crozat *et al.*, 1985; [132]Durand, Cathiard *et al.*, 1985). Exogenous administration of ACTH during the hypo-responsive period (~90-125 d of gestation), leads to a transient increase in the mRNA for CYP11A1 and CYP17 but not CYP21A1 ([408]Tangalakis, Coghlan *et al.*, 1990). In this study, the cessation of ACTH administration lead to a drop in gene expression for both CYP11A1 and CYP17 enzymes.

Overwhelming evidence suggests that, during the last 3-4 weeks of gestation, the adrenal becomes increasingly responsive to both endogenous and exogenous ACTH ([433]Wintour, Brown *et al.*, 1975; [168]Glickman and Challis, 1980; [371]Rose, Meis *et al.*, 1982; [374]Saez, Durand and Cathiard, 1984; [312]Norman, Lye *et al.*, 1985). The coordinated expression of the steroidogenic enzymes during the two weeks prior to birth is critical in mediating the increased corticotrophic drive to corticosteroid secretion ([312]Norman, Lye *et al.*, 1985). While it is clear that parturition in the sheep is preceded by an increase in the output of cortisol from the fetal adrenal, the time course of changes in steroidogenic enzyme gene expression within the fetal adrenal during the 10-15 d immediately preceding delivery have not been characterised.

The first main aim of this thesis, therefore, was to generate a gestational growth profile of the fetal sheep adrenal and to investigate the late-gestation activation of steroidogenic enzyme gene expression within the adrenal gland of the sheep fetus. In Chapter 2, a large cohort of fetal sheep (*Study 1*) was used to construct a gestational profile of the growth rate of the fetal adrenal gland. In a separate group of animals (*Study 2*), the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 was measured at three time frames within the 15 d preceding delivery, 130-134 d, 135-139 d and 140-145 d of gestation.

1.4   FETAL HYPOTHALAMO-PITUITARY-ADRENAL INTERACTIONS

1.4.1   POMC derived peptides

ACTH plays an important role in the initiation of parturition in the sheep via induction of adrenocortical steroidogenesis ([31]Bassett and Thorburn, 1969; [264]Madill and Bassett, 1973). The importance of ACTH-induced steroidogenesis in initiating parturition was first demonstrated by the failure of hypophysectomised ([247]Liggins, Kennedy and Holm, 1967; [246]Liggins and Kennedy, 1968) or adrenalectomised ([128]Drost and Holm, 1968) sheep fetuses to deliver spontaneously. Ovine fetuses which are surgically hypophysectomised not only remain *in utero* past normal term but their adrenal glands are also developmentally immature, exhibiting marked hypoplasia of the *zona fasciculata*. In contrast, intra-fetal infusion of ACTH or glucocorticoids (cortisol or dexamethasone) *in utero* elevate plasma glucocorticoid levels and lead to premature delivery ([245]Liggins, Fairclough *et al.*, 1973). In these studies, the timing of parturition is unaltered by maternal hypophysectomy, or by infusion of ACTH or cortisol into the mother ([245]Liggins, Fairclough *et al.*, 1973). Disconnection of the fetal hypothalamus from the pituitary (hypothalamo-pituitary disconnection, HPD) also results in prolonged pregnancy ([16]Antolovich, Clarke *et al.*, 1990).

ACTH can be detected within the pars distalis (anterior pituitary) and pars intermedia (neuro-intermediate lobe) of the ovine fetal pituitary as early as 40 d and 60 d of gestation respectively ([293]Mulvogue, McMillen *et al.*, 1986). In addition to ACTH, the fetal pituitary contains a number of biologically active peptides derived from the multi-hormone precursor POMC. The complete amino acid sequence of the POMC molecule was inferred from its mRNA-DNA sequence ([301]Nakanishi, Inoue *et al.*, 1979). POMC is synthesised as a 31,000 Dalton (31 kDa) common precursor molecule in the corticotrophs of the pars distalis and the melanotrophs of the pars intermedia and contains the sequence of a glycopeptide at its *N*-terminus (the 16 kDa fragment, or pro-γ-MSH), ACTH in the mid-region and β-lipotrophin (β-LPH) at its carboxy terminus ([359]Roberts and Herbert, 1977; [140]Eipper and Mains, 1978; [301]Nakanishi, Inoue *et al.*, 1979; [83]Chang, Cochet and Cohen, 1980) (*Figure 1.4*). These studies also indicate that the POMC molecule is processed differently in the pars distalis and in the pars intermedia. The pars distalis of the fetal sheep contains the 16 kDa amino (*N*)-terminal fragment, ACTH, β-LPH and β-endorphin (β-END), as well as high molecular weight forms of ACTH (POMC and pro-ACTH) ([395]Silman, Holland *et al.*, 1979; [293]Mulvogue, McMillen *et al.*, 1986). In the pars intermedia, however, ACTH is further cleaved to yield α-MSH and CLIP.

Hydrophobic leader sequence Cysteine bridges Ⓞ Galactosamine Ⓝ Glucosamine

-20 -10 1 10 20 30 40 50 60 70 80 90 100

H₂N MPRLCSSRSGALLLALLLQASMEVRGWCLESSQCQDLTTESNLLACIRACKPDLSAETPVFPGNGDEQPLTENPRKYVMGHFRWDRFGRRNGSSSSGVGGAAQKREEEVAVGEGPGPRGDDAETGPREDKR

Abbreviations

pro-ACTH
1 → 144

ACTH — adrenocorticotrophin
CLIP — corticotrophin-like intermediate lobe peptide
END — endorphin
ENK — enkephalin
JP — joining peptide
LPH — lipotrophin
MSH — melanocyte stimulating hormone
POMC — pro-opiomelanocortin

N-POMC (16 kDa fragment, or pro-γ-MSH)
1 — 103

N-POMC(1-77)   JP
1 — 77   80 — 103

N-POMC(1-49)   lys-γ₃-MSH
1 — 49   50 — 77

γ₃-MSH
51 — 77

γ₂-MSH
51 — 62

γ₁-MSH
51 — 61

Amino acid	Three letter symbol	One letter symbol	Amino acid	Three letter symbol	One letter symbol
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Ⓝ Nitrogen-linked glycosylation
Ⓞ Oxygen-linked glycosylation
▮ dibasic amino acid residues

110 120 130 140 150 160 170 180 190 200 210 220 230

SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAQAFPLEFKRELTGERLEQARGPEAQAESAAARAELEYGLVAEAEAEAAEKKNSGPYKMEHFRWGSPPKDKRYGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ COOH

pro-ACTH ←
1 — 144

β-LPH
147 — 239

ACTH(1-39)
106 — 144

γ-LPH
147 — 206

β-END
209 — 239

α-MSH   CLIP
106 — 118   123 — 144

β-MSH
189 — 206

β-END(1-27)
209 — 235

Met-ENK
209 — 213

Figure 1.4   Diagramatic representation of the bovine POMC peptide sequence inferred from the mRNA-cDNA sequence.

The complete peptide sequence of the bovine corticotrophin / β-lipotrophin precursor (pro-opiomelanocortin - POMC), as described by [301]Nakanishi, Inoue *et al.*, 1979; [102]Cohen and Chang, 1980; and [387]Seger and Bennett, 1986. Processing of the precursor molecule, POMC, in the anterior and intermediate lobes of the pituitary gland occurs at dibasic amino acid residues, giving rise to a number of biologically active peptides. Initial processing of POMC is similar in both lobes, but additional cleavage in the intermediate lobe produces smaller final products. Amino acids in yellow denote common sequences in α-, β- and γ-MSH. Each segment shares Tyr and Met residues as well as the characteristic tetra-peptide sequence His-Phe-Arg-Trp, which is required for melanotrophic activity.

The synthesis of POMC-derived peptides within the corticotrophs of the pars distalis and the melanotrophs of the pars intermedia is a highly ordered process ([41]Birch, Estivariz *et al.*, 1991). Post-translational modification of POMC involves proteolytic cleavage by endopeptidases at paired basic amino acid residues, Lysine (Lys, K) and Arginine (Arg, R) (*see Figure 1.4*) to liberate smaller, biologically active peptide hormones ([93]Chretien, Boileau *et al.*, 1984; [36]Bennett, 1986; [387]Seger and Bennett, 1986; [406]Tanaka, Nomizu and Kurosumi, 1991). The first of these proteolytic enzymes to be isolated was kexin, a serine protease capable of processing the yeast α-mating factor precursor ([161]Fuller, Brake and Thorner, 1989), followed by bacterial subtilisin ([388]Seidah, Chretien and Day, 1994). A family of mammalian subtilisin-like proteases has also been identified and named pro-hormone convertase (PC) enzymes. A number of mammalian PC enzymes have been cloned: PC1, PC2, PC4, PC5/6A, PC6B ([389]Seidah, Marcinkiewicz *et al.*, 1991), PACE4 ([265]Mains, Berard *et al.*, 1997); and furin, which was found to have homology with yeast kexin ([162]Fuller *et al.*, 1989b). These endopeptidases are localised within the pituitary corticotrophs of the adult rat ([406]Tanaka, Nomizu and Kurosumi, 1991) and fetal sheep ([33]Bell, Myers and Myers, 1998), and catalyse the cleavage of POMC in the fetal and adult pituitary.

1.4.2 Cortisol and the maturation of fetal pituitary corticotrophs

1.4.2.1 Fetal pituitary corticotroph morphology

The main corticotrophic cell observed between 90 d and 130 d of gestation is a tall, columnar 'fetal' cell type, whereas after 135 d of gestation a small, stellate or 'adult' cell type predominates ([338]Perry, Mulvogue *et al.*, 1985; [293]Mulvogue, McMillen *et al.*, 1986). Antolovich and colleagues have demonstrated that cortisol mediates the morphological maturation of the corticotrophs within the fetal pars distalis ([19]Antolovich, Perry *et al.*, 1989). After fetal adrenalectomy at 120 d of gestation, the fetal pituitary corticotrophs remain morphologically immature, and the pituitaries of adrenalectomised fetuses contain the same proportion of corticotrophs at 135 d of gestation as pituitaries of intact fetuses at 115 d of gestation. ([19]Antolovich, Perry *et al.*, 1989). After fetal adrenalectomy, palisades of 'fetal' type corticotrophs are prominent, and the histological appearance is typical of pituitaries of intact fetuses at 115 d of gestation. These data suggest that the normal changes in the corticotroph population that occur between 115 d and 135 d of gestation are arrested following fetal adrenalectomy. Conversely, infusion of cortisol (2 mg·24 h^{-1}), from 109-115 d of gestation, accelerates these maturational changes, increasing the ratio of 'adult' to 'fetal' type corticotrophs to levels similar to those in the pars distalis of 135 d old fetuses. These authors concluded that cortisol produced by the fetal adrenal mediates the

change from a predominantly 'fetal' type to an 'adult' type corticotroph population between 115 d and 135 d of gestation and that exogenous cortisol from 109-115 d of gestation can accelerate this process.

Although Antolovich and co-workers demonstrated that infusion of cortisol from 109-115 d of gestation into HPD fetal sheep results in a significant fall in the proportion of 'fetal' type corticotrophs (saline infused HPD: 10.24 %; cortisol infused HPD: 7.60 %), this change is smaller than that observed in intact fetal sheep (saline infused: 14.91 %; cortisol infused: 3.78 %). Furthermore, there is no significant change in the ratio of 'adult' : 'fetal' type corticotrophs in the pars distalis of the HPD fetus infused with cortisol. These findings suggest that an intact HP axis may be essential for cortisol to promote the full morphological maturation of the pars distalis corticotrophs in the fetal sheep. In addition, Durand and colleagues have shown that the ratio of bioactive : ir-ACTH and the proportion of 'little' ACTH released from anterior pituitary cells is enhanced by the presence of cortisol *in vitro*, suggesting that the latter may stimulate the functional maturation of the pars distalis corticotrophs directly ([62]Brieu and Durand, 1987; [63]Brieu and Durand, 1989). A similar effect of cortisol on the secretory profile of corticotrophs *in vivo*, however, has not been demonstrated.

1.4.2.2   Fetal pituitary corticotroph function

Since the placenta is impermeable to many hormones, particularly peptide hormones, maternal ACTH does not cross the placenta during gestation. Fetal plasma ACTH concentrations, therefore, depend entirely on an intact fetal hypothalamo-pituitary (HP) unit. The fetal hypothalamo-hypophysial portal blood vessel system is patent from 45 d of gestation ([240]Levidiotis, Wintour *et al.*, 1989) and provides the functional link through which hypothalamic releasing factors can influence anterior pituitary secretion. The release of pituitary hormones is regulated by hypothalamic releasing / inhibiting factors.

Biosynthesis and secretion of ACTH and other POMC derived peptides are modulated, in part, by the paraventricular nucleus (PVN) in the fetal hypothalamus. CRH and AVP released from the PVN stimulate POMC gene expression in corticotrophs within the pars distalis and also stimulate the release of a range of peptide factors, including ACTH(1-39) ([259]Lu, Yang and Challis, 1994). CRH has been shown to stimulate the release of pro-γ-MSH and ACTH ([166]Gillies, Estivariz and Lowry, 1979) and the concentration of these peptides in human plasma are correlated ([38]Bertagna, Girard *et al.*, 1980; [193]Hope, Ratter *et al.*, 1981). In late-gestation, an increase in CRH mRNA is observed in the parvocellular neurons within the PVN of the hypothalamus

(296Myers, Ding and Nathanielsz, 1991), while AVP appears to have greater potency in corticotroph stimulation earlier in fetal life (311Norman and Challis, 1987).

Several studies have demonstrated that the sensitivity of the fetal sheep pituitary to hypothalamic stimulation changes in late-gestation. Durand and colleagues (130Durand, Cathiard *et al.*, 1986) and Lu and co-workers (259Lu, Yang and Challis, 1994) have found that, at term, the pituitary ACTH response to CRH is greater than to AVP. Norman and co-workers have also shown that the ovine fetal pituitary responds separately and synergistically to AVP and CRH at 110-115 d of gestation *in vivo*, and that the relative role of AVP in stimulating ACTH release decreases with increasing gestational age (311Norman and Challis, 1987). In contrast to these findings, Fora and colleagues demonstrated that AVP produces a greater response than CRH in anterior pituitary cells from late-gestation fetal sheep (152Fora, Butler *et al.*, 1996).

1.4.2.3   Glucocorticoid modulation of fetal corticotroph function

Studies using the reverse haemolytic plaque assay, or a specific cytotoxic analog of CRH have shown that there are separate sub-populations of corticotrophic cells in the adult pituitary, distinguishable on the basis of their response to hypothalamic secretagogues (304Neill, Smith *et al.*, 1987; 386Schwartz and Vale, 1988; 211Jia, Canny *et al.*, 1991). In the adult rat, cow and sheep pituitary there are corticotrophs which are responsive to either CRH or AVP alone, or to both CRH and AVP (304Neill, Smith *et al.*, 1987; 386Schwartz and Vale, 1988; 211Jia, Canny *et al.*, 1991). Interestingly, there appear to be major functional differences between these corticotrophic cell types in the synthetic and secretory pathway for ACTH and their responsiveness to glucocorticoids (384Schwartz, Ash *et al.*, 1994).

Schwartz and colleagues have demonstrated that CRH responsive corticotrophs within adult ovine pituitaries are required for dexamethasone to inhibit CRH or AVP stimulated ACTH(1-39) secretion *in vitro* (384Schwartz, Ash *et al.*, 1994). Using a CRH-target-specific cytotoxin to selectively eliminate CRH responsive corticotrophs, Schwartz and colleagues demonstrated that dexamethasone decreases the ACTH(1-39) secretory response to AVP or CRH in intact populations of ovine pituitary cells, but does not decrease the residual ACTH(1-39) response to AVP in populations previously treated with the cytotoxin. These authors postulated that either (*1*) the ACTH response to AVP is decreased by dexamethasone, only in those corticotrophs that are CRH / AVP dually responsive, or (*2*) that glucocorticoid inhibition of the response to AVP is indirect, mediated by separate CRH-target cells and involves a paracrine interaction between the corticotrophs (384Schwartz, Ash *et al.*, 1994). It is possible that a transition from a

CRH-predominant (glucocorticoid sensitive) to an AVP-predominant (glucocorticoid insensitive) target cell population, within the fetal pituitary in late-gestation ([152]Fora, Butler *et al.*, 1996), allows the pituitary to respond to the negative feedback effects of cortisol at the fetal pituitary. Butler and colleagues have demonstrated, however, that there is no difference in the proportion of ACTH that is stored within CRH or AVP responsive corticotrophs in the fetal pituitary between 116 d and 140 d of gestation. This result also suggests that in late-gestation the stimulatory influence of the fetal hypothalamus counteracts a negative feedback effect of cortisol on ACTH synthesis in CRH responsive cells. While cortisol inhibits ACTH synthesis in CRH responsive corticotrophs, it may also mediate corticotroph maturation in conjunction with hypothalamic stimulation, to result in a sustained elevation in plasma ACTH(1-39) concentrations which precedes parturition.

1.4.3   *ACTH, cortisol and adrenocortical growth and function*

In addition to the late-gestation increase in adrenocortical ACTH receptor binding, and the increase in activity and gene expression of the steroid-synthesising enzymes, the gestational increase in the responsiveness of the fetal adrenal gland to ACTH may also involve changes in the type of ACTH secreted during late-gestation. ACTH is present in the fetal sheep circulation in a variety of different molecular weight forms throughout gestation. The ratio of the high molecular weight (HMW) forms of ACTH (POMC and pro-ACTH) to ACTH(1-39) is higher in the fetal than the adult pituitary ([395]Silman, Holland *et al.*, 1979), and plasma ACTH(1-39) levels increase during the last two weeks of gestation ([224]Jones and Roebuck, 1980; [70]Carr, Jacobs *et al.*, 1995; [342]Phillips, Ross *et al.*, 1996). Indeed, Castro and colleagues have demonstrated that the ratio of bioactive : ir-ACTH from near-term fetal sheep is higher than that found in plasma from immature fetal lambs ([77]Castro, Valego *et al.*, 1992). In addition, Brieu and colleagues have demonstrated that the proportion of HMW forms of ACTH is higher in the incubation medium of fetal than neo-natal pituitary cells ([63]Brieu and Durand, 1989), and it has been demonstrated that the HMW ACTH-containing peptides can inhibit the cortisol response of fetal ovine adrenocortical cells to ACTH(1-24) ([368]Roebuck, Jones *et al.*, 1980; [385]Schwartz, Kleftogiannis *et al.*, 1995). Thus, the change in the ratio of HMW ACTH-containing peptides : ACTH(1-39) throughout gestation has the potential to enhance adrenal function, and a decrease in the concentration of HMW ACTH-containing peptides pre-partum would presumably diminish their antagonistic effect on ACTH(1-39) induced adrenocortical steroidogenesis.

The necessity of an intact HPA axis for normal adrenocortical development has previously been demonstrated in our laboratory, using the model of fetal HPD. Disconnection of the fetal hypothalamus and pituitary between 106 d and 120 d of gestation abolishes the pre-partum rise in circulating ir-ACTH ([16]Antolovich, Clarke *et al.*, 1990) and ACTH(1-39) which normally occurs after 135 d of gestation ([342]Phillips, Ross *et al.*, 1996). Levels of adrenal CYP11A1, CYP17 and 3βHSD mRNAs are also significantly lower in fetal sheep at 140 d of gestation, after HPD at around 110 d of gestation, when compared to fetuses with an intact hypothalamo-pituitary axis ([342]Phillips, Ross *et al.*, 1996). These data are consistent with the lack of a pre-partum cortisol surge in the HPD sheep fetus ([16]Antolovich, Clarke *et al.*, 1990; [327]Ozolins, Young and McMillen, 1992) and indicate that the increase in circulating ACTH(1-39) concentrations in the sheep fetus is essential for the increase in adrenal steroidogenesis preceding delivery.

Consistent with this finding, McDonald and co-workers have found previously that bilateral stereotaxic lesions of the PVN at around 120 d of gestation abolishes the pre-partum increase in both fetal ACTH and cortisol ([277]McDonald and Nathanielsz, 1991). Bilateral lesions of the fetal PVN also significantly reduce the adrenal mRNA expression of CYP11A1 and CYP17 at 157 d of gestation, *i.e.* in post-term fetuses ([298]Myers, McDonald and Nathanielsz, 1992b). In contrast, these authors found no difference in the mRNA levels of 3βHSD and CYP21A1 between intact and PVN-lesioned fetal sheep ([298]Myers, McDonald and Nathanielsz, 1992b). Similarly, placement of dexamethasone adjacent to the ovine fetal PVN after 126-128 d of gestation prevents the ontogenic up-regulation of adrenal CYP11A1 and CYP17 mRNA expression in late-gestation, leaving 3βHSD and CYP21A1 mRNA levels unaffected ([298]Myers, McDonald and Nathanielsz, 1992b). These authors hypothesised that normal functioning of the fetal PVN is necessary for the increase in mRNA for some of the fetal adrenocortical steroid hydroxylases during this period of gestation. In contrast to these results, Phillips and co-workers demonstrated that adrenal 3βHSD mRNA expression is significantly lower in HPD fetal sheep than in intact fetuses at 140 d of gestation ([342]Phillips, Ross *et al.*, 1996). These authors suggested that there are factors present in the circulation of the sheep fetus after HPD, but not after lesion of the fetal PVN, which exert an inhibitory effect on the expression of this steroidogenic enzyme. The profile of the ACTH-containing peptides in the circulation of the fetal sheep after PVN lesion, however, has not been defined.

The relative concentrations of the HMW ACTH precursors and ACTH(1-39) are maintained in the fetal circulation between 120 d and 136 d of gestation, after HPD at 110 d of gestation ([342]Phillips, Ross *et al.*, 1996). In contrast, after 136 d of gestation, the

circulating concentrations of ACTH(1-39) increase in intact, but not in HPD, fetuses ([342]Phillips, Ross *et al.*, 1996). McMillen and colleagues have shown previously that the basal secretion rate of ACTH(1-39) from perifused slices of anterior pituitaries, collected from fetal sheep with an intact hypothalamo-pituitary axis, also increases between 136 d and 140 d of gestation ([279]McMillen, Merei *et al.*, 1995). The increased pituitary output of ACTH(1-39), but not of the ACTH precursors, in late-gestation may be a consequence of a change in the processing of the ACTH precursor, POMC, in the pars distalis of the fetal sheep in the two weeks preceding delivery. In addition to alterations in the corticotroph secretory profile during late-gestation, HPD fetuses have smaller adrenals and lower circulating levels of cortisol at 140 d of gestation than in age-matched intact fetuses ([327]Ozolins, Young and McMillen, 1992; [342]Phillips, Ross *et al.*, 1996; [372]Ross, Phillips *et al.*, 1997). Indeed, in both hypophysectomised and HPD fetal sheep, disruption of pituitary function is associated with low circulating levels of fetal glucocorticoids during late-gestation ([28]Barnes, Comline and Silver, 1977; [342]Phillips, Ross *et al.*, 1996).

Glucocorticoids stimulate the morphological development of fetal pituitary corticotrophs ([19]Antolovich, Perry *et al.*, 1989), increase the ratio of bioactive : ir-ACTH released from anterior pituitary cells *in vitro* ([62]Brieu and Durand, 1987; [63]Brieu and Durand, 1989) and reduce the proportion of ACTH stored in CRH responsive corticotrophs *in vivo* ([67]Butler, Schwartz and McMillen, 1999). Thus, endogenous glucocorticoids may mediate the changes in fetal corticotroph morphology and function, which ultimately result in the enhanced adrenocortical growth and functional maturation required to stimulate fetal organ maturation and initiate parturition. Although it is clear that ACTH(1-39) and the HMW ACTH precursors are important in modulating fetal adrenocortical steroidogenesis, the role of cortisol in mediating the trophic influence of ACTH on fetal adrenal growth and function remains unclear.

The second main aim of this thesis, therefore, was to investigate the effect of cortisol replacement after fetal HPD on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the late-gestation sheep fetus. In studies described in Chapter 2, fetal hypothalamo-pituitary disconnection was performed at 110 d of gestation (*Study 3*), to determine the effect of subsequent cortisol replacement on adrenal growth and the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1, compared with saline infused HPD fetuses.

1.4.4 Auto / paracrine effect of cortisol on adrenal maturation

1.4.4.1 Adrenal glucocorticoid receptors

In adrenals of weanling and adult rats, the existence of glucocorticoid receptors supports an autocrine / paracrine role for glucocorticoids in this gland ([375]Saez, Morera and Gallet, 1977; [253]Loose, Do *et al.*, 1980). Darbeida and co-workers have demonstrated that glucocorticoids increase the number of ACTH receptors in cultured adrenocortical cells from adult sheep ([116]Darbeida and Durand, 1990). The increase in adrenal ACTH receptor numbers in response to glucocorticoids *in vitro* is associated with an increase in ACTH receptor mRNA abundance within adult ovine adrenocortical cells ([345]Picard-Hagen, Penhoat *et al.*, 1997). These results indicate that glucocorticoids stimulate the transcription of the ACTH receptor gene. It has therefore been suggested that the ontogenic increase in ACTH receptor numbers in the adrenal gland of the ovine fetus, which occurs concomitantly with increasing circulating concentrations of ACTH(1-39) during late-gestation, may also be partly mediated by endogenous glucocorticoids ([374]Saez, Durand and Cathiard, 1984).

In contrast to the *in vitro* findings, infusion of exogenous cortisol (5 $\mu g \cdot min^{-1}$ for 96 h) into ovine fetuses results in a significant elevation in fetal plasma cortisol concentrations, with no effect on adrenal ACTH receptor mRNA expression ([158]Fraser, Jeffray and Challis, 1999). Thus, while cortisol has been shown to stimulate adrenal ACTH receptor expression in cultured adult adrenocortical cells, cortisol does not appear to stimulate fetal adrenocortical responsiveness *in utero* via changes in the transcription rate of the ACTH receptor gene.

1.4.4.2 Effect of cortisol on adrenal growth and maturation

Two studies have suggested a role for cortisol in the morphological development of the fetal adrenal prior to birth ([243]Liggins, 1968; [56]Boshier, Holloway and Liggins, 1981). Liggins and co-workers demonstrated that the adrenal hyperplasia induced by a 10 d infusion of ACTH(1-24) (0.1 $mg \cdot 24 \, h^{-1}$) from 115 d of gestation, is blocked in fetuses co-infused with metyrapone (2-methyl-1,2-bis(3-pyridyl)-1-propanone, a CYP11B1 enzyme inhibitor) ([243]Liggins, 1968). Boshier and co-workers infused cortisol (20 $mg \cdot 24 \, h^{-1}$) into hypophysectomised fetuses from 150-153 d of gestation ([56]Boshier, Holloway and Liggins, 1981). While the inner cortical *zonae* of hypophysectomised fetal adrenals were not significantly thicker following cortisol administration, morphological changes included increased cytoplasm in the cortical parenchymal cells and cytodifferentiation suggestive of a direct effect of cortisol on the inner cortex

([56]Boshier, Holloway and Liggins, 1981). Thus, cortisol may play a role in mediating the trophic effects of ACTH on fetal adrenal growth prior to birth.

The third main aim of this thesis, therefore, was to investigate the impact of intra-fetal cortisol infusion on adrenal growth, prior to the endogenous pre-partum cortisol surge. In studies described in Chapter 3, cortisol was infused (2.0-3.0 mg·24 h⁻¹) from 109-116 d of gestation to determine the effect of a premature elevation of fetal circulating cortisol levels on adrenal growth in fetuses with an intact hypothalamo-pituitary axis.

1.4.4.3 *Effect of cortisol on adrenal function*

In addition to cytological effects on the fetal adrenal cortex, glucocorticoids also enhance pregnenolone production, as demonstrated in isolated ovine adrenocortical cells in culture ([344]Picard-Hagen, Darbeida and Durand, 1995). Mitochondria isolated from dexamethasone-treated ovine adrenocortical cells contain more cholesterol and produce more pregnenolone than mitochondria from control cells, suggesting that at least part of the additional cholesterol present in these mitochondria is available for steroidogenesis ([344]Picard-Hagen, Darbeida and Durand, 1995). Thus, glucocorticoids may increase the pool of intra-mitochondrial cholesterol as well as mitochondrial ability to synthesise pregnenolone *in vitro*. Taken together, these results suggest that glucocorticoids are involved in the maturation of the fetal ovine adrenal gland.

Lye and colleagues have demonstrated a role for glucocorticoids in the functional maturation of fetal adrenocortical cells ([262]Lye and Challis, 1984). The ability of cultured fetal adrenal cells to accumulate cAMP in response to ACTH(1-24) *in vitro* was compared in ovine fetuses at 132 d of gestation, following a 100 h *in vivo* pulsatile infusion of ACTH(1-24) (66.6 ng·min⁻¹ for 15 min·2 h⁻¹) or ACTH(1-24) plus metyrapone (31.3 mg·h⁻¹) *in utero*, prior to the *in vitro* experiment ([262]Lye and Challis, 1984). Infusion of metyrapone plus ACTH(1-24) *in vivo* prevents the cAMP accumulation in cultured adrenal cells, following ACTH stimulation *in vitro* ([262]Lye and Challis, 1984). These authors concluded that cortisol may mediate the increase in adrenal responsiveness to subsequent ACTH stimulation *in vitro* which results following pulsatile ACTH(1-24) infusion *in vivo*.

A further study demonstrated that exposure to glucocorticoids or to inhibitors of steroid synthesis *in vitro* can also effect the cAMP and / or corticosteroid response to ACTH by cultured adrenal cells from 120-138 d ovine fetuses

(^{117}Darbeida, Naaman and Durand, 1987). The cAMP response to ACTH(1-24), by cells cultured for 24 h in the presence of ACTH(1-24) prior to stimulation, was twice that of control cells. In contrast, the response of cells cultured in the presence of ACTH(1-24) plus metyrapone or aminoglutethimide (an inhibitor of CYP11A1 activity) was lower that that of cells cultured in the presence of ACTH(1-24) alone. Conversely, cells cultured for 48 h in the presence of dexamethasone or cortisol released more cAMP than control cells when stimulated by ACTH(1-24). These authors suggested that glucocorticoids are involved in the maturation of the fetal adrenal gland by a direct effect on fetal *fasciculata / reticularis* cells. The increase in cortisol production subsequent to the pre-partum rise in circulating ACTH(1-39) may therefore act in an autocrine and / or paracrine fashion to enhance adrenocortical steroidogenesis.

A further aim of this thesis, therefore, was to investigate the effect of a 7 d intra-fetal infusion of cortisol on the expression of mRNA for the steroidogenic enzymes within the adrenal gland of the ovine fetus at 116 d of gestation. In studies described in Chapter 3, cortisol was infused (2.0-3.0 mg·24 h^{-1}) from 109-116 d of gestation to determine the effect of elevated circulating cortisol concentrations, prior to the endogenous pre-partum cortisol surge, on the adrenal expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 in fetuses with an intact hypothalamo-pituitary axis.

1.4.4.4   *Cortisol and adrenal 11βHSD*

Recent studies have shown that the action of cortisol in fetal tissues may be regulated via an intra-cellular microsomal enzyme, 11β hydroxysteroid dehydrogenase (11βHSD). The two isoforms (11βHSD-1 and -2) inter-convert the bioactive glucocorticoids (cortisol and corticosterone) and their inactive metabolites (cortisone and 11-dehydrocorticosterone) (^{288}Monder and Shackleton, 1984) (*see Figure 1.3, step 9a and 9b on pg. 16*). 11βHSD may therefore regulate the activity of intra-cellular glucocorticoids (^{431}Whorwood, Franklyn *et al.*, 1992). Yang and colleagues have demonstrated that mRNA for 11βHSD-2 is present in multiple fetal tissues during development, most abundantly in fetal kidney and adrenal, suggesting that fetal 11βHSD-2 may play an important role in regulating tissue-specific functions of glucocorticoids during fetal life (^{443}Yang, Smith *et al.*, 1992; ^{68}Campbell, Yu and Yang, 1996). 11βHSD-2 protects renal mineralocorticoid receptors (the type-1 GR) for binding via aldosterone via rapid local conversion of cortisol to its inactive metabolite, cortisone (^{139}Edwards, Benediktsson *et al.*, 1993). While the expression of mRNA for 11βHSD-2 in the ovine fetal kidney increases with advancing

gestational age ([237]Langlois, Matthews *et al.*, 1995), gene expression of this enzyme decreases in the fetal adrenal as gestation progresses ([281]McMillen, Warnes *et al.*, 1999). The factors which regulate the ontogenic expression of 11βHSD-2 mRNA in fetal tissues including the adrenal, however, remain to be determined.

The decrease in expression of mRNA for 11βHSD-2 within the fetal adrenal occurs concomitantly with the pre-partum increase in fetal plasma cortisol concentrations. A previous study has shown that dexamethasone, a potent synthetic glucocorticoid, up-regulates the hepatic expression of 11βHSD-1 mRNA in ovine fetuses during late-gestation ([440]Yang, Berdusco and Challis, 1994). Infusion of cortisol, the endogenous glucocorticoid, from 109-116 d of gestation into ovine fetuses also results in a 4 fold increase in hepatic expression of mRNA for 11βHSD-1, with no effect on the expression of 11βHSD-2 mRNA in the fetal kidney compared to control fetuses ([107]Coulter, Adams *et al.*, 1999). The effect of cortisol on the expression of mRNA for 11βHSD-2 within the ovine fetal adrenal, however, has not been examined. It is possible that fetal glucocorticoids are responsible for the observed ontogenic decrease in fetal adrenal 11βHSD-2 mRNA expression.

A further aim of this thesis, therefore, was to investigate the effect of a 7 d intra-fetal infusion of cortisol on the expression of mRNA for 11βHSD-2 within the adrenal gland of the ovine fetus at 116 d of gestation. In studies described in Chapter 3, cortisol was infused (2.0-3.0 mg·24 h^{-1}) from 109-116 d of gestation to determine the effect of elevated circulating cortisol concentrations, prior to the endogenous pre-partum cortisol surge, on the adrenal expression of mRNA for 11βHSD-2 in fetuses with an intact hypothalamo-pituitary axis.

1.5   N-TERMINAL POMC PEPTIDES AND ADRENAL MATURATION

Although synthetic preparations of ACTH stimulate mitosis in rat adrenals *in vivo* ([148]Estivariz, Iturriza *et al.*, 1982) cell division is only a secondary, delayed response to the hypertrophic effects of ACTH ([112]Dallman, 1985). Indeed, the action of ACTH *in vitro* is anti-mitogenic, inhibiting cell proliferation in bovine, rat, human, and tumoural adrenocortical cells ([148]Estivariz, Iturriza *et al.*, 1982; [195]Hornsby, 1985a). These results contrast with the widely accepted view that ACTH is directly mitogenic *in vivo*. These observations have led to the view that ACTH acts as an 'indirect' mitogen, possibly by increasing adrenal blood flow ([420]Vinson, Pudney and Whitehouse, 1985; [76]Carter, Richardson *et al.*, 1993) to facilitate the access of mitogens and growth stimulators to the adrenal parenchymal cells ([196]Hornsby, 1985b). A further possibility is that peptide

hormones that are co-secreted with ACTH from the pituitary corticotrophs may also mediate adrenocortical growth.

The mouse tumour cell line, AtT20, and rat pituitary cells secrete the 16 kDa *N*-terminal fragment of POMC (pro-γ-MSH), ACTH and β-END related peptides in approximately equimolar amounts ([226]Keutmann, Eipper and Mains, 1979; [266]Mains and Eipper, 1979). The concomitant secretion of the 16 kDa fragment and ACTH have also been reported in the rat and human, and the relative concentration of these peptides in the plasma are correlated ([38]Bertagna, Girard *et al.*, 1980; [193]Hope, Ratter *et al.*, 1981). The major *N*-terminal POMC peptide localised within the human anterior pituitary gland is *N*-POMC(1-76) ([147]Estivariz, Hope *et al.*, 1980). In adult rats and humans, it is interesting that the pars distalis produces mainly larger, mitogenically inactive *N*-POMC peptides that are co-secreted with ACTH ([146]Estivariz, Gillies and Lowry, 1981). There is evidence however, in the neo-natal rat ([382]Sato and Mains, 1988), fetal human ([394]Silman, Chard *et al.*, 1976) and fetal rhesus monkey ([396]Silman, Holland *et al.*, 1978), *i.e.* periods characterised by rapid adrenal growth, that proteolytic processing of POMC is more complete and that corticotrophs secrete proportionately more of the smaller peptides compared to the adult. This may be relevant in relation to the rapid adrenal growth that occurs during late-gestation and early post-natal life.

The 16 kDa *N*-terminal fragment of POMC (pro-γ-MSH) can be enzymatically cleaved to yield several molecular weight forms of the peptides called γ-MSH related peptides, and the *N*-terminal fragment, *N*-POMC(1-49). These peptides have been isolated from the pituitaries of various species including cow, sheep, mouse, rat, pig and human ([405]Tanaka, Nakai *et al.*, 1980; [391]Shibasaki, Masui *et al.*, 1981; [141]Ekman, Hakanson *et al.*, 1982; [39]Bertagna, Seurin *et al.*, 1983). Lowry and Estivariz have demonstrated the mitogenic potential of these smaller peptides in perifused cultures of adult rat adrenocortical cells ([256]Lowry, Estivariz *et al.*, 1985; [145]Estivariz, Carino *et al.*, 1988). In addition, Pedersen and colleagues have demonstrated that γ$_3$-MSH has the capacity to modulate adrenal function in the rat by stimulating pathways different from those responsive to ACTH(1-24) ([332]Pedersen, Brownie and Ling, 1980b). Based on these observations, Silman and colleagues have suggested that *N*-terminal POMC-derived peptides may serve to drive the fetal adrenal gland during the course of pregnancy and may explain the increase in the production of adrenal corticosteroids at the low basal concentration of ACTH observed during the last third of gestation ([394]Silman, Chard *et al.*, 1976) (*Figure 1.5*).

POMC
derived
peptides

| POMC | pro-ACTH | pro-γ MSH | *N*-POMC (1-77) | *N*-POMC (1-49) & (51-77) | ACTH (1-39) | α-MSH | CLIP |

cortisol
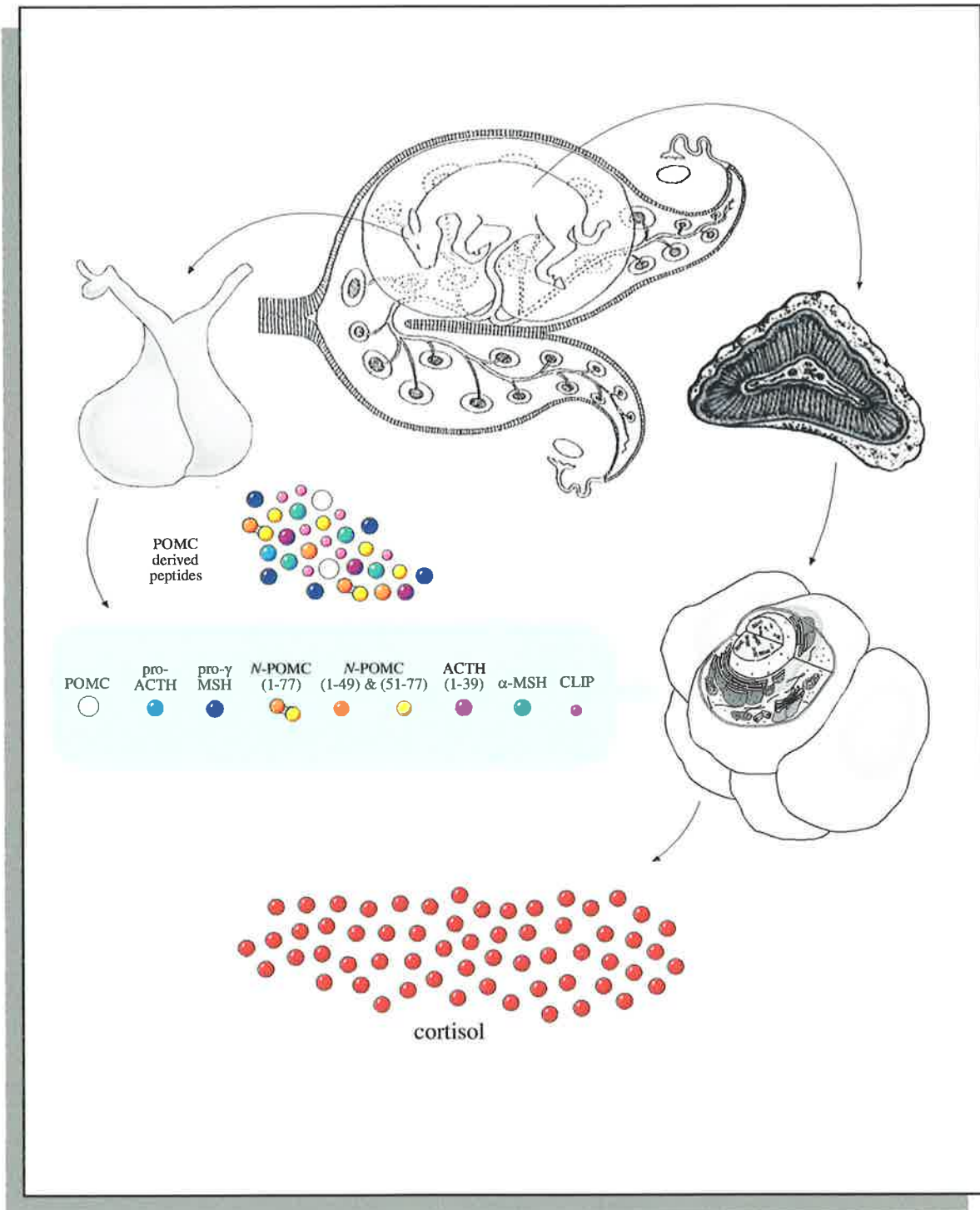
Figure 1.5   **Pituitary control of fetal adrenal steroidogenesis.**

POMC derived peptides secreted from the corticotrophs of the fetal pituitary mediate steroid synthesis within the fetal adrenal cortex.

1.5.1   N-POMC peptides and adrenocortical growth

While previous studies have provided conflicting evidence about the physiological role of ACTH as an adrenal mitogen, two separate reports strongly argue against a mitogenic role for ACTH. Chronic treatment of adult ([354]Rao, Long and Ramachandran, 1978) or young rats ([148]Estivariz, Iturriza *et al.*, 1982) with ACTH antisera reduces plasma corticosteroid levels but does not inhibit adrenal proliferative activity. In contrast, the administration of anti-*N*-POMC antiserum to young and adult rats causes significant inhibition of adrenal growth and mitosis, without altering plasma corticosterone levels ([148]Estivariz, Iturriza *et al.*, 1982). In accordance with the role of *N*-POMC peptides in adrenal growth, infusion of peptides arising from the non-γ_3-MSH sequence of *N*-POMC [*N*-POMC(1-28)] produce a potent mitogenic response *in vivo*, in dexamethasone-treated ([148]Estivariz, Iturriza *et al.*, 1982) and hypophysectomised rats ([256]Lowry, Estivariz *et al.*, 1985; [145]Estivariz, Carino *et al.*, 1988). In addition, while full length *N*-POMC(1-76) has no effect on adrenal growth, a tryptic digest of this peptide results in *N*-POMC fragments which induce proliferative activity in adrenals of adult rats ([148]Estivariz, Iturriza *et al.*, 1982). Finally, enhanced incorporation of tritiated thymidine in rat adrenal cell cultures has been demonstrated using synthetic peptides that include the first 28 amino acids of *N*-POMC ([145]Estivariz, Carino *et al.*, 1988). Thus, the first 49 amino acid residues of the *N*-terminal fragment of POMC appear to encode a potent stimulus to adrenocortical cell division.

1.5.1.1   Adrenal regeneration

Bilateral adrenal enucleation (extrusion of the adrenal parenchyma from both glands), or enucleation of one gland and adrenalectomy of the other, leaving only the connective tissue capsule with a few adherent parenchymal cells, stimulates rapid adrenal regeneration ([190]Holzwarth, Shinsako and Dallman, 1980). In the rat, complete regeneration of the adrenocortical parenchyma and full restoration of normal zonation occurs within 6 weeks. Adrenal regeneration is dependent on the pituitary since hypophysectomy prevents the regenerative process ([145]Estivariz, Carino *et al.*, 1988). Regeneration may be triggered by a diminished corticosteroid feedback at the hypothalamus or pituitary, since administration of corticosterone blocks adrenal regeneration. It is clear that the gland regenerates via a dramatic proliferative activity of the remaining cells following enucleation, under trophic stimulation from the pituitary gland ([145]Estivariz, Carino *et al.*, 1988).

There is clear evidence that *N*-terminal POMC peptides are important mitogens in adrenal regeneration following bilateral or unilateral adrenal enucleation. Estivariz and co-workers have demonstrated that daily injection or continuous infusion of *N*-POMC(1-28) into

adrenal enucleated rats partially restores mitosis when regeneration is arrested by hypophysectomy, whereas ACTH has no effect on adrenal mitotic activity in this model ([145]Estivariz, Carino *et al.*, 1988; [149]Estivariz, Morano *et al.*, 1988). Conversely, ACTH administration to hypophysectomised rats inhibits mitosis within the regenerating adrenal following adrenal enucleation ([145]Estivariz, Carino *et al.*, 1988). In rats 2 and 3 weeks after adrenal enucleation, administration of *N*-POMC antisera decreases cell mitosis in the regenerating cortex, whereas ACTH antiserum, while lowering plasma corticosterone levels, does not affect cell division ([145]Estivariz, Carino *et al.*, 1988). These experiments clearly suggest that the mitogenic drive to the regenerating adrenal gland originates in the pituitary corticotrophic sector, and is mediated by *N*-POMC peptides. In accordance with this hypothesis, Estivariz and co-workers demonstrated that plasma levels of *N*-POMC(1-76) are elevated during adrenal regeneration, concomitant with the rise in circulating ACTH levels ([149]Estivariz, Morano *et al.*, 1988).

1.5.1.2   Compensatory adrenal growth

In the adult rat, compensatory adrenal growth after unilateral adrenalectomy is mediated by afferent and efferent neural pathways ([143]Engeland and Dallman, 1975; [114]Dallman, Engeland and Shinsako, 1976). Afferent nerve connections from the adrenal gland to the hypothalamus are activated upon manipulation or removal of one of the adrenal glands, triggering compensatory growth of the contralateral gland ([114]Dallman, Engeland and Shinsako, 1976). Spinal cord hemi-section in the thoracic region inhibits compensatory adrenal growth when the contralateral adrenal is removed, while pre-treatment of the left adrenal with a local anaesthetic before removal inhibits the subsequent compensatory growth in the right adrenal ([114]Dallman, Engeland and Shinsako, 1976).

The involvement of the pituitary corticotrophs in the neurally mediated response to adrenal compensatory growth has also been demonstrated using antisera to different portions of the *N*-terminal region of POMC ([257]Lowry, Silas *et al.*, 1983). Lowry and co-workers reported that treatment with anti-ACTH antisera in the adult rat after unilateral adrenalectomy does not effect the increase in adrenal weight and DNA content, but decreases adrenal RNA content and steroidogenesis ([257]Lowry, Silas *et al.*, 1983). In contrast, treatment of unilaterally adrenalectomised rats with antisera raised against *N*-POMC(1-77) or *N*-POMC(1-28) does not affect the increase in weight or RNA content of the remaining adrenal, but abolishes the increase in adrenal DNA content. Finally, antisera raised against *N*-POMC(50-74) have a predominantly suppressive effect on adrenal RNA content. Lowry and colleagues suggested, therefore, that in compensatory adrenal growth, adrenal

hyperplasia and hypertrophy are mediated by neural activation of a proteolytic enzyme which cleaves *N*-POMC(1-77) to give rise locally to the mitogenic peptide *N*-POMC(1-49) ([257]Lowry, Silas *et al.*, 1983; [256]Lowry, Estivariz *et al.*, 1985) and to *N*-POMC(50-77) (lys-γ_3-MSH) which stimulates RNA synthesis and cellular hypertrophy ([150]Farese, Ling *et al.*, 1983). Recently, Lowry and colleagues have isolated and cloned a novel 28 kDa protease that is up-regulated in the adrenal gland during compensatory adrenal growth and contains the conserved His / Asp / Ser catalytic triad of the trypsin family ([40]Bicknell, Hutchinson *et al.*, 1998). *In situ* hybridisation was used to demonstrate that the expression of this adrenal protease is confined to the outer rim of the cortex, with a negative centripetal gradient in the outer *zona fasciculata*. Thus, the biological activity of the 16 kDa *N*-POMC fragment and the smaller *N*-POMC(1-77) peptide may be mediated by local proteolytic cleavage at the adrenal, yielding *N*-POMC(1-49) and *N*-POMC(50-77).

1.5.2   *N-POMC peptides and adrenocortical function*

Pedersen and co-workers have reported that ACTH stimulation of rat adrenal steroidogenesis is potentiated by either the products of a tryptic digest of the 16 kDa fragment of murine POMC, or by synthetic bovine γ_3-MSH ([328]Pedersen and Brownie, 1980; [332]Pedersen, Brownie and Ling, 1980b). Al-Dujaili and co-workers observed a similar potentiation of ACTH induced steroidogenesis by human *N*-POMC(1-76) on perifused rat and human adrenocortical cells ([9]Al-Dujaili, Hope *et al.*, 1981).

Although both ACTH and γ_3-MSH stimulate adrenocortical steroidogenic function, their effects can be distinguished *in vitro*. While γ_3-MSH administered alone to adult rat adrenal cells is only weakly steroidogenic, this peptide markedly potentiates the steroidogenic effect of ACTH ([332]Pedersen, Brownie and Ling, 1980b; [150]Farese, Ling *et al.*, 1983). Durand and co-workers demonstrated that it is necessary to culture ovine fetal adrenal cells for at least 4 d in the presence of γ_3-MSH before corticosteroid production is stimulated in the absence of ACTH ([137]Durand, Cathiard *et al.*, 1984b), whereas when co-incubated with ACTH, γ_3-MSH potentiates the steroidogenic response of ovine fetal adrenal cells after only 3 d in culture. The fetal adrenal, therefore, may require elevated ACTH concentrations before γ_3-MSH stimulates an increase in steroidogenesis *in vivo*. γ_3-MSH appears to act via a cAMP-independent mechanism to stimulate the activity of cholesterol ester hydrolase, however, unlike ACTH, γ_3-MSH has no effect on CYP11A1 enzyme activity ([332]Pedersen, Brownie and Ling, 1980b). Pro-γ-MSH also has adrenocorticotrophic actions independent of ACTH, such as increased cholesterol ester hydrolase activity, mitochondrial cholesterol accumulation ([332]Pedersen, Brownie and Ling, 1980b; [9]Al-Dujaili, Hope *et al.*, 1981) and accelerated RNA transcription

(^{10}Al-Dujaili, Williams *et al.*, 1982). This potentiating action of pro-γ-MSH may play an important role in modulating ACTH induced steroidogenesis.

These observations suggest that various forms of γ-MSH are capable of potentiating ACTH-induced steroidogenesis and that the biologically active sequences of these peptides may reside in the sequence of γ₃-MSH. The molecular weight of isolated ir-γ-MSH has been reported to be between 6 kDa and 12 kDa (^{390}Shibasaki, Ling and Guillemin, 1980; ^{405}Tanaka, Nakai *et al.*, 1980; ^{39}Bertagna, Seurin *et al.*, 1983). This would suggest that ir-γ-MSH is either in the form of glycosylated pro-γ-MSH or glycosylated γ₃-MSH. Presumably the circulating form of high molecular weight ir-γ-MSH peptides serve as stable precursor molecules which may be enzymatically cleaved at the target organ to yield the active peptides.

1.5.3   *N-POMC peptides and fetal adrenal maturation*

Previously, our laboratory has identified cells in the anterior and intermediate lobes of the fetal sheep pituitary which contain ACTH(1-39), β-endorphin, *N*-POMC(1-77) and the *N*-POMC(51-77) peptide γ₃-MSH (^{293}Mulvogue, McMillen *et al.*, 1986). Saphier and colleagues recently reported that the molar ratio of *N*-POMC(1-77) : ACTH(1-39) is between twenty and fifty in the fetal sheep during late-gestation, whereas this ratio is two in the adult ewe (^{377}Saphier, Glynn *et al.*, 1993). Moreover, the high circulating concentrations of *N*-POMC(1-77) are present in the fetal sheep circulation during the periods of minimal (110-130 d of gestation) and maximal (135-147 d of gestation) fetal adrenal growth (^{377}Saphier, Glynn *et al.*, 1993). In the sheep adrenal, splanchnic innervation is not complete until 130-135 d of gestation. This raises the possibility that *N*-POMC(1-77) cannot act at the fetal adrenal prior to the development of functional splanchnic innervation because its actions are dependent on a neurally dependent proteolytic cleavage to *N*-POMC(1-49) and *N*-POMC(50-77). Indeed, Saphier and colleagues also found that there is a decrease in the plasma levels of *N*-POMC(1-77) concurrent with an increase in *N*-POMC(50-74), after 138 d of gestation. Thus, the *N*-terminal region of POMC, including *N*-POMC(1-77) and its proteolytic cleavage products, may have a role in the stimulation of fetal adrenal growth and function prior to delivery.

The fourth main aim of this thesis, therefore, was to investigate the effect of intra-fetal infusion of peptides derived from the *N*-terminal region of bovine POMC on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the late-gestation sheep fetus. In studies described in Chapter 4,

N-POMC(1-77) and *N*-POMC(1-49) were administered to fetal sheep between 136-138 d of gestation, *i.e.* after the development of adrenal innervation, to determine the separate effects of these peptides on the growth of the fetal adrenal, and on the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 within the adrenal gland of the ovine fetus.

1.6   *INSULIN-LIKE GROWTH FACTORS AND THE FETAL ADRENAL*

The somatomedins, or insulin-like growth factors (IGFs), form a family of growth promoting peptide hormones with a wide range of actions. They consist of IGF-I and IGF-II, as well as several variant forms (*for review see* [188]Hill and Hogg, 1989). The IGF axis in both man and experimental animals is most extensively characterised in terms of promoting linear bone growth by stimulating cell proliferation and hypertrophy, and inhibiting apoptosis within the growth plate ([203]Isgaard, Nilsson *et al.*, 1986; [184]Hill, 1992; [73]Carrascosa and Audi, 1993), while promoting protein synthesis and inhibiting protein degradation within soft tissues ([205]Jacob, Hu *et al.*, 1996; [23]Bark, McNurlan *et al.*, 1998). *In vitro*, IGFs have been shown to be the universal progression factor in the cell cycle, essential for cells to enter the S phase of DNA synthesis and to mediate differentiation in a range of cell types ([379]Sara and Hall, 1990; [29]Baserga, 1992; [30]Baserga, Porcu *et al.*, 1993). *In vivo*, the IGFs and their specific receptors are present in early development from the oocyte stage onwards in many species ([439]Xia, Tekpetey and Armstrong, 1994; [271]Matsui, Takahashi *et al.*, 1995; [242]Lighten, Hardy *et al.*, 1997; [357]Rieger, Luciano *et al.*, 1998), are produced by, and / or act at, a wide variety of cell types and are essential for growth and maturation as shown by gene deletion and other studies ([340]Philipps, Persson *et al.*, 1988; [120]DeChiara, Efstratiadis *et al.*, 1990; [22]Baker, Liu *et al.*, 1993; [251]Liu, Baker *et al.*, 1993a; [252]Lok, Owens *et al.*, 1996). Fetal adrenal cells synthesise IGF-I and -II ([178]Han, Lu *et al.*, 1992) and express specific IGF receptors ([333]Penhoat, Chatelain *et al.*, 1988), suggesting that these peptides may play an important role in fetal adrenal growth and development. The co-localisation of IGF-II mRNA and peptide with immunoreactivity for 3βHSD within *zona fasciculata* cells of the developing fetal adrenal cortex also implicates IGF-II in the regulation of fetal adrenal steroidogenesis ([178]Han, Lu *et al.*, 1992).

1.6.1   *The insulin-like growth factor axis*

In the fetal sheep, as in other species, the most abundant IGF is IGF-II, which is present throughout fetal life. In contrast, the tissue expression and circulating levels of IGF-I increase in late-gestation and persist post-natally in large mammalian species ([71]Carr, 1994). The biological actions of the IGFs are mediated by specific membrane-bound

receptors. Two IGF receptors, called type 1 and type 2, have been identified in a variety of adult and fetal tissues ([355]Rechler and Nissley, 1985). The type 1 IGF receptor is abundant in developing tissues and binds IGF-I with an order of magnitude greater affinity than it does IGF-II ([167]Gilmour, Prosser *et al.*, 1988). The mitogenic actions of IGF-I and IGF-II appear to be mediated predominantly by the type 1 IGF receptor. The type 2 IGF receptor, by comparison, is identical to the cation independent mannose-6-phosphate (M6P) receptor, which is important in the regulation of lysosomal enzyme trafficking ([232]Koster, Saftig *et al.*, 1993). The type 2 IGF receptor binds IGF-II with greater affinity than IGF-I, and does not bind insulin ([308]Nissley and Rechler, 1984; [307]Nissley, Haskell *et al.*, 1985; [355]Rechler and Nissley, 1985; [356]Rechler and Nissley, 1986; [227]Kiess, Yang *et al.*, 1994). A naturally occurring mutant lacking the type 2 IGF / M6P receptor has been identified in mice lacking the 'Tme' locus ([27]Barlow, Stoger *et al.*, 1991). This gene deletion results in lethality at the embryo stage. Thus, it is unclear whether the type 2 IGF / M6P receptor mediates IGF-II signalling. While the biological action of this receptor is unclear, its role as a clearance receptor for the IGF peptides has been suggested ([227]Kiess, Yang *et al.*, 1994).

In vivo, the IGFs are usually complexed to one of six distinct binding proteins, (IGFBPs-1 to -6). The IGFBPs are found both in serum and extra-cellular fluids, and serve as carrier proteins to extend the biological half-life of the ligands and modulate their biological actions ([100]Clemmons, 1993). While all six IGFBPs have a conserved core structure, differences in their amino- and carboxy-terminal ends, and a range of post-translational modifications (*i.e.* phosphorylation and glycosylation) confer individual specific binding affinities for IGF-I and IGF-II, and an ability to interact with both extra-cellular matrix and the cell surface ([276]McCusker and Clemmons, 1992). It has been suggested that the membrane-bound forms serve to attract the IGFs to their cognate receptor, whereas the circulating IGFBPs inhibit receptor-ligand interaction. Thus, a complex system exists by which the level of physiologically active IGFs at the cell surface can be determined by both soluble and membrane associated IGFBPs, the expression of IGF-I and -II peptide and the number and type of IGF receptors. It is this mechanism that allows these two peptides to have important multi-functional roles in growth, development and metabolic homeostasis.

In post-natal mammalian life, normal growth is controlled by the pituitary polypeptide growth hormone (GH), which acts at the tissue and cellular level by stimulating the production of IGF-I. Insulin, thyroxine (T_4), glucocorticoids and sex steroids, as well as substrate supply also modulate the production and actions of IGF-I

(^{101}Clemmons, Klibanski *et al.*, 1981; ^{79}Cavaliere, Knobel and Medeiros-Neto, 1987). In contrast to the adult, the IGF axis of the fetus appears to be modulated by different mechanisms. Mesiano and colleagues demonstrated that fetal plasma IGF-I, but not IGF-II, levels are reduced following fetal hypophysectomy at 120 d of gestation (^{284}Mesiano, Young *et al.*, 1989). Furthermore, the IGF-I concentrations in Hx fetuses that received T$_4$ replacement were significantly increased compared with those in Hx fetuses that did not receive T$_4$ replacement, while IGF-II concentrations in Hx fetuses were unchanged following T$_4$ administration (^{284}Mesiano, Young *et al.*, 1989). These data show that circulating IGF-I concentrations in the fetal lamb are under some pituitary and thyroid control, whereas IGF-II concentrations are independent of pituitary or thyroid status. Intra-fetal infusion of GH also has no effect on fetal growth or IGF levels (^{170}Gluckman, 1984). In addition, GH deficiency or GH receptor deficiency in humans does not substantially affect size at birth (^{172}Gluckman and Holdaway, 1976). Fetal growth is subject to different influences compared to life after birth, and less is known of the control of fetal growth and maturation during this period.

1.6.2   *The IGF axis and fetal growth*

There is compelling evidence that IGF-I is a major determinant of fetal growth. In recent years it has become clear that the IGFs are synthesised in all fetal tissues, where they act as paracrine hormones on neighbouring cells to stimulate growth (^{124}D'Ercole, Applewhite and Underwood, 1980; ^{125}D'Ercole, Hill *et al.*, 1986). In addition, circulating IGF-I correlates positively with fetal body weight, consistent with a possible role in influencing fetal growth (^{322}Owens, Kind *et al.*, 1994; ^{228}Kind, Owens *et al.*, 1995). Measurements in fetal sheep have demonstrated a gradual increase in circulating IGF-I levels throughout late-gestation, continuing into the post-partum period. In contrast, IGF-II levels are high in fetal life and fall to adult levels in the immediate peri-partum period (^{171}Gluckman and Butler, 1983). Observations *in vivo* have confirmed the presence of IGF receptors and the growth promoting action of IGFs in a variety of fetal tissues (^{355}Rechler and Nissley, 1985; ^{356}Rechler and Nissley, 1986; ^{179}Han, Lund *et al.*, 1988). The anabolic actions of IGFs include a potent stimulation of fetal cell proliferation, protein and glycogen synthesis, and cellular differentiation. These anabolic actions have been demonstrated in a number of cell types from several species, and with the availability of recombinant IGF-I it has been possible to confirm the growth promoting actions of IGF-I *in vivo* in neo-natal rats (^{340}Philipps, Persson *et al.*, 1988) and fetal sheep (^{252}Lok, Owens *et al.*, 1996).

While IGF-II in early-gestation and both IGF-I and -II in late-gestation are widely expressed, the expression of the various IGFBP species is developmentally regulated in a

tissue specific manner ([261]Lund, Moats-Stoats *et al.*, 1986; [179]Han, Lund *et al.*, 1988; [61]Brice, Cheetham *et al.*, 1989; [51]Bondy, Werner *et al.*, 1990). In late first and early second trimester, IGF-II and much lower levels of IGF-I mRNA are widely expressed within the human fetus ([176]Han, D'Ercole and Lund, 1987). IGF peptides are localised to the epithelia of the lung, gut, kidney, liver parenchymal cells and adrenal cortex ([177]Han, Hill *et al.*, 1987). The sites of IGF peptide immunoreactivity coincide with the localisation of IGFBP mRNA and peptides ([187]Hill and Clemmons, 1989; [186]Hill and Clemmons, 1992; [122]Delhanty, Hill *et al.*, 1993), and suggest that these growth factors are present *in vivo*, complexed with their specific binding proteins.

Gene expression for a range of IGFBPs is most prevalent in regions of active cell replication and differentiation, such as the epidermis of the skin, the crypt epithelia of the developing gut and the uteric bud of the kidney ([122]Delhanty, Hill *et al.*, 1993; [175]Han, Asano *et al.*, 1994). In the fetal rat lung, IGF-I protein is found associated with alveolar cells, whereas the mRNA coding for IGF-I is detected in fibroblasts, suggesting that alveolar IGFBPs have determined its location ([230]Klempt, Hutchins *et al.*, 1992). Han and colleagues have investigated the ontogeny of IGFBP expression in the sheep fetus, demonstrating localisation of IGF-II and IGFBP-5 mRNAs predominantly in mesenchymal cells, while IGFBP-2, -3, -4 and -6 are found both in mesenchymal and epithelial cells ([175]Han, Asano *et al.*, 1994). These data strongly suggest that a major level of control of the IGF axis in development is the regulation, localisation and availability of IGFs by IGFBPs ([185]Hill, 1995).

1.6.2.1 Gene knockout studies

The role of IGF-I and IGF-II in fetal growth has also been studied by Efstratiadis and colleagues using gene 'knockout' studies in mice ([120]DeChiara, Efstratiadis *et al.*, 1990; [22]Baker, Liu *et al.*, 1993; [251]Liu, Baker *et al.*, 1993a). Homologous recombination has been used to disrupt the IGF-I, IGF-II, or type 1 IGF receptor gene loci in mice. Inter-breeding of the mutant strains has also led to the creation of combination gene 'knockouts'. Deletion of the IGF-I gene yields null mutants with reduced birth weights (approximately 60 % of the wild type strain), of which some die within 6 h after birth ([251]Liu, Baker *et al.*, 1993a). Interestingly, growth restriction does not commence until embryonic day (E) 13.5 suggesting that IGF-I becomes important for fetal growth only after that age. Using a similar strategy to delete IGF-II, it was found that IGF-II 'knockouts' have similar growth deficiency at birth to animals lacking IGF-I. The growth profile of these mice, however, shows growth restriction from approximately E11, but after

E18 the growth rate parallels that of control fetuses ([120]DeChiara, Efstratiadis *et al.*, 1990). These data are consistent with the suggestion of a dominant role for IGF-II earlier in gestation, while IGF-I is critical during late-gestation and post-natally in small rodents.

Deletion of the type 1 IGF receptor in the mouse yields homozygous animals which are only 45 % of normal weight at delivery, and die within minutes of birth, due to respiratory muscle hypoplasia and failure to breathe ([22]Baker, Liu *et al.*, 1993), similar to mutants lacking the IGF-I gene. Double gene 'knockout' involving both IGF-I and the type 1 IGF receptor produces a similar phenotype to that found after deletion of the receptor or ligand genes alone. In mice with co-deletion of IGF-II and the type 1 IGF receptor, however, animals are only 30 % of normal birth weight at term and show marked skeletal immaturity. These results suggest that an additional receptor to the type 1 IGF receptor may also contribute to IGF-II signalling.

1.6.3 The IGF axis and the adrenal gland

IGF-I has been demonstrated to modulate steroid biosynthesis and cell proliferation in ovine fetal adrenal cells *in vitro* ([299]Naaman, Chatelain *et al.*, 1989), as occurs in a range of steroidogenic tissue, *e.g.* in cultured ovarian granulosa ([4]Adashi, Resnick *et al.*, 1984; [118]Davorean, Hsueh and Li, 1985; [418]Veldhuis, Rodgers *et al.*, 1986), bovine adrenal *zona fasciculata* ([333]Penhoat, Chatelain *et al.*, 1988), and rat Leydig cells ([250]Lin, Haskell *et al.*, 1986). Specific receptors for IGF-I have been reported in most of these steroid producing cells ([250]Lin, Haskell *et al.*, 1986; [2]Adashi, Resnick *et al.*, 1988; [333]Penhoat, Chatelain *et al.*, 1988; [347]Pillion, Yang and Grizzle, 1988; [346]Pillion, Arnold *et al.*, 1989; [392]Shigematsu, Niwa *et al.*, 1989). Reports have also demonstrated receptors for IGF-I in adult human ([346]Pillion, Arnold *et al.*, 1989; [392]Shigematsu, Niwa *et al.*, 1989), bovine ([333]Penhoat, Chatelain *et al.*, 1988) and rat adrenal glands ([347]Pillion, Yang and Grizzle, 1988).

1.6.3.1 Distribution of IGF receptors in the adrenal

The distribution of IGF-I receptors has been studied in adult human adrenals ([346]Pillion, Arnold *et al.*, 1989; [392]Shigematsu, Niwa *et al.*, 1989) using autoradiography of thin human adrenal slices. Specific binding of IGF-I was detected in one study throughout the entire cortex and medulla ([392]Shigematsu, Niwa *et al.*, 1989). In contrast, a different study reported prominent binding in the *zona reticularis* ([346]Pillion, Arnold *et al.*, 1989). The reasons for the discrepancy between the findings of these two studies are not clear. Arafah and colleagues have also investigated the zonal distribution of receptors for IGF-I within adult rat *zonae glomerulosa*, *fasciculata*, *reticularis* and the medulla, demonstrating that

receptors for IGF-I are uniformly distributed throughout the adrenal ([20]Arafah, 1991). Weber and colleagues have also confirmed the presence of specific IGF-I and IGF-II receptors in adult bovine adrenal cells ([426]Weber, Kiess *et al.*, 1994). These authors demonstrated that [^{125}I]-labelled IGF-I binding to dispersed adrenocortical cells is characteristic of the type 1 IGF receptor, and IGF-I binding kinetics are similar in cortical and medullary membranes, confirming the results of Arafah and co-workers ([20]Arafah, 1991). While there are no differences in the binding of [^{125}I]-labelled IGF-II to cortical and medullary membrane fractions, binding activity for the type 2 IGF receptor is much more abundant in both adrenal cortex and medulla than for the type 1 IGF receptor ([426]Weber, Kiess *et al.*, 1994).

Coulter and colleagues have investigated the expression of mRNA for the type 1 IGF receptor within the primate fetal adrenal cortex from mid- to late-gestation ([108]Coulter, Goldsmith *et al.*, 1996). While mRNA for the type 1 IGF receptor is expressed abundantly throughout the definitive, transitional and fetal *zonae* of the adrenal cortex, this transcript is not detectable within the adrenal medulla ([108]Coulter, Goldsmith *et al.*, 1996). Han and colleagues, using *in situ* hybridisation with immunohistochemistry, demonstrated that IGF-II mRNA is present in both adrenocortical as well as adrenomedullary cells ([178]Han, Lu *et al.*, 1992), indicating that IGF-II may exert autocrine and / or paracrine actions on both types of cell. Moreover, since IGF-II is a mitogen as well as a differentiation promoting factor ([355]Rechler and Nissley, 1985), it is possible that IGF-II may be responsible for both hyperplasia as well as hypertrophy of adrenal cells. While previous studies have demonstrated that IGFs stimulate adrenal medullary cell growth *in vitro* ([174]Hall and Ekanayake, 1991; [160]Frodin and Gammeltoft, 1994), the lack of expression of the type 1 IGF receptor within the fetal adrenal medulla *in vivo* ([108]Coulter, Goldsmith *et al.*, 1996) suggests that the IGFs do not play a role in adrenal medullary development during gestation.

1.6.3.2   *Effects of ACTH and AII on the adrenal IGF axis*

In cultured adult bovine adrenocortical cells, treatment with AII or ACTH for 48 h increases the abundance of receptors for IGF-I, which can be partially reproduced by the activation of either the cAMP or the phosphatidyl inositide pathways ([255]Louveau, Penhoat and Saez, 1989). This up-regulation of receptors for IGF-I by steroidogenic peptide hormones has also been demonstrated in two other steroid-synthesising cell types, *e.g.* human chorionic gonadotrophin (*h*CG) up-regulates receptors for IGF-I in both adult pig ([337]Perrard-Sapori, Chatelain *et al.*, 1987) and rat Leydig cells

([249]Lin, Blaisdell and Haskell, 1988), and follicle stimulating hormone (FSH) enhances IGF-I receptor expression in adult rat granulosa cells both *in vitro* ([3]Adashi, Resnick and Svoboda, 1986) and *in vivo* ([2]Adashi, Resnick *et al.*, 1988). Thus, it appears that receptors for IGF-I are positively regulated by peptide hormones specific for steroidogenic cells.

The physiological significance of ACTH and AII stimulated IGF receptor expression, and the potential autocrine / paracrine role of IGFs in regulating adrenocortical cell function is supported by the observation that cultured adult bovine adrenocortical cells secrete IGF-I and IGFBPs, and these secretions are stimulated by both ACTH and AII ([336]Penhoat, Naville *et al.*, 1989; [335]Penhoat, Leduque *et al.*, 1991). In addition, the amount of IGF-I accumulation in the cell medium, following ACTH or AII stimulation, is comparable to the range in which exogenous IGF-I exerts a maximal effect on the steroidogenic responses to ACTH and AII ([336]Penhoat, Naville *et al.*, 1989; [335]Penhoat, Leduque *et al.*, 1991). Penhoat and colleagues have demonstrated that the main effect of both ACTH and AII on IGF-I secretion by cultured adult bovine adrenocortical cells is due to an increase in the number of cells engaged in IGF-I biosynthesis, rather than an increase in IGF-I secretion ([335]Penhoat, Leduque *et al.*, 1991). In addition, the lack of secretory granules and diffuse immunostaining of the cytoplasm suggest that the release of synthesised IGF-I is constitutive rather than regulated ([335]Penhoat, Leduque *et al.*, 1991). In support of this hypothesis is the fact that the cellular content of IGF-I in both control and hormone stimulated cells is very low compared with the IGF-I secreted into the cell medium ([335]Penhoat, Leduque *et al.*, 1991).

1.6.3.3 Effects of IGFs on adrenal responsiveness to ACTH and AII

Long-term treatment of cultured adult bovine adrenocortical cells with IGF-I at nanomolar concentrations, or insulin at micromolar concentrations, enhances cAMP accumulation and steroidogenesis in response to both ACTH and AII by 4-5 fold ([333]Penhoat, Chatelain *et al.*, 1988). The enhanced steroidogenic responsiveness of cultured adult bovine adrenocortical cells following incubation with IGF-I can be explained in part by an up-regulation of receptors for ACTH and AII ([333]Penhoat, Chatelain *et al.*, 1988; [334]Penhoat, Jaillard and Saez, 1989). Indeed, a 72 h treatment with IGF-I increases the number of AII and ACTH receptors in a dose dependent manner without changing ligand binding affinity. Similarly, treatment of adult porcine Leydig cells with IGF-I enhances the number of *h*CG receptors ([37]Bernier, Chatelain *et al.*, 1986). IGF-I also enhances cAMP accumulation and steroidogenesis in response to incubation with ACTH in ovine fetal adrenal cells *in vitro* ([299]Naaman, Chatelain *et al.*, 1989).

The effect of IGF-I on receptors for steroidogenic peptides in cultured adult bovine adrenocortical cells is associated with a 2-4 fold increase in the stimulatory subunits of G proteins ([32]Begeot, Langlois and Saez, 1989; [236]Langlois, Hinsch *et al.*, 1990). The increase in AII and ACTH receptor numbers and the coupling proteins should increase the response of the specific intra-cellular effectors of each hormone. Indeed, pre-treatment of adult bovine adrenocortical cells for 72 h with IGF-I *in vitro* enhances both basal and AII induced inositol phosphate accumulation ([236]Langlois, Hinsch *et al.*, 1990) as well as ACTH- or cholera toxin-induced cAMP production ([333]Penhoat, Chatelain *et al.*, 1988; [32]Begeot, Langlois and Saez, 1989; [299]Naaman, Chatelain *et al.*, 1989). Thus, IGFs stimulate the responsiveness of steroid-synthesising tissue to trophic stimulation by steroidogenic peptide hormones. Taken together, these results and those described previously suggest a reciprocal positive trophic effect between steroidogenic peptide hormones and IGF-I on the regulation of their specific membrane receptors in steroid-producing cells.

1.6.3.4 *Effects of IGF-I on adrenal steroidogenic enzymes*

The enhanced steroidogenic responsiveness of IGF-I-treated adrenal cells is due not only to the effects of this growth factor on membrane-bound receptors and their coupling proteins, but also to its effects on the steroidogenic pathway downstream of cAMP formation, since IGF-I increases the output of corticosteroids from cultured fetal ovine adrenal cells stimulated by 8bromo-cAMP ([299]Naaman, Chatelain *et al.*, 1989). This effect could be due either to an increased formation of pregnenolone from endogenous substrate or to an increased conversion of pregnenolone to glucocorticoids, or to both.

While IGF-I induces CYP11A1 and adrenodoxin enzyme activities in porcine ovarian granulosa cells *in vitro* ([418]Veldhuis, Rodgers *et al.*, 1986), the action of IGF-I in cultured adult bovine adrenocortical cells ([333]Penhoat, Chatelain *et al.*, 1988) and fetal ovine adrenal cells ([299]Naaman, Chatelain *et al.*, 1989) *in vitro* is not mediated through changes in the activity of these enzymes, since the conversion of 22R-hydroxy cholesterol to pregnenolone is not modified by prior treatment with IGF-I. Since the amount of pregnenolone formed from endogenous cholesterol, after ACTH stimulation *in vitro* is enhanced in IGF-I pre-treated adult bovine adrenocortical cells compared with untreated cells ([333]Penhoat, Chatelain *et al.*, 1988), it is likely that IGF-I increases the amount of endogenous cholesterol available for steroidogenesis. Chatelain and colleagues have demonstrated, however, that IGF-I enhances the conversion of cholesterol to glucocorticoids in cultured adult bovine adrenocortical cells by increasing the enzyme

activities of 3βHSD, CYP21A1 and CYP11B1, but not CYP17 ([86]Chatelain, Penhoat *et al.*, 1988; [333]Penhoat, Chatelain *et al.*, 1988). More recently, it has been demonstrated that IGF-I is required by these cells for the induction, by cAMP, of CYP21A1 and CYP11B1 but not CYP17 ([302]Naseeruddin and Hornsby, 1990; [84]Chang, Naseeruddin and Hornsby, 1991; [87]Cheng and Hornsby, 1992).

The effects of ACTH and IGF-I on the steroidogenic responsiveness of both adult bovine and fetal ovine adrenal cells in culture are synergistic ([299]Naaman, Chatelain *et al.*, 1989; [334]Penhoat, Jaillard and Saez, 1989). This is due not only to their effects on ACTH receptors ([334]Penhoat, Jaillard and Saez, 1989) but also to their complementary actions on the expression of genes encoding the steroidogenic enzymes. A synergistic effect between IGF-I and steroidogenic peptide hormones has also been observed in adult rat granulosa cells, where IGF-I potentiates the effect of FSH on luteinising hormone (LH) receptors ([5]Adashi, Resnick *et al.*, 1985) and steroidogenic responsiveness to LH ([417]Veldhuis and Rodgers, 1987). With respect to mitosis, IGF-I is active as a mitogen in adult bovine ([194]Horiba, Nomura *et al.*, 1987; [333]Penhoat, Chatelain *et al.*, 1988) and fetal ovine ([299]Naaman, Chatelain *et al.*, 1989) adrenocortical cells, but has no effect on the proliferation of fetal rat adrenocortical cells ([416]van Dijk, Tansswell and Challis, 1988). Taken together, these factors strongly support a role for IGF-I as an endocrine and / or paracrine factor in the development of fetal adrenal glands.

1.6.3.5   Paracrine role of IGF-II in adrenocortical development

IGF-II stimulates fetal adrenal cell mitosis *in vitro* ([416]van Dijk, Tansswell and Challis, 1988). In addition, tissue levels of IGF-I and IGF-II peptide are increased in adult rat adrenal glands undergoing the rapid growth that occurs during either adrenal regeneration following adrenal enucleation, or compensatory adrenal growth following unilateral adrenalectomy ([412]Townsend, Dallman and Miller, 1990). However, since no increase in circulating IGF levels was detected in these models of adrenal growth, the local rise in IGF peptides likely reflects an increase in the local production of these growth factors; and may also represent a response to elevated circulating *N*-POMC peptides, which have been implicated in the adrenal mitogenic response in these perturbations ([257]Lowry, Silas *et al.*, 1983; [145]Estivariz, Carino *et al.*, 1988; [149]Estivariz, Morano *et al.*, 1988). While Townsend and co-workers measured no change in adrenal IGF-I and IGF-II mRNA levels during compensatory adrenal growth ([412]Townsend, Dallman and Miller, 1990), the increase in IGF-I and IGF-II peptide concentrations ([204]Jackson, Hodgkinson *et al.*, 1991) could occur at the translational or post-translational level. Finally, IGF-II mRNA increases in

association with increases in mRNA for steroidogenic enzymes such as CYP11A1 and CYP17 in response to ACTH stimulation in cultured human fetal adrenal cells ([421]Voutilainen and Miller, 1987). IGF-II may, therefore, play a role in adrenal cell proliferation and functional maturation *in utero*.

The increase in fetal plasma cortisol levels, which precedes parturition in the sheep, occurs coincident with a decline in serum levels and hepatic expression of IGF-II. Lu and colleagues have demonstrated that infusion of either ACTH(1-24) or cortisol into fetal sheep from 120-125 d of gestation also decreases the level of adrenal IGF-II mRNA ([258]Lu, Han *et al.*, 1994). Cultured fetal human adrenal cells primarily secrete the adrenal androgen dehydroepiandrosterone (DHEA) upon ACTH stimulation ([270]Mason, Hemsell and Korte, 1983). In contrast, the fetal ovine adrenal cortex secretes cortisol, which inhibits adrenal IGF-II mRNA expression ([258]Lu, Han *et al.*, 1994). Thus, while ACTH directly stimulates the intra-adrenal production of IGF-II peptide via an increase in IGF-II mRNA expression, the cortisol produced concomitantly may limit adrenal IGF-II mRNA expression, serving as a local regulatory mechanism for adrenal IGF-II mRNA expression. It is possible that the pre-partum cortisol surge in the fetal sheep, associated with rapid adrenal growth and steroidogenic development, may initiate a peri-natal decline in the hepatic expression of IGF-II at the same time that ACTH stimulates the synthesis of IGF-II within the adrenal. In this regard, Fowden and colleagues have demonstrated that infusion of cortisol into the ovine fetus results in a decrease in the mRNA expression of IGF-II in the fetal liver ([241]Li, Saunders *et al.*, 1993). In addition, cortisol may modulate the adrenal expression of the IGFBPs, modulating the availability of the IGF peptides to the adrenocortical cells. *In vitro*, ACTH clearly causes cellular hypertrophy and hyperplasia of adrenocortical cells; however, *in vivo* it does not appear to be a direct mitogen ([353]Ramachandran and Suyama, 1975). A possible explanation of this paradox is that ACTH may stimulate the local production of growth factors such as IGF-II that then induce adrenocortical cell division by a paracrine effect ([422]Voutilainen and Miller, 1988).

The abundance of IGF-II mRNA in the adrenal is higher than that of IGF-I, and immunoreactive IGF-II is localised predominantly to the *zona fasciculata* of the adrenal cortex ([178]Han, Lu *et al.*, 1992). It has, therefore, been proposed that locally synthesised IGF-II secreted in response to ACTH may stimulate the growth and differentiation of the adrenocortical cells in late-gestation ([422]Voutilainen and Miller, 1988). Coulter and colleagues have shown that removal of glucocorticoid negative feedback at the pituitary, by infusion of metyrapone into fetal rhesus monkeys, causes an increase in adrenal growth and a parallel increase in the expression of mRNA for IGF-II and the type 1 IGF receptor

in fetal adrenocortical cells ([108]Coulter, Goldsmith *et al.*, 1996). It is not known, however, whether the increased endogenous ACTH secretion, which occurs during the 2 weeks before delivery, stimulates adrenal IGF-II mRNA levels in this way in the late-gestation sheep fetus.

The fifth main aim of this thesis, therefore, was to determine the role of the IGF axis in adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the ovine fetus. In studies described in Chapter 5, *rh*IGF-I was infused into fetal sheep between 120-130 d of gestation to determine the separate effects of this peptide growth factor on the growth of the adrenal, and on the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 within the fetal sheep adrenal.

A further aim of this thesis was to investigate the late-gestation ontogeny of mRNA expression for IGF-II and the major IGF-II binding protein, IGFBP-2, within the fetal adrenal during the last 15 d of gestation (Chapter 2, *Study 2*). Since I hypothesised that HPD alters the bioactivity of circulating ACTH in the fetal sheep, I also investigated the effect of fetal HPD at around 110 d of gestation, in the presence and absence of subsequent cortisol replacement, on the expression of mRNA for IGF-II within the adrenal gland of the ovine fetus in late-gestation (Chapter 2, *Study 3*). In addition, I investigated the effect of intra-fetal cortisol infusion into fetuses with an intact HP axis, prior to the endogenous pre-partum cortisol surge, on the expression of mRNA for IGFBP-2 within the adrenal gland of the ovine fetus (Chapter 3).

1.7 FETAL GROWTH RESTRICTION AND ADRENAL DEVELOPMENT

Exposure to cortisol at specific times in fetal and neo-natal life can irreversibly trigger the programmed development of a range of fetal tissues including the brain, lung, liver and gut. Thus, it is critical that during fetal life, adrenal activity is tightly regulated to limit exposure of the developing tissues to cortisol. It has previously been shown that when energy supply to the fetus is chronically restricted in late-gestation, there is activation of the pituitary-adrenal axis of the fetus and an increase in the synthesis and secretion of cortisol ([343]Phillips, Simonetta *et al.*, 1996). It has also been shown that cortisol inhibits IGF-II gene expression in fetal tissues and inhibits fetal growth ([258]Lu, Han *et al.*, 1994), thereby matching the energy demand of the growing fetus to the available level of energy supply. The mechanisms by which fetal adrenal steroid synthesis is modulated in response to changes in fetal energy supply, however, are poorly understood.

1.7.1 Human intra-uterine growth restriction

Normal growth can be described as "the expression of the genetic potential to grow, which is neither abnormally constrained nor promoted by internal or external factors" ([360]Robinson, 1989). Variations from the norm, in particular reduced growth *in utero*, are associated with increased risk of peri-natal mortality and morbidity and poor subsequent physical and neurological development ([14]Allen, 1984; [50]Bonds, Gabbe *et al.*, 1984; [361]Robinson, Falconer and Owens, 1985). The term 'restricted fetal growth' is used increasingly in preference to 'intra-uterine growth retardation', since the latter has unfortunate connotations for consumers of health services.

The major substrates for fetal growth and development are O_2, glucose, lactate and amino acids in all mammalian species studied so far ([153]Fowden, 1994). Fetal growth and development are largely determined by the interaction between genome and the availability of these substrates, which are essential for growth. The placenta as interface between fetus and mother determines substrate availability to the fetus, due to (*1*) its substrate transfer functions, (*2*) its high metabolic rate, which makes it a competitor with the fetus for the very substrates it supplies and (*3*) its modification of those nutrients, prior to exchange with the fetus ([182]Hay and Wilkening, 1994). In addition, the placenta secretes a variety of hormones and factors into the fetal and maternal circulations to coordinate and influence their metabolic and physiological adaptations during pregnancy. Since maternal placental blood flow is the only source of O_2 and nutrients for the fetus, reductions in placental blood flow can be detrimental to the fetus.

There is a progressive increase in placental blood flow during pregnancy. If this increase is attenuated, intra-uterine growth restriction (IUGR) may result. Nylund and co-workers demonstrated, in pregnant women, that a 23 % reduction in birth weight was associated with a 56 % reduction in utero-placental blood flow index, while placental weight, and the volume and surface area for exchange are also major correlates of fetal size in the second half of gestation ([314]Nylund, Lunell *et al.*, 1983). In one study of a large cohort of human infants, fetal growth restriction was associated with disproportionate growth, increased length and head circumference for weight, and increased variability in body proportions as indicated by anthropometric measurements ([233]Kramer, Olivier *et al.*, 1990). This disproportionate, or asymmetric, growth was evident throughout development, at early gestational ages, term and post-term, and increased with the severity of fetal growth restriction ([233]Kramer, Olivier *et al.*, 1990).

In asymmetric human fetal growth-restriction brain growth is largely spared, while liver and lymphoid tissues are disproportionately reduced in size ([64]Brooke, Wood and Butters, 1984). Human infants with this pattern of asymmetrical fetal growth restriction have a higher incidence of peri-natal morbidity and mortality, including asphyxia during labour and acidosis, hypoglycaemia and hypothermia after birth ([151]Fay and Ellewood, 1993). In addition, small size and asymmetrical growth at birth are associated with increased risk of death from cardiovascular disease and of non-insulin dependent diabetes, hypertension and hyperlipidaemia in later life ([24]Barker, 1994).

The metabolic disturbances in small human fetuses are accompanied by a variety of endocrine changes; generally a reduction in the abundance of anabolic factors and an increase in inhibitory or catabolic factors. Fetal hypoinsulinaemia also occurs to a greater extent than expected from the degree of hypoglycaemia present in IUGR fetuses. Reduced circulating concentrations of IGF-I ([238]Lassarre, Hardouin *et al.*, 1991) and increased concentrations of IGFBP-1, a binding protein which inhibits the stimulatory actions of IGF-I on glucose utilisation ([424]Wang, Lim *et al.*, 1991), also occur in the small fetus. In addition, in small hypoxaemic fetuses, the circulating concentration of cortisol is increased, as is the concentration of noradrenaline.

In summary, growth restriction in the human fetus is characterised by asymmetric growth. In this condition, body weight is affected more than length, and the brain and heart are developed at the expense of the abdominal organs. Restriction in the supply of O_2 and nutrients leads to alterations in endocrine signals and metabolism, and a re-programming of tissue development such that fetal growth is matched with the available level of energy supply ([222]Jones, Lafeber *et al.*, 1987). The cause-effect relationship between impaired placental growth, surface area for exchange and perfusion, and the molecular mechanisms involved in the development of IUGR, however, remains unclear.

1.7.2 *Experimental intra-uterine growth restriction*

The experimental approaches used to restrict fetal growth in animals reflect the different questions being asked. Some interventions were devised to mimic known or suspected causes of naturally occurring fetal growth restriction, and to determine the extent to which such factors are causative by imposing them for varying periods at different stages of development. Some perturbations can be imposed chronically, and are used to examine the consequences for the individual in the medium to long-term. Others are designed to delineate the specific physiological, cellular and molecular mechanisms by which perturbations restrict fetal growth and alter development of key fetal organs and tissues.

The consequences of experimental restriction of fetal growth and their relevance to the human condition have been reviewed previously and referenced in detail ([324]Owens, Owens and Robinson, 1989; [317]Owens, 1991; [365]Robinson, Owens and Owens, 1994).

Several different approaches have been used to produce animal models of placental insufficiency with resultant asymmetric IUGR. The primary perturbations employed include maternal food or O_2 restriction ([283]Mellor, 1983; [85]Charlton and Johengen, 1987); or a reduction in uterine blood flow either by vascular ligation ([235]Lafeber, Rolph and Jones, 1984; [208]Jansson, Thordstein and Kjellmer, 1986; [419]Vileisis and D'Ercole, 1986; [74]Carter and Detmer, 1990), placental destruction by repetitive embolisation ([110]Creasy, Barrett *et al.*, 1972; [98]Clapp, Szeto *et al.*, 1980; [97]Clapp, McLaughlin *et al.*, 1984; [85]Charlton and Johengen, 1987), or limitation of placental growth through surgical carunclectomy ([11]Alexander, 1964; [180]Harding, Jones and Robinson, 1985; [361]Robinson, Falconer and Owens, 1985; [318]Owens, Falconer and Robinson, 1986; [320]Owens, Falconer and Robinson, 1987b).

1.7.2.1   Maternal hypoglycaemia and hypoxaemia

Either chronic maternal hypoglycaemia or hypoxaemia alone can reduce fetal growth or birth weight in most species examined ([321]Owens, Falconer and Robinson, 1989). In sheep, moderate maternal undernutrition for most of gestation (maternal arterial glucose, ~70 % of control values), which reduces the availability of glucose to the fetus, reduces fetal growth by approximately 30 % ([283]Mellor, 1983), while a comparable decrease in maternal oxygenation (chronic maternal hypobaric hypoxaemia: maternal p_aO_2, 64 % of control values) reduces fetal body weight in late-gestation by only 21 % ([206]Jacobs, Robinson *et al.*, 1988). In other species, such as the rat, maternal undernutrition and hypoxaemia also reduce fetal growth and often placental growth ([410]Tapanainen, Bang *et al.*, 1994). When undernutrition or hypobaric hypoxaemia are imposed only in the last third of gestation in the sheep, fetal growth is restricted to almost the same degree as when limitations are imposed for most of pregnancy ([283]Mellor, 1983; [206]Jacobs, Robinson *et al.*, 1988). In contrast, placental weight is affected less by perturbations in late-gestation. Thus, the fetus and placenta appear most susceptible to reduced substrate availability during periods of their most rapid growth in terms of mass: the first half of gestation for the placenta and late-gestation for the fetus.

1.7.2.2   Reduced uterine or umbilical blood flow

Ligation of the uterine artery in rats and guinea pigs results in reduced uterine blood flow (RUBF) and placental transfer of O_2, glucose and amino acids, fetal hypoxaemia,

hypoglycaemia and fetal growth restriction ([223]Jones and Parer, 1983; [235]Lafeber, Rolph and Jones, 1984; [221]Jones, Harding *et al.*, 1988; [207]Jansson and Persson, 1990). Mechanical occlusion of a maternal iliac artery in the pregnant sheep in late-gestation ([99]Clark, Durnwald and Austin, 1982) or umbilical artery ligation of the sheep fetus during the last half of gestation ([142]Emmanoulides, Townsend and Bauer, 1968) also produces fetal hypoxaemia and substantially reduces fetal growth.

Reducing uterine blood flow in the sheep in late-gestation, by repetitive embolisation of the utero-placental circulation with microspheres, increases the ratio of fetal weight to placental weight ($F_w : P_w$) by impairing placental growth ([110]Creasy, Barrett *et al.*, 1972; [85]Charlton and Johengen, 1987), and in one study reduced fetal body weight ([164]Gagnon, Challis *et al.*, 1994). The placenta appears to be more susceptible to weight reduction by reduced blood flow than to a decrease in availability of substrates from the mother, particularly in late-gestation, and the consequences for the fetus at that stage are accordingly greater. Fetal hypoxaemia and marginal hypoglycaemia develop, but the magnitude of RUBF and the length of treatment may be insufficient to reduce growth consistently or substantially and, in late-gestation, can result in fetal death ([142]Emmanoulides, Townsend and Bauer, 1968).

The initial mechanism invoked by vascular ligation and embolisation appears similar: reduced placental perfusion. The difference in outcome for fetal growth and viability between the various models of RUBF induced via vascular ligation and utero-placental embolisation, however, may reflect differences in the magnitude of the increase in placental vascular resistance and reduction in umbilical blood flow, and the gestational age at the time of onset, as well as the much more rapid onset of such changes in the embolised placenta.

1.7.2.3   *Surgical restriction of placental growth*

Placental growth can be restricted from early in pregnancy in sheep by reducing the number of sites available for implantation prior to pregnancy ([364]Robinson, Kingston *et al.*, 1979). Removal of the majority of endometrial caruncles (carunclectomy) is performed before mating and involves the excision of the majority of endometrial caruncles lining the non-gravid uterus (*Figure 1.6*). When placental growth (as indicated by placental weight in late-gestation) is restricted to approximately 50 % of control, fetal growth is reduced in late-gestation to a greater extent than is achieved by either chronic maternal nutrient or O_2 deprivation alone ([318]Owens, Falconer and Robinson, 1986; [319]Owens, Falconer and Robinson, 1987a; [320]Owens, Falconer and Robinson, 1987b). Carunclectomised fetal

Placentomes develop at the sites of the maternal endometrial caruncles. Removal of these caruncles prior to mating results in reduced placental size and intra-uterine growth restriction of the fetus.
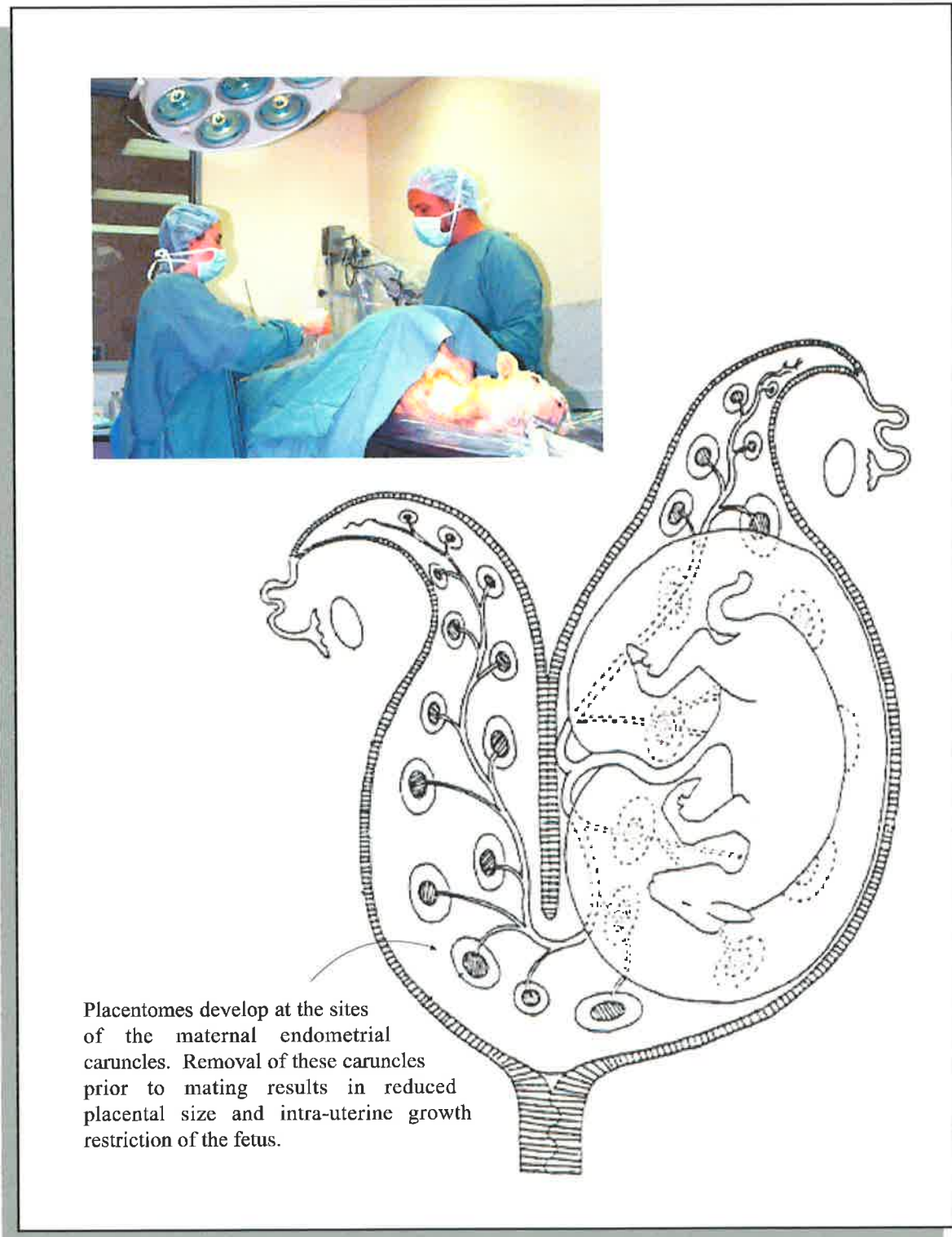
Figure 1.6   Surgical induction of intra-uterine growth restriction.

Removal of the majority of maternal endometrial caruncles prior to mating results in a small placenta and fetal growth restriction during pregnancy.   Ewes and fetuses are subsequently catheterised for measurement of circulating hormone concentrations.

sheep are chronically hypoxaemic and hypoglycaemic in late-gestation ([364]Robinson, Kingston *et al.*, 1979; [319]Owens, Falconer and Robinson, 1987a; [320]Owens, Falconer and Robinson, 1987b), and the subsequent long-term reduction in uterine blood flow (by ~69 %) observed in one study was associated with a reduction in fetal body weight of 42 % ([318]Owens, Falconer and Robinson, 1986). An increase in the ratio of $F_w : P_w$ is also observed when placental growth is restricted via carunclectomy, which contrasts with the normal $F_w : P_w$ ratio observed with reduced maternal substrate availability. These observations reinforce the view that, in general, placental growth is more susceptible to restriction during the first half of gestation, when it is growing most rapidly, while the fetus is more responsive in mid- to late-gestation. The increased $F_w : P_w$ ratio seen when placental size and / or function are reduced by carunclectomy or placental embolisation suggests that compensatory mechanisms increase placental efficiency to maintain fetal growth.

All of the various experimental procedures discussed above reduce the rate and alter the pattern of fetal growth, with body weight being reduced to a greater extent than crown-rump length or girth ([364]Robinson, Kingston *et al.*, 1979; [318]Owens, Falconer and Robinson, 1986; [206]Jacobs, Robinson *et al.*, 1988; [324]Owens, Owens and Robinson, 1989). Typically, brain weight is maintained relative to body weight, while the relative weights of liver, spleen and lymphoid tissue are disproportionately reduced in the experimentally growth restricted fetal sheep. Other organs essential in adaptation to post-natal life, such as the lungs and gastrointestinal tract, are reduced in proportion to body weight. Growth of the adrenal and thyroid glands are maintained or even increased, and consequently are often increased in size relative to body weight, while that of the thymus is reduced relative to the body weight of the growth restricted sheep fetus. This pattern of asymmetric growth, which is also seen in human IUGR, suggests that there are common responses of organ growth rates to environmental perturbations in many species.

1.7.3   *Endocrine consequences of intra-uterine growth restriction*

A wide range of endocrine changes occur in the experimentally growth restricted fetus ([362]Robinson, Hart *et al.*, 1980; [180]Harding, Jones and Robinson, 1985; [218]Jones, 1989). Many hormones, particularly polypeptide hormones, do not cross the placenta rendering the fetus essentially autonomous in its endocrinology. Consequently, the hormonal changes observed in fetal growth restriction substantially reflect the fetal response to perturbation of its environment, specifically, changes in the supply of O_2 or glucose, with subsequent changes in fetal metabolism and growth.

The fetal hormonal profiles of the carunclectomised model of IUGR have been investigated ([361]Robinson, Falconer and Owens, 1985). As might be anticipated with the chronic stress of placental insufficiency, the levels of catabolic or stress hormones such as catecholamines ([397]Simonetta, Rourke *et al.*, 1997), cortisol ([343]Phillips, Simonetta *et al.*, 1996), glucagon, and β-endorphins in the fetal circulation are increased, while the levels of most growth-promoting factors (T_3, T_4, somatomedins, insulin, and prolactin) are decreased. ACTH ([343]Phillips, Simonetta *et al.*, 1996), IGF-II, ovine placental lactogen, and GH levels are unchanged. Enhanced adrenal growth, either in absolute terms or relative to fetal body weight, also occurs with the increased fetal circulating cortisol levels, and appears to be due to O_2 deficit, since it occurs with chronic fetal hypoxaemia ([206]Jacobs, Robinson *et al.*, 1988), but not with hypoglycaemia alone.

The response of the adrenal cortex to acute episodes of hypoxaemia is also greater in growth restricted fetal sheep, which respond with a larger increase in circulating cortisol than control fetuses ([363]Robinson, Jones and Kingston, 1983). This accelerated maturation of the fetal adrenal cortex may enhance development of other cortisol dependent systems in the fetus. Interestingly, while restriction of placental growth from conception via carunclectomy results in an increase in fetal plasma cortisol and an increase in adrenal weight relative to body weight ([343]Phillips, Simonetta *et al.*, 1996), these adaptations occur without consistently elevated fetal plasma ACTH levels ([362]Robinson, Hart *et al.*, 1980; [343]Phillips, Simonetta *et al.*, 1996). Thus, the mechanisms underlying the enhanced fetal adrenal growth and steroidogenic response to placental restriction are unknown.

The sixth main aim of this thesis, therefore, was to investigate the effect of surgical restriction of placental growth from conception, via carunclectomy, on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the ovine fetus in late-gestation. In studies described in Chapter 6, maternal carunclectomy was performed prior to mating, to determine the subsequent effects of chronic fetal growth restriction on the growth of the fetal adrenal, and on the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 within the adrenal gland of the ovine fetus in late-gestation.

1.7.3.1 Sensitivity of the IUGR fetal adrenal to ACTH

It is clear from a number of studies that an increase in fetal cortisol is a consistent response to prolonged or repeated fetal hypoxaemia in late-gestation. Furthermore, this increase is not always associated with an increase in fetal ACTH ([192]Hooper, Coulter *et al.*, 1990; [59]Braems, Matthews and Challis, 1996; [343]Phillips, Simonetta *et al.*, 1996). In experiments

in which fetal hypoxaemia is induced by a reduction in utero-placental blood flow for 24 h, fetal ACTH concentrations are increased only at 2 h after the onset of hypoxaemia and then return to baseline values. In contrast, fetal cortisol concentrations are elevated by 2 h after the onset of hypoxaemia and remain elevated throughout the 24 h period ([192]Hooper, Coulter *et al.*, 1990). Similarly, when maternal hypoxaemia is induced for 48 h through a reduction in the fraction of inspired O_2, fetal cortisol concentrations are increased in the absence of a sustained fetal ACTH response ([59]Braems, Matthews and Challis, 1996). Cordocentesis studies in small for gestational age human fetuses have also found that plasma cortisol concentrations are higher and plasma ACTH concentrations lower than in normally grown fetuses at 18-38 weeks of gestation ([138]Economides, Nicolaides *et al.*, 1988). Thus, it is possible that the fetal hypothalamo-pituitary axis adapts to the effects of prolonged, sustained hypoxaemia (induced through either maternal hypoxaemia, reduction of utero-placental blood flow or placental restriction), via enhanced sensitivity of the fetal adrenal to ACTH. I propose that the enhanced sensitivity of the adrenal gland to ACTH, observed in chronically growth restricted fetal sheep, is mediated via an increase in the expression of the adrenal ACTH receptor.

It has recently been demonstrated that steroidogenic factor-1 (SF-1), is essential for basal expression of the ACTH receptor in the human and mouse adrenal ([104]Commas, Pullinger *et al.*, 1997; [269]Marchal, Naville *et al.*, 1998). SF-1 is an orphan nuclear receptor and transcription factor that is essential for the differentiation and normal function of steroidogenic cells within the adrenal ([434]Wong, Ikeda *et al.*, 1997). SF-1 consensus sequences are present in the 5' region of the P450 enzymes involved in steroidogenesis and 3βHSD. In addition, other factors which stimulate ACTH receptor expression in ovine adrenocortical cells include ACTH and glucocorticoids, and it has also been shown that IGF-I and -II increase the expression of the ACTH receptor in adult human adrenocortical cells ([131]l'Allemand, Penhoat *et al.*, 1996; [345]Picard-Hagen, Penhoat *et al.*, 1997). Thus, I propose that the enhanced adrenal responsiveness of the chronically growth restricted ovine fetus may also be mediated via an increase in the expression of mRNA for the transcription factor SF-1.

A further aim of this thesis, therefore, was to investigate the effect of maternal carunclectomy and subsequent chronic fetal growth restriction on the expression of mRNA for the ACTH receptor and steroidogenic factor-1 within the adrenal gland of the ovine fetus during late-gestation.

Finally, Lu and co-workers have found, in the sheep, that intra-fetal infusion of ACTH or cortisol for 84 h, beginning at 120-125 d of gestation, results in a decrease in fetal adrenal IGF-II mRNA levels ([258]Lu, Han *et al.*, 1994). It has also been reported that cortisol infusion inhibits the expression of mRNA for IGF-II in other fetal tissues including the liver ([258]Lu, Han *et al.*, 1994). Interestingly, Braems and colleagues have recently reported that reducing the fraction of O_2 in maternal inspired air for 48 h also results in a significant decrease in the expression of adrenal IGF-II mRNA in fetuses after 134 d of gestation ([58]Braems, Han and Challis, 1998). I propose, therefore, that the increase in adrenal cortisol output which occurs in the hypoxaemic growth-restricted sheep fetus after 125 d of gestation ([343]Phillips, Simonetta *et al.*, 1996) will inhibit the expression of adrenal IGF-II mRNA, or alter the expression of the IGF-II binding protein, IGFBP-2.

An additional aim of this thesis, therefore, was to investigate the effect of maternal carunclectomy and subsequent chronic fetal growth restriction on the expression of mRNA for IGF-II and IGFBP-2 within the adrenal gland of the ovine fetus during late-gestation.

1.8   SUMMARY

The aims of this thesis are:

Chapter 2.

1. **To characterise adrenal growth throughout gestation, and to investigate the activation of steroidogenic enzyme gene expression within the adrenal gland of the sheep fetus during the last 15 d of gestation.**

   - *Study 1*: A large cohort of fetal sheep were used to construct a gestational profile of fetal adrenal gland growth.

   - *Study 2*: The expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 was examined at 130-134 d, 135-139 d and 140-145 d of gestation.

   - The late-gestation ontogeny of IGF-II and IGFBP-2 mRNA expression within the fetal adrenal was measured during the last 15 d of gestation.

2. **To investigate the effect of cortisol replacement after fetal HPD on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the late-gestation sheep fetus.**

   - *Study 3*: Fetal hypothalamo-pituitary-disconnection was performed at 110 d of gestation to determine the effect of subsequent cortisol replacement on adrenal growth and the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1, compared with saline-infused HPD fetuses.

   - The effect of fetal HPD at 110 d of gestation, with or without subsequent cortisol replacement, on the expression of IGF-II mRNA in the fetal sheep adrenal was determined.

Chapter 3.

3. **To investigate the impact of intra-fetal cortisol infusion, prior to the endogenous pre-partum cortisol surge, on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes and 11βHSD-2 within the adrenal glands of fetuses with an intact hypothalamo-pituitary axis.**

   - Cortisol (2.0-3.0 mg·24 h^{-1}) was infused from 109-116 d of gestation to determine the effects of a premature elevation of circulating cortisol on adrenal growth and on the expression of mRNA for CYP11A1, CYP17, 3βHSD, CYP21A1 and 11βHSD-2.

   - The impact of intra-fetal cortisol infusion on the expression of mRNA for IGFBP-2 within the fetal sheep adrenal gland was also investigated.

Chapter 4.

4. To investigate the effect of intra-fetal infusion of peptides derived from the *N*-terminal region of bovine POMC on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the late-gestation sheep fetus.

- *N*-POMC(1-77) and *N*-POMC(1-49) were administered to fetal sheep between 136-138 d of gestation, *i.e.* after the development of adrenal innervation, to determine the separate effects of these peptides on the growth of the fetal adrenal, and on the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 within the fetal sheep adrenal.

Chapter 5.

5. To investigate the effect of intra-fetal infusion of IGF-I on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the ovine fetus at 130 d of gestation.

- *rh*IGF-I was infused into fetal sheep between 120-130 d of gestation to determine the effects of this peptide growth factor on the growth of the fetal adrenal, and on the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 within the adrenal gland of the ovine fetus.

Chapter 6.

6. To investigate the effects of surgical restriction of placental growth from conception, via carunclectomy, on adrenal growth and the expression of mRNA for the adrenal steroid-synthesising enzymes in the ovine fetus in late-gestation.

- Maternal carunclectomy was performed prior to mating, to determine the subsequent effects of chronic placental restriction on the growth of the fetal adrenal, and on the expression of mRNA for CYP11A1, CYP17, 3βHSD and CYP21A1 within the fetal sheep adrenal.

- The impact of maternal carunclectomy on the expression of mRNA for IGF-II, IGFBP-2, the ACTH receptor and steroidogenic factor-1 in the fetal sheep adrenal was determined.

CHAPTER 2.
ADRENAL MATURATION AFTER FETAL HYPOTHALAMO-PITUITARY DISCONNECTION: EFFECT OF INTRA-FETAL CORTISOL INFUSION IN LATE-GESTATION

2.1   *INTRODUCTION*

The dramatic increases in growth and steroidogenic activity of the fetal sheep adrenal gland between mid- and late-gestation have been well characterised ([80]Challis and Brooks, 1989). Boshier and colleagues have described the morphological maturation of the fetal sheep adrenal throughout gestation between 53 d and 144 d of gestation, and at 2 d post-partum ([53]Boshier and Holloway, 1989). These authors have described a doubling in weight and volume of the adrenal gland between 130 d and 144 d of gestation, due primarily to an increase in the volume of secretory cells within the *zona fasciculata* ([53]Boshier and Holloway, 1989). In the first study of this chapter, the gestational profile of adrenal gland weight from approximately 400 fetal sheep between 42 d and 147 d of gestation has been quantitatively characterised. This profile provides a baseline for the evaluation of the effect of different treatments on adrenal growth described throughout this thesis.

Several studies have measured CYP11A1, CYP17 and CYP21A1 mRNA levels in the fetal sheep adrenal between 50 d and 136 d of gestation ([216]John, Simpson *et al.*, 1987; [407]Tangalakis, Coghlan *et al.*, 1989; [298]Myers, McDonald and Nathanielsz, 1992b). A qualitative study of adrenal steroid hydroxylase gene expression has shown an increase in hybridisation intensity for CYP11A1 and CYP17 mRNA in the fetal adrenal between 114 d and 132 d of gestation ([407]Tangalakis, Coghlan *et al.*, 1989). A >2 fold increase in adrenal CYP11A1 and CYP17 mRNA abundance was also observed between 128 d and 136 d of gestation, with a further increase in the neo-natal period (<2 h after birth) ([298]Myers, McDonald and Nathanielsz, 1992b). These results correlate well with the increase in adrenal cortisol : corticosterone (F : B) output observed by Wintour and colleagues, which occurs between 100 d and 145 d of gestation ([433]Wintour, Brown *et al.*, 1975). Enzyme kinetic studies have also demonstrated that this increase in F : B ratio is due to increased adrenal CYP17 enzyme activity ([131]Durand, Cathiard *et al.*, 1982; [267]Manchester and Challis, 1982).

While these studies have shown that an increase in adrenal steroid hydroxylase activity and gene expression occurs between the quiescent phase of adrenal function (100-120 d of gestation) and activation of the adrenal in late-gestation, there have been no quantitative studies which have characterised the ontogenic changes in adrenal steroidogenic enzyme gene expression throughout the final 2 weeks of gestation, *i.e.* during the period of rapid adrenocortical growth and cytological maturation which precedes birth. The second study in this chapter examines the time-course of changes in the expression of mRNA for the adrenal steroidogenic enzymes at three time intervals throughout the last 15 d of gestation (130-135 d, 136-140 d and 141-145 d of gestation).

The insulin-like growth factors (IGFs) are considered important regulatory factors modulating the development of the fetus throughout gestation in human and other species ([123]D'Ercole, 1987). The fetal sheep adrenal is a major site of synthesis of the IGFs, and both IGF-II immunoreactivity and mRNA expression are predominantly localised within cells of the *zona fasciculata* within the adrenal cortex ([178]Han, Lu *et al.*, 1992). Han and colleagues have measured adrenal IGF-II mRNA expression at 125-130 d of gestation and at term (145-147 d of gestation). The availability of IGFs within the developing adrenal may also be modulated by changes in the production of adrenal IGF binding proteins (IGFBPs), which act to regulate the half-life, tissue delivery and localisation of the IGF peptides ([71]Carr, 1994). There have been no detailed quantitative studies of adrenal IGFBP-2 mRNA expression during reactivation of adrenal growth (120-145 d of gestation). In the second study in this chapter, I have measured the changes in the expression of mRNA for IGF-II and IGFBP-2 in the fetal adrenal at three time intervals during the last 15 d of gestation (130-135 d, 136-140 d and 141-145 d of gestation).

Fetal hypophysectomy abolishes the pre-partum increase in adrenal growth and steroidogenesis, and prolongs gestation in pregnant sheep ([247]Liggins, Kennedy and Holm, 1967; [28]Barnes, Comline and Silver, 1977). Surgical disconnection of the fetal hypothalamus from the pituitary gland also results in smaller adrenals and a lower adrenal steroid-synthetic capacity than in intact fetuses at the same gestational age ([342]Phillips, Ross *et al.*, 1996). Exogenous infusion of ACTH into intact and hypophysectomised fetuses at 130-134 d of gestation increases the thickness of the *zona fasciculata* by ~2 fold, and the proportion of histologically mature adrenocortical cells ([366]Robinson, Comline *et al.*, 1983). Steroid output and steroidogenic enzyme activity are also stimulated by a 72 h infusion of ACTH(1-24) (0.5 $\mu g \cdot h^{-1}$) ([263]Lye, Sprague *et al.*, 1983; [268]Manchester, Lye and Challis, 1983). Tangalakis and co-workers have demonstrated that infusion of ACTH(1-24) into fetal sheep for 24 h (12 $\mu g \cdot 24 h^{-1}$) from 102-106 d of gestation results in

a substantial increase in the adrenal expression of CYP11A1 and CYP17 mRNA, as determined by hybridisation histochemistry and Northern blot analysis ([408]Tangalakis, Coghlan *et al.*, 1990). In adrenals of fetuses left *in utero* for 24 h or 72 h after cessation of the ACTH infusion, however, the abundance of adrenal CYP11A1 and CYP17 mRNA declined to pre-infusion levels ([408]Tangalakis, Coghlan *et al.*, 1990). Thus, ACTH is required to induce and maintain the expression of mRNA for the steroidogenic enzymes within the fetal adrenal.

It remains unclear, however, whether ACTH(1-39) is the sole factor responsible for the coordination of adrenal growth and steroidogenesis throughout late-gestation. Chromatographic studies have shown that ir-ACTH is present within fetal circulation in a range of molecular weight forms including the bioactive ACTH(1-39) and the high molecular weight ACTH precursors (POMC and pro-ACTH) ([217]Jones, 1980). Schwartz and colleagues have recently demonstrated that these large molecular forms of ACTH inhibit the steroidogenic response of fetal adrenocortical cells to ACTH(1-24) *in vitro* ([385]Schwartz, Kleftogiannis *et al.*, 1995). One possibility, therefore, is that in late-gestation the coordinate regulation of fetal adrenal growth, steroidogenesis and cortisol output is dependent on changes in the post-translational processing of POMC in the fetal pituitary. A second possibility is that the functional changes in the pituitary-adrenal axis in late-gestation are, in part, dependent on the actions of the pre-partum increase in cortisol, which is absent in hypophysectomised and HPD fetuses.

Glucocorticoid receptors are present in adult sheep ([375]Saez, Morera and Gallet, 1977) and fetal sheep ([441]Yang and Challis, 1989) adrenocortical cells, and glucocorticoids may therefore act in an autocrine or paracrine manner to modulate ACTH induced activation of fetal adrenal function. Liggins and colleagues demonstrated that infusion of metyrapone (a CYP11B1 inhibitor) with ACTH(1-24), inhibits the hyperplasia of the fetal sheep adrenal which normally occurs in response to ACTH administration ([243]Liggins, 1968). Furthermore, metyrapone treatment *in vivo* prevents the increase in fetal adrenal weight observed following an intra-fetal 100 h pulsatile infusion of ACTH(1-24) (66.6 ng·min^{-1} for 15 min·2 h^{-1}) from 127-132 d of gestation ([262]Lye and Challis, 1984). In addition, the increased accumulation of cAMP in response to ACTH(1-24) *in vitro*, following the 100 h intra-fetal infusion of ACTH(1-24), is abolished by concurrent administration of metyrapone *in vivo*, prior to collection of tissue ([262]Lye and Challis, 1984). Thus, the effects of intra-adrenal exposure to glucocorticoids may synergise with endocrine signals from the fetal pituitary to coordinate the increase in adrenocortical responsiveness to ACTH, resulting in the enhanced adrenal growth and steroidogenesis observed prior to

birth in the sheep fetus. It has been demonstrated previously that surgical disconnection of the fetal hypothalamus and pituitary inhibits fetal adrenal growth and steroidogenesis ([342]Phillips, Ross *et al.*, 1996). This decrease may be due to a reduction in circulating ACTH(1-39) concentrations, or alternatively it may be a consequence of the lower circulating cortisol levels in the HPD fetus. In the third study in this chapter, I have investigated the effect of a 5 d infusion of cortisol from 135-140 d of gestation, after disconnection of the fetal hypothalamus and pituitary at around 110 d of gestation, on circulating ir-ACTH and ACTH(1-39) concentrations, on adrenal growth and on expression of mRNA for the cytochrome P450 enzymes: CYP11A1, CYP17, CYP21A1 and 3βHSD in the ovine fetal adrenal.

In cultured human fetal adrenal cells, ACTH(1-24) increases IGF-II mRNA accumulation ([421]Voutilainen and Miller, 1987; [202]Ilvesmaki, Blum and Voutilainen, 1993b). It has therefore been proposed that ACTH acts via local or intra-adrenal growth factors to stimulate adrenocortical growth and to stimulate the activity of key steroidogenic enzymes in late-gestation ([80]Challis and Brooks, 1989). There is also evidence that cortisol modulates the IGF axis in fetal tissues, including the adrenal, by inhibiting the expression of mRNA for IGF-II ([241]Li, Saunders *et al.*, 1993; [258]Lu, Han *et al.*, 1994). Thus, cortisol and ACTH may regulate the mRNA and peptide expression of IGF-II within the fetal adrenal. In the third study in this chapter, I have investigated the effect of HPD at around 110 d of gestation, with and without subsequent cortisol replacement, on the expression of mRNA for IGF-II within the adrenal at 140 d of gestation.

Thus, I wished to determine the gestational profile of total adrenal weight and the ratio of adrenal : fetal body weight, the expression of mRNA for the adrenal steroidogenic enzymes, IGF-II and IGFBP-2; and the impact of surgical disconnection of the fetal hypothalamus and pituitary with subsequent cortisol replacement on circulating levels of ACTH(1-39), adrenal growth and the expression of mRNA for the steroidogenic enzymes and IGF-II.

2.2   MATERIALS AND METHODS

2.2.1   Animal protocols and surgery

2.2.1.1   Study 1. Gestational profile of adrenal gland weight

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation. Data from 422 fetuses from a variety of experimental studies were collected in order to construct gestational profiles of total adrenal weight (the

combined weight of left and right adrenals) and the ratio of adrenal to fetal body weight. Fetuses were selected from one of three control protocols for the purposes of other studies, which included:

(*A*)   Fetuses killed at designated gestational ages without prior surgery or treatment.

(*B*)   Catheterised fetuses which underwent routine blood sampling.

(*C*)   Catheterised fetuses which underwent saline infusion and routine blood sampling.

2.2.1.2   *Study 2. Late-gestation gene expression*

Sixteen pregnant Border-Leicester × Merino ewes, with eighteen fetuses between 130-145 d of gestation were used in the late-gestation study. Ewes were housed in individual pens in animal holding rooms, with a 12 h light / dark lighting regimen, and fed once daily with 1 kg of lucern chaff (Rye and Grains) and 1 kg of Baramil joint stock rations (Ridley Agri Products) between 0900 and 1300 h with water *ad libitum*. No fetal or maternal surgery or blood sampling was carried out in this study. Ewes and their fetal sheep were killed with an intra-venous overdose of Lethabarb (sodium pentobarbitone: 25 ml at 325 mg·ml^{-1}; Virbac Australia) and fetuses (130-135 d, n=6; 136-140 d, n=6; 141-145 d of gestation, n=6) were removed, weighed and killed by decapitation. Fetal adrenal glands were quickly removed, weighed, snap frozen in liquid nitrogen (N_2) and stored at -80 °C until total RNA was extracted.

2.2.1.3   *Study 3. Fetal HPD*

2.2.1.3.1   *Fetal vascular catheterisation surgery*

Twenty-three pregnant Border-Leicester × Merino ewes (singleton and twin pregnancies) were used in the fetal HPD study. Surgery was performed, using aseptic techniques, on twenty-three fetuses between 104-124 d of gestation. Surgery was performed on only one fetus of twin pregnancies. General anaesthesia was induced with intra-venous Pentothal (sodium thiopentone: 20 ml, 0.1 g·ml^{-1}; Rhone Merieux Australia) and maintained with fluothane inhalation anaesthetic (0.5-4.0 %; ICI) in medical grade O_2 (Linde Gas). During surgery, the uterus was exposed by a mid-line laparotomy incision and the fetal head was located by palpating the uterus. The fetal head and neck were then delivered through an incision in the uterine wall and fetal membranes. Single lumen polyvinyl catheters (Critchley Electrical Products) were inserted into the fetal carotid artery and external jugular vein (catheter outer diameter: 1.52 mm; inner diameter: 0.86 mm) and the amniotic sac (catheter outer diameter: 2.70 mm; inner diameter: 1.50 mm) as described previously ([119]Dawes, Fox *et al.*, 1972). A catheter (outer diameter: 2.70 mm; inner diameter: 1.50 mm) was also inserted into the maternal external jugular vein.

All ewes and fetal sheep received a 2 ml intra-muscular injection of Ilium Penstrep (250 mg·ml^{-1} procaine penicillin, 250 mg·ml^{-1} dihydrostreptomycin sulphate, 20 mg·ml^{-1} procaine hydrochloride; Troy Laboratories). The fetal head was then repositioned within the uterus and the uterine wall and fetal membranes were closed in a single layer using 2/0 chromic cat gut (Ethicon; Johnson & Johnson). A layer of inverting sutures was subsequently placed in the myometrium using 2/0 chromic cat gut. All catheters were filled with 50 IU·ml^{-1} heparinised saline (heparin sodium; David Bull Laboratories; saline: 0.9 % w·v^{-1} sodium chloride, NaCl solution; Baxter Healthcare) and fetal catheters were exteriorised via an incision in the ewe's flank. The maternal abdomen was closed in two layers, the peritoneum and rectus sheet, followed by the sub-cutaneous tissue and skin, using 3/0 chromic cat gut (Ethicon; Johnson & Johnson) and Vetafil Bengen (WDT) respectively. Austrapen (500 mg; sodium ampicillin, Commonwealth Serum Laboratories) was administered intra-amniotically to all fetal sheep, daily for 4 d post-operatively.

2.2.1.3.2   Fetal HPD surgery

The procedures for fetal hypothalamo-pituitary disconnection and vascular catheterisation used in this study have been described in full previously ([16]Antolovich, Clarke *et al.*, 1990). Briefly, a midline incision was made in the nose of seventeen fetal sheep and the nasal bone opened just left of the intra-nasal septum. The optic chiasm was located and exposed to allow access to the median eminence. The neural tissues of both internal and external laminae of the median eminence were then removed, using gentle suction, above the level of the hypophysial portal circulation. A small piece of Gelfoam soaked in Thrombostat (thrombin; Parke-Davis) was introduced to maintain physical separation of the hypothalamus from the pituitary. A sham procedure was carried out in six fetal sheep (Intact group) in which vascular and amniotic catheters were inserted. A three day recovery period after surgery preceded collection of blood samples.

The effectiveness of disconnection of the fetal hypothalamus and pituitary was confirmed as described previously, by the lack of a fetal prolactin response to intra-venous administration of the dopamine antagonist, chlorpromazine (Largactil, Rhone-Poulent Rorer Australia), in all HPD fetal sheep ([199]Houghton, Young and McMillen, 1995a). Between 139 d and 141 d of gestation, ewes were killed with an overdose of Lethabarb (25 ml at 325 mg·ml^{-1}) and fetal adrenal glands were quickly removed, weighed and snap frozen in liquid N$_2$ and stored at -80° C until total RNA was extracted. Macroscopic examination of the HPD lesion at post-mortem also confirmed the anatomical completeness of disconnection in all HPD fetuses.

2.2.2 Infusions, blood sampling and hormone assays. Study 3. Fetal HPD

2.2.2.1 Infusion regimen and blood sampling protocol

Infusion regimen. Cortisol (hydrocortisone succinate, Solucortef: 3.5 mg in 4.8 ml·24 h⁻¹; UpJohn), was infused via the jugular vein catheter into HPD fetuses (HPD+F group, n=5 fetuses) for 120 h, from 134/135 d of gestation until 139/141 d of gestation ([341]Phillips, Fielke *et al.*, 1996). Saline (0.9 % w·v⁻¹ NaCl: 4.8 ml·24 h⁻¹) was infused into the remaining HPD fetuses (HPD group, n=12 fetuses) and intact fetuses (Intact group, n=6 fetuses) from 134/135 d of gestation until 139/141 d of gestation.

Blood sampling protocol. Fetal arterial blood samples (2 ml) were collected from fetuses in the fetal HPD study into chilled collection tubes every one or two days between 130 d and 140 d of gestation for ir-ACTH and cortisol radioimmunoassays, and an immunoradiometric assay (IRMA) for ACTH(1-39). Blood for cortisol assay was collected into tubes containing 125 IU lithium heparin (Sarstedt Australia). Blood for ir-ACTH and ACTH(1-39) assays were collected into tubes containing ethylenediaminetetraacetic acid (EDTA, 18.6 g·l⁻¹ of whole blood) (Sarstedt Australia) and aprotinin (100 kallikrein inhibitor units, KIU in 100 μl·ml⁻¹ of whole blood; Sigma-Aldrich). Blood samples were centrifuged at 1800 g for 10 min at 4 °C before separation and storage of plasma at -20 °C for subsequent assay. Fetal arterial blood (0.5 ml) was collected on alternate days for measurement of whole blood p_aO_2, p_aCO_2, pH, O_2 saturation and haemoglobin content using an ABL 550 acid base analyser and OSM2 haemoximeter (Radiometer Pacific).

2.2.2.2 Radioimmunoassays

Cortisol radioimmunoassay. Cortisol concentrations were measured in fetal plasma samples collected from the Intact group (n=5 fetuses; *n*=10 samples), HPD group (n=5 fetuses; *n*=11 samples), and HPD+F group (n=5 fetuses; *n*=14 samples). Total cortisol concentrations in fetal sheep plasma were measured using a radioimmunoassay, validated for fetal sheep plasma, (Orion Diagnostica). Prior to assay, cortisol was extracted from plasma with dichloromethane (BDH Laboratory Supplies) using a method described previously ([46]Bocking, McMillen *et al.*, 1986). Recovery efficiency of [125I]cortisol from fetal plasma using this method was >90 %. The sensitivity of the assay was 0.78 nmol·l⁻¹ and cross-reactivity of the rabbit anti-cortisol antibody was <1 % with cortisone and 17α-hydroxy progesterone and <0.001 % with pregnenolone, aldosterone, progesterone and estradiol. The inter- and intra-assay coefficients of variation (COVs) were <10 %.

ACTH radioimmunoassay. ir-ACTH concentrations were measured in fetal plasma samples from the Intact group (n=4 fetuses; *n*=34 samples), HPD group (n=6 fetuses; *n*=48 samples) and HPD+F group (n=5 fetuses; *n*=43 samples), using a radioimmunoassay (ICN Biomedicals Australasia) which has previously been validated for fetal sheep plasma ([278]McMillen, Antolovich *et al.*, 1990). The sensitivity of the assay was 7 pg·ml^{-1} and the rabbit anti-human ACTH(1-39) had a cross-reactivity of <0.1 % with β-endorphin, α-MSH, α-lipotrophin and β-lipotrophin. The inter-assay COV was 14.6 % and the intra-assay COV was <10 %.

ACTH(1-39) immunoradiometric assay. ACTH(1-39) concentrations were measured in fetal plasma samples from the Intact group (n=6 fetuses; *n*=16 samples), HPD group (n=6 fetuses; *n*=17 samples), and HPD+F group (n=5 fetuses; *n*=15 samples), using an IRMA with one radiolabelled monoclonal antibody (MAb) that is specific for the sequence ACTH (10-18), and a second MAb, coupled to Sephacryl S-300 as a solid phase, that is specific for the sequence ACTH (25-39). The IRMA assay was performed by Drs. Anne White and Sarah Gibson (Department of Medicine, University of Manchester, Salford, UK). The ACTH(1-39) assay has a cross-reactivity with POMC and pro-ACTH of <1 % at the concentrations measured and there is no cross reactivity with fragments of ACTH ([111]Crosby, Stewart *et al.*, 1988). Plasma (100 µl) was incubated with radiolabelled MAb for either 2 h at 21 °C or 16 h at 4 °C, followed by incubation with solid phase MAbs for a further 2 h at 21 °C with constant agitation. Bound and free radiolabelled MAbs were separated by sucrose layering, using a two-pass system.

2.2.3 Tissue analysis. Studies 2. Late-gestation gene expression and 3. Fetal HPD

2.2.3.1 cDNA and antisense oligonucleotide probes and probe labelling

cDNA probes. An 1821 bp human (*h*) CYP11A1 cDNA probe ([272]Matteson, Chung and Miller, 1984; [95]Chung, Matteson *et al.*, 1986) and a 1754 bp *h*CYP17 cDNA probe ([275]Matteson, Picado *et al.*, 1986; [96]Chung, Picado-Leonard *et al.*, 1987), subcloned into the *Eco*R1 restriction site of the multiple cloning region within the pUC18 plasmid vector (Clontech Laboratories) ([285]Messing, 1983) (*Figures 2.1 and 2.2*), were generously provided by Professor W Miller (Department of Pediatrics, UCSF, San Francisco, CA, USA). A 435 bp *h*3βHSD cDNA probe ([254]Lorence, Murray *et al.*, 1990), subcloned into the multiple cloning region spanned by the *Bam*H1 and *Eco*R1 restriction sites within the pBluescript II (SK+) phagemid vector (Stratagene) ([393]Short, Fernandez *et al.*, 1988) (*Figure 2.3*), was donated by Dr. R Rodgers (Department of Medicine, Flinders University, SA, Australia). A 1141 bp *h*CYP21A1 cDNA probe ([430]White, New and DuPont, 1986;

The **pUC18** plasmid is a 2686 bp plasmid derived from pBR322, M13mp18 and pUC12.

ColE1 origin: Plasmid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant plasmids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the plasmid vector.

*Hind*III 400
*Bsp*MI 405
*Sph*I 410
*Pst*I 416
*Sal*I 418
*Acc*I 419
*Hind*II 420
*Hinc*II 420
*Xba*I 424
*Bam*HI 430
*Xma*I 435
*Psp*AI 435
*Ava*I 435
*Sma*I 437
*Asp*718 439
*Acc*65I 439
*Kpn*I 443
*Sac*I 449
*Ban*II 449
*Eco*R1 451

*Nde*I 185
*Kas*I 236
*Nar*I 237
*Ehe*I 238
*Bbe*I 240

*Bsm*BI 46

*Eco*O109I 4497
*Aat*II 4443
*Ssp*I 4325
*Xmn*I 4120
*Sca*I 4001

*Cfr*10I 3601
*Gsu*I 3592
*Bpm*I 3591
*Bsa*I 3582
*Ahd*I 3521

Amp

lacZ-

pUC18-CYP11A1
4507 bp

CYP11A1
insert
1821 bp

*Alw*NI 3044
*Ple*I 3007
*Afl*III 2628
*Sap*I 2512

ColE1

lac
promoter

-lacZ

*Eco*R1 2272

Figure 2.1   **Plasmid map of the pUC18 plasmid vector containing the 1821 bp *h*CYP11A1 cDNA insert.**

E. coli DH5α was transformed with the pUC18 plasmid containing the 1821 bp *h*CYP11A1 cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Eco*R1 restriction enzyme digestion.

The **pUC18** plasmid is a 2686 bp plasmid derived from pBR322, M13mp18 and pUC12.

ColE1 origin: Plasmid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant plasmids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the plasmid vector.

*Nde*I 185
*Kas*I 236
*Nar*I 237
*Ehe*I 238
*Bbe*I 240

*Hin*dIII 400
*Bsp*MI 405
*Sph*I 410
*Pst*I 416
*Sal*I 418
*Acc*I 419
*Hin*cII 420
*Hin*dII 420
*Xba*I 424
*Bam*HI 430
*Psp*AI 435
*Ava*I 435
*Xma*I 435
*Sma*I 437
*Acc*65I 439
*Asp*718 439
*Kpn*I 443
*Ban*II 449
*Sac*I 449
*Xmn*I 455
*Eco*R1 451

*Bsm*BI 46

*Eco*O109I 4430
*Aat*II 4376
*Ssp*I 4258
*Xmn*I 4053
*Sca*I 3934

*Cfr*10I 3534
*Gsu*I 3525
*Bpm*I 3524
*Bsa*I 3515
*Ahd*I 3454

Amp

lacZ-

pUC18-CYP17
4440 bp

CYP17
insert
1754 bp

*Alw*NI 2977
*Ple*I 2940
*Afl*III 2561
*Sap*I 2445

ColE1

lac
promoter

-lacZ

*Eco*R1 2205

Figure 2.2   **Plasmid map of the pUC18 plasmid vector containing the 1754 bp *h*CYP17 cDNA insert.**

E. coli DH5α was transformed with the pUC18 plasmid containing the 1754 bp *h*CYP17 cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Eco*R1 restriction enzyme digestion.

The **pBluescript II (SK+)** phagemid is a 2961 bp phagemid derived from pUC19. The SK designation indicates the multiple cloning region is oriented such that *lacZ* transcription proceeds from *Sac*I to *Kpn*I.

f1 origin: f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

ColE1 origin: Phagemid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant phagemids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the phagemid vector.

NgoMI 133
BsaAI 238
DraIII 241

KpnI 658
EcoO109I 661
ApaI 664
SalI 675
ClaI 685
HindIII 690
EcoRV 698
EcoRI 702

*Xmn*I 3063
*Hin*II 3001
*Sca*I 2944

f1

Amp

lacZ

pBlue-3βHSD (SK+)
3375 bp

3βHSD
insert
435 bp

*Bsa*I 2525
*Ahd*I 2464

*Bam*HI 1137
*Spe*I 1143
*Xba*I 1149
*Xma*III 1156
*Sac*II 1168
*Bst*XI 1169
*Sac*I 1177

*Hgi*EII 2150
*Alw*NI 1987

ColE1

*Sap*I 1455
*Afl*III 1571
*Nsp*HI 1575

Figure 2.3   Plasmid map of the pBluescript II (SK+) phagemid vector containing the 435 bp *h*3βHSD cDNA insert.

E. coli DH5α was transformed with the pBluescript II (SK+) phagemid containing the 435 bp *h*3βHSD cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Bam*H1 / *Eco*R1 restriction enzyme digestion.

[274]Matteson, Phillips *et al.*, 1987), subcloned into the *Eco*R1 restriction site of the multiple cloning region within the pBR322 plasmid vector (Clontech Laboratories) ([49]Bolivar, Rodriguez *et al.*, 1977; [403]Sutcliffe, 1979) (*Figure 2.4*), was also generously provided by Professor W Miller. An ovine (*o*) IGF-II cDNA probe, encoding 260 bp of exon 4 ([241]Li, Saunders *et al.*, 1993) and subcloned into the multiple cloning region spanned by the *Hind*III and *Eco*R1 restriction sites within the pBluescript II (KS+) phagemid vector (Stratagene) ([393]Short, Fernandez *et al.*, 1988) (*Figure 2.5*), was a kind gift from Dr. R S Gilmour (Institute of Animal Physiology and Genetics, Cambridge, UK). A 1092 bp bovine (*b*) IGFBP-2 cDNA probe (named CL2) ([415]Upton, Szabo *et al.*, 1990), subcloned into the *Eco*R1 restriction site of the pBluescript II (KS+) phagemid vector (*Figure 2.6*), was generously provided by Dr. Jill Carr (Department of Biochemistry, The University of Adelaide, SA, Australia). The *b*IGFBP-2 cDNA clone was isolated by Dr. Z Upton and colleagues by screening a bovine kidney cell line (MDBK) λ gt11 cDNA library with a synthetic oligonucleotide complementary to the first 17 residues of the 'internal' MDBK binding protein peptide sequence, reported by Szabo and colleagues ([404]Szabo, Mottershead *et al.*, 1988).

Bacterial transformation and cDNA probe purification. Escherichia coli (*E. coli*) DH5α ([169]Glover, 1985) was made competent by the calcium chloride protocol, originally described by Cohen and colleagues ([103]Cohen, Chang and Hsu, 1972), and modified by Sambrook and colleagues ([376]Sambrook, Fritsch and Maniatis, 1989). Plasmid DNA (50 ng) in 10 μl of sterile water was added to 200 μl of 100 mM calcium chloride solution (CaCl$_2$·6H$_2$O; BDH Laboratory Supply), containing competent *E. coli* DH5α and kept on ice for 30 min. Bacteria were subsequently 'heat shocked' in a water bath at 42 °C for 90 sec and placed on ice for a further 1-2 min. SOC medium (800 μl) (*see Appendix I*) was added to the bacteria, which were incubated at 37 °C for 45 min with constant agitation (180 cycles·min^{-1}). Aliquots of the transformed bacteria were plated (100 μl·plate^{-1}) onto Luria-Bertani (LB) agar plates (*see Appendix I*) containing either 60 μg·ml^{-1} ampicillin (Boehringer Mannheim Australia) or 50 μg·ml^{-1} tetracycline (Boehringer Mannheim Australia), to select bacteria expressing the appropriate antibiotic resistance marker. LB agar plates were incubated at 37 °C overnight.

A single bacterial colony was then selected from the LB agar plate, inoculated into 10 ml of LB broth (*see Appendix I*) containing either 60 μg·ml^{-1} ampicillin or 50 μg·ml^{-1} tetracycline and grown to late log-phase (optical density at 600 nm: OD$_{600}$ ~0.4-0.6) at 37 °C with vigorous shaking (300 cycles·min^{-1}). Upon reaching late log-phase, 1 ml of the 10 ml starter culture was inoculated into 100 ml of LB broth containing either 60 μg·ml^{-1}

The **pBR322** plasmid is a 4363 bp plasmid derived from pSC101.

ColE1 origin: Plasmid origin of replication used in the absence of helper phage.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the plasmid vector.

Tet **gene**: Tetracycline resistance gene for antibiotic selection of the plasmid vector.

EcoR1 5501

ClaI 25
HindIII 30
EcoRV 188
NheI 230
BamHI 376
SphI 567
EcoNI 627
SalI 652

XmaIII 940
EagI 940
NruI 975
BspMI 1055
BsmI 1360
StyI 1370
AvaI 1426
MscI 1447
BsgI 1636
BspMII 1665
BsaBI 1673
PvuII 2067
BsmBI 2117
Tth111I 2221
BsaAI 2228
XcaI 2247
Bst107 I 2247
NdeI 2297
SapI 2358
AflIII 2474

CYP21A1 insert *1141 bp*

Tet

EcoR1 4360
AatII 4289
SspI 4171

pBR-CYP21A1
5504 bp

Amp

ROP

ScaI 3847
PvuI 3737
PstI 3612
VspI 3539
BsaI 3428
AhdI 3367
AlwNI 2890

ColE1

Figure 2.4   **Plasmid map of the pBR322 plasmid vector containing the 1141 bp *h*CYP21A1 cDNA insert.**

E. coli DH5α was transformed with the pBR322 plasmid containing the 1141 bp *h*CYP21A1 cDNA insert. Bacterial colonies were selected on the basis of tetracycline resistance and the insert was recovered from the plasmid via *Eco*R1 restriction enzyme digestion.
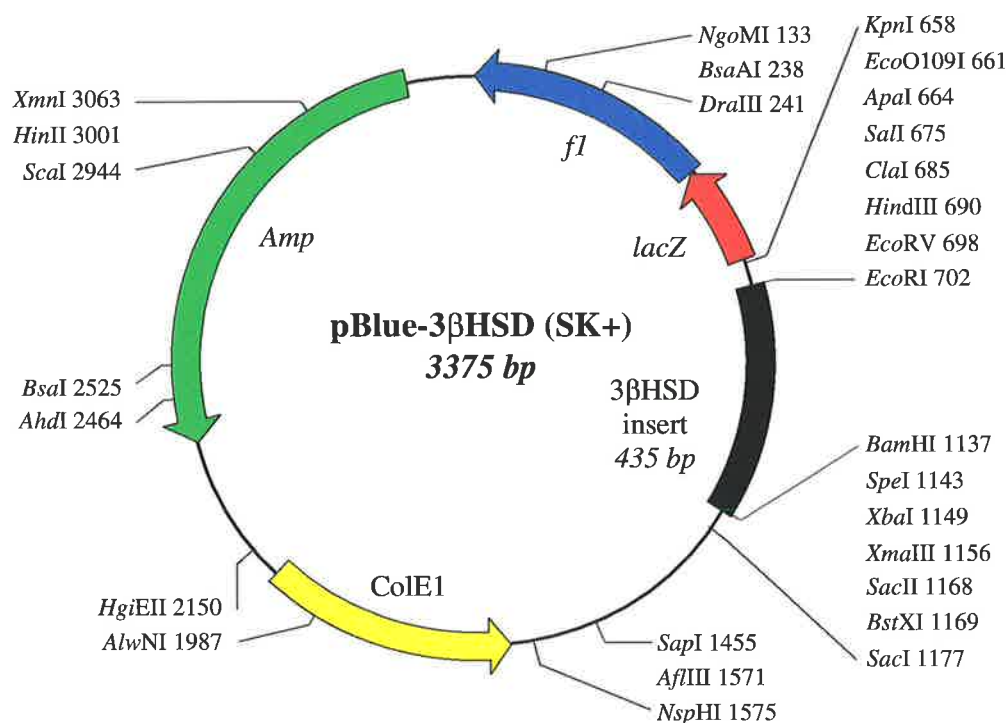
The **pBluescript II (KS+)** phagemid is a 2961 bp phagemid derived from pUC19. The KS designation indicates the multiple cloning region is oriented such that *lacZ* transcription proceeds from *Kpn*I to *Sac*I.

f1 origin: f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

ColE1 origin: Phagemid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant phagemids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the phagemid vector.

Figure 2.5   Plasmid map of the pBluescript II (KS+) phagemid vector containing the 260 bp *o*IGF-II cDNA insert.

E. coli DH5α was transformed with the pBluescript II (KS+) phagemid containing the 260 bp *o*IGF-II cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Hin*dII / *Eco*R1 restriction enzyme digestion.

The **pBluescript II (KS+)** phagemid is a 2961 bp phagemid derived from pUC19. The KS designation indicates the multiple cloning region is oriented such that *lacZ* transcription proceeds from *Kpn*I to *Sac*I.

f1 origin: f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

ColE1 origin: Phagemid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant phagemids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the phagemid vector.

*Xmn*I 3738
*Aha*II 3676
*Bsa*HI 3676
*Hin*II 3676
*Sca*I 3619

*Gsu*I 3210
*Bpm*I 3209
*Bsa*I 3200
*Ahd*I 3139

*Hgi*EII 2825
*Alw*NI 2662

*Nsp*HI 2250
*Afl*III 2246
*Nsp*7524I 2246

*Sap*I 2130

*Bsa*AI 228
*Ngo*MI 329
*Nae*I 331
*Bsm*FI 443

*Sac*I 658
*Sac*II 665
*Xma*III 671
*Eag*I 671
*Not*I 671
*Xba*I 678
*Spe*I 684
*Bam*HI 690
*Sma*I 698
*Pst*I 706
*Eco*RI 708

*Eco*RI 1800
*Eco*RV 1808
*Hind*III 1812
*Cla*I 1819
*Sal*I 1827
*Eco*O109I 1842
*Kpn*I 1852

Amp

f1

lacZ

ColE1

pBlue-IGFBP-2 (KS+)
4053 bp

IGFP-2
insert
1092 bp

Figure 2.6   Plasmid map of the pBluescript II (KS+) phagemid vector containing the 1092 bp *b*IGFBP-2 cDNA insert.

E. coli DH5α was transformed with the pBluescript II (KS+) phagemid containing the 1092 bp *b*IGFBP-2 cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Eco*R1 restriction enzyme digestion.

ampicillin or 50 $\mu g \cdot ml^{-1}$ tetracycline and grown to saturation (12-16 h) at 37 °C with constant agitation (300 cycles·min^{-1}). Bacteria were pelleted by centrifugation at 4,000 g and plasmid DNA was purified by alkaline lysis using a Maxiprep plasmid purification kit (Qiagen). Purified plasmid DNA was resuspended in 10 mM tris-EDTA (TE) buffer (pH 8.0) (*see Appendix I*) and the maximum absorbance at OD_{260} nm and OD_{280} nm was determined using a Beckman DU-50 spectrophotometer (Beckman Coulter Australia) to quantify nucleic acid purity, concentration and yield.

The cDNA inserts were isolated from plasmid DNA by digestion with the appropriate restriction enzymes (RE; MBI Fermentas) in appropriate reaction buffers (MBI Fermentas). Plasmid DNA (10 μg) was digested for 16 h at 37 °C using 1 IU·ng^{-1} plasmid DNA of the appropriate RE i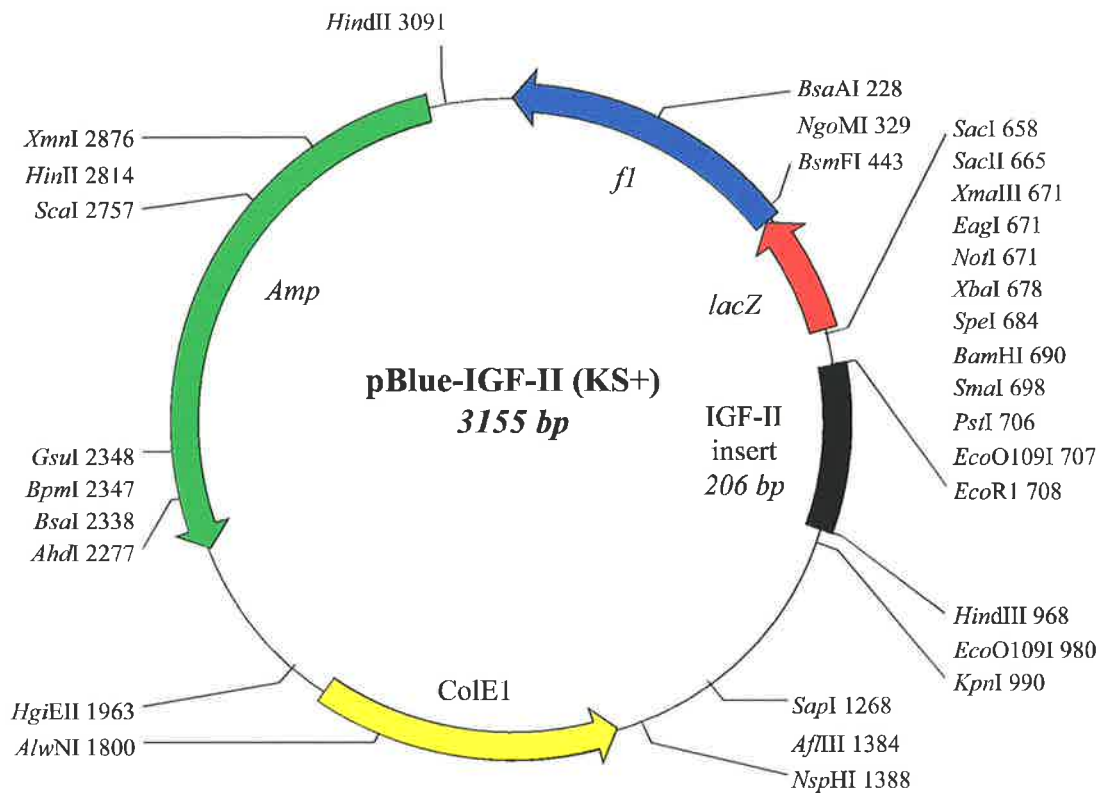n a final solution of 1× reaction buffer in 100 μl. For digestions involving two restriction enzymes, the *Eco*R1 digestion was always performed last, with the addition of tris-HCl (Sigma-Aldrich) to the reaction mixture, prior to the second digestion, to approximate the *Eco*R1 reaction buffer tris-HCl concentration. The cDNAs were separated from the digested plasmid DNA by electrophoresis on a 1 % low melting-point agarose gel (BDH Laboratory Supplies), using 0.5× tris borate electrophoresis buffer (TBE; pH 8.0) (*see Appendix I*). The cDNA inserts were stained with ethidium bromide (EtBr; BDH Laboratory Supplies) and identified under ultra violet (UV) light on the basis of size comparison with λ DNA size markers cut with *Eco*1301 and *Sty*I (MBI Fermentas). cDNA inserts were excised from the gel using a sterile scalpel blade and purified from the gel slices using a Bresa-Clean nucleic acid purification kit (GeneWorks).

cDNA probe labelling. cDNAs were radiolabelled with α-[^{32}P] dCTP (3,000 Ci·mmol^{-1}; GeneWorks), to a specific activity of 10^9 cpm·μg^{-1} or greater by the random priming method, using Klenow fragment (6.4 U·μl^{-1}; Pharmacia) and the Gigaprime DNA labelling kit (GeneWorks). Briefly, 2 μl of the cDNA solution (~100 ng of cDNA) and 7 μl of sterile water were added to a sterile 1.5 ml eppendorf tube (Eppendorf), then heat denatured by incubation in a water bath at 100 °C for 3 min. The tube was then placed on ice and spun briefly in a microfuge to collect the contents. Decanucleotide solution (6 μl; GeneWorks) was then added, followed by 6 μl of α-[^{32}P] dCTP and 1 μl of Klenow fragment. The labelling reaction was incubated in a water bath at 37 °C for 60 min and terminated by incubating at 65 °C for 5 min.

Oligonucleotide probes. A 30 mer antisense oligonucleotide probe for rat 18S rRNA, complementary to nucleotides 151-180 ([82]Chan, Gutell *et al.*, 1984; GenBank accession

number: 2624399), was synthesised (GeneWorks) and supplied as desalted, lyophilised stocks (*Figure 2.7*). The oligonucleotide probe was resuspended in sterile water and further purified by elution from NICK columns containing Sephadex G-50 (Pharmacia) with 10 mM TE buffer (pH 8.0) (*see Appendix I*).

Oligonucleotide probe labelling. Oligonucleotide probes were end-labelled with T4 polynucleotide kinase (T4PNK, 7.9 U·μl^{-1}; Pharmacia) and γ-[^{32}P] ATP (4,000 Ci·mmol^{-1}; GeneWorks) as substrate. Briefly, 3 μl of oligonucleotide solution (2 pmol·μl^{-1}) was added to a sterile 1.5 ml eppendorf tube, followed by 2 μl of 10× One-Phor-All buffer (Pharmacia), 0.5 μl of sterile water, 13.5 μl of γ-[^{32}P] ATP and 1 μl of T4PNK. The labelling reaction was mixed and incubated in a water bath at 37 °C for 60 min.

Purification of radiolabelled cDNA and oligonucleotide probes. cDNA and oligonucleotide probes were separated from unincorporated radionucleotides by elution from NICK columns containing Sephadex G-50 with 10 mM TE buffer (pH 8.0) (*see Appendix I*). Probe activity was determined by adding 2 μl of the labelled probe solution to 4 ml of aqueous counting scintillant (Amersham International) and counting the activity using an LS-3801 liquid scintillation β-counter (Beckman Coulter Australia).

2.2.3.2 Total RNA isolation

Total RNA was extracted from one adrenal from each of the eighteen fetal sheep in the late-gestation gene expression study, and from one adrenal per fetus in the Intact (n=6 fetuses), HPD (n=6 fetuses) and HPD+F (n=5 fetuses) groups of the fetal HPD study, using a modification of the method previously described by Chirgwin and colleagues ([90]Chirgwin, Przybyla *et al.*, 1979). Individual adrenals were homogenised in 3 ml of 4 M guanidine hydrochloride solution (*see Appendix I*) using a Polytron PT 3000 laboratory homogeniser with a PT-DA 3007/2 generator (Kinematica AG) at 30,000 rpm for approximately 20 sec (*Figure 2.8*). Cesium chloride (CsCl, 1.2 g; Boehringer Mannheim Australia) was added to the adrenal homogenate, which was layered onto a solution of 5.7 M CsCl in 100 mM EDTA in an 11 mm × 60 mm polyallomer centrifuge tube (Beckman Coulter Australia). The samples were ultracentrifuged for 16 h at 36,000 rpm (130,000 g) using an LE-80K Optima ultracentrifuge (Beckman Coulter Australia) equipped with an SW60 swing-bucket rotor (Beckman Coulter Australia).

Following ultracentrifugation the supernatant was decanted and tubes were air dried at room temperature (RT) for 5-10 min. RNA pellets were then resuspended in 100 μl of

A

```
   1   TACCTGGTTG ATCCTGCCAG TAGCATATGC TTGTCTCAAA GATTAAGCCA
  51   TGCATGTCTA AGTACGCACG GCCGGTACAG TGAAACTGCG AATGGCTCAT
 101   TAAATCAGTT ATGGTTCCTT TGGTCGCTCG CTCCTCTCCT ACTTGGATAA
 151   CTGTGGTAAT TCTAGAGCTA ATACATGCCG ACGGGCGCTG ACCCCCCTTC
 201   CCGTGGGGGG AACGCGTGCA TTTATCAGAT CAAAACCAAC CCGGTCAGCC
 251   CCCTCCCGGC TCCGGCCGGG GGTCGGGCGC CGGCGGCTTT GGTGACTCTA
 301   GATAACCTCG GGCCGATCGC ACGTCCCCGT GGCGGCGACG ACCCATTCGA
 351   ACGTCTGCCC TATCAACTTT CGATGGTAGT CGCCGTGCCT ACCATGGTGA
 401   CCACGGGTGA CGGGGAATCA GGGTTCGATT CCGGAGAGGG AGCCTGAGAA
 451   ACGGCTACCA CATCCAAGGA AGGCAGCAGG CGCGCAAATT ACCCACTCCC
 501   GACCCGGGGA GGTAGTGACG AAAAATAACA ATACAGGACT CTTTCGAGGC
 551   CCTGTAATTG GAATGAGTCC ACTTTAAATC CTTTAACGAG GATCCATTGG
 601   AGGGCAAGTC TGGTGCCAGC AGCCGCGGTA ATTCCAGCTC CAATAGCGTA
 651   TATTAAAGTT GCTGCAGTTA AAAAGCTCGT AGTTGGATCT TGGGAGCGGG
 701   CGGGCGGTCC GCCGCGAGGC GAGCTCACCG CCCTGTCCCC AGCCCCTGCC
 751   TCTCGGCGCC CCCTCGATGC TCTTAGCTGA GTGTCCCGCG GGGCCCGAAG
 801   CGTTTACTTT GAAAAAATTA GAGTGTTCAA AGCAGGCCCG AGCCGCCTGG
 851   ATACCGCAGC TAGGAATAAT GGAATAGGAC CGCGGTTCTA TTTTGTTGGT
 901   TTTCGGAACT GAGGCCATGA TTAAGAGGGA CGGCCGGGGG CATTCGTATT
 951   GCGCCGCTAG AGGTGAAATT CTTGGACCGG CGCAAGACGA ACCAGAGCGA
1001   AAGCATTTGC CAAGAATGTT TTCATTAATC AAGAACGAAA GTCGGAGGTT
1051   CGAAGACGAT CAGATACCGT CGTAGTTCCG ACCATAAACG ATGCCGACTG
1101   GCGATGCGGC GGCGTTATTC CCATGACCCG CCGGGCAGCT TCCGGGAAAC
1151   CAAAGTCTTT GGGTTCCGGG GGGAGTATGG TTGCAAAGCT GAAACTTAAA
1201   GGAATTGACG GAAGGGCACC ACCAGGAGTG GAGCCTGCGG CTTAATTTGA
1251   CTCAACACGG GAAACCTCAC CCGGCCCGGA CACGGACAGG ATTGACAGAT
1301   TGATAGCTCT TTCTCGATTC CGTGGGTGGT GGTGCATGGC CGTTCTTAGT
1351   TGGTGGAGCG ATTTGTCTGG TTAATTCCGA TAACGAACGA GACTCTCGGC
1401   ATGCTAACTA GTTACGCGAC CCCCGAGCGG TCGGCGTCCC CCAACTTCTT
1451   AGAGGGACAA GTGGCGTTCA GCCACCGAGA TTGAGCAATA ACAGGTCTGT
1501   GATGCCCTTA GATGTCCGGG GCTGCACGCG CGCTACACTG AACTGGCTCA
1551   GCGTGTGCCT ACCCTACGCC GGCAGGCGCG GGTAACCCGT TGAACCCCAT
1601   TCGTGATGGG GATCGGGGAT TGCAATTATT CCCCATGAAC GAGGAATTCC
1651   CAGTAAGTGC GGGTCATAAG CTTGCGTTGA TTAAGTCCCT GCCCTTTGTA
1701   CACACCGCCC GTCGCTACTA CCGATTGGAT GGTTTAGTGA GGCCCTCGGA
1751   TCGGCCCCGC CGGGGTCGGC CCACGGCCTT GCGGAGGCC TGAGAAGACG
1801   GTCGAACTTG ACTATCTAGA GGAAGTAAAA GTCGTAACAA GGTTTCCGTA
1851   GGTGAACCTG CGGAAGGATC ATTA
```

B

Figure 2.7   30 mer rat 18S rRNA antisense oligonucleotide probe, complementary to nucleotides 159-188.

(*A*) Rat 18S rRNA gene sequence. (*B*) Sequence of the 30 mer rat 18S rRNA antisense oligonucleotide probe.

BREAKING CELLS AND TISSUES

The first step in the purification of RNA is to disrupt tissues and cells in a controlled fashion. Using gentle mechanical homogenisation, the plasma membranes of cells can be ruptured to release the cell contents.

The resulting homogenate contains large and small molecules from the cytosol, such as enzymes, RNA and metabolites, as well as all the membrane-bounded organelles.

tissue in
4 M guanidine
hydrochloride solution

shear cells with a
rotating generator
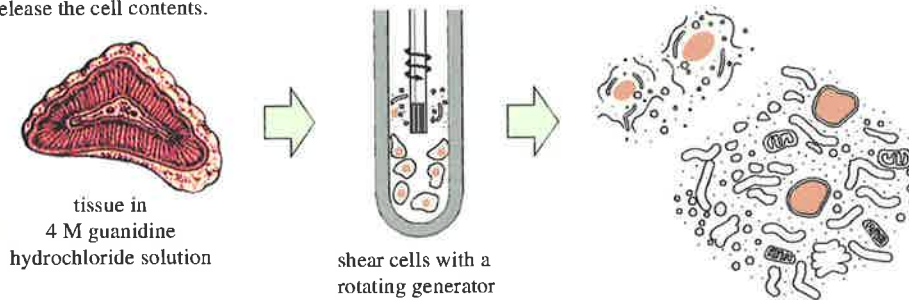
EQUILIBRIUM SEDIMENTATION

The ultracentrifuge is used to separate cellular components on the basis of their buoyant density. The sample is layered on top of a 5.7 M cesium chloride cushion. Each subcellular component will move up or down when centrifuged until it reaches its buoyant density. A series of distinct bands will eventually be produced, with those nearest the bottom of the tube containing the components of highest density.

THE CENTRIFUGE

armored chamber

swing bucket
rotor

sedimenting
material

refrigeration   vacuum
motor

swing bucket rotor

centrifugation

tube

metal bucket

The metal buckets that hold the tubes are free to swing outward as the rotor turns.
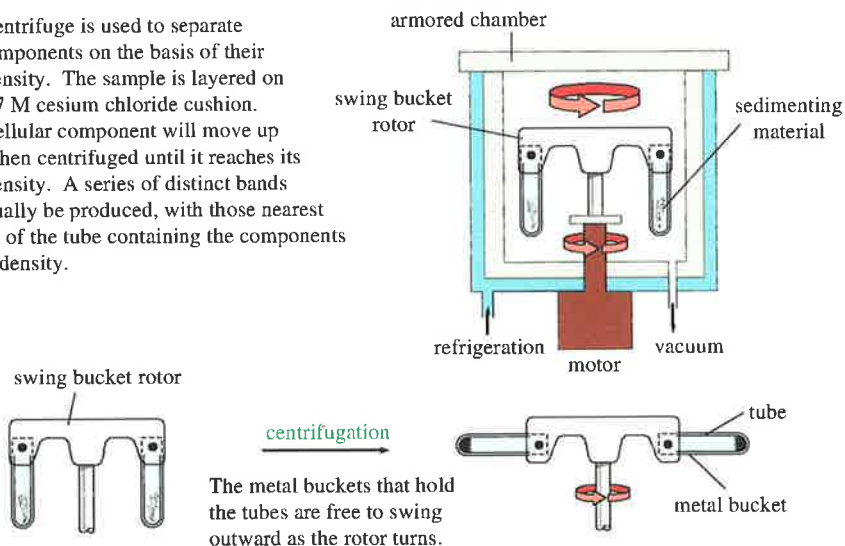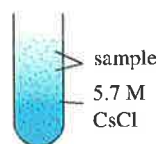
START   **BEFORE EQUILIBRIUM**   **EQUILIBRIUM**

centrifugation

centrifugation

low density
component

sample

5.7 M
CsCl
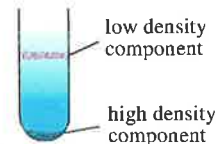
At equilibrium,
components have
migrated to a region
in the gradient that
matches their own density

high density
component

Figure 2.8   Tissue homogenisation and RNA extraction from ovine fetal adrenals.

sterile water and transferred to sterile 1.5 ml eppendorf tubes. Total adrenal RNA was further purified by addition of 0.1× and 2.5× volumes of 3 M sodium acetate (Ajax Chemicals) and cold absolute ethanol (Chem-Supply) respectively, and precipitation on dry ice for 30 min. The precipitated RNA was pelleted by centrifugation at 12,000 g for 30 min at 4 °C. The supernatant was aspirated and the pellet dried using a SpeedVac SC110 vacuum centrifuge concentrator (Savant Instruments) for 10-15 min. Dried pellets were resuspended in a volume of sterile distilled water estimated to give a final concentration of approximately 5 $\mu g \cdot \mu l^{-1}$ of total RNA. Maximum absorbance at OD_{260} nm and OD_{280} nm was determined using a Beckman DU-50 spectrophotometer to quantify nucleic acid purity and concentration.

Prior to Northern or slot blot analysis, the integrity of total RNA preparations was verified by subjecting 1 μl of each RNA sample to gel electrophoresis in electrophoresis tanks (Owl Scientific Plastics). Samples were run on 30 ml, 7 cm × 8 cm 1 % agarose denaturing gels (*see Appendix I*) with molecular biology grade agarose (BDH Laboratory Supplies), in 1× tris-acetate EDTA (TAE; pH 8.0) (*see Appendix I*) as the running buffer, and stained with EtBr.

2.2.3.3   Northern and slot blot analysis

For Northern blots, total RNA samples (20 μg of adrenal RNA in 5.5 μl of sterile water) were made up to 20 μl with 3.5 μl deionised formaldehyde (APS Ajax Finechem), 10 μl deionised formamide (BDH Laboratory Supplies) and 1.0 μl 20× Northern running buffer (*see Appendix I*) and denatured at 55 °C for 15 min. Samples were chilled on ice and 2 μl of 1× RNA loading buffer (*see Appendix I*) was added to the samples. Total RNA was separated by gel electrophoresis in 100 ml, 12 cm × 14 cm 1 % agarose denaturing gels (*see Appendix I*), using 1× Northern running buffer (*see Appendix I*). To determine the size of RNA detected by Northern hybridisation, one lane of the gel was reserved for 0.5-9 kb RNA Millennium size markers (Ambion) (*Figure 2.9A*). Electrophoresis was carried out at 70 V for 4-6 h, in electrophoresis tanks using a peristaltic Miniplus 3 pump (Gilson) to circulate the Northern running buffer. Following electrophoresis, the RNA size marker lane was cut off, stained with EtBr and photographed. Total RNA was then transferred overnight by capillary blotting to Zetaprobe nitrocellulose membranes (Biorad Laboratories) using Teri wipers (Kimberly-Clark) and 10× SSC (*see Appendix I*) as the transfer buffer (*Figure 2.9B*). For slot blots, RNA (10 μg of adrenal RNA in 5.5 μl of sterile water) was made up to 20 μl in a solution containing 6.15 M formaldehyde and 10× SSC, and denatured by incubation in a water bath at 55 °C for 15 min. Samples were

(A) RNA FRAGMENTS SEPARATED BY AGAROSE GEL ELECTROPHORESIS

unlabelled total RNA

electrophoresis

RNA fragments of known sizes used as molecular weight marker

stain RNA markers with EtBr and photograph prior to blotting

agarose gel

(B) RNA FRAGMENTS BLOTTED ONTO NITROCELLULOSE MEMBRANE

dry stack of paper towels

nitrocellulose membrane

agarose gel

saturated stack of Teri wipers

10x SSC solution

(F) BAND DENSITY QUANTITATED AND TRANSCRIPT SIZES CONFIRMED

position of stained markers on photograph confirm size of transcripts

X-ray film or phosphorimage

labelled bands on autoradiograph or phosphorimage screen

(C) RNA FIXED ON NITROCELLULOSE MEMBRANE WITH UV LIGHT

remove nitrocellulose membrane with bound RNA and cross-link to membrane with UV light

agarose gel

(E) NITROCELLULOSE MEMBRANE EXPOSED TO X-RAY FILM OR PHOSPHORIMAGE SCREEN

sealed cassette stored at -70 °C for X-ray film

X-ray film or phosphorimage screen

labelled nitrocellulose membrane

(D) LABELLED cDNA PROBE HYBRIDISED TO COMPLEMENTARY RNA BANDS

sealed plastic bag

labelled cDNA or oligonucleotide probe in hybridisation buffer

Figure 2.9   Gel electrophoresis and Northern blot of fetal adrenal RNA.

(*A*) Adrenal RNA is separated by gel electrophoresis in a 1 % agarose formaldehyde gel. RNA molecular weight markers are run in the gel and photographed. (*B*) RNA fragments are blotted onto a nitrocellulose membrane in 10× SSC. (*C*) RNA is fixed to the membrane by exposure to UV light. (*D*) Membranes are prehybridised in hybridisation buffer prior to the addition of radiolabelled cDNA or oligonucleotide probes. (*E*) Membranes are washed, sealed in plastic and exposed to X-ray film or phosphorimage plates. (*F*) Images are quantified and transcript sizes confirmed with RNA markers.

chilled on ice and applied to a Zetaprobe nitrocellulose membrane via a slot-blot apparatus (Hoefer Scientific) using 10× SSC as the transfer buffer.

Membranes were washed in 10× SSC, 0.1 % sodium dodecyl sulfate (SDS; BDH Laboratory Supplies) for 10 min at RT and baked for 1 h at 80 °C or exposed to UV light [12 sec at 120 Joules(J)·cm^{-2}] (*Figure 2.9C*), prior to overnight incubation at 42 °C in 30 ml of either cDNA or antisense oligonucleotide hybridisation buffer (*see Appendix I*). Membranes were then hybridised sequentially with cDNA or oligonucleotide probes for 16 h (42 °C for cDNA probes or 50 °C for oligonucleotide probes) in 30 ml of fresh hybridisation buffer, containing either 1-2×10^6 cpm·ml^{-1} of the cDNA probe or 5×10^5 cpm·ml^{-1} of the 30 mer antisense 18S rRNA oligonucleotide probe (*Figure 2.9D*). Prior to exposure to X-AR film (Kodak Australasia) or phosphorimage plates (Berthold Australia), membranes were washed once (10 min) at RT in 1× SSC, 0.1 % SDS; then twice (10 min each time) in 1× SSC, 0.1 % SDS at 42 °C; and twice (10 min each time) in 0.1× SSC, 0.1 % SDS at 42 °C, then briefly air-dried and sealed in a plastic bag.

Membranes were exposed either to X-AR film at -70 °C in an X-ray cassette fitted with intensifying screens, or to phosphorimage plates in BAS 2040 cassettes (Berthold Australia) (*Figure 2.9E*). cDNA probes were stripped from membranes between hybridisations by washing in 0.01× SSC, 0.5 % SDS for 10 min at 80 °C. Consistency of lane or slot loading for each membrane was verified by a final hybridisation of each membrane with 5×10^5 cpm·ml^{-1} of the 30 mer antisense 18S rRNA oligonucleotide probe and exposure to X-AR film for the late-gestation gene expression study and phosphorimage plates for the fetal HPD study. Autoradiograph exposures on X-AR film were quantified on a scanning laser densitometer using ImageQuant software (Molecular Dynamics). Phosphorimage plate exposures were quantified on a Fuji-BAS 1000 phosphorimage scanner using Fuji MacBAS software (MacBAS 2.2; Berthold Australia) (*Figure 2.9F*). A ratio of the density of each specific band with the density of the corresponding 18S rRNA band was calculated before comparisons were made.

Study 2. Late-gestation gene expression. Radiolabelled *h*CYP11A1, *h*CYP17, *h*CYP21A1, *h*3βHSD and *b*IGFBP-2 cDNAs were used to probe three Northern blots of fetal adrenal total RNA from the late-gestation gene expression study. The *o*IGF-II cDNA was used to probe a Northern and slot blot of fetal adrenal total RNA from this study. Northern and slot blots of fetal adrenal RNA were exposed to X-AR film at -70 °C in an X-ray cassette fitted with intensifying screens. One Northern blot membrane was hybridised sequentially with the *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNA probes, while the second membrane

was hybridised with the *b*IGFBP-2 cDNA probe. A third Northern blot membrane was hybridised separately with *h*3βHSD and *o*IGF-II cDNA probes. Slot blot analysis was used to quantify the relative abundance of total IGF-II mRNA transcripts in the late-gestation gene expression study.

Study 3. Fetal HPD. Radiolabelled *h*CYP11A1, *h*CYP17, *h*CYP21A1, *h*3βHSD and *o*IGF-II cDNAs were used to probe two Northern blots of fetal adrenal total RNA from the fetal HPD study. One Northern blot of fetal adrenal RNA was hybridised with *h*CYP11A1 and *h*3βHSD cDNA probes and exposed to X-AR film at -70 °C in a cassette fitted with intensifying screens. The second Northern blot of fetal adrenal RNA was hybridised with *h*CYP17, *h*CYP21A1 and *o*IGF-II cDNA probes and exposed to phosphorimage plates.

2.2.4   *Statistical analysis*

Study 1. Gestational profile of adrenal gland weight. Data from 422 fetuses from 42-147 d of gestation, including total adrenal weight (the sum of the weights of the left and right adrenals; mg), and the ratio of adrenal : fetal body weight (mg·kg^{-1}), were used to calculate regression equations. Logarithmic transformations of both dependent (total adrenal weight and the ratio of adrenal : fetal body weight) and independent (gestational age and fetal weight) variables best described associations between variables. Multiple linear regression analysis revealed a significant interaction between the independent variables log[gestational age] and log[fetal weight], when either log[adrenal weight] or log[adrenal : fetal body weight] were chosen as the dependent variable. Thus, simple linear regression models were used to assess correlations between log[adrenal weight] versus (vs.) log[gestational age] or log[fetal weight] separately, and between log[adrenal : fetal body weight] vs. log[gestational age] or log[fetal weight]. Rearrangement of the linear regression equations yielded power relationships between the dependent and independent variables, which are used for comparison with those in different experimental studies described throughout this thesis.

Studies 2 and 3. Late-gestation gene expression and fetal HPD. Data are presented as mean ± standard error of the mean (SEM) for the late-gestation gene expression and fetal HPD studies. In the late-gestation gene expression study, the total adrenal weight, the ratio of adrenal : fetal body weight and the relative levels of adrenal IGF-II, IGFBP-2 and steroidogenic enzyme mRNA : 18S rRNA were compared at 130-135 d, 136-140 d and 141-145 d of gestation using one-way Analysis of Variance (ANOVA) and Duncan's multiple range post-hoc test. In the fetal HPD study, plasma concentrations of ir-ACTH were compared using a two-way ANOVA with repeated measures and treatment group

(*e.g.* Intact, HPD and HPD+F) and age group (*e.g.* 130-134 d and 135-140 d of gestation) as the specified factors. Mean plasma concentrations of cortisol and ACTH(1-39) were calculated for each fetus between 135 d and 140 d of gestation and these mean hormone concentrations were compared between the three treatment groups using a one-way ANOVA. Similarly, total adrenal weight, the ratio of total adrenal : fetal body weight and the relative levels of adrenal steroidogenic enzymes and IGF-II mRNA : 18S rRNA were also compared between the three treatment groups using a one-way ANOVA. Where the ANOVA identified significant differences between the groups, Duncan's multiple range post-hoc test was used to identify differences between mean values. A probability of $<5\%$ ($p<0.05$) was taken to be significant.

2.3   RESULTS

2.3.1   Study 1. Gestational profile of fetal adrenal gland weight

Total adrenal weight and the ratio of adrenal : fetal body weight. 422 fetal sheep were used to construct gestational age profiles of the total adrenal weight and the ratio of adrenal : fetal body weight (*Figure 2.10A and 2.10B*). Total adrenal weight (mg) increased with both gestational age (days) and fetal body weight (kg) (*Figure 2.11A*). In contrast to total adrenal weight, the ratio of adrenal : fetal body weight (mg·kg^{-1}) decreased with increasing gestation and fetal body weight (*Figure 2.12A*). Plotting these data on logarithmic scales demonstrates the changes in total adrenal weight and the ratio of adrenal : fetal body weight with both gestational age and fetal body weight (*Figures 2.11B and 2.12B*). The increase in adrenal weight and decrease in the ratio of adrenal : fetal body weight with increasing gestational age is described by the equations:

$$\log[\text{Adrenal weight}] = 2.98733 \times \log[\text{Gestational age}] - 3.81341 \quad\quad (2.1)$$
($r=0.92$, $p<0.000005$)

$$\log[\text{Adrenal : Fetal weight}] = -1.90883 \times \log[\text{Gestational age}] + 6.05219 \quad\quad (2.2)$$
($r=0.84$, $p<0.000005$)

The increase in adrenal weight and decrease in the ratio of adrenal : fetal body weight with increasing fetal weight is described by the equations:

$$\log[\text{Adrenal weight}] = 0.60582 \times \log[\text{Fetal weight}] + 2.20776 \quad\quad (2.3)$$
($r=0.94$, $p<0.000005$)

$$\log[\text{Adrenal : Fetal weight}] = -0.39417 \times \log[\text{Fetal weight}] + 2.20776 \quad\quad (2.4)$$
($r=0.87$, $p<0.000005$)
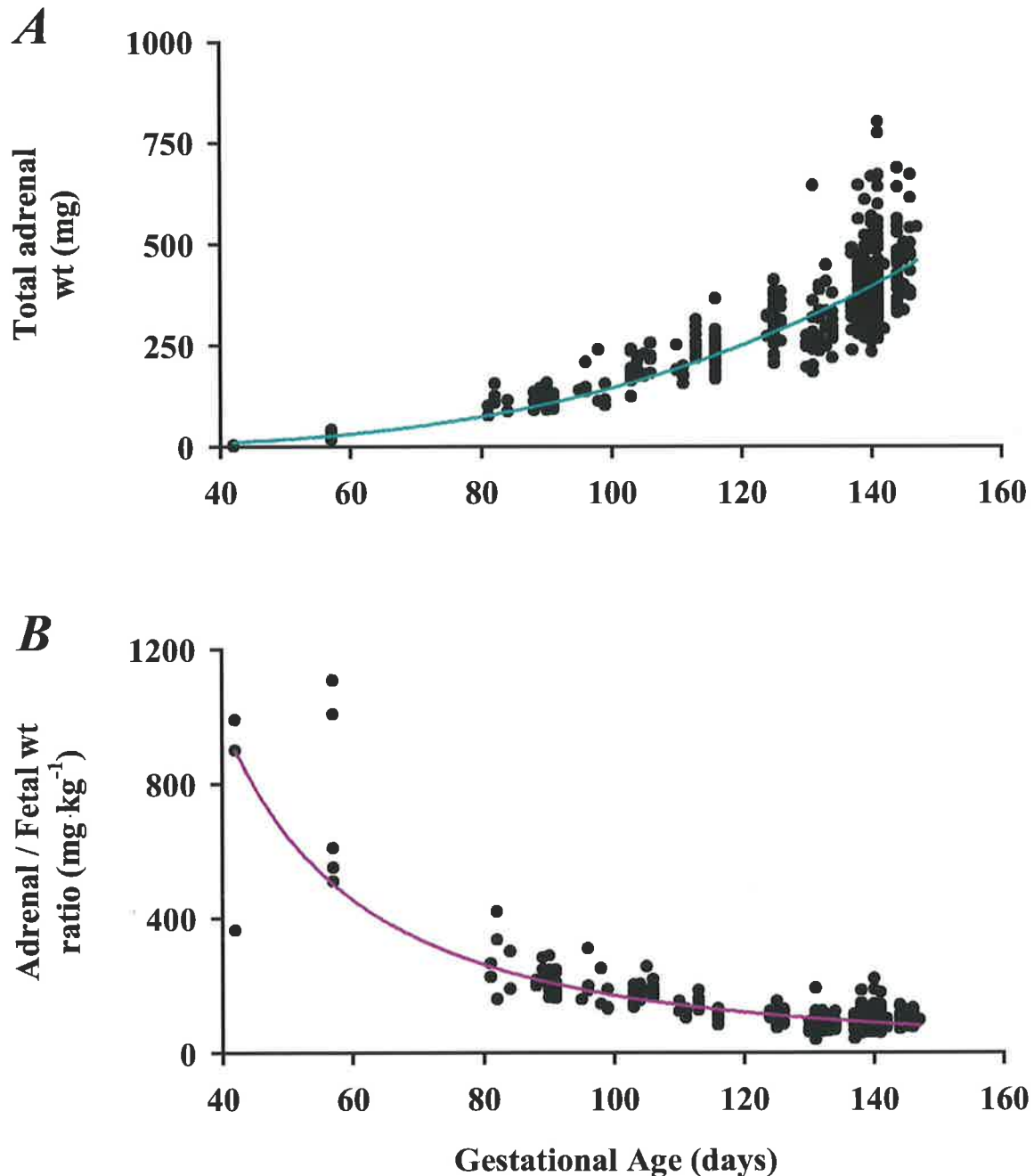
Figure 2.10   **Gestational profile of total adrenal weight and the ratio of adrenal : fetal body weight in fetuses between 42-147 d of gestation.**

(*A*) Total adrenal weight of control fetuses (●) increases with increasing gestational age, according to the equation: **[Adrenal weight] = 10$^{-3.81341}$ × [Gestational age]$^{2.98733}$** (R^2=0.85), (*see equations 2.1 and 2.6 on pp. 91 and 95*). (*B*) The ratio of adrenal : fetal body weight decreases until ~120 d of gestation as fetal growth exceeds that of the adrenal until mid-gestation, and then is matched by adrenal growth in late-gestation. The regression equation: **[Adrenal : Fetal weight] = 10$^{6.05219}$ × [Gestational age]$^{-1.90883}$** (R^2=0.70), describes this relationship (*see equations 2.2 and 2.7 on pp. 91 and 95*).
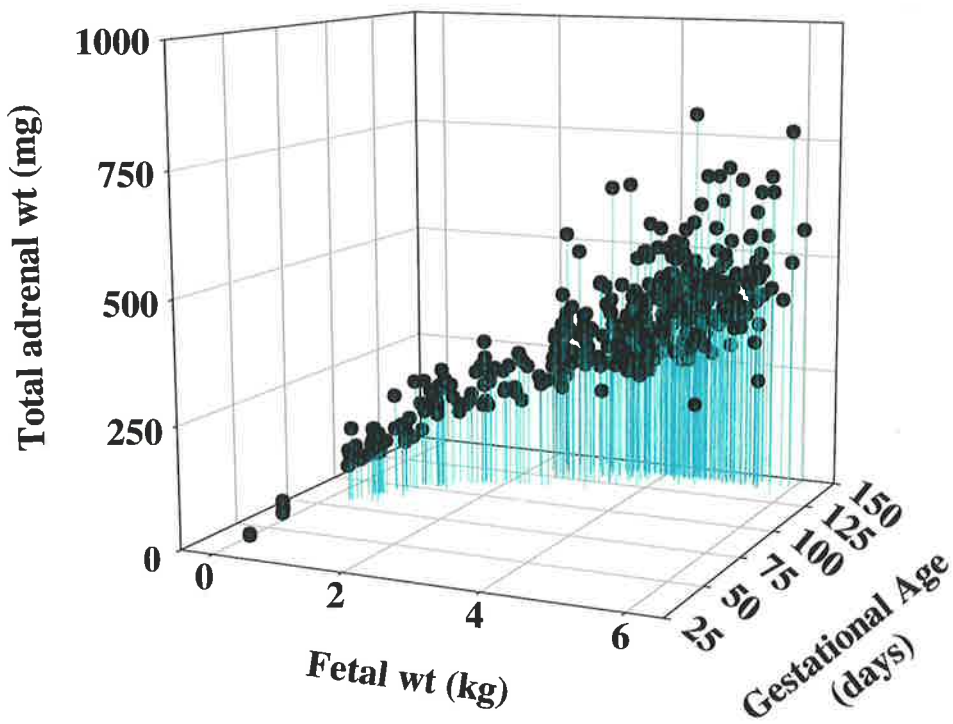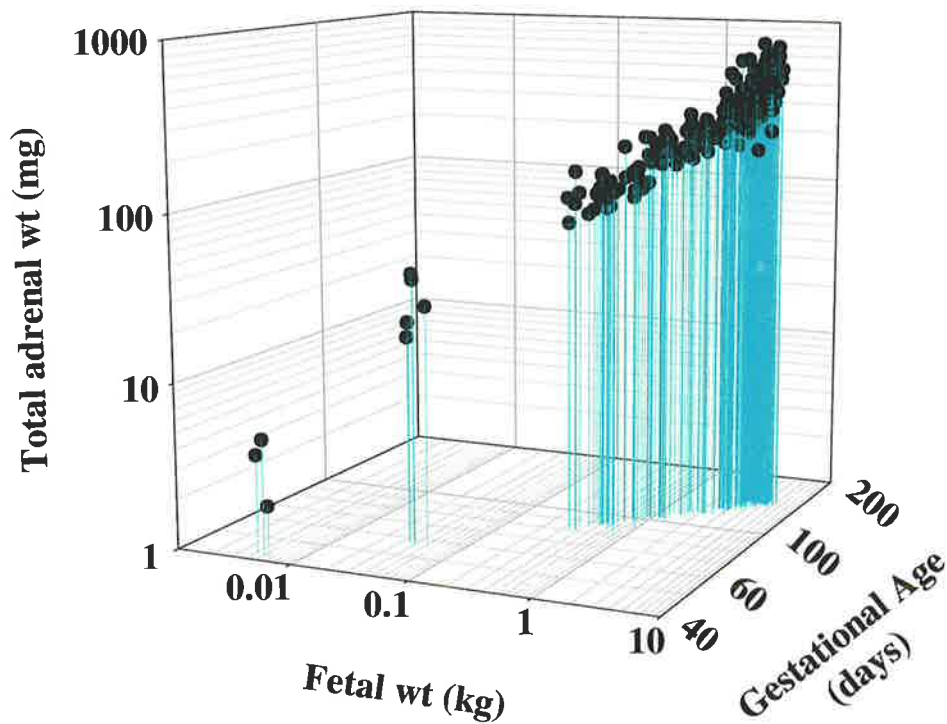
Figure 2.11   **Gestational age and fetal body weight profiles of total adrenal weight in fetuses between 42-147 d of gestation.**

(*A*) Total adrenal weight of control fetuses (●) increases with increasing gestational age and fetal body weight. (*B*) When data are plotted on logarithmic axes, significant linear associations are observed between paired variables (*see equations 2.1, 2.3 and 2.5 on pg. 91*).
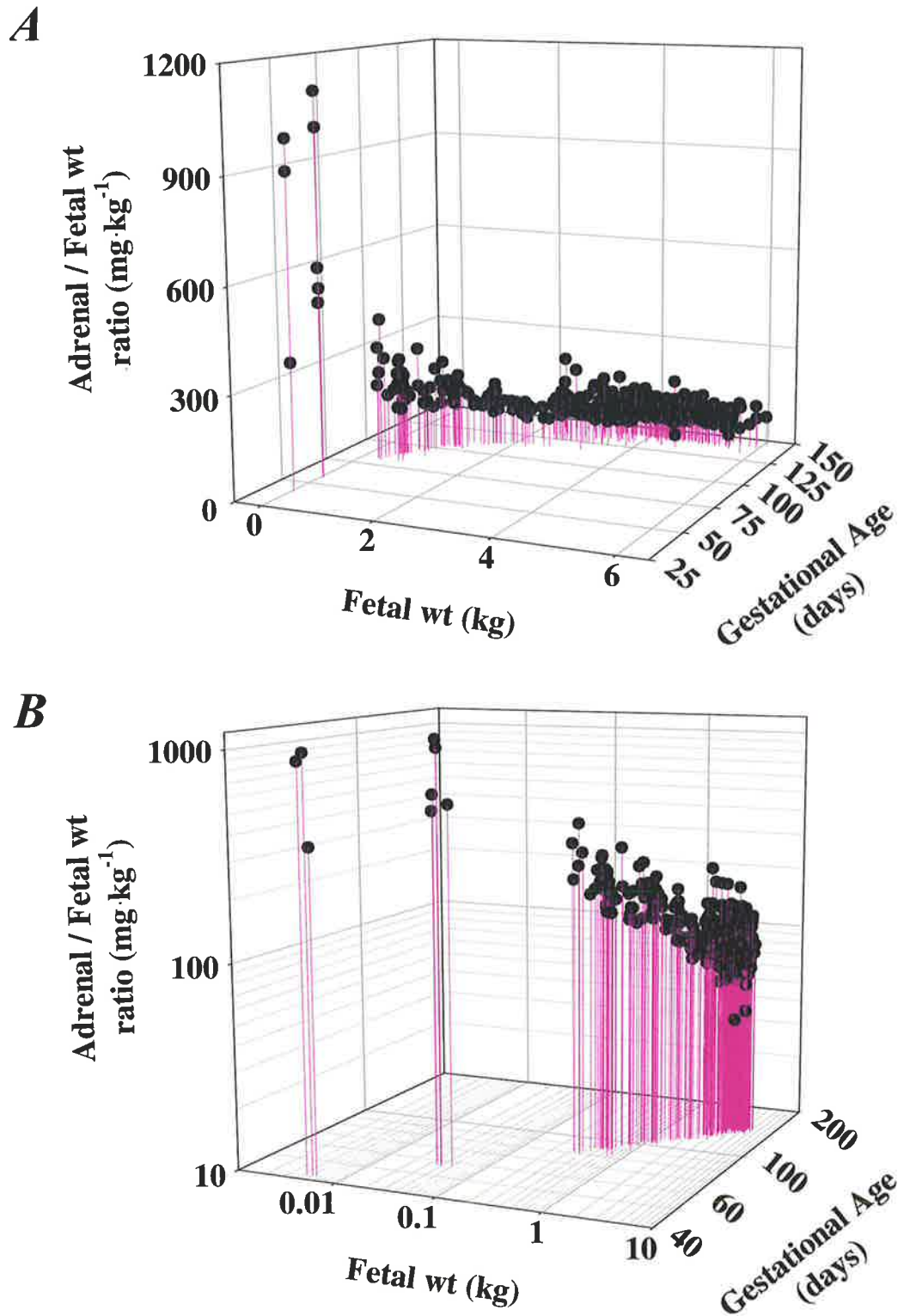
Figure 2.12   **Gestational age and fetal body weight profiles of the ratio of adrenal : fetal body weight in fetuses between 42-147 d of gestation.**

(*A*) The ratio of adrenal : fetal body weight in control fetuses (●) decreases with increasing gestational age and fetal body weight. (*B*) When data are plotted on logarithmic axes, significant linear associations are observed between paired variables (*see equations 2.2, 2.4 and 2.5 on pg. 91*).

Finally, fetal weight increases with increasing gestational age according to the equation:

$$\log[\text{Fetal weight}] = 4.89618 \times \log[\text{Gestational age}] - 9.86563 \qquad (2.5)$$

(r=0.98, p<0.000005)

Algebraic rearrangement of these linear regression equations yields power relationships between the untransformed dependent and independent variables as follows:

$$[\text{Adrenal weight}] = 10^{-3.81341} \times [\text{Gestational age}]^{2.98733} \qquad (R^2=0.85)\ (2.6)$$

$$[\text{Adrenal : Fetal weight}] = 10^{6.05219} \times [\text{Gestational age}]^{-1.90883} \qquad (R^2=0.70)\ (2.7)$$

$$[\text{Adrenal weight}] = 10^{2.20776} \times [\text{Fetal weight}]^{0.60582} \qquad (R^2=0.88)\ (2.8)$$

$$[\text{Adrenal : Fetal weight}] = 10^{2.20776} \times [\text{Fetal weight}]^{-0.39417} \qquad (R^2=0.75)\ (2.9)$$

$$[\text{Fetal weight}] = 10^{-9.86563} \times [\text{Gestational age}]^{4.89618} \qquad (R^2=0.96)\ (2.10)$$

2.3.2   *Study 2. Late-gestation gene expression*

Adrenal weights, IGF-II and IGFBP-2 mRNA expression. Total adrenal weight increased significantly from 278 ± 17 mg (130-135 d; n=6 fetuses) to 359 ± 29 mg (136-140 d; n=6 fetuses) (p<0.05) and reached 392 ± 32 mg at 141-145 d of gestation (n=6 fetuses) (*Figure 2.13A*). The ratio of adrenal : fetal body weight, in contrast, did not change significantly across this period of gestation (130-135 d: 80.9 ± 3.5 mg·kg^{-1}; 136-140 d: 75.4 ± 4.2 mg·kg^{-1}; 141-145 d of gestation: 84.6 ± 4.1 mg·kg^{-1}) (*Figure 2.13B*). The *o*IGF-II cDNA probe identified six IGF-II mRNA transcripts in the size range 6.0 to 1.8 kb by Northern blot analysis of total RNA from the fetal adrenals. Slot blot analysis was used to quantitate the relative abundance of total IGF-II mRNA transcripts in adrenal total RNA in each gestational age group (*Figure 2.13C*). While total fetal adrenal weight increased in late-gestation, the ratio of adrenal IGF-II mRNA : 18S rRNA expression did not change across this gestational age range (130-135 d: 0.28 ± 0.02; 136-140 d: 0.32 ± 0.05; 141-145 d of gestation: 0.28 ± 0.03). A single transcript of ~1.4 kb was observed in Northern blots of fetal adrenal total RNA probed with the *b*IGFBP-2 cDNA. In contrast to the profile of IGF-II mRNA abundance, the adrenal expression of IGFBP-2 mRNA decreased significantly during this 15 d period. The ratio of adrenal IGFBP-2 mRNA : 18S rRNA expression was markedly lower (p<0.05) by 136-140 d, compared to the level at 130-135 d, and remained low during the subsequent 5 d period from 141-145 d of gestation (130-135 d: 22.1 ± 3.5; 136-140 d: 11.1 ± 0.6; 141-145 d of gestation: 10.3 ± 0.6) (*Figure 2.13D*).

CYP11A1 and CYP21A1. A single specific hybridisation signal (1.9 kb) was detected for adrenal CYP11A1 mRNA (*Figure 2.14*). Hybridisation signals of ~1.8 kb and 2.2 kb were observed for CYP21A1 mRNA at all ages (*Figure 2.14*). Adrenal expression of mRNA for

A Total adrenal weight (mg)

B Adrenal / Fetal wt ratio (mg·kg⁻¹)

C IGF-II mRNA : 18S rRNA

D IGFBP-2 mRNA : 18S rRNA

Gestational Age (days)

130-135   136-140   141-145
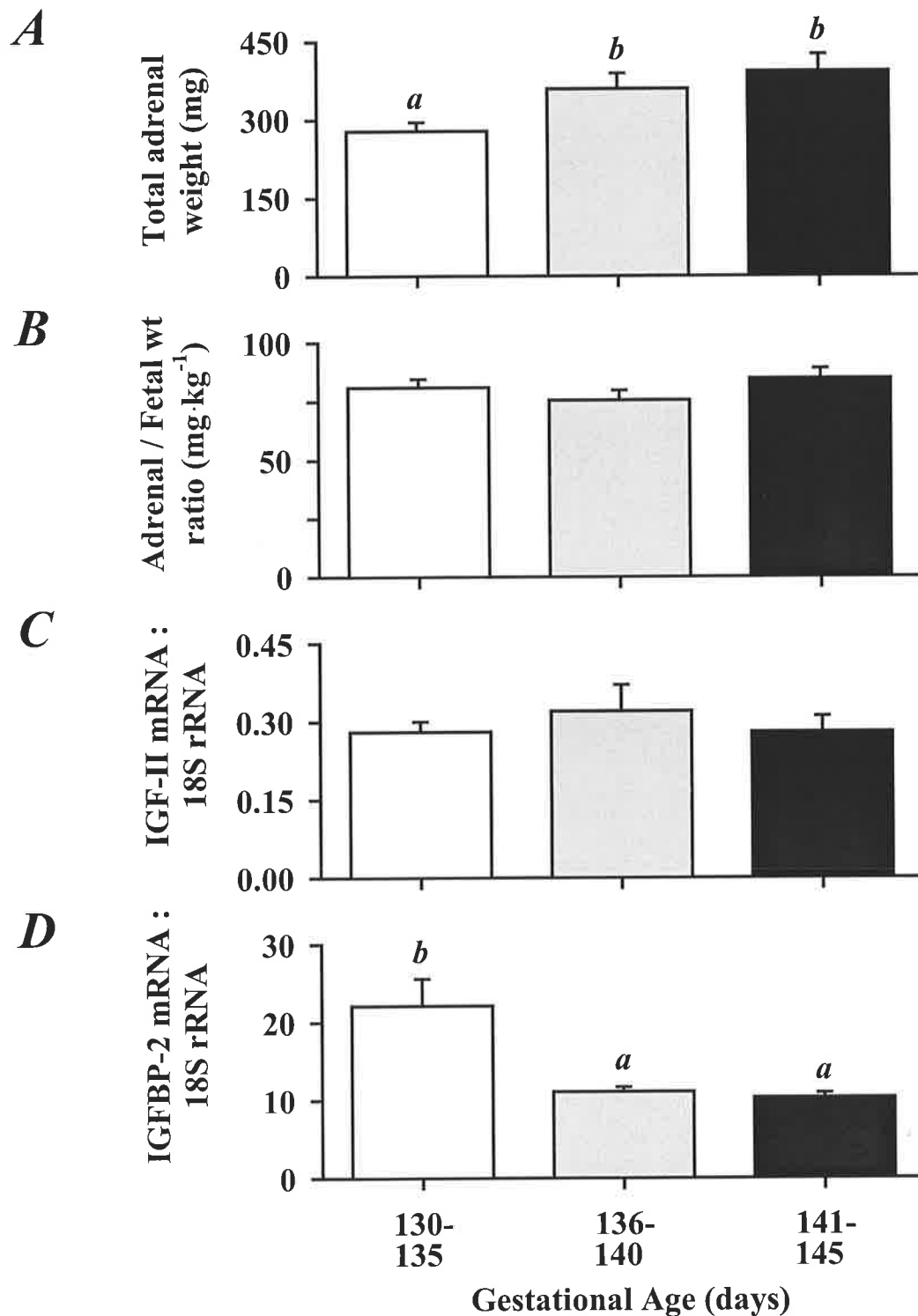
Figure 2.13   Ontogeny of total adrenal weight, adrenal : fetal body weight ratio, adrenal IGF-II and IGFBP-2 mRNA expression in the late-gestation ovine fetus.

(*A*) Total adrenal weight, (*B*) the ratio of adrenal : fetal body weight, (*C*) the ratio of IGF-II mRNA : 18S rRNA and (*D*) the ratio of IGFBP-2 mRNA : 18S rRNA in fetal adrenals collected between 130-145 d of gestation. Significant differences (p<0.05) between the different gestational ages are denoted by different superscripts, *e.g.* a < b (p<0.05).
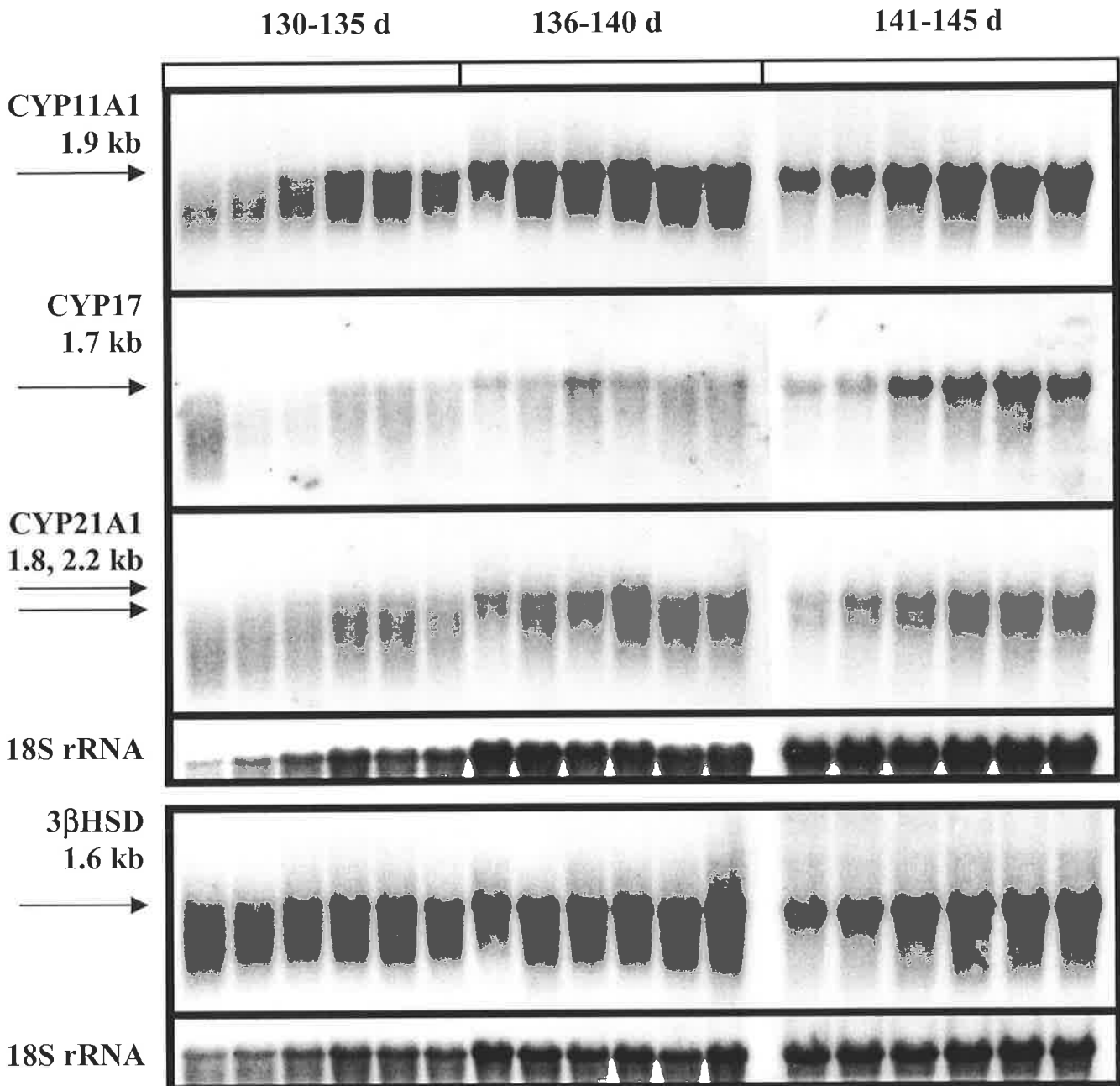
Figure 2.14   Northern blot of adrenal steroidogenic enzyme mRNA expression from fetal sheep during late-gestation.

Northern blot analyses of total RNA (20 µg·lane^{-1}) prepared from adrenal glands of fetal sheep between 130-145 d of gestation, and hybridised sequentially with the radiolabelled *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNA and the 30 mer antisense 18S rRNA oligonucleotide probes. A second Northern blot was probed with the radiolabelled *h*3βHSD cDNA and the 30 mer antisense 18S rRNA oligonucleotide probes.

both CYP11A1 and CYP21A1 increased between 130-135 d (CYP11A1, 0.19 ± 0.06; CYP21A1, 0.14 ± 0.01) and 136-140 d of gestation (CYP11A1, 0.68 ± 0.06; CYP21A1, 0.25 ± 0.03) (p<0.05). The adrenal expression of mRNA for these steroidogenic enzymes remained high after 140 d of gestation (141-145 d of gestation: CYP11A1, 0.65 ± 0.07; CYP21A1, 0.28 ± 0.03) (*Figure 2.15A and 2.15D*).

CYP17. A single hybridisation signal was detected for adrenal CYP17 mRNA (1.7 kb) (*Figure 2.14*). The relative abundance of adrenal CYP17 mRNA remained constant between 130-135 d (0.09 ± 0.01) and 136-140 d of gestation (0.12 ± 0.02) and increased significantly after 140 d of gestation (141-145 d of gestation: 0.32 ± 0.06) (p<0.05) (*Figure 2.15B*).

3βHSD. A single hybridisation signal (1.6 kb) for adrenal 3βHSD mRNA was detected (*Figure 2.14*). The relative abundance of adrenal 3βHSD mRNA in the adrenal did not change between 130-145 d of gestation (130-135 d: 1.18 ± 0.17; 136-140 d: 1.22 ± 0.10; 141-145 d of gestation: 1.14 ± 0.16) (*Figure 2.15C*).

2.3.3 Study 3. Fetal HPD

Fetal plasma cortisol concentrations. Plasma cortisol concentrations were higher (p<0.05) at 135-139 d of gestation in the Intact group (18.7 ± 3.8 nmol·l^{-1}) than the HPD group (3.5 ± 1.6 nmol·l^{-1}). Plasma cortisol concentrations in the HPD+F group, however, were higher (p<0.05) during the period of cortisol infusion (97.4 ± 23.7 nmol·l^{-1}), than in either the Intact or HPD groups.

Fetal plasma ir-ACTH and ACTH(1-39) concentrations. There were no differences in the plasma concentrations of ir-ACTH in the Intact (116 ± 12 pg·ml^{-1}), HPD (103 ± 16 pg·ml^{-1}) and HPD+F (96 ± 9 pg·ml^{-1}) groups at 130-134 d of gestation. Plasma ir-ACTH concentrations did not change between 130-134 d and 135-140 d of gestation in any group, *i.e.* there was no significant effect of either saline or cortisol infusion on plasma ir-ACTH concentrations in the Intact (122 ± 4 pg·ml^{-1}), HPD (106 ± 20 pg·ml^{-1}) or HPD+F (93 ± 10 pg·ml^{-1}) groups after 135 d of gestation. Finally, there were no significant differences between the plasma concentrations of ACTH(1-39) in the Intact (4.8 ± 0.4 pmol·l^{-1}), HPD (4.1 ± 0.3 pmol·l^{-1}) and HPD+F (4.1 ± 0.6 pmol·l^{-1}) groups between 135-140 d of gestation, *i.e.* during the saline or cortisol infusion.

Adrenal weights and IGF-II mRNA expression. Total adrenal weight was significantly greater (p<0.05) in the Intact (351.7 ± 21.2 mg) and HPD+F groups (318.0 ± 20.1 mg) than
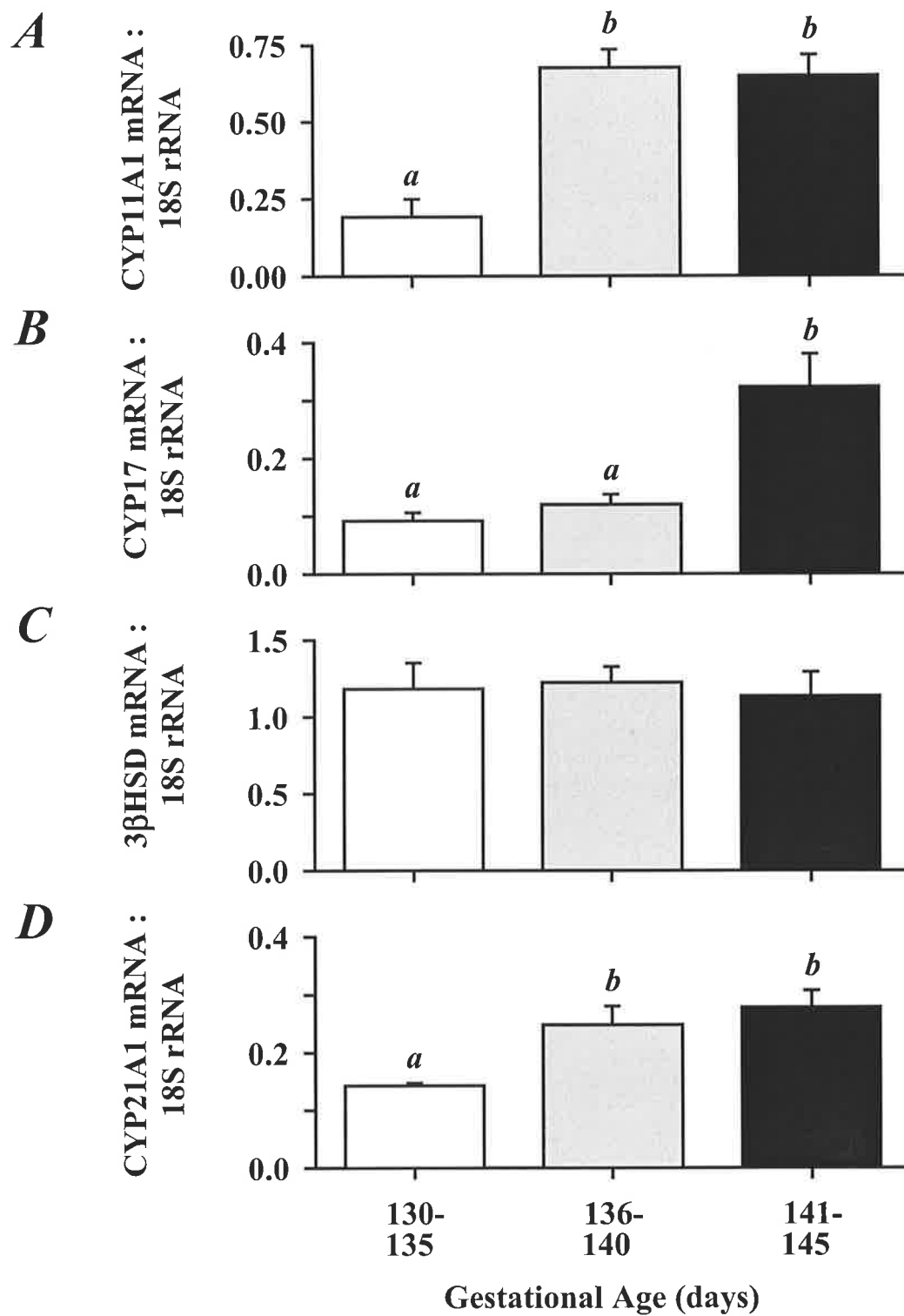
Figure 2.15   **Ontogeny of adrenal steroidogenic enzyme mRNA expression in the sheep fetus during late-gestation.**

The ratios of (*A*) CYP11A1, (*B*) CYP17, (*C*) 3βHSD and (*D*) CYP21A1 mRNA : 18S rRNA in fetal adrenals collected between 130-145 d of gestation. Significant differences (p<0.05) between the ratios at different gestational ages are denoted by different superscripts, *e.g.* a < b (p<0.05).

in the HPD group (259.4 ± 16.9 mg) (*Figure 2.16A*). The ratio of the adrenal : fetal body weight was also significantly greater (p<0.05) in the Intact group (84.1 ± 5.6 mg·kg^{-1}) and HPD+F group (88.4 ± 8.7 mg·kg^{-1}) than in the HPD group (63.7 ± 5.4 mg·kg^{-1}) (*Figure 2.16B*). These measures of adrenal growth in HPD fetuses are also shown with respect to the gestational profiles of adrenal weight and the ratio of adrenal : fetal body weight between 100 d and 147 d of gestation (*from Study 1*) for comparison (*Figure 2.17A and 2.17B*). On the basis of weight, the power relationship:

[Adrenal weight] $= 10^{-3.81341} \times$ **[Gestational age]**$^{2.98733}$

(*equation 2.6 on pg. 95, see also Figure 2.10A on pg. 92*)

indicates that the weight of the adrenal glands in HPD fetuses are equivalent to those of adrenals in intact fetuses at approximately 114-128 d of gestation (*see Figure 2.17A*).

Six transcripts in the size range 1.8-6.0 kb were detected with the *o*IGF-II cDNA probe on Northern blots of total RNA from the fetal adrenal glands. The ratio of adrenal IGF-II mRNA : 18S rRNA was similar in the Intact (0.48 ± 0.09), HPD (0.78 ± 0.09) and HPD+F (0.71 ± 0.11) groups (*Figure 2.16C*).

Adrenal steroidogenic enzyme mRNA expression. The ratios of CYP11A1, 3βHSD and CYP21A1 mRNA : 18S rRNA were significantly lower (p<0.05) in adrenals from the HPD group (CYP11A1: 0.14 ± 0.04; 3βHSD: 0.09 ± 0.01; CYP21A1: 0.46 ± 0.06) than those in the Intact group (CYP11A1: 0.37 ± 0.07; 3βHSD: 0.18 ± 0.02; CYP21A1: 0.81 ± 0.12) (*Figure 2.18A, 2.18C and 2.18D respectively*), and were not restored to normal levels by cortisol infusion. The ratios of adrenal CYP11A1, 3βHSD and CYP21A1 mRNA : 18S rRNA were 2.6, 2.0 and 1.8 times lower, respectively, in the HPD group than the Intact group; and 4.1, 2.3 and 1.7 times lower in the HPD+F group (CYP11A1: 0.09 ± 0.01; 3βHSD: 0.08 ± 0.01; CYP21A1: 0.47 ± 0.10) than in the Intact group. The ratio of adrenal CYP17 mRNA : 18S rRNA was significantly greater (p<0.05) in Intact fetuses than the HPD+F, but not the HPD group (Intact: 0.08 ± 0.02; HPD: 0.04 ± 0.02; HPD+F: 0.02 ± 0.01) (*Figure 2.18B*).

2.4   DISCUSSION

In this chapter, I have reported that fetal adrenal weight increases cubically (*see equation 2.6 on pg. 95*) with increasing gestational age. While there was an increase in fetal adrenal weight during the final 15 d of gestation, there was no change in either the ratio of adrenal : fetal body weight or the ratio of adrenal IGF-II mRNA : 18S rRNA expression during this period. I have also demonstrated that there is a differential pattern
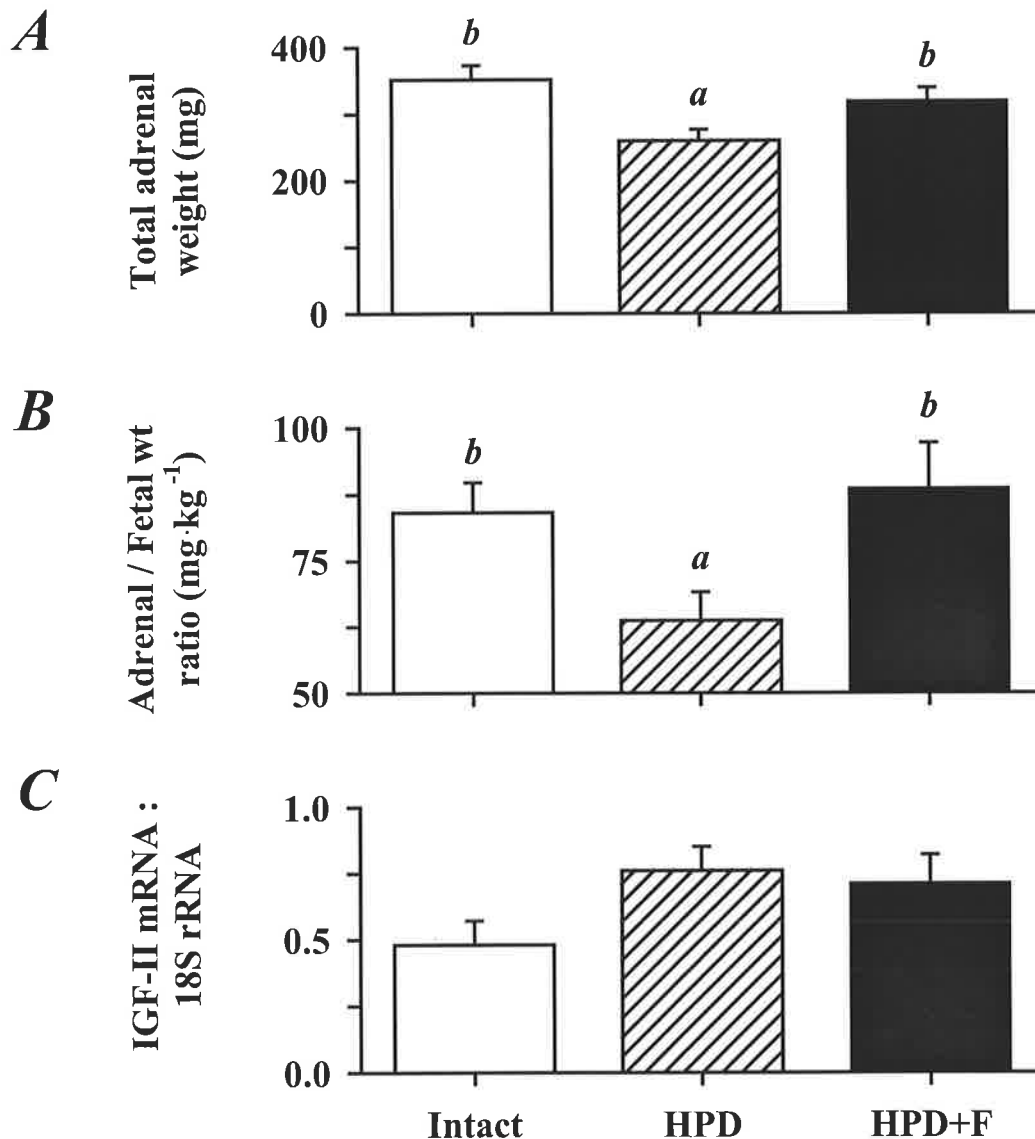
Figure 2.16 **Total adrenal weight, the ratio of adrenal : fetal body weight and adrenal IGF-II mRNA expression in Intact, HPD and HPD+F fetal sheep at 139-141 d of gestation.**

(*A*) Total adrenal weight and (*B*) the ratio of adrenal : fetal body weight were significantly greater in the Intact (open bar) and HPD+F (black bar) groups when compared with the HPD (striped bar) group. (*C*) There was no difference in the ratio of IGF-II mRNA : 18S rRNA in fetal adrenals from Intact, HPD and HPD+F fetal sheep at 139-141 d of gestation. Significant differences between the Intact, HPD and HPD+F groups are denoted by different superscripts, *e.g.* a < b (p<0.05).
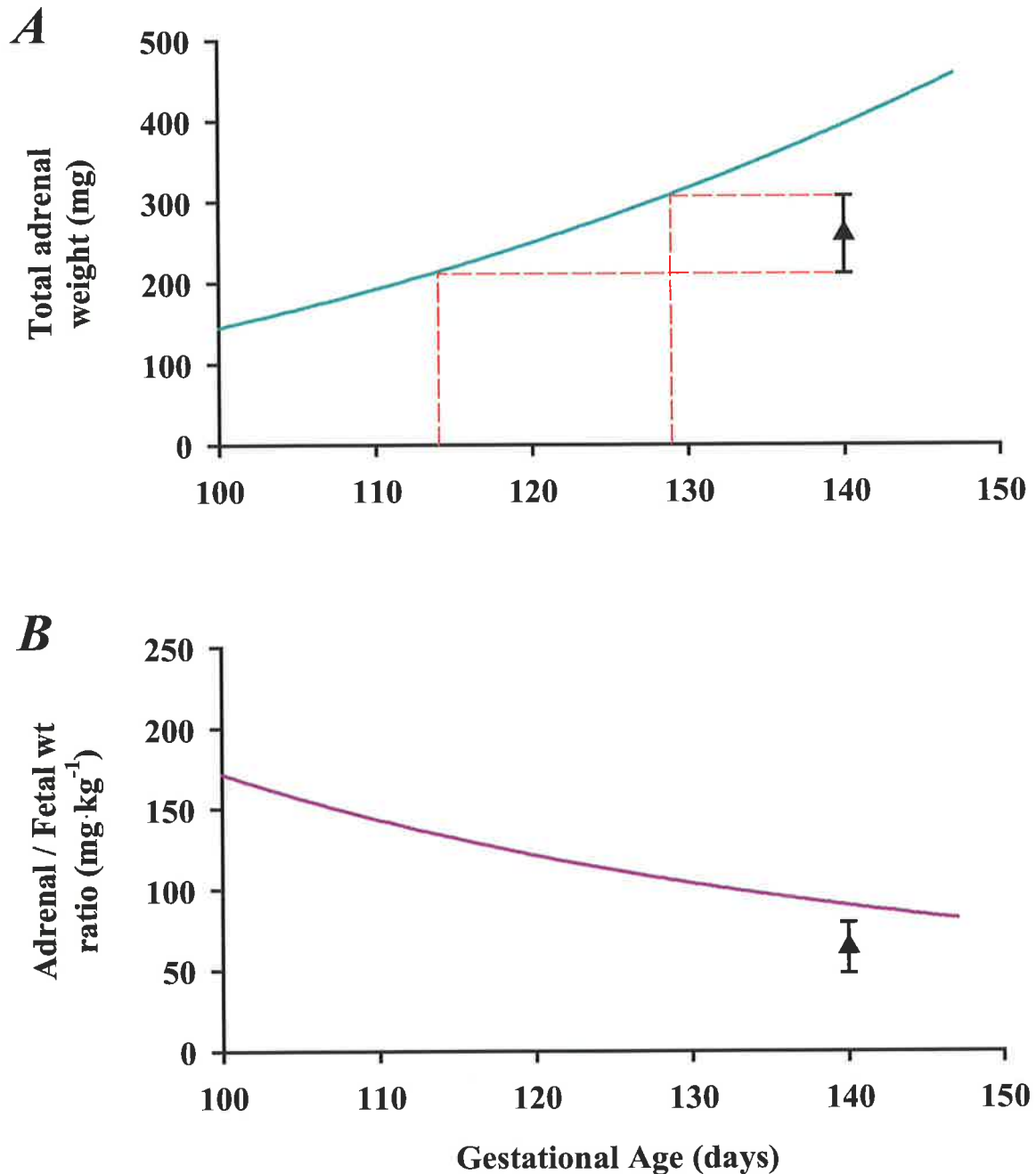
Figure 2.17   **Total adrenal weight and the ratio of adrenal : fetal body weight in HPD fetal sheep at 139-141 d of gestation compared with the respective gestational profiles between 100-147 d of gestation.**

(*A*) Total adrenal weight and (*B*) the ratio of adrenal : fetal body weight were lower in the HPD (▲) group when compared with the Intact and HPD+F groups and the gestational profile data from Study 1. The standard deviation of total adrenal weight of HPD fetuses is displayed for estimation of comparative adrenal developmental age (dashed red lines) using the power regression equation:

[Adrenal weight] = $10^{-3.81341}$ × [Gestational age]$^{2.98733}$ (*equation 2.6 on pg. 95*).
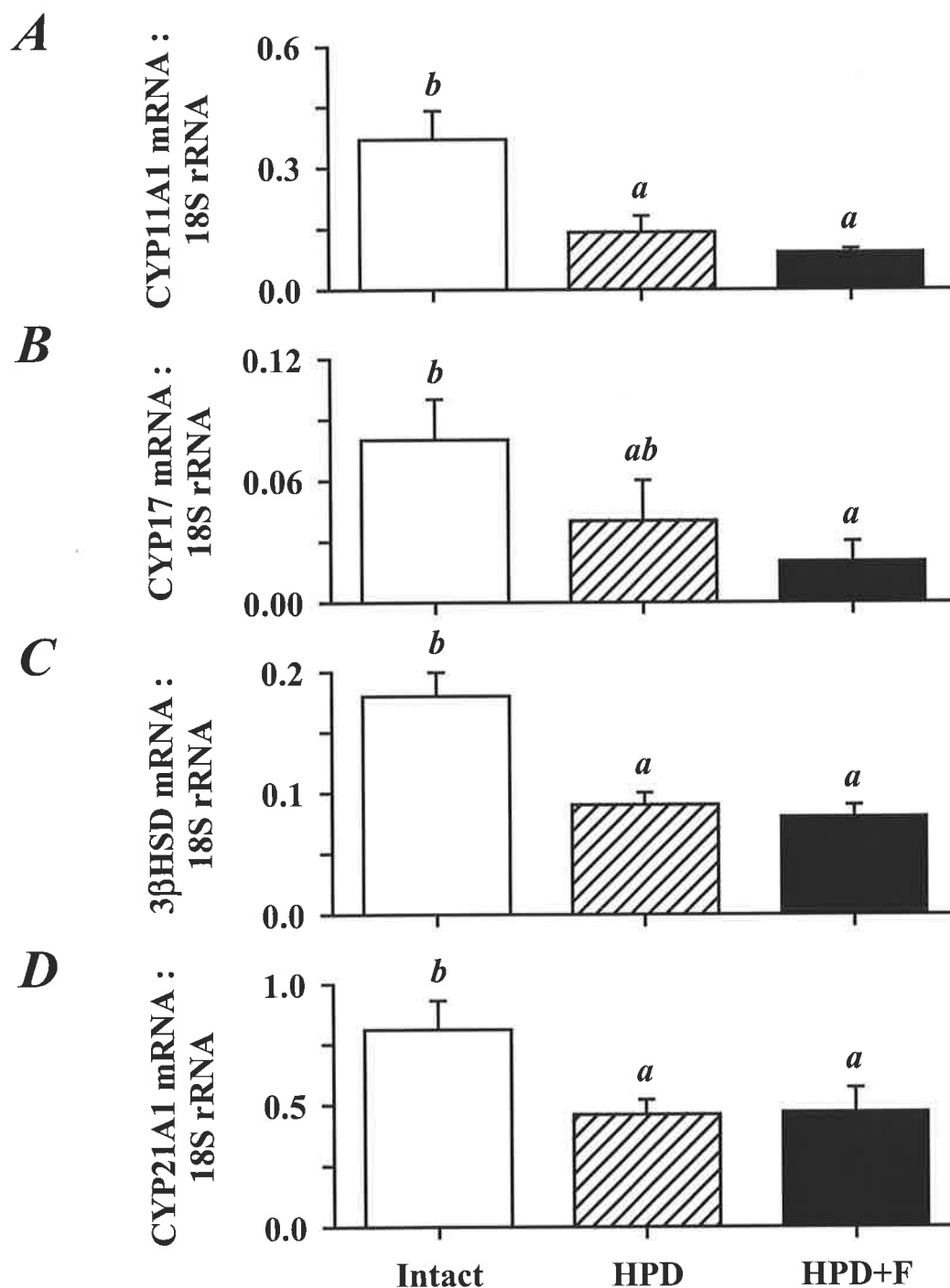
Figure 2.18 **Adrenal steroidogenic enzyme mRNA expression in Intact, HPD and HPD+F fetal sheep at 139-141 d of gestation.**

The ratios of (*A*) CYP11A1, (*B*) CYP17, (*C*) 3βHSD and (*D*) CYP21A1 mRNA : 18S rRNA in fetal adrenals from Intact (open bar), HPD (striped bar) and HPD+F (black bar) fetal sheep at 139-141 d of gestation. Significant differences (p<0.05) in the ratios between the Intact, HPD and HPD+F groups are denoted by different superscripts, *e.g.* a < b (p<0.05).

of expression of mRNA for the adrenal steroidogenic enzymes across this period of gestation. Disconnection of the fetal sheep hypothalamus and pituitary at around 110 d of gestation resulted in significantly lower total adrenal weights and adrenal : fetal body weight ratios at 139-141 d of gestation, when compared to intact fetuses, but did not alter the adrenal levels of IGF-II mRNA. Fetal HPD at ~110 d of gestation also resulted in significantly lower circulating cortisol levels and adrenal CYP11A1, CYP21A1 and 3βHSD mRNA levels at 139-141 d of gestation. Cortisol infusion during late-gestation in HPD fetuses increased the adrenal gland weight to values comparable to intact fetal sheep. Cortisol infusion, however, had no effect on either adrenal steroidogenic enzyme or IGF-II mRNA levels. It would appear, therefore, that there is a differential effect of cortisol on adrenal growth and steroidogenesis in the HPD fetal sheep during late-gestation.

2.4.1   Late-gestation study

2.4.1.1   Adrenal growth

Wintour and colleagues have demonstrated previously that the fetal adrenal gland forms a larger proportion of body weight in relatively young fetuses (40-80 d of gestation) than older fetuses ([433]Wintour, Brown *et al.*, 1975). As gestation continues, the rate of fetal growth steadily increases and, by late-gestation, matches the growth rate of the adrenal gland. I have shown that the total adrenal weight of fetuses increased cubically with increasing gestational age: $[\text{Adrenal weight}] = 10^{-3.81341} \times [\text{Gestational age}]^{2.9873}$ (*see equation 2.6 on pg. 95*), in contrast to the exponential relationship proposed by Boshier and colleagues ([53]Boshier and Holloway, 1989). The lower rate of change of adrenal growth with increasing gestational age in this study may reflect the greater number of fetuses and time points included in our study (n=422 fetuses, 39 time points) compared with that of Boshier and colleagues (n=26 fetuses, 5 time points).

Boshier and colleagues have shown that the increase in adrenal mass during the first two weeks of gestation is accompanied by a ~2.6 fold increase in the *total volume* of secretory cells within the *zona fasciculata* from 56 mm^3 to 146 mm^3 ([53]Boshier and Holloway, 1989). This increase in the *total volume* of *zona fasciculata* secretory cells during the two weeks prior to delivery is due predominantly to cellular hypertrophy, as the mean *individual volume* of adrenocortical secretory cells increases from 420 μm^3 at 130 d of gestation to 915 μm^3 at 144 d of gestation. The increase in the mean *individual volume* of secretory cells continues into the post-partum period, reaching 1130 μm^3 at 2 d post-partum. There is also a small increase in the adrenocortical secretory *cell number* between 130 d (133×10^6 cells) and 144 d of gestation (160×10^6 cells). The majority of the

hyperplastic growth within the adrenal cortex, however, occurs between 144 d of gestation and 2 d post-partum, when the adrenocortical secretory *cell number* reaches 250×10^6 ([53]Boshier and Holloway, 1989).

The adrenal medulla also increases in *total volume* from 56 mm^3 to 109 mm^3 between 130 d and 144 d of gestation ([52]Boshier, Gavin and Holloway, 1989). This growth is a combination of cellular hypertrophy, predominantly in the juxta-cortical region of the medulla, and hyperplasia within the central medullary region ([52]Boshier, Gavin and Holloway, 1989). While it is clear that growth occurs within both adrenal compartments during the last two weeks of gestation, the rapid increase in adrenal weight prior to birth is due primarily to the increase in cortical thickness ([53]Boshier and Holloway, 1989). Finally, the adrenocortical growth observed in late-gestation is dependent on an intact fetal pituitary. The adrenocortical thickness of hypophysectomised fetuses at 155 d of gestation (*i.e.* post-mature), was one quarter to one third of the adrenocortical thickness of intact fetuses at 147 d of gestation ([55]Boshier, Holloway and Liggins, 1980; [56]Boshier, Holloway and Liggins, 1981). Fetal pituitary stimulation of adrenal growth in late-gestation appears to be specific for the adrenal cortex as the growth of the adrenal medulla is unaffected following fetal hypophysectomy ([109]Coulter, Young *et al.*, 1989).

2.4.1.2   *Adrenal steroidogenic enzyme mRNA expression*

Several studies have measured CYP11A1, CYP17 and CYP21A1 mRNA levels in the fetal sheep adrenal between 50 d and 136 d of gestation ([216]John, Simpson *et al.*, 1987; [407]Tangalakis, Coghlan *et al.*, 1989; [298]Myers, McDonald and Nathanielsz, 1992b). There have been no previous detailed studies, however, of the changes in expression of the steroidogenic enzymes during the 10-15 d immediately preceding delivery, at the time of the pre-partum cortisol surge.

Myers and colleagues have demonstrated that adrenal expression of CYP11A1 and CYP17 mRNA increases by ~3 fold between 120 d to 128 d of gestation and increases again by ~3 fold at 136 d ([298]Myers, McDonald and Nathanielsz, 1992b). These investigators reported a further 1.5-2.0 fold increase in the adrenal mRNA abundance of these enzymes between 136 d of gestation and 2 h post-partum ([298]Myers, McDonald and Nathanielsz, 1992b). Myers and colleagues demonstrated that the overall increases in the adrenal expression of CYP11A1 and CYP17 mRNA are approximately 15-20 fold between 120 d of gestation and 2 h post-partum. In the present study, I observed a 3.6 fold increase in adrenal CYP11A1 mRNA abundance between 130-135 d and 136-140 d of gestation, and a 3.6 fold increase in adrenal CYP17 mRNA expression after 140 d of gestation. It would

appear from the results of the present study, and those of Myers and colleagues, that there is a substantial increase in the expression of CYP11A1 and CYP17 mRNA within the fetal adrenal gland during late-gestation. This is the first time, however, that a differential time-course of adrenal steroid hydroxylase gene expression has been demonstrated during the final 15 d of gestation.

Tangalakis and colleagues did not measure any differences in adrenal CYP21A1 mRNA expression between 114 d and 132 d of gestation using *in situ* hybridisation, although there appeared to be a qualitative late-gestation increase in adrenal CYP21A1 mRNA in this age range by Northern analysis ([407]Tangalakis, Coghlan *et al.*, 1989). Myers and co-workers measured a significant ~2 fold increase in adrenal CYP21A1 mRNA between 120 d and 128 d, and found no further changes at 136 d of gestation or at 2 h after birth ([298]Myers, McDonald and Nathanielsz, 1992b). Our finding of a ~2 fold increase in adrenal CYP21A1 mRNA between 130-135 d and 141-145 d of gestation would appear to contrast those of Myers and colleagues. These authors, however, did not measure adrenal CYP21A1 mRNA expression between 136 d and 2 h post-partum. One interpretation of our data and those of Myers and co-workers, is that there is a ~4 fold increase in adrenal CYP21A1 mRNA expression between 120-145 d of gestation, and that CYP21A1 mRNA expression then falls post-partum. Durand and colleagues have demonstrated that CYP21A1 enzyme activity in dispersed ovine adrenocortical cells is not suppressed to the same degree as CYP17 during mid-gestation ([131]Durand, Cathiard *et al.*, 1982), thus a smaller increase in adrenal CYP21A1 enzyme activity and gene expression compared with that of CYP11A1 and CYP17 might be expected in late-gestation.

Immunoreactivity for 3βHSD is detectable in steroidogenic cells of the adrenal gland throughout gestation, from 43 d of gestation until term, and is localised to the *zonae glomerulosa* and *fasciculata* as gestation progresses ([358]Riley, Boshier *et al.*, 1992). In contrast to changes in gene expression of the steroid hydroxylases, I found no change in the expression of 3βHSD mRNA in the fetal adrenal between 130 d and 145 d gestation. Thus, it would appear that while ACTH stimulates 3βHSD activity in isolated fetal sheep adrenocortical cells *in vitro* ([131]Durand, Cathiard *et al.*, 1982), adrenal 3βHSD mRNA abundance is not altered by trophic stimulation of the adrenal during late-gestation. It may be that 3βHSD mRNA is maximally expressed by 130 d of gestation and that ACTH alters the level of activity of the enzyme, rather than its gene expression.

These data suggest that steroidogenic enzyme gene expression is differentially regulated in the fetal adrenal in the two weeks before delivery. It is well established that expression of

adrenocortical steroidogenic enzymes is directly regulated by ACTH ([400]Simpson and Waterman, 1988). Our data suggest that expression of the different steroidogenic enzymes in the fetal adrenal is, in fact, increased at different times before delivery, possibly due to the differential effects of ACTH(1-39) on steroidogenic enzyme gene expression. Naaman-Reperant and colleagues have demonstrated that adrenocortical cells from ovine fetuses show a spontaneous increase in the activity of the CYP11A1 enzyme during the first 48 h of culture under standard conditions ([300]Naaman-Reperant, Cathiard and Durand, 1992). This development was inhibited when 5 % ovine fetal serum was added to the culture medium. It is possible, therefore, that fetal adrenal steroidogenic enzyme activity is not only ACTH dependent, but may also be inhibited by factors present in the fetal circulation.

POMC-derived peptides other than ACTH(1-39) are present in the fetal circulation and may also be implicated in the differential regulation of the adrenal steroidogenic enzymes. Schwartz and colleagues demonstrated that the high molecular weight ACTH-containing peptides (POMC and pro-ACTH) inhibit the steroidogenic response of cultured fetal ovine adrenal cells to ACTH(1-24) ([385]Schwartz, Kleftogiannis *et al.*, 1995). Pro-γ-MSH has also been proposed as a potential mediator of steroid synthesis within the fetal adrenal gland ([328]Pedersen and Brownie, 1980). These authors demonstrated that treatment of isolated rat adrenocortical cells with pro-γ-MSH significantly potentiates the steroidogenic effect of ACTH(1-24). In addition, prior treatment of the 16 kDa fragment of POMC with trypsin for 30 sec dramatically increases the dose dependent synergism with ACTH(1-24) ([328]Pedersen and Brownie, 1980). Trypsinised 16 kDa fragment of POMC and γ3-MSH each stimulate cholesterol ester hydrolase activity in the adrenal gland of hypophysectomised female rats, but have no effect on CYP11A1 enzyme activity ([328]Pedersen and Brownie, 1980). Conversely, ACTH inhibits cholesterol esterification by acyl CoA : cholesterol acyltransferase (ACAT) ([330]Pedersen and Brownie, 1987a). Thus, these authors have suggested that γ3-MSH and ACTH together may coordinate a shift in the cholesterol ester ↔ free cholesterol set-point within the adrenocortical cell, favouring free cholesterol. Since ACTH also activates synthesis of StAR mRNA and protein ([239]Lehoux, Fleury and Ducharme, 1998), this coordinate effect on cholesterol substrate flux and transfer to the inner mitochondrial membrane would result in the potentiation of adrenal corticosteroidogenesis. Thus, it is possible that the gene expression and activity of fetal adrenal steroidogenic enzymes may be under the coordinate regulation of inhibitory and stimulatory peptides from the fetal pituitary gland. It is also possible that the adrenal steroid hydroxylases and 3βHSD are each differentially sensitive to the array of

pituitary derived factors within the fetal circulation, resulting in the differential changes in gene expression observed during late-gestation in the present study.

2.4.1.3   *Adrenal IGF-II and IGFBP-2 mRNA expression*

The fetal adrenal gland of sheep and humans expresses high levels of IGF-II mRNA ([179]Han, Lund *et al.*, 1988; [121]Delhanty and Han, 1993). The present study has extended the observations of Han and colleagues, which demonstrated IGF-II mRNA expression in the fetal sheep adrenal between 60 d and 130 d of gestation, and at term (145-147 d of gestation) ([178]Han, Lu *et al.*, 1992). In the present study, despite a 1.4 fold increase in adrenal weight between 130-135 d and 141-145 d of gestation, there was no change in the adrenal mRNA abundance of IGF-II during this period. It appears, therefore, that while IGF-II may be a modulator of adrenocortical growth and function, adrenal growth does not appear to depend on changes in the abundance of steady state mRNA levels for IGF-II. Lu and co-workers recently reported that intra-fetal infusion of ACTH(1-24) (n=4 fetuses) or cortisol (n=3 fetuses) for 84 h beginning at 120-125 d of gestation results in a decrease in the levels of adrenal IGF-II mRNA in ovine fetuses ([258]Lu, Han *et al.*, 1994). It may be that rapid or short-term increases in circulating ACTH or cortisol induce adrenal IGF-II mRNA responses which differ from those induced by a sustained increase in these hormones, such as occurs during the last 15 d of gestation.

The present finding that adrenal IGFBP-2 mRNA decreased by greater than 50 % between 130-135 d and 141-145 d of gestation is consistent with the results of Carr and co-workers, who measured a decrease in heart and lung IGFBP-2 mRNA levels throughout late-gestation ([72]Carr, Owens *et al.*, 1995). One possibility is that the late-gestation increase in circulating cortisol acts to inhibit the expression of IGFBP-2 mRNA in tissues that are sensitive to glucocorticoids at this time. In this way, IGF-II peptide may have an enhanced autocrine / paracrine effect on the adrenal gland, mediating the rapid growth that occurs in this gland prior to birth.

2.4.2   *Fetal HPD study*

2.4.2.1   *Adrenal growth*

While it has been demonstrated previously that adrenal growth is maintained in HPD fetal sheep until 135-138 d of gestation ([17]Antolovich, McMillen *et al.*, 1991), I found in the present study that fetal adrenal weight at 139-141 d of gestation was significantly lower after fetal HPD at around 110 d of gestation, than in age-matched control fetuses. The gestational profile of total adrenal weight indicates that the weight of the adrenal after HPD

is consistent with that of adrenals of intact fetuses at approximately 114-128 d of gestation. It may be that adrenal growth until ~120 d of gestation is maintained by the actions of ACTH(1-39) and / or other POMC related peptides, which are present in the circulation of intact and HPD fetal sheep at this stage. The lower adrenal weight in HPD fetal sheep at 139-141 d of gestation may be related to the lack of the normal ontogenic increase in circulating ACTH(1-39) after 136 d of gestation in the HPD sheep fetus ([342]Phillips, Ross *et al.*, 1996). While the present results indicate equivalent levels of plasma ACTH(1-39) in HPD and intact fetuses at 135-140 d of gestation, the gestational range and more limited number of fetal plasma samples in the present study may have contributed to the inability to demonstrate the relatively modest differences in circulating ACTH(1-39) observed by Phillips and colleagues ([342]Phillips, Ross *et al.*, 1996).

From the results of the present study, I propose that the pre-partum phase of adrenal growth requires the presence of a functional hypothalamus, and may in turn be dependent on changes in either post-translational processing of POMC in the fetal pituitary, or to the pre-partum increase in circulating cortisol.

2.4.2.2   *Adrenal steroidogenic enzyme mRNA expression*

Adrenal levels of CYP11A1, CYP21A1 and 3βHSD mRNA were all significantly lower at 139-141 d of gestation after disconnection of the fetal hypothalamus and pituitary at around 110 d of gestation. I have shown, in a previous study, that the abundance of adrenal CYP17 mRNA is also significantly lower following fetal HPD ([342]Phillips, Ross *et al.*, 1996). The larger between-animal variability in the present study may explain the lack of significant difference in adrenal CYP17 mRNA expression between HPD and intact fetuses. These data are consistent with the lack of a pre-partum cortisol surge in the HPD sheep fetus and indicate that an intact hypothalamo-pituitary axis is essential for the increase in adrenal steroidogenesis preceding delivery. Consistent with this, Myers and co-workers demonstrated that bilateral lesion of the PVN in the fetal hypothalamus at around 120 d of gestation prevents the pre-partum increase in fetal cortisol and reduces adrenal mRNA levels of CYP11A1 and CYP17 at 157 d of gestation, *i.e.* post-term ([297]Myers, McDonald and Nathanielsz, 1992a). These authors found, however, that there is no difference between the levels of adrenal 3βHSD mRNA in intact and PVN lesioned sheep ([297]Myers, McDonald and Nathanielsz, 1992a). It is interesting, in the present study, that while there is no change in the adrenal level of 3βHSD mRNA in intact fetal sheep between 130 d and 145 d of gestation, the level of adrenal 3βHSD mRNA is significantly lower in the HPD than in the intact group at 139-141 d of gestation. This implies that there

are factors present in the circulation of the HPD sheep fetus, but not after lesion of the fetal PVN, which exert an inhibitory effect on the expression of this steroidogenic enzyme within the fetal adrenal. Alternatively, fetal HPD may remove a pituitary factor which is required for the maintenance of adrenal 3βHSD gene expression throughout late-gestation and which is normally present after PVN lesion. The profile of ACTH-containing and other POMC-derived peptides in the circulation of fetal sheep after PVN lesion has not been determined.

2.4.2.3   *Adrenal IGF-II mRNA expression*

There was no difference in adrenal IGF-II mRNA levels between HPD and intact fetal sheep at 139-141 d of gestation. Our study, therefore, indicates that adrenal IGF-II mRNA levels are not directly related to either adrenal growth or steroidogenic capacity in late-gestation. Clearly, the distribution, peptide level and actions of IGF-II within the fetal adrenal may also be determined by the location and abundance of IGF binding proteins. The mRNA expression of IGFBP-2 was not measured in the adrenal glands from HPD fetuses and therefore remains as an area for further study.

2.4.3   *Cortisol infusion into HPD fetuses*

2.4.3.1   *Plasma ACTH*

The ACTH sequence is present in peptides within the fetal circulation in a range of molecular weights, including bioactive ACTH(1-39) and the larger molecular weight ACTH precursors (POMC and pro-ACTH) ([217]Jones, 1980; [342]Phillips, Ross *et al.*, 1996). After 135 d of gestation, there is a relative increase in the output of ACTH(1-39) : ACTH precursors from the perifused fetal pars distalis and in the levels of ACTH(1-39) in the fetal circulation ([279]McMillen, Merei *et al.*, 1995). Changes in the relative output of ACTH(1-39) in late-gestation may represent changes in post-translational processing of ACTH in a single corticotrophic cell type, or a change in the predominant type of corticotrophic cell in the fetal pituitary, after 136 d of gestation. Infusion of cortisol before 120 d of gestation is associated with a premature maturation of the relative proportions of 'fetal' and 'adult' corticotrophic cell types within the fetal pars distalis ([18]Antolovich, McMillen *et al.*, 1992). In the present study, however, I have found no evidence to suggest that cortisol can act at the fetal pituitary *in vivo*, following surgical disconnection from the hypothalamus, to alter post-translational processing of POMC to ACTH(1-39).

2.4.3.2   Adrenal growth and steroidogenesis

In the present study, I have provided evidence that fetal cortisol may play an important role in the increase in fetal adrenal weight, which occurs prior to birth in intact fetal sheep. While adrenal growth was increased in HPD fetuses that received a 5 d intra-fetal infusion of cortisol, I found no evidence that cortisol infusion in HPD fetuses altered adrenal IGF-II mRNA expression. The effect of exogenous cortisol on fetal adrenal growth may be mediated through the release of other POMC-derived peptides that do not contain the ACTH(1-39) sequence. The *N*-terminal region of POMC contains peptides that are involved in adrenal regeneration following bilateral adrenal enucleation in adult rats ([145]Estivariz, Carino *et al.*, 1988). It may be that fetal HPD results in lower circulating levels of these *N*-POMC peptides, resulting in smaller adrenals and altered steroidogenic function due to the changes in the ratio of *N*-POMC-related : ACTH-related peptides. It is tempting to speculate that exogenous cortisol may alter the processing or secretion of *N*-POMC peptides from the pituitary of the HPD fetus, which then mediate the increase in adrenal growth observed in the present study. In contrast to the effect of cortisol on adrenal growth after fetal HPD, I found no evidence for cortisol acting at the fetal adrenal after HPD to increase adrenal steroidogenic enzyme mRNA expression. Our data confirm the observation that an intact fetal hypothalamo-pituitary axis is essential for the pre-partum increases in the mRNA expression of the adrenal steroidogenic enzymes and plasma cortisol concentrations ([342]Phillips, Ross *et al.*, 1996). Thus, cortisol may act at the fetal pituitary *in vivo*, following surgical disconnection from the hypothalamus, to stimulate the release of adrenal growth promoting peptides derived from the *N*-terminal region of POMC which are, in the absence of elevated circulating levels of ACTH(1-39), unable to stimulate adrenal steroidogenesis.

2.4.4   Summary

In summary, I have demonstrated increases in the growth of the fetal adrenal, and in the adrenal level of CYP11A1 and CYP21A1 mRNAs after 135 d and CYP17 mRNA after 140 d of gestation. The mRNA levels of CYP11A1, CYP17, CYP21A1 and 3βHSD in the fetal adrenal are clearly dependent on a functional hypothalamo-pituitary axis, and the expression of these steroidogenic enzymes in the fetal adrenal appears to be differentially regulated in the 10-15 d preceding delivery in the sheep. It appears that there is no direct relationship between adrenal IGF-II mRNA expression and either fetal adrenal weight or adrenal steroidogenic enzyme mRNA expression between 130-145 d of gestation. In addition, I have demonstrated that IGF-II expression is maintained in the fetal adrenal after surgical disconnection of the fetal hypothalamus and pituitary. I have demonstrated a

late-gestation decrease in adrenal IGFBP-2 mRNA expression, while the role of cortisol and the fetal pituitary on this binding protein has yet to be determined.

Finally, I have demonstrated that cortisol stimulates fetal adrenal growth after HPD in the absence of any effects on adrenal IGF-II or steroidogenic enzyme mRNA levels. Cortisol may act directly at the adrenal to modulate the activity of locally produced growth factors and / or their binding proteins, or to suppress a local inhibitor of adrenal growth. There is evidence that other growth factors such as epidermal growth factor ([195]Hornsby, 1985a), fibroblast growth factor ([399]Simonian and Gill, 1981; [195]Hornsby, 1985a) and transforming growth factor-β ([198]Hotta and Baird, 1986; [351]Rainey, Oka *et al.*, 1991), can also influence fetal adrenal growth and function. Alternatively, cortisol may act at the surgically disconnected pituitary to stimulate the secretion of growth promoting factors such as peptides arising from the *N*-terminal region of POMC, or to inhibit the release of adrenal growth inhibitors. Independently of the mechanisms, these data provide evidence that an intact hypothalamo-pituitary axis and cortisol each play important separate roles in the stimulation of adrenal growth and steroidogenesis which occurs before delivery in the sheep.

CHAPTER 3.
EFFECT OF PRECOCIOUS ELEVATION OF FETAL PLASMA CORTISOL CONCENTRATIONS ON ADRENAL GROWTH AND STEROIDOGENIC FUNCTION IN INTACT FETAL SHEEP

3.1   INTRODUCTION

It has been postulated that ACTH is the major trophic hormone driving fetal adrenal growth and steroidogenesis before birth ([80]Challis and Brooks, 1989), as intra-fetal administration of ACTH after fetal hypophysectomy restores adrenocortical growth and steroidogenesis ([243]Liggins, 1968). In both the hypophysectomised and HPD fetal sheep, however, disruption of pituitary function is also associated with low fetal circulating levels of cortisol during late-gestation ([325]Ozolins, Antolovich *et al.*, 1991; [342]Phillips, Ross *et al.*, 1996). Interestingly, Liggins and co-workers demonstrated that the adrenal hyperplasia following infusion of ACTH(1-24) does not occur in fetuses concurrently infused with an inhibitor of adrenal cortisol biosynthesis, metyrapone ([243]Liggins, 1968). Furthermore, in Chapter 2 of this thesis, I demonstrated that cortisol replacement in HPD fetal sheep in late-gestation restores the weight of the fetal adrenal to values comparable with those measured in intact fetal sheep ([372]Ross, Phillips *et al.*, 1997).

Boshier and colleagues also demonstrated that cortisol administration to hypophysectomised fetuses results in an enhanced degree of cytodifferentiation of adrenocortical cells ([56]Boshier, Holloway and Liggins, 1981), and Lye and colleagues reported that concurrent infusion of metyrapone with ACTH(1-24) in fetal sheep *in vivo* prevents the cAMP accumulation measured following subsequent ACTH stimulation in adrenal cells *in vitro* ([262]Lye and Challis, 1984). These authors concluded that cortisol may mediate the increase in adrenal responsiveness to ACTH stimulation *in vitro* which results following pulsatile ACTH(1-24) infusion *in vivo* ([262]Lye and Challis, 1984). While the actions of cortisol on intra-cellular events distal to cAMP generation are unknown, it appears that during a number of different conditions, intra-adrenal cortisol may play a role in mediating the actions of ACTH on fetal adrenal growth and steroidogenesis in late-gestation.

Recent studies have shown that the action of cortisol in fetal tissues may be regulated via the two isoforms of the intra-cellular microsomal enzyme, 11βHSD. The reversible

NADP(H)-dependent isoform, 11βHSD type-1, can act either as a dehydrogenase or as a reductase, favouring the formation of cortisol from cortisone *in vivo* (*see Figure 1.3, step 9a on pg. 16*). 11βHSD type-2, however, is a unidirectional NAD-dependent enzyme which catalyses the conversion of the biologically active cortisol to the inert cortisone ([163]Funder, Pearce *et al.*, 1988) (*see Figure 1.3, step 9b on pg. 16*). Thus, the level of expression and direction of activity of 11βHSD within a tissue may regulate tissue exposure to glucocorticoid action. It has recently been shown that there is a decrease in the levels of 11βHSD type-2 mRNA in the fetal sheep adrenal during the last 10 d of gestation, concomitant with the pre-partum increase in fetal plasma cortisol ([281]McMillen, Warnes *et al.*, 1999). I have hypothesised that this decrease in the adrenal expression of mRNA for 11βHSD type-2 may be a consequence of the increase in cortisol, which could result in a positive feedback system operating within the fetal adrenal in the pre-partum period, such that there is increasing tissue exposure to endogenously generated glucocorticoids.

The fetal ovine adrenal expresses mRNA for IGF-II throughout gestation ([178]Han, Lu *et al.*, 1992; [121]Delhanty and Han, 1993) and I have demonstrated, in studies described in Chapter 2, that the profile of adrenal IGF-II mRNA abundance remains constant throughout the two weeks prior to delivery ([342]Phillips, Ross *et al.*, 1996). I have also demonstrated, in Chapter 2, that adrenal IGFBP-2 mRNA expression decreases after 135 d of gestation, concomitant with the late-gestation increase in fetal circulating levels of glucocorticoids. The factors that regulate the ontogenic expression of IGFBP-2 in fetal tissues remain to be determined. In the present study, therefore, cortisol was infused (2.0-3.0 mg·24 h^{-1}) from 109-116 d of gestation into intact fetal sheep, prior to the normal pre-partum cortisol surge, to investigate the impact of cortisol on adrenal growth and the expression of mRNA for 11βHSD type-2, the key steroid-synthesising enzymes, IGF-II and IGFBP-2.

3.2   MATERIALS AND METHODS

3.2.1   Animal protocols and surgery

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation. Twenty five pregnant Border-Leicester × Merino ewes and their singleton fetuses were used in this study. The ewes were housed in individual pens in animal holding rooms, with a 12 h light / dark lighting regimen, and fed once daily with 1 kg of lucern chaff and 1 kg of Baramil joint stock rations between 0900 and 1300 h with water *ad libitum*. Catheterisation surgery was carried out at either 103 d or 104 d of

gestation under general anaesthesia and using aseptic techniques, as described in Chapter 2 (*see section 2.2.1.3.1 on pg. 72*).

There was a recovery period of at least three days after surgery before fetal blood samples were collected. At 116 d of gestation, ewes were killed with an overdose of Lethabarb (25 ml at 325 mg·ml^{-1}) and fetal sheep were removed, weighed and killed by decapitation. One adrenal gland from each fetus was quickly removed, weighed, snap frozen in liquid N_2 and stored at -80 °C until total RNA was extracted.

3.2.2 Infusion regimen and blood sampling protocol

Infusion regimen. Cortisol (F: hydrocortisone succinate, Solucortef: 2.0-3.0 mg in 4.4 ml·24 h^{-1}) was infused into fetal sheep from 109-116 d of gestation (cortisol infused group; n=12 fetuses). Four fetuses received 2.0 mg·24 h^{-1}, seven fetuses received 2.5 mg·24 h^{-1} and one fetus received 3.0 mg·24 h^{-1} of F throughout the 7 d infusion period. Control animals received an infusion of saline (4.4 ml·24 h^{-1}) from 109-116 d of gestation (saline infused group; n=13 fetuses).

Blood sampling protocol. Fetal arterial blood samples (2 ml) were collected into chilled collection tubes daily from fetuses in the saline and cortisol infused groups between 107 d and 116 d of gestation for cortisol radioimmunoassays. Blood for cortisol assay was collected into tubes containing 125 IU lithium heparin. Blood for ir-ACTH assay was collected into plain tubes containing EDTA (18.6 g·l^{-1} of whole blood) and aprotinin (100 KIU in 100 μl·ml^{-1} of whole blood). Blood samples were centrifuged at 1800 g for 10 min at 4 °C before separation and storage of plasma at -20 °C for subsequent assay. Fetal arterial blood (0.5 ml) was collected on alternate days for measurement of whole blood p_aO_2, p_aCO_2, pH, O_2 saturation and haemoglobin content using an ABL 550 acid base analyser and OSM2 haemoximeter.

3.2.3 Radioimmunoassays

Cortisol radioimmunoassay. Cortisol concentrations were measured in fetal plasma samples from the saline infused group (n=13 fetuses; *n*=125 samples) and cortisol infused group (n=12 fetuses; *n*=107 samples). Total cortisol concentrations in fetal sheep plasma were measured using a radioimmunoassay, validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 74*). The inter- and intra-assay coefficients of variation (COVs) were <10 %.

ACTH radioimmunoassay. ir-ACTH concentrations were measured in fetal plasma samples from a subset of the saline infused group (n=6 fetuses; *n*=34 samples) and cortisol

infused group (n=9 fetuses; *n*=54 samples). The concentrations of ir-ACTH were measured using a radioimmunoassay which has previously been validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 75*). The inter-assay COV was 14.6 % and the intra-assay COV was <10 %.

3.2.4   cDNA and antisense oligonucleotide probes

cDNA probes. *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNA probes were generously provided by Professor W Miller (Department of Pediatrics, UCSF, San Francisco, CA, USA) (*see section 2.2.3.1 on pg. 75 and Figures 2.1, 2.2 and 2.4 on pp. 76, 77 and 80 respectively*). A *h*3βHSD cDNA probe was donated by Dr. R Rodgers (Department of Medicine, Flinders University, SA, Australia) (*see section 2.2.3.1 on pg. 75 and Figure 2.3 on pg. 78*). A *b*IGFBP-2 cDNA probe was generously provided by Dr. Jill Carr (Department of Biochemistry, The University of Adelaide, SA, Australia) (*see section 2.2.3.1 on pg. 79 and Figure 2.6 on pg. 82*). cDNAs were radiolabelled with α-[^{32}P] dCTP (3,000 Ci·mmol^{-1}) by the random priming oligomer method to a specific activity of 10^9 cpm·μg^{-1} or greater, as described in Chapter 2 (*see section 2.2.3.1 on pg. 83*).

Oligonucleotide probes. A 45 mer antisense oligonucleotide probe for ovine 11βHSD-2, complementary to nucleotides 1066-1110 ([68]Campbell, Yu and Yang, 1996; GenBank accession number: S83516), was synthesised (GeneWorks) and supplied as desalted, lyophilised stocks (*Figure 3.1*). A 30 mer antisense oligonucleotide probe for rat 18S rRNA, complementary to nucleotides 151-180, was also synthesised (GeneWorks) (*see section 2.2.3.1 on pg. 83 and Figure 2.7 on pg. 85*). Oligonucleotide probes were end-labelled using T4PNK and γ-[^{32}P] ATP (4,000 Ci·mmol^{-1}) as substrate, as described in Chapter 2 (*see section 2.2.3.1 on pg. 84*).

3.2.5   Total RNA isolation

Total RNA was extracted from one adrenal from each of nine saline infused (n=9 fetuses) and nine cortisol infused (n=9 fetuses) fetal sheep by homogenisation in 4 M guanidine hydrochloride solution and ultracentrifugation overnight at 36,000 rpm, through a cushion of 5.7 M CsCl in 100 mM EDTA ([90]Chirgwin, Przybyla *et al.*, 1979), as described in Chapter 2 (*see section 2.2.3.2 on pg. 84 and Figure 2.8 on pg. 86*).

3.2.6   Northern blot analysis

Total RNA samples (20 μg of adrenal RNA) were separated by electrophoresis in 1 % agarose denaturing gels (*see Appendix I*), using 1× Northern running buffer (*see Appendix I*) and transferred by capillary blotting to Zetaprobe nitrocellulose

A

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   1   CTACCCAGGC TGTGACTCTG GTTTTGGCAA CGCGACGGCC AAGAAGCTTG
  51   ACGCCATGGG CTTCACAGTG TTGGCCACCG TGTTGGATCT GAATAGCCCT
 101   GGCGCCCTAG AGCTGCGTGC CTGCTGTTCT TCTCGTCTGC AGCTGCTGCA
 151   GATGGACCTG ACCAAGCCAG CAGACATTAG CCGTGTGCTG GAGTTCACCA
 201   AGGTCCACAC CGCCAGCACT GGTCAGTAAG CTCAACTTCG CCAGGAAAGG
 251   GCATGGCTGG GCAGATGTGA GGGGGCCAGG ATTGGAGGTG TGCGGTGGGA
 301   CTGACCCGCA GCTTCCTCTG GCTCCATGCC CTCAGGTCTG TGGGGCCTGG
 351   TCAACAATGC GGGCCAGAAC ATCTTTGTGG CGGATGCAGA GCTGTGTCCG
 401   GTGGCCACTT TCCGCACCTG CATGGAGGTG AACTTCTTTG GTGCACTAGA
 451   GATGACCAAA GGCCTCTTGC CACTGCTGCG TCGTTCGAGT GGTCGCATTG
 501   TGACCGTAAG CAGCCCAGCA GGTGAGGGAT CTCCTCCCGC ACTGGAGCAG
 551   AAAAGATGCC TCTGCTGGGC AGGGAGAGAT ACTGGAGTCC CCGGCCCAGG
 601   CTGAGGCATC CGCCACTCCC AATCCTATCC CCAGAGGAGA CATGCCGTTT
 651   CCATGCTTGG CTGCCTATGG GACCTCCAAA GCAGCCTTGG CATTGCTCAT
 701   GGGCAATTTT AGCTGTGAAC TTCTGCCCTG GGGTGTCAAG GTCAGCATCA
 751   TCCAGCCTGC CTGCTTCAAG ACAGGTGAGG CTCAGGGATT TGGGGGTGGC
 801   CTGTCGGGGA GAGGAGGAAG GCAGGGTGGG TGTGGTCTTC TCTGGAGTGG
 851   GATTGGGTCT GAGGAATGGG CCTGACTTTG GCTGGGGCGG GTCTCAGGTT
 901   GTGAGTTAGA GACCTGGGTC TGGGTTTGGC TTCCCTCTCT GACCCCTTTC
 951   TGGCCTTGGC ACTCCTTCCG CAGAGTCAGT GAAGGACGTG CACCAGTGGG
1001   AAGAGCGCAA GCAGCAGCTG CTGGCCACCC TGCCGCAAGA GCTGCTGCAG
1051   GCCTATGGTG AGGAC**TACAT** **CGAGCACTTG** **AATGGGCAGT** **TCCTGCACTC**
1101   **TCTGAGCCAG** GCCCTGCCAG ACCTCAGCCC GGTGGTAGAT GCCATCACCG
1151   ATGCGCTGCT GGCGGCCCAG CCACGCCGCC GCTATTACCC AGGTCATGGC
1201   CTGGGGCTCA TATACTTCAT CCACTACTAC CTGCCTGAGG GCCTGCGGCA
1251   GCGTTTCCTG CAGTCCTTCT TCATCAGTCC CTATGTGCCA AGAGCACTAC
1301   AGGCTGGCCA GCCTGGCCTT ACCTCTGCCC GGGACATAGC CCAGGACCAA
1351   GGCCCCAGAC TGGACCCCTC TCCCACTGCC CAGTGAGCAG GGCACATGTA
1401   GAGCAGCTCC AGCAGAGGAG GTTCCTTGTG CCCTTGCTCT TCTTCCAGGT
1451   ATTCTGACCC CCAAGGTCTG CCCTAGAGCC TGGCCCAAAG GACCCCAACC
1501   CATGCACTGC CGATGCCACA GGCCAGGCCT GGTGAGGTGA AGGCTTTCCA
1551   GTGAACTCTG GGCCTCTCCT GCTTCATGAG CCCAAACAGA CCCTCCTGGG
1601   CACAAGGCTC CACCATGCAG CTTGCAGAAC CTAGCTGGAT GGGAAGTTTA
1651   GTTCAAGGCT TAACTAGAGC CTTAGCTAGG ATCCTACAGA CAGTGCCTCT
1701   GCAAACTAAG GCGAGATTAG GTTGGTTGGG GACCCCCTCA GGATTATTTC
1751   TTGGCACCAG TGCCTCAGTG CTGTGATTTG AAGGGTGAAT CCTGTTTCTT
1801   GACTGGCTCA AGGATTAGGG CCCTGACCAC CCACCCCAAA CCCCTCAAGC
1851   CACAGGGAGG CTACATACTC ACCTGATTGA CACTTTTTGA ATAAAGATAA
1901   ATTTTTATTT CTCCTAGAAT GGGGGAAATG CTCTGTTTGC CTAGTGACAG
1951   GCTGTGGCGT GTGACAGACC AGAACT
```

B

sense strand
of gene

1066   1110

5' — TAC ATC GAG CAC TTG AAT GGG CAG TTC CTG CAC TCT CTG AGC CAG — 3'

3' — ATG TAG CTC GTG AAC TTA CCC GTC AAG GAC GTG AGA GAC TCG GTC — 5'

antisense
oligonucleotide
probe

Figure 3.1   **A 45 mer antisense oligonucleotide probe for ovine 11βHSD-2, complementary to nucleotides 1066-1110.**

(*A*) Sense strand of the ovine 11βHSD-2 gene sequence. (*B*) Sequence of the 45 mer ovine 11βHSD-2 antisense oligonucleotide probe.

membranes as described in Chapter 2 (*see section 2.2.3.3 on pg. 87 and Figure 2.9 on pg. 88*). Membranes were washed in 10× SSC, 0.1 % SDS for 10 min at RT and baked for 1 h at 80 °C or exposed to UV light (12 sec at 120 J·cm^{-2}), prior to overnight incubation at 42 °C in 30 ml of either cDNA or antisense oligonucleotide hybridisation buffer (*see Appendix I*). Membranes were then hybridised sequentially with cDNA or oligonucleotide probes for 16 h (42 °C for cDNA probes or 50 °C for oligonucleotide probes) in 30 ml of fresh hybridisation buffer, containing either 1-2×10^6 cpm·ml^{-1} of the cDNA probe or 5×10^5 cpm·ml^{-1} of the 45 mer antisense *o*11βHSD-2 oligo probe, or the 30 mer antisense 18S rRNA oligonucleotide probe, as described in Chapter 2 (*see section 2.2.3.3 on pg. 89*). Membranes were washed once (10 min) at RT in 1× SSC, 0.1 % SDS; then twice (10 min each time) in 0.1× SSC, 0.1 % SDS at 42 °C, briefly air-dried and sealed in a plastic bag. Membranes were exposed to phosphorimage plates in BAS 2040 cassettes for 24-48 h and images were quantified as described in Chapter 2 (*see section 2.2.3.3 on pg. 89*).

The radiolabelled *h*CYP11A1, *h*CYP17, *h*CYP21A1, *h*3βHSD and *b*IGFBP-2 cDNA and the antisense *o*11βHSD-2 oligonucleotide probes were used to probe two Northern blots of fetal adrenal total RNA from a subset of the saline infused (n=9 fetuses) and cortisol infused (n=9 fetuses) groups. One Northern blot membrane was hybridised sequentially with the *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNAs, while the second membrane was hybridised sequentially with the *h*3βHSD and *b*IGFBP-2 cDNA and the antisense *o*11βHSD-2 oligonucleotide probes.

3.2.7 *Statistical analysis*

All data are presented as the mean ± SEM. The ratios of CYP11A1, CYP17, CYP21A1, 3βHSD, IGFBP-2 and 11βHSD-2 mRNA : 18S rRNA were compared between the saline and cortisol infused groups using two-tailed, unpaired Student's *t*-tests. Student's *t*-tests were also used to compare fetal body weight, adrenal weight and the ratio of adrenal : fetal body weight between saline and cortisol infused fetuses. Plasma concentrations of ir-ACTH and cortisol were compared using a two-way ANOVA with repeated measures, with treatment group (*e.g.* saline and cortisol infusion) and time (*e.g.* pre-infusion and infusion) as the specified factors. If a significant interaction between two major factors was identified in an ANOVA, then the data were split on the basis of the interacting factor and re-analysed. Significant differences between hormonal values in different treatment groups or time-points were identified using Student's *t*-test, and a probability of <5 % (p<0.05) was considered to be significant.

3.3 RESULTS

3.3.1 Fetal plasma cortisol and ir-ACTH levels

Fetal plasma cortisol concentrations. Cortisol infusion into fetal sheep from 109-116 d of gestation resulted in a significant increase ($p<0.0005$) in plasma cortisol concentrations throughout the infusion period, when compared with the saline infused group (cortisol: 39.3 ± 2.8 nmol·l^{-1}; saline: 1.6 ± 0.1 nmol·l^{-1}) (*Figure 3.2A*). In cortisol infused fetal sheep, plasma cortisol concentrations were also significantly higher ($p<0.0005$) throughout the infusion, when compared with pre-infusion levels (pre-infusion: 1.5 ± 0.1 nmol·l^{-1}; infusion: 39.3 ± 2.8 nmol·l^{-1}) (*Figure 3.2A*).

Fetal plasma ir-ACTH concentrations. Plasma ir-ACTH concentrations did not change before or during the infusion of either saline (pre-infusion: 41.4 ± 4.2 pg·ml^{-1}; infusion: 32.7 ± 2.5 pg·ml^{-1}) or cortisol (pre-infusion: 35.3 ± 3.9 pg·ml^{-1}; infusion: 30.8 ± 3.1 pg·ml^{-1}). There was no significant difference in the plasma ir-ACTH concentrations between the two treatment groups (*Figure 3.2B*).

3.3.2 Total fetal adrenal weight and fetal body weight

Adrenal and fetal body weights. There was no effect of cortisol infusion on fetal body weight (cortisol: 2.21 ± 0.08 kg; saline: 2.17 ± 0.13 kg) or total adrenal weight (cortisol: 223.00 ± 11.18 mg; saline: 238.15 ± 15.50 mg) when compared with saline infused animals. Similarly, there was no significant effect of cortisol infusion on the ratio of adrenal : fetal body weight (cortisol: 101.70 ± 5.31 mg·kg^{-1}; saline: 108.22 ± 4.33 mg·kg^{-1}) when compared with the saline infused group.

3.3.3 Adrenal 11βHSD-2 mRNA expression

11βHSD-2 mRNA expression. A single 11βHSD-2 mRNA transcript of ~2.0 kb was measured in Northern blots of fetal adrenal total RNA (*Figure 3.3A*). The ratio of adrenal 11βHSD-2 mRNA : 18S rRNA expression was significantly lower in fetuses infused with cortisol (cortisol: 0.75 ± 0.20) compared with saline (saline: 1.65 ± 0.14) (*Figure 3.3B*).

3.3.4 Adrenal steroidogenic enzyme and IGFBP-2 mRNA expression

Adrenal steroidogenic enzyme mRNA expression. Specific hybridisation signals were detected for the CYP11A1 (1.9 kb), CYP17 (1.7 kb), CYP21A1 (1.8 and 2.2 kb) and 3βHSD (1.6 kb) mRNA transcripts, using the Northern blots of adrenal total RNA from cortisol and saline infused fetal sheep (*Figure 3.4*). There was no significant effect of the cortisol infusion on the adrenal mRNA abundance of any of these enzymes (*Figure 3.5*).
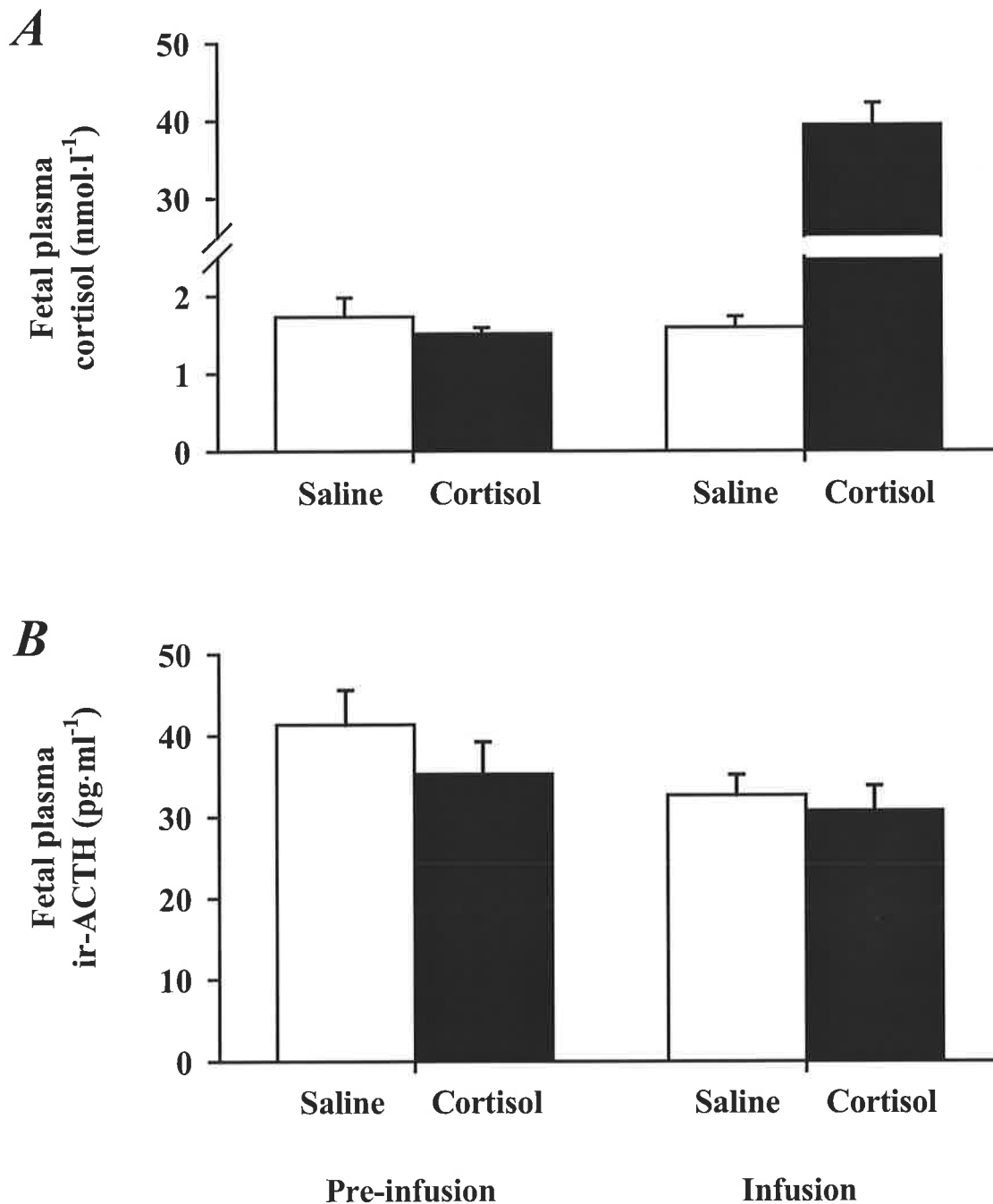
Figure 3.2   **Plasma concentrations of cortisol and ir-ACTH in fetal sheep prior to and during a 7 d infusion of either saline or cortisol from 109-116 d of gestation.**

(*A*) The plasma concentrations of cortisol were significantly higher in fetuses infused with cortisol (black bars) for 7 d when compared with fetuses infused with saline (open bars). (*B*) Plasma ir-ACTH concentrations did not change prior to or during the 7 d infusion of either saline or cortisol and there was no significant difference in the plasma ir-ACTH concentrations between the two treatment groups.

A

| Saline | Cortisol |

11βHSD-2
2.0 kb
→

18S rRNA

B
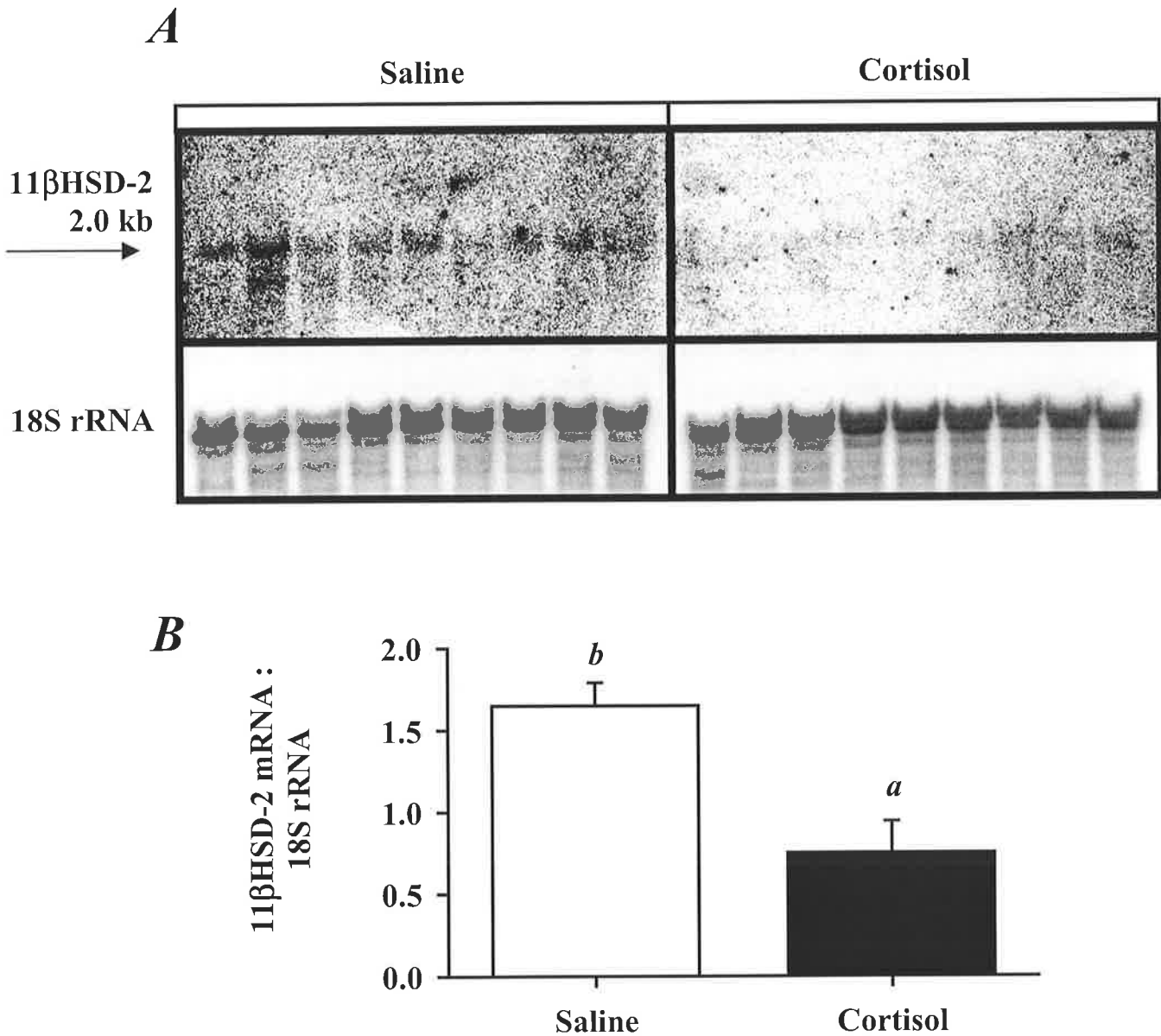
Figure 3.3   Northern blot of adrenal 11βHSD-2 mRNA expression from fetal sheep following a 7 d infusion of either saline or cortisol from 109-116 d of gestation.

(*A*) Northern blot analysis of total RNA (20 µg·lane^{-1}) prepared from adrenal glands of fetal sheep following a 7 d infusion of either saline or cortisol. The Northern membrane was hybridised sequentially with the radiolabelled 45 mer antisense *o*11βHSD-2 oligonucleotide and the 30 mer antisense 18S rRNA oligonucleotide probes. (*B*) Adrenal 11βHSD-2 mRNA : 18S rRNA expression in saline (open bar) and cortisol (black bar) infused fetal sheep. Significant differences between groups are denoted by different superscripts, *e.g.* a<b (p<0.05).
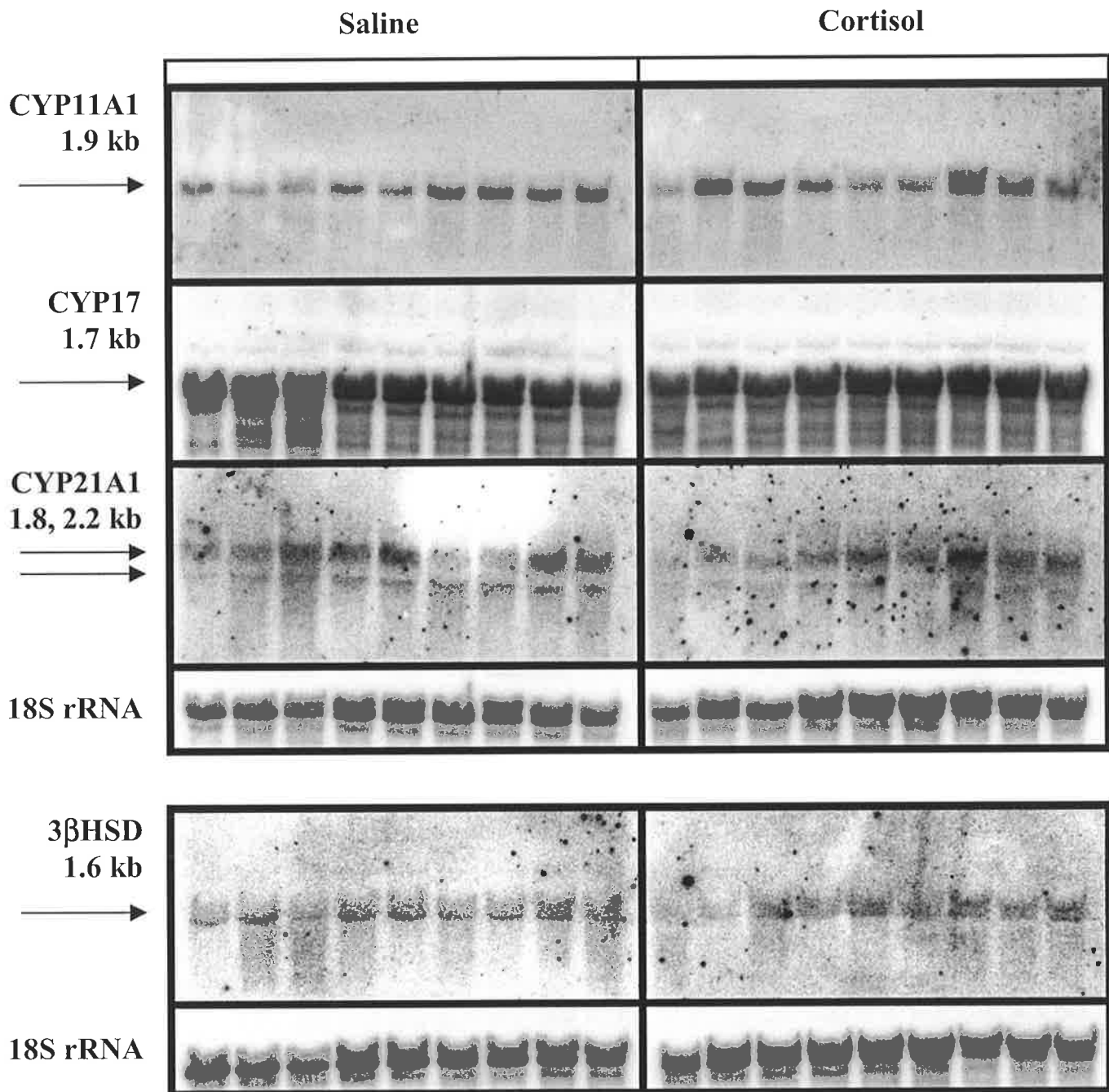
Figure 3.4   **Northern blot of adrenal steroidogenic enzyme mRNA expression from fetal sheep following a 7 d infusion of either saline or cortisol from 109-116 d of gestation.**

Northern blot analyses of total RNA (20 μg·lane^{-1}) prepared from adrenal glands of fetal sheep following a 7 d infusion of either saline or cortisol from 109-116 d of gestation. Northern membranes were hybridised sequentially with the radiolabelled *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNA and the 30 mer antisense 18S rRNA oligonucleotide probes. A second Northern blot was probed sequentially with the radiolabelled *h*3βHSD cDNA and the 30 mer antisense 18S rRNA oligonucleotide probes.

Figure 3.5   **Steroidogenic enzyme mRNA expression in adrenals of fetal sheep following a 7 d infusion of either saline or cortisol from 109-116 d of gestation.**

The ratios of (*A*) CYP11A1, (*B*) CYP17, (*C*) 3βHSD and (*D*) CYP21A1 mRNA : 18S rRNA in fetal adrenals from saline infused (open bar), and cortisol infused (black bar) fetal sheep at 116 d of gestation. There was no effect of a 7 d intra-fetal infusion of cortisol on the adrenal mRNA expression of the steroidogenic enzymes.

IGFBP-2 mRNA expression. A single IGFBP-2 mRNA transcript of ~1.4 kb was observed in Northern blots of fetal adrenal total RNA (*Figure 3.6A*). The ratio of adrenal IGFBP-2 mRNA : 18S rRNA expression was significantly lower in fetuses infused with cortisol (cortisol: 9.41 ± 0.22) compared with saline (saline: 8.16 ± 0.20) (*Figure 3.6B*).

3.4   DISCUSSION

In this study, I have demonstrated that a 7 d intra-fetal infusion of cortisol (2-3 mg·24 h^{-1}) into intact fetuses from 109-116 d of gestation significantly reduced the mRNA expression of the 11βHSD-2 enzyme within the fetal adrenal gland. Adrenal growth and the mRNA expression of the adrenal steroid-synthesising enzymes, however, remained unchanged following the intra-fetal infusion of cortisol, compared with saline infused fetuses. Intra-fetal cortisol administration, prior to the normal pre-partum cortisol surge did, however, result in a decrease in adrenal expression of mRNA for IGFBP-2.

In the adult sheep, 11βHSD-2 mRNA is localised exclusively to the adrenal cortex and is expressed highly in the *zonae fasciculata* and *reticularis* with relatively low expression in the *zona glomerulosa* ([442]Yang and Matthews, 1995). It has been shown previously that there is a decrease in adrenal expression of mRNA for 11βHSD-2 after 125 d of gestation in the fetal sheep ([281]McMillen, Warnes *et al.*, 1999). The findings of the present study indicate that a physiological elevation in fetal cortisol concentrations from 109 d to 116 d of gestation results in a premature decrease in the expression of adrenal 11βHSD-2 mRNA. It appears that the decrease in 11βHSD-2 mRNA levels in response to cortisol is not mediated through an indirect action of cortisol at the fetal pituitary, as there was no change in circulating ACTH in response to the intra-fetal cortisol infusion. This data is consistent with the finding that the HPA axis is relatively insensitive to negative feedback by physiological elevations in circulating glucocorticoids in fetal sheep at this gestational age range under basal conditions ([326]Ozolins, Young and McMillen, 1990).

It is possible that 11βHSD-2 within the fetal sheep adrenal protects the adrenocortical cells from locally produced glucocorticoids up until around 125 d of gestation. The decrease in adrenal 11βHSD-2 mRNA expression in late-gestation is, however, coincident with the pre-partum increase in adrenocortical growth and steroid output. This pre-partum increase in fetal adrenocortical function may, therefore, require enhanced intra-adrenal exposure to glucocorticoids. In the present study, however, there was no increase in fetal adrenal weight or in steroidogenic enzyme expression in association with the decrease in adrenal 11βHSD-2 mRNA levels at 116 d of gestation. It is possible that while an increase in
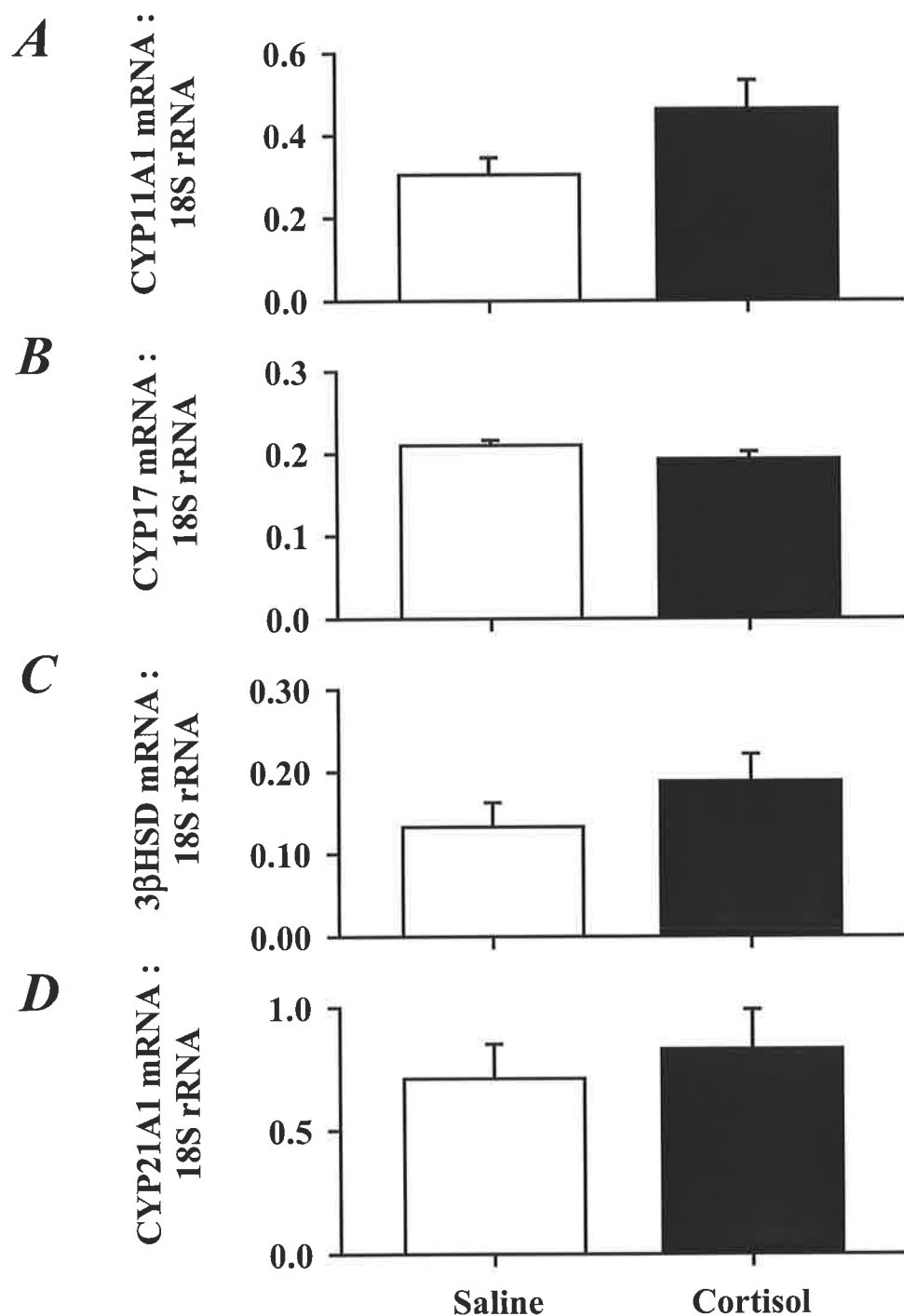
A

B
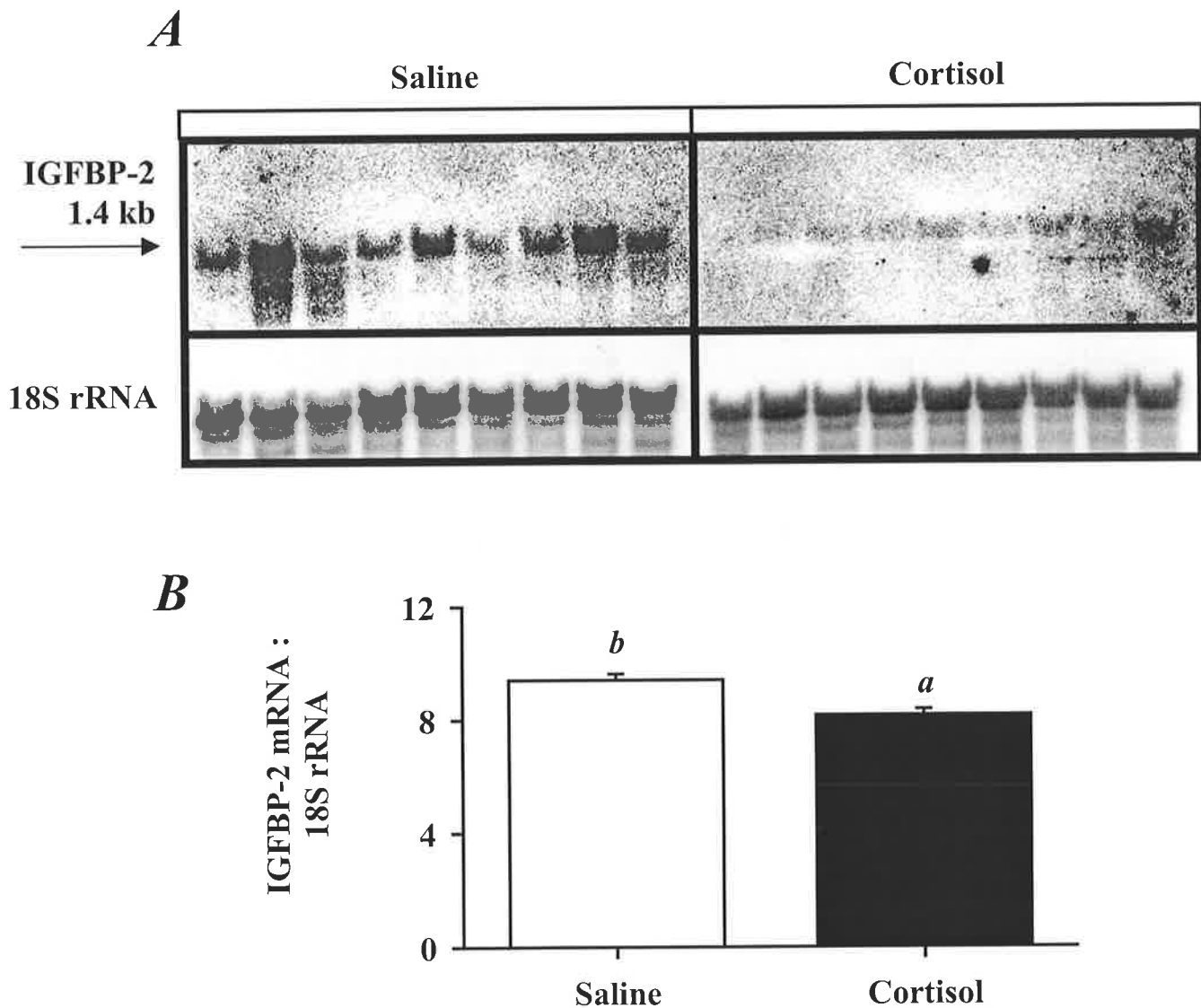
Figure 3.6   **Northern blot of adrenal IGFBP-2 mRNA expression from fetal sheep following a 7 d infusion of either saline or cortisol from 109-116 d of gestation.**

(*A*) Northern blot analysis of total RNA (20 µg·lane^{-1}) prepared from adrenal glands of fetal sheep following a 7 d infusion of either saline or cortisol from 109-116 d of gestation. The Northern membrane was hybridised sequentially with the radiolabelled *b*IGFBP-2 cDNA and 30 mer antisense 18S rRNA oligonucleotide probes.   (*B*) Adrenal IGFBP-2 mRNA : 18S rRNA expression in saline (open bar) and cortisol (black bar) infused fetal sheep.   Significant differences between groups are denoted by different superscripts, *e.g.* a<b (p<0.05).

circulating cortisol concentrations is sufficient to inhibit the expression of adrenal 11βHSD-2 mRNA, the trophic effects of cortisol may require high circulating concentrations of ACTH, as occurs during the two weeks prior to birth.

In the present study, I have demonstrated a reduction in adrenal IGFBP-2 mRNA expression following a 7 d intra-fetal cortisol infusion. This finding, taken with those of the previous chapter may indicate that the level of gene expression for IGFBP-2 within the fetal adrenal is modulated by either intra-adrenal or circulating cortisol. It is interesting to speculate that while cortisol and ACTH may regulate the mRNA and peptide expression of IGF-II within the fetal adrenal ([258]Lu, Han *et al.*, 1994), endogenous cortisol also regulates the tissue delivery and localisation of this growth factor through modulation of the gene expression of its major binding protein, IGFBP-2.

In contrast to the current findings, I reported in Chapter 2 that cortisol infusion in HPD fetuses stimulated an increase in fetal adrenal weight to values comparable with those in intact fetal sheep ([372]Ross, Phillips *et al.*, 1997). Infusion of cortisol into hypophysectomised fetuses, however, increased cytodifferentiation within the adrenal cortex and did not increase fetal adrenal weight ([56]Boshier, Holloway and Liggins, 1981). One interpretation of these findings is that while ACTH stimulates adrenal growth and function in intact and hypophysectomised fetuses, cortisol only stimulates adrenal growth in the presence of a surgically disconnected fetal pituitary. A number of hypotheses arise from the present data to account for the differential impact of cortisol and ACTH on the adrenal glands of intact, HPD and hypophysectomised fetal sheep.

3.4.1   *Model 1. Cortisol acts directly at the fetal adrenal*

One possibility is that cortisol acts directly at the adrenal in the HPD fetus. In this model the action of cortisol requires the presence of the fetal pituitary, and the removal of a pituitary derived 'inhibitory' factor which is normally present in intact fetuses and is not present in HPD fetal sheep (*Figure 3.7*). In this model, cortisol would be unable to stimulate adrenal growth in the intact fetus during mid-gestation, due to the presence of a pituitary derived 'inhibitory' factor which interferes with the autocrine / paracrine effect of cortisol at the fetal adrenal. Cortisol would also be unable to stimulate adrenal growth in hypophysectomised fetuses ([56]Boshier, Holloway and Liggins, 1981) due to the absence of a 'permissive / priming' factor derived from the fetal pituitary which is present in the HPD fetus. Disconnection of the fetal hypothalamus and pituitary, however, may result in lower circulating levels of the putative 'inhibitory' factor, while levels of the 'permissive /

Intact	HPD	Hypophysectomy
F infusion @ (109-116 d) 2-3 mg·24 h^{-1} Chapter 3	F infusion @ (135-140 d) 3.5 mg·24 h^{-1} Chapter 2	F infusion @ (150-153 d) 20 mg·24 h^{-1} Boshier, Holloway and Liggins, 1981

ACTH precursors Present — ACTH (1-39) Permissive factor — Pituitary inhibitor of steroid action on adrenal growth — No effect

ACTH precursors Present — ACTH (1-39) Permissive factor — Growth

ACTH precursors None — ACTH (1-39) None — No effect

Cortisol

| Inhibitor present
ACTH present | Inhibitor absent
ACTH present | Inhibitor absent
ACTH absent |
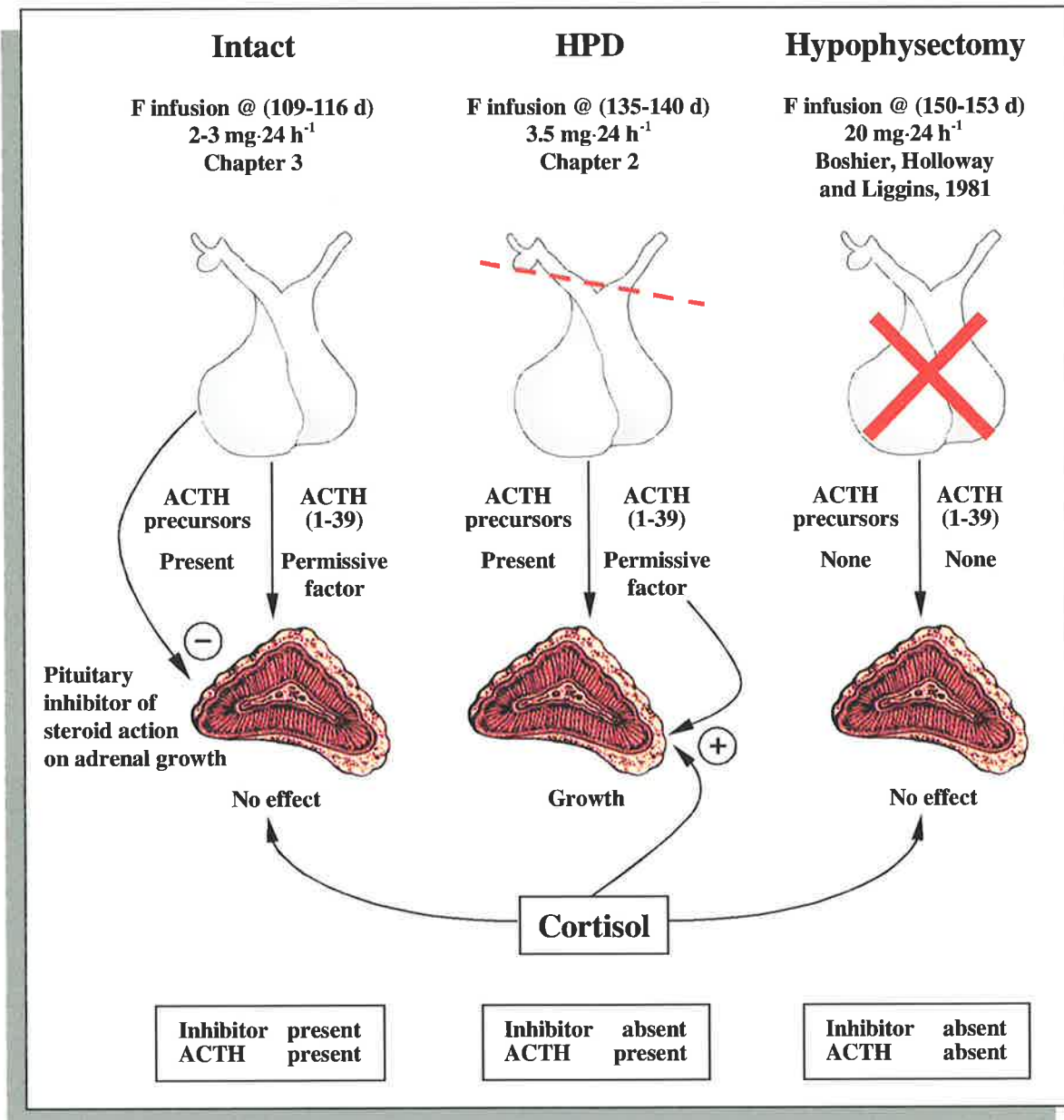
Figure 3.7   Model 1.   Cortisol may act directly at the fetal adrenal gland to promote adrenal growth *in utero*.

The circulating levels of a pituitary derived factor, which normally inhibits the autocrine / paracrine effect of cortisol on adrenal growth in intact fetuses, may be diminished by the surgical disconnection of the fetal pituitary from the hypothalamus. The levels of this inhibitory factor may persist in intact animals throughout most of gestation and diminish in late-gestation, or the inhibition may be overcome by the elevated fetal circulating levels of cortisol in late-gestation.   The ACTH precursors, POMC and pro-ACTH, may also act to inhibit the steroidogenic effects of ACTH(1-39) at the fetal adrenal throughout gestation.   Alternatively, the actions of cortisol at the fetal adrenal may require the prolonged exposure of the adrenal to the 'permissive / priming' effects of ACTH(1-39), prior to the development of an autocrine / paracrine trophic influence of endogenous cortisol.

priming' factor remain unchanged, thus enabling the direct trophic effect of cortisol at the fetal adrenal.

3.4.1.1   Candidate for pituitary derived permissive factor: ACTH(1-39)

Poore and colleagues demonstrated that continuous, low-dose infusion of ACTH(1-24) (43 ng·kg^{-1}·h^{-1}) into hypophysectomised fetuses from 125 d of gestation resulted in parturition at normal term (term=148 ± 2 d of gestation) ([348]Poore, Young *et al.*, 1998). These authors also demonstrated that fetal plasma cortisol concentrations in hypophysectomised fetuses infused with low-dose ACTH(1-24) were similar to the plasma cortisol concentrations in intact fetuses in late-gestation. In addition, total adrenal weights were similar in hypophysectomised fetuses infused with low-dose ACTH(1-24) compared with intact fetuses ([348]Poore, Young *et al.*, 1998).

Poore and co-workers proposed that ACTH may only play a permissive role in the activation of adrenal growth and function since adrenal growth, steroid synthesis and parturition at normal term were accomplished using a continuous low-dose infusion of ACTH(1-24) in the absence of any other pituitary factors, and did not require the late-gestation increase in circulating ACTH measured in intact fetuses ([348]Poore, Young *et al.*, 1998). These and other investigators have suggested that an inhibitory mechanism may act to decrease the ability of ACTH to stimulate growth and steroidogenesis in the adrenal glands of intact fetal sheep until late-gestation, when increasing concentrations of bioactive ACTH(1-39) ([224]Jones and Roebuck, 1980; [279]McMillen, Merei *et al.*, 1995) overcome the tonic inhibition from the fetal pituitary.

3.4.1.2   Candidates for pituitary derived inhibitory factors

Jones and co-workers reported that immunoreactive ACTH-containing peptides in fetal plasma were present in three main peaks on gel chromatography, corresponding to molecular weight ranges of >50 kDa, 30 kDa and 4.5 kDa. These authors also reported an increase in the ratio of the bioactive 4.5 kDa ACTH(1-39) peptide to the higher molecular weight forms of ACTH after 138 d of gestation ([224]Jones and Roebuck, 1980). POMC and pro-ACTH are present in high concentrations in the fetal circulation ([224]Jones and Roebuck, 1980), have low steroidogenic capacity ([165]Gasson, 1979) and inhibit the ACTH induced secretion of cortisol from calf adrenal glands *in vivo* ([220]Jones, Edwards and Tindell, 1992), and ovine fetal adrenal cells *in vitro* ([385]Schwartz, Kleftogiannis *et al.*, 1995). Thus, the inhibitory effects of the ACTH precursor peptides may be two-fold: firstly the inhibition of ACTH(1-39) induced cortisol synthesis, and secondly the inhibition

of the 'permissive / priming' role of ACTH(1-39) in cortisol mediated adrenal growth. Indeed, Phillips and colleagues have demonstrated that while fetal HPD at around 110 d of gestation abolishes the late-gestation increase in circulating levels of ACTH(1-39), the levels of the ACTH precursors remain unchanged between 120 d and 140 d of gestation ([342]Phillips, Ross *et al.*, 1996). Thus, fetal HPD abolishes the late-gestation increase in the ratio of bioactive ACTH(1-39) : ACTH precursors within the fetal circulation.

It is possible that the IRMA used to detect the levels of these precursors in fetal plasma may not detect changes which have occurred in post-translational processing prior to secretion from the pituitary. Thus, while the circulating levels of these peptides detected using the IRMA may be unchanged in the fetal circulation following HPD, their biological activity at the fetal adrenal may be substantially altered following fetal HPD. The high ratio of ACTH precursors : ACTH(1-39) in the circulation of HPD fetuses may inhibit the steroidogenic actions of ACTH(1-39), with less effect on the permissive influence of ACTH(1-39) on adrenal growth.

Since cortisol infusion into fetuses with a surgically disconnected pituitary results in substantial growth of the adrenal ([372]Ross, Phillips *et al.*, 1997), it seems reasonable to suggest that the putative 'inhibitory' factor, proposed in the present model, may be absent or at lower levels within the circulation of HPD fetuses than in intact fetuses. Since the circulating levels of the ACTH precursors (POMC and pro-ACTH) do not appear to be altered following disconnection of the fetal hypothalamus and pituitary gland ([342]Phillips, Ross *et al.*, 1996), it is possible that another pituitary derived 'inhibitory' factor may exist, the circulating levels of which are lower following the surgical disconnection of the fetal hypothalamus and pituitary. While this factor appears to originate from the fetal pituitary, it may originate from cells other than the fetal corticotrophs. One candidate hormone that fulfils the requirements of this putative 'inhibitory' factor is fetal prolactin. A recent study in our laboratory by Houghton and colleagues demonstrated that fetal plasma concentrations of prolactin increase progressively throughout gestation ([200]Houghton, Young and McMillen, 1995b). Phillips and colleagues have demonstrated that disconnection of the fetal hypothalamus and pituitary at around 110 d of gestation results in significantly lower levels of prolactin mRNA in the pars distalis and prolactin peptide in the fetal circulation from 135-140 d of gestation (8.0 ± 3.3 ng·ml^{-1}), when compared with levels in intact fetuses (28.3 ± 3.9 ng·ml^{-1}) ([341]Phillips, Fielke *et al.*, 1996).

A number of studies have established the presence of prolactin receptors within the fetal adrenal ([159]Freemark, Driscoll *et al.*, 1997). In the early-gestation human fetus and the

mid-gestation fetal rat, the prolactin receptor is expressed in a rim of capsular like mesenchymal cells and neocortical like parenchymal cells lying near the surface of the gland ([159]Freemark, Driscoll *et al.*, 1997). Cells of this definitive zone have high mitotic activity, thus, while it has been suggested that lactogens may play a role in adrenal differentiation early in development, it is also possible that prolactin may interact with the autocrine / paracrine effect of cortisol on the adrenal. If prolactin does inhibit the autocrine / paracrine effect of cortisol on the fetal adrenal, the lowered circulating levels of this hormone following fetal HPD at around 110 d of gestation may remove a 'brake' from the fetal adrenal, such that cortisol can stimulate adrenal growth in a fashion which is not possible in intact fetuses until circulating cortisol levels increase and the adrenal is exposed to the 'permissive / priming' factor, possibly ACTH(1-39), for a sufficient duration. It would appear that while fetal HPD may remove an inhibitory factor for the trophic effect of cortisol on adrenal growth, neither the removal of this putative 'inhibitory' factor, or cortisol infusion, restored the steroidogenic capacity of the adrenal of the HPD fetus.

It is therefore proposed that ACTH precursors (POMC and pro-ACTH) may serve as inhibitors of the steroidogenic effects of ACTH(1-39) on the fetal adrenal, while another factor (possibly prolactin) inhibits the growth promoting effect of cortisol at the adrenal gland in the intact fetus, while the circulating levels of this factor are reduced following surgical disconnection of the fetal hypothalamus and pituitary.

3.4.1.3   *Cortisol and adrenal steroidogenesis*

Fetal ovine adrenocortical cells cultured for 48 h in the presence of dexamethasone, or cortisol, synthesise more cAMP than control cells, when both groups are stimulated by ACTH(1-24) ([117]Darbeida, Naaman and Durand, 1987). These authors also found that the potentiating effect of glucocorticoids on ACTH stimulated cAMP accumulation in adult adrenocortical cells requires at least 21 h of treatment with dexamethasone *in vitro*, and is concentration dependent and steroid specific ([115]Darbeida and Durand, 1987). In addition, corticosteroid production by fetal adrenal cells stimulated by ACTH(1-24), forskolin or Bt_2cAMP is also enhanced by prior dexamethasone treatment *in vitro* ([117]Darbeida, Naaman and Durand, 1987). These authors have suggested that glucocorticoids can affect the maturation of ovine fetal adrenocortical cells by an autocrine / paracrine process and that chronic exposure to glucocorticoids may be necessary for the full expression of the cAMP response to ACTH(1-24) ([117]Darbeida, Naaman and Durand, 1987). Exposure of adult ovine adrenal cells to dexamethasone *in vitro* also increases the number of cellular ACTH receptors and enhances the ACTH stimulated translocation of cholesterol from the

cytoplasm into the mitochondria ([116]Darbeida and Durand, 1990). A further study by these authors confirmed that the increase in ovine adrenocortical ACTH receptors, following chronic glucocorticoid treatment *in vitro,* is mediated via an increase in the mRNA abundance for the ACTH receptor ([345]Picard-Hagen, Penhoat *et al.,* 1997). Thus, glucocorticoids may mediate the capacity of the fetal adrenal cortex to respond to ACTH stimulation by increasing the expression of the ACTH receptor and the intra-cellular production of cAMP in response to ACTH stimulation. A recent study by Fraser and co-workers, however, has shown no change in the mRNA expression of adrenal ACTH receptors following a 96 h infusion of cortisol (5 μg·min^{-1}) *in vivo* from 126-129 d of gestation at ([158]Fraser, Jeffray and Challis, 1999). Thus, there appears to be a differential effect of cortisol on adrenocortical cells *in vitro* and *in vivo,* with respect to ACTH receptor expression.

3.4.1.4   *Duration of exposure to permissive factors may be critical*

The finding, in the present study, that cortisol infusion into intact fetuses did not result in adrenal growth, may be due to a pituitary derived factor which inhibits the actions of exogenous glucocorticoids at the fetal adrenal. Alternatively, insufficient duration of exposure to a pituitary derived permissive factor, such as ACTH(1-39), may leave the adrenal gland inadequately 'primed' and unable to respond to the exogenous cortisol infusion. In the present study, and in the previous chapter, I have measured no changes in the circulating levels of ACTH(1-39) or ir-ACTH in response to fetal HPD, or in response to cortisol infusion into HPD and intact fetal sheep. It is possible that the action of ACTH on the fetal adrenal is two-fold: firstly to stimulate the cytological maturation necessary for steroid synthesis, and secondly to 'prime' the adrenal by stimulating adrenal IGF-II mRNA expression, allowing cortisol to stimulate adrenal growth distal to the actions of ACTH. The direct action of cortisol at the fetal adrenal may, therefore, be dependent on developmental changes mediated by the non-steroidogenic 'permissive / priming' actions of ACTH(1-39).

In this model, the existence of a pituitary derived inhibitory factor need not be invoked to explain the absence of an effect of exogenous cortisol infusion in mid-gestation on adrenal growth. The autocrine / paracrine effects of cortisol may occur only after prolonged adrenal exposure to ACTH(1-39). It is possible that the increase in circulating levels of ACTH(1-39) in the fetal sheep during late-gestation serves to stimulate cytological maturation and the synthesis of glucocorticoids. These steroids, in turn, mediate the local milieu of growth factors that bring about the rapid hyperplasia, characteristic of
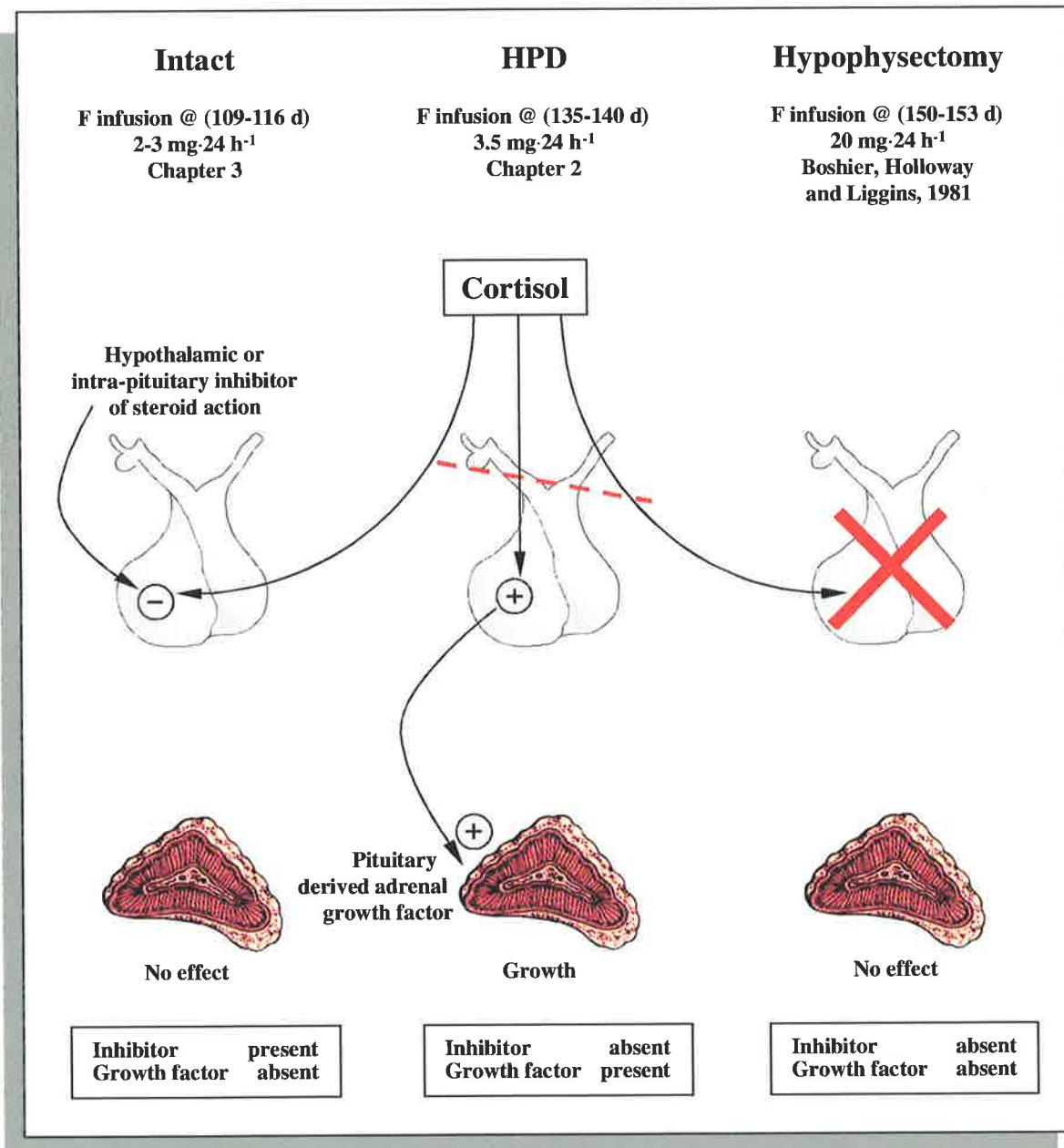
adrenocortical growth prior to birth. The IGF axis may, therefore, be modulated within the adrenal gland by the combined actions of ACTH and cortisol. In addition, cortisol may direct the actions of this growth factor via alterations in the gene expression of the IGF cognate binding proteins.

3.4.2   Model 2. Cortisol acts indirectly via the fetal pituitary

While ACTH, in the presence of endogenous cortisol, acts at the adrenal of the hypophysectomised fetus to stimulate adrenal growth, cortisol infusion into hypophysectomised fetuses, in the absence of a pituitary and ACTH-containing peptides, does not stimulate adrenal growth ([56]Boshier, Holloway and Liggins, 1981). A second possibility, therefore, is that cortisol may stimulate growth of the adrenal gland via an indirect action at the fetal pituitary (*Figure 3.8*). In this model, cortisol would not be able to stimulate adrenal growth in the intact fetus during mid-gestation due to the presence of an hypothalamic or intra-pituitary 'inhibitory' factor which blocks the actions of cortisol at the pituitary. Cortisol would also not stimulate adrenal growth in hypophysectomised fetuses ([56]Boshier, Holloway and Liggins, 1981) as removal of the pituitary would abolish the action of cortisol via the fetal pituitary. Disconnection of the fetal hypothalamus and pituitary, however, may remove the putative inhibitory factor, allowing cortisol to stimulate the secretion of a pituitary derived 'adrenal growth factor'. In this model, while ACTH(1-39) is important in inducing the adrenal cytological maturation leading to the enhanced adrenal steroidogenesis observed during late-gestation ([56]Boshier, Holloway and Liggins, 1981; [80]Challis and Brooks, 1989), cortisol stimulates growth within the adrenal indirectly by stimulating the release of non ACTH-containing pituitary derived peptides, which are trophic for the fetal adrenal.

3.4.3   Summary

In summary, I have demonstrated a decrease in the expression of mRNA for 11βHSD-2 and IGFBP-2 within the fetal adrenal following a 7 d intra-fetal infusion of cortisol from 109-116 d of gestation. I have measured no change, however, in adrenal weight or in the mRNA expression of the adrenal steroidogenic enzymes in cortisol infused fetuses when compared with saline infused fetuses. Increased intra-adrenal exposure to cortisol at this stage of gestation is, therefore, not sufficient to promote adrenal growth or steroidogenic enzyme gene expression. It is possible that under conditions when the fetal adrenal is stimulated by increased fetal ACTH, such as in the week before birth ([80]Challis and Brooks, 1989), or during chronic intra-uterine stress ([343]Phillips, Simonetta *et al.*, 1996), a fall in adrenal 11βHSD-2 mRNA expression and increased intra-adrenal exposure to

Intact

F infusion @ (109-116 d)
2-3 mg·24 h⁻¹
Chapter 3

HPD

F infusion @ (135-140 d)
3.5 mg·24 h⁻¹
Chapter 2

Hypophysectomy

F infusion @ (150-153 d)
20 mg·24 h⁻¹
Boshier, Holloway
and Liggins, 1981

Cortisol

Hypothalamic or
intra-pituitary inhibitor
of steroid action

Pituitary
derived adrenal
growth factor

No effect

Growth

No effect

| Inhibitor | present |
| Growth factor | absent |

| Inhibitor | absent |
| Growth factor | present |

| Inhibitor | absent |
| Growth factor | absent |

Figure 3.8   Model 2. Cortisol may act indirectly, via the fetal pituitary, to promote the secretion of an adrenal trophic factor *in utero*.

The presence of an hypothalamic or intra-pituitary inhibitor of steroid action may inhibit the action of cortisol at the fetal adrenal. In the absence of a functional connection between the pituitary and hypothalamus, however, cortisol may stimulate the secretion of a pituitary derived adrenal trophic factor, which stimulates adrenal growth in the absence of a change in the circulating levels of the ACTH-containing peptides.

glucocorticoids may be important in enhancing the local growth factor and steroidogenic responses to the prevailing high ACTH concentrations.

It is interesting to speculate that while the circulating levels of cortisol were sufficient to inhibit the adrenal mRNA expression of IGFBP-2, there may have been an insufficient duration of exposure to the 'permissive / priming' effects of ACTH by 116 d of gestation to allow the changes in IGFBP-2 to exert an effect on adrenal growth or function. Thus, while some elements of the intra-adrenal growth factor axis were modulated by the mid-gestation cortisol infusion, these changes appear to have been insufficient to stimulate growth within the fetal adrenal gland.

CHAPTER 4.
EFFECT OF N-TERMINAL POMC DERIVED PEPTIDE INFUSION ON FETAL ADRENAL GROWTH AND FUNCTION

4.1   INTRODUCTION

A number of studies have demonstrated the mitogenic and steroidogenic potentiating effects of peptides arising from the N-terminal region of POMC, on isolated rat adrenocortical cells in culture ([148]Estivariz, Iturriza et al., 1982; [150]Farese, Ling et al., 1983). Administration of trypsinised N-POMC(1-77) to seven week old rats stimulates adrenocortical mitosis, and N-POMC(1-28) and N-POMC(2-59) stimulate adrenal growth in vivo and DNA synthesis in adrenocortical cells in vitro ([148]Estivariz, Iturriza et al., 1982). γ₃-MSH [N-POMC(51-77)] or intact N-POMC(1-77), however, are inactive in this in vitro model, suggesting that adrenal growth and mitosis are a consequence of post-secretional cleavage of N-POMC(1-77) to release mitogenically active N-terminal fragments and γ₃-MSH ([148]Estivariz, Iturriza et al., 1982). γ₃-MSH has been shown to potentiate the steroidogenic action of ACTH on isolated adrenocortical cells from adult rats ([150]Farese, Ling et al., 1983) without independently affecting steroid output. Thus, the N-terminal region of POMC contains peptides that affect different aspects of adrenal activity. N-POMC(1-28) and N-POMC(2-59) stimulate DNA synthesis while γ₃-MSH potentiates ACTH stimulated steroidogenesis.

Studies in the fetal sheep have demonstrated that pro-γ–MSH and ACTH are co-localised within fetal corticotrophs from as early as 38 d of gestation ([293]Mulvogue, McMillen et al., 1986) and POMC and pro-ACTH are present in ~20 fold higher concentrations in fetal sheep circulation than ACTH(1-39) ([342]Phillips, Ross et al., 1996). Saphier and co-workers have also reported that the molar ratio of N-POMC(1-77) : ACTH(1-39) is around 20-50 in the fetal sheep in late-gestation, whereas this ratio is 2 in the adult ewe ([377]Saphier, Glynn et al., 1993). These authors also found that there is a decrease in the plasma levels of N-POMC(1-77) concomitant with an increase in the plasma levels of N-POMC(50-74) after 138 d of gestation. These investigators suggested that the N-POMC(1-77) peptide may undergo proteolytic cleavage within the fetal circulation, liberating smaller biologically active peptides during late-gestation. Thus, a potential role

for these peptides, in the growth and development of the fetal adrenal cortex *in utero*, has been suggested.

While the *N*-POMC peptides have potent mitogenic and steroidogenic effects on adult rat adrenocortical cells *in vivo* and *in vitro*, little is known about their effect on fetal adrenal development *in utero*. I have investigated the impact of intra-fetal infusion of purified bovine *N*-POMC(1-77) or *N*-POMC(1-49), on fetal adrenal growth in late-gestation. I have also investigated the effect of these peptides on the circulating level of cortisol and on the expression of mRNA for the steroidogenic enzymes within the fetal adrenal gland in late-gestation.

4.2 MATERIALS AND METHODS

4.2.1 Animal protocols and surgery

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation. Twenty-seven pregnant Border Leicester × Merino ewes with singleton fetuses were used in this study. The ewes were housed in individual pens in animal holding rooms, with a 12 h light / dark lighting regimen, and fed once daily with 1 kg of lucern chaff and 1 kg of Baramil joint stock rations between 0900 and 1300 h with water *ad libitum*. Catheterisation surgery was carried out between 116 d and 125 d of gestation under general anaesthesia and using aseptic techniques, as described in Chapter 2 (*see section 2.2.1.3.1 on pg. 72*). There was a recovery period of at least three days after surgery before fetal blood samples were collected.

4.2.2 Isolation and preparation of bovine N-POMC peptides

Peptides derived from the *N*-terminal region of POMC were generously provided by Professor Hugh Bennett (Endocrine Laboratory, Royal Victoria Hospital and Department of Medicine, McGill University, Montreal, Canada). *N*-POMC(1-77) and *N*-POMC(1-49) were extracted from bovine neurointermediate pituitaries (Pel-Freez Biologicals) using an acidic extraction procedure followed by reversed-phase batch fractionation ([66]Browne, Bennett and Solomon, 1981). The peptides were purified to homogeneity by sequential steps of reversed phase HPLC using solvent systems containing trifluoroacetic acid and heptafluorobutyric acid as ion-pairing reagents, as described previously ([35]Bennett, 1983). The identity and integrity of each peptide was confirmed by mass spectrometry ([411]Toney, Bateman *et al.*, 1993). The extraction and purification procedures were performed by Dr. Susan James (Endocrine Laboratory, Royal Victoria Hospital and Department of Medicine, McGill University, Montreal, Canada).

4.2.3 Infusion regimen and blood sampling protocol

Infusion regimen. N-POMC(1-77) (2 $\mu g \cdot ml^{-1} \cdot h^{-1}$; n=9 fetuses), N-POMC(1-49) (2 $\mu g \cdot ml^{-1} \cdot h^{-1}$; n=7 fetuses) or saline (1 $ml \cdot h^{-1}$; n=5 fetuses) was infused into fetal sheep for 48 h from 136-138 d of gestation. Infusion started at 0900 h at 136 d of gestation (designated time 0). An additional group (n=6 fetuses) of non-infused control fetuses were used, in conjunction with the saline infused group, in the estimation of adrenal weight and area in control fetuses.

Blood sampling protocol. Fetal arterial blood samples (2 ml) were collected into chilled collection tubes at -120 min, -30 min, +4 h, +24 h, +40 h and +48 h. Blood for cortisol assay was collected into tubes containing 125 IU lithium heparin. Blood for ir-ACTH assay was collected into plain tubes containing EDTA (18.6 $g \cdot l^{-1}$ of whole blood) and aprotinin (100 KIU in 100 $\mu l \cdot ml^{-1}$ of whole blood). Blood samples were centrifuged at 1800 g for 10 min at 4 °C before separation and storage of plasma at -20 °C for subsequent assay. Fetal arterial blood (0.5 ml) was collected on alternate days for measurement of whole blood p_aO_2, p_aCO_2, pH, O_2 saturation, and haemoglobin content using an ABL 550 acid base analyser and OSM2 haemoximeter.

4.2.4 Tissue collection

At 138 d of gestation, ewes were killed with an overdose of Lethabarb (25 ml at 325 $mg \cdot ml^{-1}$) and fetal sheep were removed, weighed and killed by decapitation. The left adrenal gland from each fetus was quickly removed, weighed, snap frozen in liquid N_2 and stored at -80 °C until total RNA was extracted. The right fetal adrenal gland was fixed in phosphate buffered 4 % paraformaldehyde (*see Appendix I*) at 4 °C. Following fixation for 24 h, adrenals were washed in ice cold 100 mM phosphate buffered saline (PBS tablets, Sigma-Aldrich) (2× 24 h) and stored in 70 % ethanol at 4 °C prior to embedding in paraffin wax.

4.2.5 Radioimmunoassays

Cortisol radioimmunoassay. Cortisol concentrations were measured in fetal plasma samples from the N-POMC(1-77) infused group (n=9 fetuses; *n*=46 samples), the N-POMC(1-49) infused group (n=6 fetuses; *n*=34 samples) and the saline infused group (n=5 fetuses; *n*=30 samples). Total cortisol concentrations in fetal sheep plasma were measured using a radioimmunoassay, validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 74*). The intra- and inter-assay COVs were <10 % and <20 % respectively.

ACTH radioimmunoassay. ir-ACTH concentrations were measured in fetal plasma samples from the *N*-POMC(1-77) infused group (n=9 fetuses; *n*=46 samples), the *N*-POMC(1-49) infused group (n=7 fetuses; *n*=39 samples) and the saline infused group (n=5 fetuses; *n*=30 samples). The concentrations of ir-ACTH were measured using a radioimmunoassay which has previously been validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 75*). The inter-assay COV was <20 % and the intra-assay COV was <10 %.

4.2.6 cDNA and antisense oligonucleotide probes and probe labelling

cDNA probes. *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNA probes were generously provided by Professor W Miller (Department of Pediatrics, UCSF, San Francisco, CA, USA) (*see section 2.2.3.1 on pg. 75 and Figures 2.1, 2.2 and 2.4 on pp. 76, 77 and 80 respectively*). A *h*3βHSD cDNA probe was donated by Dr. R Rodgers (Department of Medicine, Flinders University, SA, Australia) (*see section 2.2.3.1 on pg. 75 and Figure 2.3 on pg. 78*). cDNAs were radiolabelled with α-[^{32}P] dCTP (3,000 Ci·mmol^{-1}) by the random priming oligomer method to a specific activity of 10^9 cpm·μg^{-1} or greater, as described in Chapter 2 (*see section 2.2.3.1 on pg. 83*).

Oligonucleotide probes. A 30 mer antisense oligonucleotide probe for rat 18S rRNA, complementary to nucleotides 151-180, was synthesised (GeneWorks) (*see section 2.2.3.1 on pg. 83 and Figure 2.7 on pg. 85*) and end-labelled using T4PNK and γ-[^{32}P] ATP (4,000 Ci·mmol^{-1}) as substrate, as described in Chapter 2 (*see section 2.2.3.1 on pg. 84*).

4.2.7 Total RNA isolation

Total RNA was extracted from one adrenal from each fetus of the *N*-POMC(1-77) infused (n=9 fetuses), *N*-POMC(1-49) infused (n=7 fetuses) and saline infused groups (n=5 fetuses). Total RNA was extracted by homogenisation in 1 ml of Tri Reagent (Sigma-Aldrich) ([92]Chomczynski and Sacchi, 1987; [91]Chomczynski, 1993) using a Polytron PT 3000 laboratory homogeniser with a PT-DA 3007/2 generator at 30,000 rpm. The homogenate was transferred to a sterile 1.5 ml eppendorf tube and 100 µl of 1-bromo-3-chloropropane (Sigma-Aldrich) was added to the homogenate. The samples were then mixed and allowed to stand at RT for 5 min. Adrenal homogenates were centrifuged at 12,000 *g* for 15 min at 4 °C using a Hawksley MBC Microfuge (Hawksley and Sons).

The upper aqueous phase was then transferred to a separate sterile 1.5 ml eppendorf tube. RNA was precipitated from the aqueous phase by the addition of 0.5 ml isopropanol

(BDH Laboratory Supplies), and samples allowed to stand at RT for 10 min, followed by centrifuge at 12,000 g for 10 min at 4 °C. The supernatant was aspirated and the RNA pellet washed with 1 ml of 75 % ethanol, centrifuged at 12,000 g for 10 min at 4 °C and dried using a Savant SpeedVac SC110 vacuum centrifuge concentrator. RNA pellets were reconstituted in sterile deionised distilled water and nucleic acid purity and concentration were quantified using a Beckman DU-50 spectrophotometer. Prior to Northern analysis, the integrity of the total RNA preparations was verified by subjecting 1 µl of each RNA sample to 1 % agarose gel electrophoresis using molecular biology grade agarose in 1× TAE (pH 8.0) (*see Appendix I*) and staining with EtBr. Total RNA preparations were stored at a concentration of approximately 5 µg·µl^{-1} at -80 °C until required for use.

4.2.8   *Northern blot analysis*

Total RNA samples (20 µg of adrenal RNA) were separated by electrophoresis in 1 % agarose denaturing gels (*see Appendix I*), using 1× Northern running buffer (*see Appendix I*), with one lane of the gel reserved for 0.5-9 kb RNA Millennium size markers (*Figure 4.1A*), as described in Chapter 2 (*see section 2.2.3.3 on pg. 87*). Total RNA was then transferred by gravity-feed blotting onto Zetaprobe nitrocellulose membranes using an inverted blotting configuration and 10× SSC (*see Appendix I*) as the transfer buffer (*Figure 4.1B*).

Membranes were washed in 10× SSC, 0.1 % SDS for 10 min at RT and exposed to UV light (12 sec at 120 J·cm^{-2}) to cross-link RNA onto the membrane (*Figure 4.1C*). Membranes were rolled in a piece of gauze and transferred into a hybridisation bottle (Hybaid). Membranes were pre-hybridised overnight at 42 °C in 30 ml of either cDNA or antisense oligonucleotide hybridisation buffer (*see Appendix I*), in a Shake 'N' Stack hybridisation oven (Hybaid). Membranes were then hybridised sequentially with cDNA and oligonucleotide probes for 16 h at 42 °C (cDNA probes) or 50 °C (oligonucleotide probes) in 30 ml of fresh hybridisation buffer, containing either 1-2×10^6 cpm·ml^{-1} of the cDNA probe or 5×10^5 cpm·ml^{-1} of the 30 mer antisense 18S rRNA oligonucleotide probe (*Figure 4.1D*). Prior to exposure to phosphorimage plates, membranes were washed once (10 min) at RT in 1× SSC, 0.1 % SDS; then twice (10 min each time) in 1× SSC, 0.1 % SDS at 42 °C; and twice (10 min each time) in 0.1× SSC, 0.1 % SDS at 42 °C, then briefly air-dried and sealed in a plastic bag.

Membranes were exposed to phosphorimage plates in BAS 2040 cassettes (*Figure 4.1E*). cDNA probes were stripped from membranes between hybridisations by washing in 0.01× SSC, 0.5 % SDS for 10 min at 80 °C. Consistency of lane loading for each
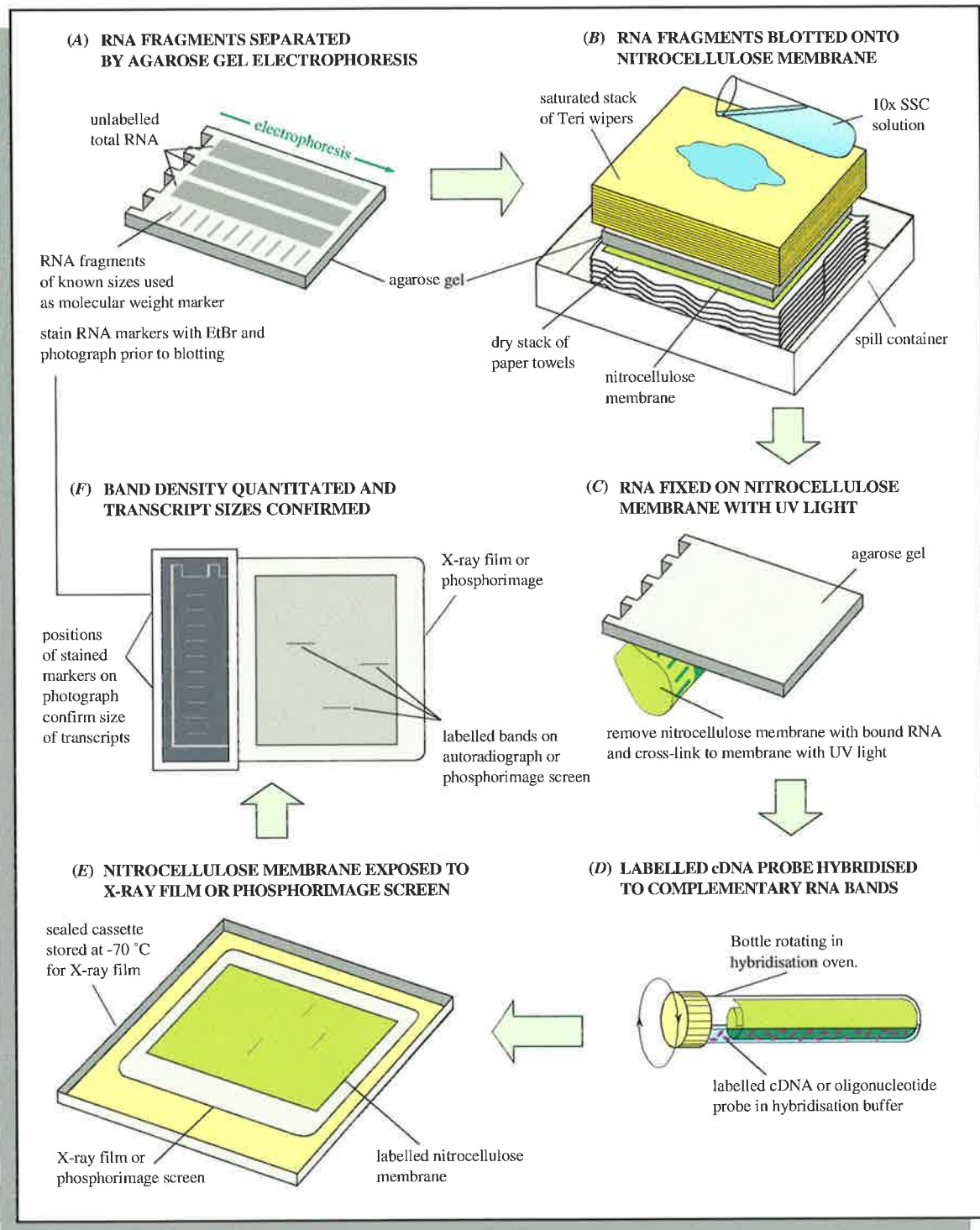
Figure 4.1   **Gel electrophoresis and Northern blot of fetal adrenal RNA.**

(*A*) Adrenal RNA is separated by gel electrophoresis in a 1 % agarose formaldehyde gel. RNA molecular weight markers are run in the gel and photographed. (*B*) RNA fragments are blotted onto a nitrocellulose membrane in 10× SSC. (*C*) RNA is fixed to the membrane by exposure to UV light. (*D*) Membranes are prehybridised in hybridisation buffer prior to the addition of radiolabelled cDNA or oligonucleotide probes. (*E*) Membranes are washed, sealed in plastic and exposed to X-ray film or phosphorimage plates. (*F*) Images are quantified and transcript sizes confirmed with RNA markers.

membrane was verified by a final hybridisation of each membrane with 5×10^5 cpm·ml^{-1} of the 30 mer antisense 18S rRNA oligonucleotide probe and exposure to phosphorimage plates. Phosphorimage plate exposures were quantified on a Fuji-BAS 1000 phosphorimage scanner using Fuji MacBAS software (MacBAS 2.2) (*Figure 4.1F*). A ratio of the density of each specific band with the density of the corresponding 18S rRNA band was calculated before comparisons were made. The radiolabelled *h*CYP11A1, *h*CYP17, *h*CYP21A1 and *h*3βHSD cDNA probes were used to probe three Northern blots of total RNA from fetal adrenals. One Northern blot membrane of adrenal total RNA, from *N*-POMC(1-77), *N*-POMC(1-49) and saline infused fetal sheep (n=9, 7 and 5 fetuses respectively), was hybridised with the *h*CYP11A1 cDNA probe. The second membrane (n=9, 5 and 5 fetuses respectively) was hybridised with the *h*CYP17 cDNA probe, and the third membrane (n=9, 7 and 5 fetuses respectively) was hybridised with the *h*CYP21A1 and *h*3βHSD cDNA probes.

4.2.9   *Immunohistochemistry*

Adrenals from the *N*-POMC(1-77) (n=6 fetuses), *N*-POMC(1-49) (n=6 fetuses) and saline infused (n=3 fetuses) groups, and additional non-infused control fetuses (n=3 fetuses), were fixed using phosphate buffered 4 % paraformaldehyde for 24 h prior to paraffin wax embedding at 60 °C.

The anti-3βHSD polyclonal antibody raised in rabbits against human placental 3βHSD was a generous gift from Dr. Ian Mason ([127]Doody, Carr *et al.*, 1990), and has previously been validated for use in ovine adrenals ([44]Bird, Zheng *et al.*, 1996). Transverse adrenal sections (5 μm) were cut on a microtome and mounted onto Knittel glass histology slides (Crown Scientific). Sections were deparaffinised (2× 10 min) in histoclear (National Diagnostics) and rehydrated in graded ethanols (2× 5 min each), and sterile, deionised distilled water (2× 2 min). Prior to incubation, sections were washed with 100 mM PBS for 20 min, followed by 100 mM PBS containing 0.5 % hydrogen peroxide (APS Ajax Finechem) for 30 min to quench endogenous peroxidase activity, then 100 mM PBS (3× 5 min). Sections were then incubated in 100 mM PBS containing 3 % normal goat serum (NGS, Vectastain ABC kit PK-4001, Vector Laboratories) and 1 % bovine serum albumin (BSA) fraction V (Sigma-Aldrich) for 30 min. Excess NGS was then blotted and the sections covered with anti-3βHSD, diluted (1 : 2,000) in 100 mM PBS containing 1 % BSA. Sections were incubated overnight in an air tight humidified container at 4 °C.

Following incubation with the primary antibody, the sections were washed with 100 mM PBS (3× 5 min) and incubated with biotinylated goat anti-rabbit secondary antibody

(Vectastain ABC kit PK-4001) for 60 min at RT. Sections were washed with 100 mM PBS (3× 5 min) and each section was incubated with avidin-biotin-peroxidase complex (Vectastain kit PK-4001) at RT for 60 min. Sections were then washed in 100 mM PBS (3× 5 min) before being covered with 0.5 mg·ml⁻¹ 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) in 100 mM PBS and 0.02 % hydrogen peroxide at RT for 10 min. Sections were finally washed in 100 mM PBS (3× 5 min) and dehydrated before Knittel glass coverslips (Crown Scientific) were mounted with DPX (BDH Laboratory Supplies).

4.2.10   *Adrenal morphometry*

The transverse areas of the adrenal cortex and medulla were determined from mid-glandular sections. Images of adrenal sections were captured using a CCD black and white video camera (SSC-M370CE; Sony) mounted on a dissecting microscope via an SZ-CTV photomount tube (Olympus Optical Company). The image was digitised as a grey scale image using an Apple Power Mac 8500/120 (Apple) equipped with Image V1.61 software (National Institutes of Health). Total adrenal areas were obtained by defining and measuring the area contained within the border of the adrenal capsule, excluding the central adrenal vein. The area of the adrenal cortex was defined as the area of the gland that stained positively with anti-3βHSD. The area of the adrenal medulla was defined by the difference between the total adrenal and adrenocortical areas.

4.2.11   *Statistical analysis*

Data are presented as the mean ± SEM. Total adrenal weight (the sum of the weights of the left and right adrenals), the ratio of adrenal : fetal body weight, the area of the right adrenal cortex as a percentage of the total area of the right adrenal, the ratio of adrenal steroidogenic enzyme mRNA : 18S rRNA, and the ratio of adrenal CYP17 : 3βHSD mRNA expression were compared between groups using a one-way ANOVA. Least Significant Difference (LSD) post-hoc test was used when the one-way ANOVA identified a significant difference between the groups. Fetal plasma concentrations of cortisol and ir-ACTH were compared using a two-way ANOVA with repeated measures, with treatment group (*e.g.* saline, *N*-POMC(1-49) or *N*-POMC(1-77) infusion) and time (*e.g.* -120 min, -30 min, +4 h, +24 h, +40 h and +48 h) as the specified factors. Linear regression models were used to assess correlations of (*1*) area of the right adrenal vs. weight of the right adrenal gland, and (*2*) mean circulating cortisol concentration during the last 24 h of the infusion period vs. the ratio of adrenal CYP17 mRNA : 18S rRNA expression in fetuses infused with saline, *N*-POMC(1-49) or *N*-POMC(1-77). A probability of <5 % (p<0.05) was considered to be significant.

4.3   RESULTS

4.3.1   Fetal plasma cortisol and ir-ACTH levels

Fetal plasma cortisol concentrations. Infusion of *N*-POMC(1-77) or *N*-POMC(1-49) into fetal sheep from 136-138 d of gestation did not alter plasma cortisol concentrations throughout the infusion period when compared with saline infused fetuses (*Figure 4.2A*).

Fetal plasma ir-ACTH concentrations. Plasma ir-ACTH concentrations were not different between the saline, *N*-POMC(1-49) and *N*-POMC(1-77) infused groups between 136-138 d of gestation and there was no change in the plasma ir-ACTH concentrations with time in any of the groups (*Figure 4.2B*).

4.3.2   Total fetal adrenal weight, fetal body weight and adrenal morphometry

Adrenal weights and fetal body weights. Fetal body weights were not significantly different after intra-fetal infusion of *N*-POMC(1-77) (4.47 ± 0.24 kg), *N*-POMC(1-49) (4.40 ± 0.12 kg) or saline (4.80 ± 0.19 kg). *N*-POMC(1-77) infusion did, however, result in a significant increase ($p<0.05$) in total adrenal weight (414.4 ± 16.3 mg), compared with the *N*-POMC(1-49) (361.4 ± 23.1 mg) or saline infused groups (361.0 ± 12.3 mg) (*Figure 4.3A*). The ratio of total adrenal : fetal body weight was also significantly greater ($p<0.05$) in the *N*-POMC(1-77) infused group (94.6 ± 5.7 mg·kg^{-1}) compared with saline (75.6 ± 1.8 mg·kg^{-1}), but not *N*-POMC(1-49) infused (82.7 ± 6.1 mg·kg^{-1}) fetal sheep (*Figure 4.3B*).

Adrenal morphometry. In adrenals from all groups, staining with anti-3βHSD was observed throughout the adrenal cortex. Positive staining with anti-3βHSD was also present in some cells adjacent to the central adrenal vein. Positive staining with anti-3βHSD was not observed in the adrenomedullary cells in any adrenal sections. The area of the right adrenal (mm^2) was positively correlated with adrenal weight (mg) in saline, *N*-POMC(1-49) and *N*-POMC(1-77) infused fetuses (*Figure 4.3C*), as described by the equation:

$$\textbf{[Adrenal area]} \qquad = \textbf{0.059[Adrenal weight]} + \textbf{3.4} \qquad\qquad (4.1)$$
(r=0.58, p<0.05)

There was no significant difference between the groups, however, in the relative areas of the adrenal cortex [saline: 52.0 ± 2.7 %; *N*-POMC(1-49): 51.3 ± 3.8 %; *N*-POMC(1-77): 48.1 ± 3.0 %].

A

B

Time Relative to Infusion
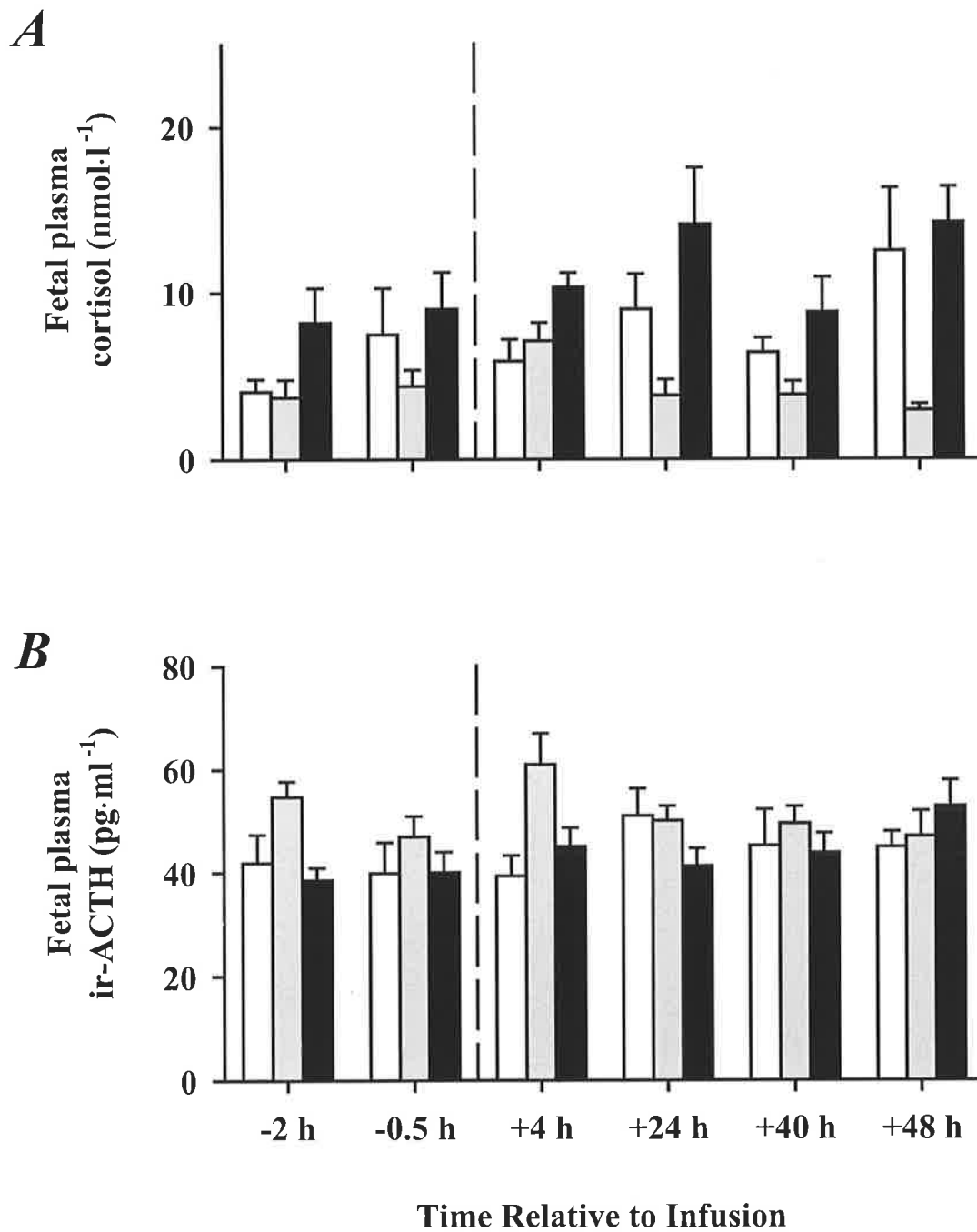
Figure 4.2   Plasma   concentrations   of   ir-ACTH   and   cortisol   in   saline,
**   N-POMC(1-49) and N-POMC(1-77) infused fetal sheep.**

Plasma concentrations of (*A*) cortisol and (*B*) ir-ACTH between 136 d and 138 d of gestation in saline (open bars), *N*-POMC(1-49) (grey bars) and *N*-POMC(1-77) (black bars) infused fetal sheep.

A

B

C
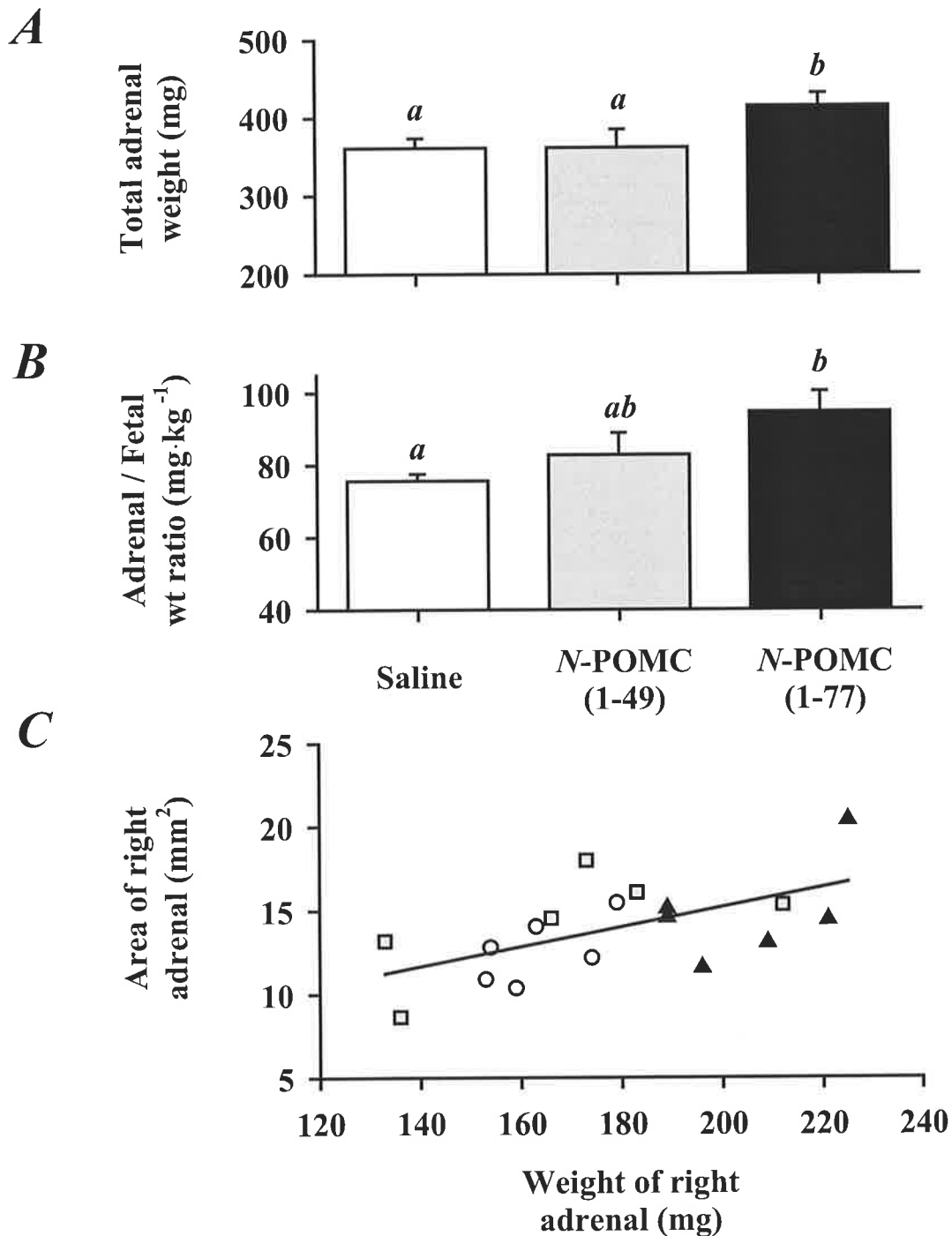
Figure 4.3   Adrenal weight, the ratio of adrenal : fetal body weight and the correlation between area and weight of the right adrenal gland in saline, *N*-POMC(1-49) and *N*-POMC(1-77) infused fetal sheep.

(*A*) Total adrenal weight (mg) and (*B*) the ratio of adrenal : fetal body weight in saline (open bar), *N*-POMC(1-49) (grey bar) and *N*-POMC(1-77) (black bar) infused fetal sheep. Significant differences between groups are denoted by different superscripts, *e.g.* a<b (p<0.05). (*C*) Correlation between the area and weight of the right adrenal in saline (open circle), *N*-POMC(1-49) (grey square) and *N*-POMC(1-77) (black triangle) infused fetal sheep. There was a significant correlation between the two variables (*see equation 4.1 on pg. 143*).

4.3.3   Adrenal steroidogenic enzyme mRNA expression

There was a significantly greater ratio of CYP17 mRNA (1.7 kb transcript) : 18S rRNA in fetal adrenals of *N*-POMC(1-77) infused fetuses, compared with either the *N*-POMC(1-49) or saline infused groups [*N*-POMC(1-77): 49.1 ± 4.7; *N*-POMC(1-49): 20.4 ± 6.4; saline: 15.2 ± 4.4] (*Figures 4.4A and 4.4B and Table 4.1*). There was no difference, however, in the ratios of adrenal CYP11A1 mRNA (1.9 kb transcript), 3βHSD mRNA (1.6 kb transcript) and CYP21A1 mRNA (2 transcripts; 2.2 and 1.8 kb) : 18S rRNA between the saline, *N*-POMC(1-49) and *N*-POMC(1-77) infused groups (*Table 4.1*). The ratio of adrenal CYP17 : 3βHSD mRNA expression was also significantly higher in *N*-POMC(1-77) infused animals than in either the *N*-POMC(1-49) or saline infused groups [*N*-POMC(1-77): 1.73 ± 0.23; *N*-POMC(1-49): 0.54 ± 0.16; saline: 0.76 ± 0.16] (*Figure 4.5B*).

Finally, there was a significant correlation between the mean circulating cortisol concentration during the last 24 h of the infusion period and the level of adrenal CYP17 mRNA : 18S rRNA expression in fetuses infused with either saline, *N*-POMC(1-49) or *N*-POMC(1-77) (*Figure 4.5A*), as described by the equation:

$$[\text{Circulating cortisol}] = 0.128[\text{CYP17 mRNA : 18S rRNA}] + 5.3 \qquad (4.2)$$

(r=0.45, p<0.05)

4.4   DISCUSSION

In this study, I have demonstrated a significant increase in the total adrenal weight and the ratio of adrenal : fetal body weight in fetuses following a 48 h intra-fetal infusion of *N*-POMC(1-77) (2 μg·ml^{-1}·h^{-1}) from 136-138 d of gestation. Neither of the *N*-POMC peptide infusions, however, altered the fetal circulating levels of ir-ACTH, compared with saline infused fetuses. I have also demonstrated an increase in the adrenal mRNA expression of CYP17 within fetuses following *N*-POMC(1-77) infusion, compared with either *N*-POMC(1-49) or saline infusion. The adrenal mRNA expression of CYP11A1, 3βHSD and CYP21A1, however, remained unchanged following the intra-fetal infusion of either *N*-POMC(1-77) or *N*-POMC(1-49), compared with saline infusion. Intra-fetal infusion of *N*-POMC(1-77) for 48 h also resulted in a 3 fold increase in the ratio of adrenal CYP17 : 3βHSD mRNA expression. While the *N*-POMC(1-77) infusion 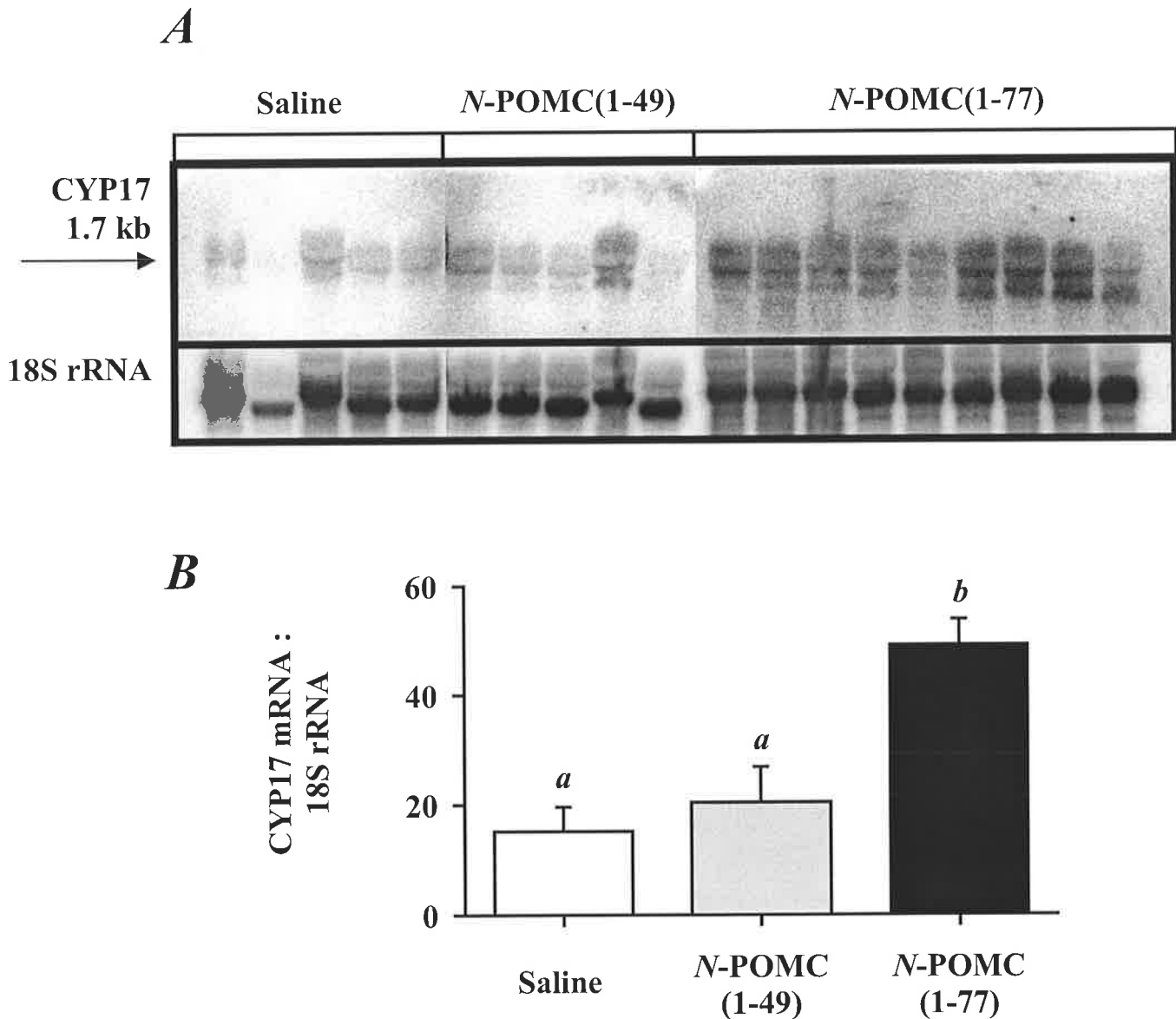was insufficient to alter the fetal circulating levels of cortisol, there was a significant correlation between the mean circulating cortisol concentrations during the last 24 h of the infusion period and the level of adrenal CYP17 mRNA : 18S rRNA expression in all fetuses.

A

B

Figure 4.4   Northern blot of adrenal CYP17 mRNA expression from fetal sheep following a 48 h infusion of either saline, *N*-POMC(1-49) or *N*-POMC(1-77).

(*A*) Northern blot analysis of total RNA (20 μg·lane^{-1}) prepared from adrenal glands of fetal sheep following a 48 h infusion of either saline, *N*-POMC(1-49) or *N*-POMC(1-77). from 136-138 d of gestation. The Northern membrane was hybridised sequentially with radiolabelled *h*CYP17 cDNA and 30 mer antisense 18S rRNA oligonucleotide probes. (*B*) Adrenal CYP17 mRNA : 18S rRNA expression in saline (open bar), *N*-POMC(1-49) (grey bar) and *N*-POMC(1-77) (black bar) infused fetal sheep. Significant differences between groups are denoted by different superscripts, *e.g.* a<b (p<0.05).

Table 4.1   Adrenal steroidogenic enzyme mRNA : 18S rRNA ratios in fetal sheep following a 48 h infusion of either saline, *N*-POMC(1-49) or *N*-POMC(1-77).

Treatment group	Adrenal Steroidogenic Enzyme mRNA : 18S rRNA			
	CYP11A1 : 18S	CYP17 : 18S	3βHSD : 18S	CYP21A1 : 18S
N-POMC(1-77)	63.4 ± 4.2	49.1 ± 4.7 [b]	29.8 ± 2.5	51.2 ± 5.3
N-POMC(1-49)	74.2 ± 10.4	20.4 ± 6.4 [a]	39.1 ± 5.5	42.3 ± 7.8
Saline	78.6 ± 11.0	15.2 ± 4.4 [a]	25.5 ± 1.7	51.9 ± 4.8

The mean ratios of adrenal CYP11A1, CYP17, 3βHSD and CYP21A1 mRNA : 18S rRNA in fetal sheep at 138 d of gestation, following a 48 h infusion of either saline, *N*-POMC(1-49) or *N*-POMC(1-77). Significant differences (p<0.05) in the mean ratios between the groups are denoted by different superscripts.

Figure 4.5   Correlation between circulating cortisol concentration and the level of adrenal CYP17 mRNA : 18S rRNA expression, and the ratio of adrenal CYP17 : 3βHSD mRNA expression in fetuses infused with either saline, N-POMC(1-49) or N-POMC(1-77).

(*A*) Correlation between the mean circulating cortisol concentration during the last 24 h of the infusion period and the level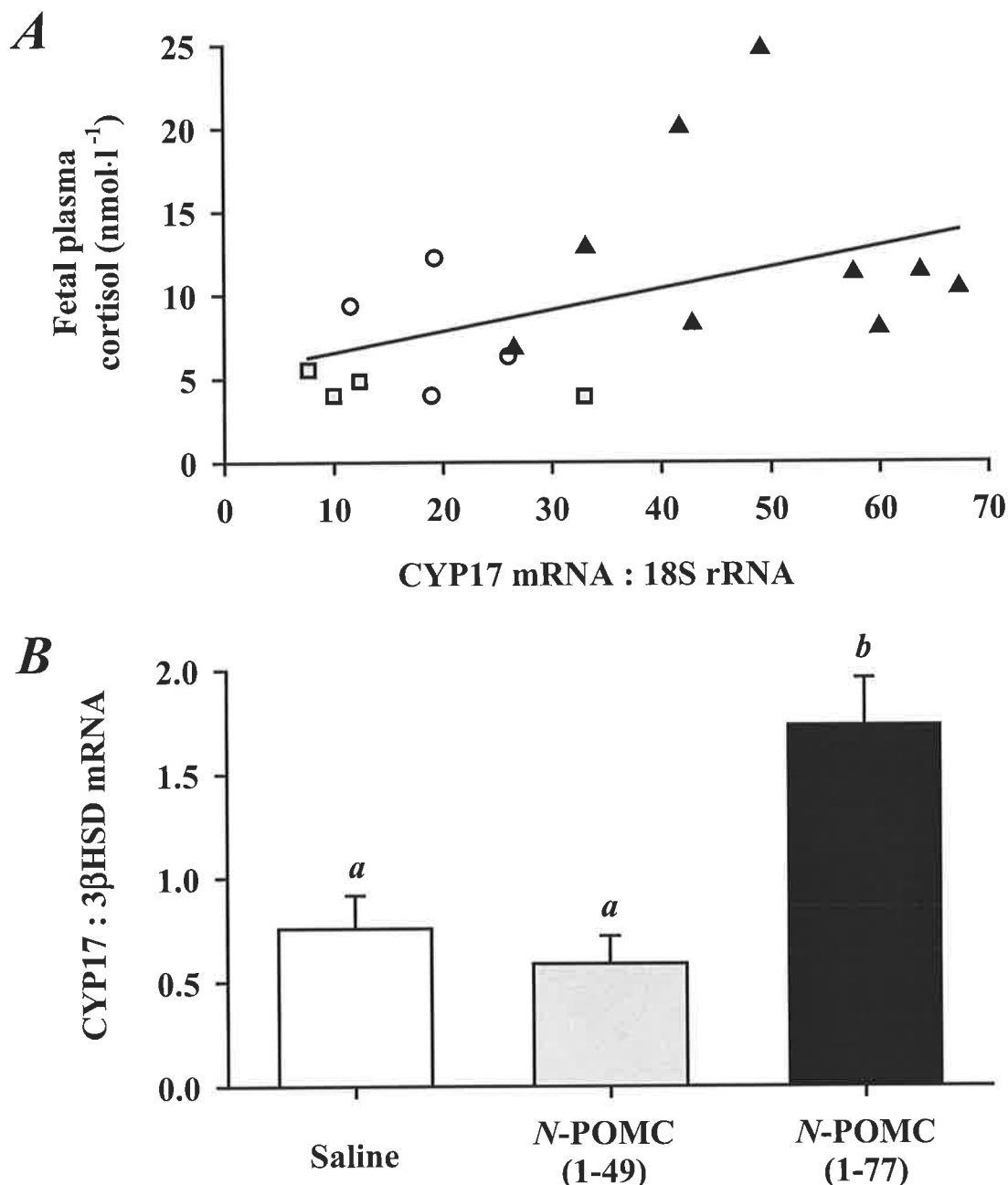 of adrenal CYP17 mRNA : 18S rRNA expression in all fetuses infused with either saline (open circle), N-POMC(1-49) (grey square) or N-POMC(1-77) (black triangle) infused fetal sheep. There was a significant correlation between the two variables (*see equation 4.2 on pg. 146*). (*B*) the ratio of adrenal CYP17 mRNA : 3βHSD mRNA in saline (open bar), N-POMC(1-49) (grey bar) and N-POMC(1-77) (black bar) infused fetal sheep. Significant differences between groups are denoted by different superscripts, *e.g.* a<b (p<0.05).

4.4.1 N-POMC peptides and models of adrenal growth

1.1.1.1 Anti-N-POMC antisera and compensatory adrenal growth

Previous studies in the rat have demonstrated that peptides derived from the *N*-terminal region of POMC may play a role in either compensatory adrenal hypertrophy following unilateral adrenalectomy, or in the adrenal regeneration which occurs after bilateral adrenal enucleation ([257]Lowry, Silas *et al.*, 1983; [145]Estivariz, Carino *et al.*, 1988; [149]Estivariz, Morano *et al.*, 1988). Selective quenching of different regions of circulating *N*-POMC peptides in adult rats, using specific antisera, demonstrated that while antisera against the extreme *N*-terminal region of POMC [anti-*N*-POMC(1-28)] has no effect on the increase in adrenal weight and RNA content, it completely abolishes the increase in DNA content in the remaining adrenal following unilateral adrenalectomy ([257]Lowry, Silas *et al.*, 1983). Anti-*N*-POMC(1-76) antiserum also abolishes the increase in DNA content without affecting adrenal weight, however, this antiserum significantly enhances the increase in adrenal RNA content above control adrenalectomised rats infused with normal rat serum (NRS) (138 % increase vs. 83 % increase) ([257]Lowry, Silas *et al.*, 1983).

In contrast to the anti-*N*-POMC antisera infusions, treatment of adrenalectomised rats with anti-ACTH antiserum has no effect on the increase in adrenal weight or DNA content, however, this antiserum inhibits the increases in plasma corticosterone concentrations and in adrenal RNA content (18 % compared with 83 % in NRS treated rats). The inhibition of hyperplasia by both of the anti-*N*-POMC antisera led Lowry and colleagues to propose that the mitogenic activity of *N*-POMC resides in the extreme *N*-terminal region ([148]Estivariz, Iturriza *et al.*, 1982; [257]Lowry, Silas *et al.*, 1983). These authors also suggested that the enhancing effect of the anti-*N*-POMC(1-76) antiserum on RNA synthesis may be due to an increased half-life of antibody-bound *N*-POMC(1-76) in the circulation, in which the steroidogenic potentiating and RNA stimulating site, γ_3-MSH, is not masked by the antibody ([257]Lowry, Silas *et al.*, 1983).

4.4.1.2 Anti-N-POMC antisera and adrenal regeneration

Estivariz and co-workers have demonstrated that bilateral adrenal enucleation of adult rats results in a rapid regeneration of the adrenal cortex and causes an increase in the ACTH and *N*-POMC content of the pas distalis ([145]Estivariz, Carino *et al.*, 1988; [149]Estivariz, Morano *et al.*, 1988). These investigators demonstrated that immuno-neutralisation with antisera against *N*-POMC peptides reduces adrenal mitotic activity in rats following adrenal enucleation, whereas antisera raised against ACTH diminishes plasma corticosterone levels only, and has no effect on adrenal mitotic activity

([149]Estivariz, Morano *et al.*, 1988). Administration of synthetic human *N*-POMC(1-28) partially reverses the rapid atrophy of regenerating adrenals in enucleated rats after subsequent hypophysectomy, whereas ACTH has no effect on adrenal mitotic activity in this model ([145]Estivariz, Carino *et al.*, 1988; [149]Estivariz, Morano *et al.*, 1988).

In rats, fragments from the extreme *N*-terminus of *N*-POMC, *i.e.* sequences (1-28) and (2-59), are active in causing adrenal growth in adult rats and DNA synthesis in cultured rat adrenocortical cells *in vitro* ([148]Estivariz, Iturriza *et al.*, 1982). In addition, Estivariz and colleagues demonstrated that administration of trypsinised *N*-POMC(1-77) stimulates adrenocortical growth and mitosis in seven week old rats *in vivo* ([148]Estivariz, Iturriza *et al.*, 1982), and DNA synthesis in perifused cultures of adult rat adrenocortical cells *in vitro* ([256]Lowry, Estivariz *et al.*, 1985; [145]Estivariz, Carino *et al.*, 1988). γ_3-MSH and the intact *N*-POMC(1-77) peptide, however, are not mitogenic in this *in vitro* model, suggesting that adrenal growth and mitosis can be controlled by cleavage of *N*-POMC(1-77) to release the mitogenically active *N*-terminal fragment and γ_3-MSH ([148]Estivariz, Iturriza *et al.*, 1982). Indeed, a novel 28 kDa protease, that is up-regulated in the adrenal gland during compensatory adrenal growth and contains the Histidine / Aspartate / Serine catalytic triad common to the trypsin family, has recently cloned ([40]Bicknell, Hutchinson *et al.*, 1998).

4.4.2   Neural activation of adrenal growth

Dallman and co-workers demonstrated that regulation of the rapid compensatory growth observed in the remaining adrenal gland following unilateral adrenalectomy is a neurally mediated process ([114]Dallman, Engeland and Shinsako, 1976; [113]Dallman, Engeland *et al.*, 1980). Mechanical manipulation of one adrenal without removal is followed by growth only in the contra-lateral gland. This finding led to the concept of neural regulation of compensatory adrenal growth via a loop from one adrenal through the hypothalamus and back to the contra-lateral gland ([114]Dallman, Engeland and Shinsako, 1976). Indeed, spinal cord hemi-section in the thoracic region inhibits compensatory adrenal growth when the contra-lateral adrenal, but not the ipsi-lateral gland, is removed ([144]Engeland and Dallman, 1976). In addition, pre-treatment of the left adrenal gland with lidocaine prior to removal inhibits the subsequent compensatory adrenal growth observed in the remaining adrenal ([144]Engeland and Dallman, 1976). These observations led Dallman to conclude that both afferent and efferent neural pathways mediate adrenal growth after unilateral adrenalectomy.

The involvement of afferent and efferent innervation in adrenal development and compensatory adrenal growth is supported by the findings of Halasz and colleagues ([173]Halasz and Szentagothai, 1959). Halasz and colleagues demonstrated that following adrenalectomy, nuclei of cell bodies in the hypothalamic ventromedial nucleus (VMN), on the side ipsi-lateral to the adrenalectomy, become smaller whereas those on the opposite side of adrenalectomy become enlarged. Following treatment causing adrenal growth, such as stress and ACTH administration, nuclear shrinkage occurs within cells of the VMN. In contrast, bilateral adrenalectomy, treatment with corticosteroids, or celiac ganglionectomy result in bilateral increases in nuclear size in VMN cell bodies ([173]Halasz and Szentagothai, 1959). Engeland and colleagues have also demonstrated that unilateral electrolytic lesions in the ventral hypothalamus of young male rats, ipsi-lateral to adrenalectomy, prevent compensatory adrenal growth of the remaining gland, while lesions on the contra-lateral side to the removed gland do not interfere with compensatory adrenal growth ([143]Engeland and Dallman, 1975).

4.4.2.1   Neurally activated proteolytic cleavage of N-POMC(1-77)

The prevention of the neurally mediated compensatory adrenal growth with antisera to the *N*-terminal region of POMC led Lowry and colleagues to propose that adrenal hypertrophy and hyperplasia are mediated by neural activation of a proteolytic enzyme. This enzyme cleaves *N*-POMC(1-77) to give rise locally to *N*-POMC(1-49) and γ_3-MSH at the adrenal, such that *N*-POMC(1-49) stimulates DNA synthesis and mitosis while γ_3-MSH stimulates RNA synthesis and hypertrophy ([257]Lowry, Silas *et al.*, 1983). In this model, secretion of *N*-POMC(1-77) and ACTH from corticotrophs would only lead to an increase in RNA synthesis and hypertrophy. DNA synthesis and subsequent hyperplasia would only occur following neural activation of the proteolytic enzyme responsible for the cleavage of *N*-POMC(1-77). Bicknell and colleagues used *in situ* hybridisation to demonstrate that expression of the novel 28 kDa trypsin-like protease is confined to the outer rim of the adrenal cortex ([40]Bicknell, Hutchinson *et al.*, 1998). These authors proposed that temporary attachment of the protease to the external cellular membrane following secretion would enable it to release the mitogenic fragments from circulating *N*-POMC(1-77) in the vicinity of the cells which mitose during compensatory adrenal growth following unilateral adrenalectomy ([40]Bicknell, Hutchinson *et al.*, 1998).

4.4.2.2   N-POMC(1-49) may stabilise N-POMC(1-77)

Seger and colleagues investigated the structure and biosynthetic origin of peptides arising from the *N*-terminal region of murine POMC ([387]Seger and Bennett, 1986). These authors

demonstrated that while the *N*-POMC(1-74) portion of murine 16 kDa pro-γ-MSH is *O*-glycosylated at Threonine$_{45}$ and *N*-glycosylated at Asparagine$_{65}$, murine *N*-POMC(1-49) has no carbohydrate content ([387]Seger and Bennett, 1986). In addition, Seger and colleagues purified *N*-POMC(1-77) from bovine pituitaries and demonstrated that this peptide possesses Cystine bridges linking Cystine residues 2 and 24, and linking Cystine residues 8 and 20 (*see Figure 1.4 on pg. 29*). In addition, the majority of the purified bovine *N*-POMC(1-77) peptide (approximately 80 %) is glycosylated at both Threonine$_{45}$ (*O*-linked) and Asparagine$_{65}$ (*N*-linked) residues. In the minor component, however, only glycosylation at Asparagine$_{65}$ is evident ([387]Seger and Bennett, 1986). These authors have proposed that the presence of *O*-linked sugars at Threonine$_{45}$ prevents proteolytic cleavage of *N*-POMC(1-77) at the dibasic amino-acid site Arginine$_{49}$-Lysine$_{50}$, which forms the *N*-terminus of γ$_3$-MSH. These authors further suggested that only *N*-POMC(1-77) lacking the Threonine$_{45}$ glycosylation is capable of being processed to form *N*-POMC(1-49) and γ$_3$-MSH. In the present study, the purified *N*-POMC(1-77) was a mixture of *O*-glycosylated and non *O*-glycosylated peptide.

Seger and colleagues have demonstrated the difference in ACTH receptor activation by synthetic *h*ACTH(1-39) (ED$_{50}$=49 ± 12 pM) and ACTH(1-24) (ED$_{50}$=6.2 ± 1.6 pM) ([387]Seger and Bennett, 1986). These authors suggested that the carboxy-terminal sequence of ACTH may stabilise the molecule *in vivo*. The effect of γ$_3$-MSH, *N*-POMC(1-49), *N*-glycosylated murine *N*-POMC(1-74) and *N*- and *O*-glycosylated murine *N*-POMC(1-74) on ACTH induced steroidogenesis has also been examined ([387]Seger and Bennett, 1986). *N*-POMC(1-49) shows no ability to potentiate the action of ACTH. In addition, while Seger and co-workers demonstrated that none of the peptides examined stimulate steroid output from dispersed adrenal cells when incubated in the absence of ACTH, both γ$_3$-MSH and *N*-glycosylated *N*-POMC(1-74) enhance the steroidogenic response of adrenal cells to ACTH. The form of *N*-POMC(1-74) containing both *N*- and *O*-linked oligosaccharides is the most effective potentiator of ACTH-induced steroidogenesis. These authors concluded that γ$_3$-MSH contains the minimum sequence required for potentiating ACTH-induced steroidogenesis. The similarity of the synergistic effects obtained with γ$_3$-MSH and the form of *N*-POMC(1-74) lacking *O*-linked oligosaccharides may be attributed in part to the proteolytic cleavage of *N*-glycosylated *N*-POMC(1-74) to γ$_3$-MSH. The greater potentiating effects obtained by the *O*-glycosylated peptide can be explained by the stability of the full-length, non-cleaved form. These authors further suggested that the extreme *N*-terminal sequence, *N*-POMC(1-49), while having no synergistic role in ACTH-induced steroidogenesis, may be analogous to the carboxy-terminal sequence of

ACTH(1-39), in terms of enhancing receptor binding and stability of the full-length peptide within the circulation.

4.4.3   N-POMC peptides and fetal adrenal growth

Interestingly, Saphier and co-workers have found that the molar ratio of *N*-POMC(1-77) : ACTH(1-39) is 10-25 times higher in the fetal than adult sheep ([377]Saphier, Glynn *et al.*, 1993). Furthermore, after 138 d of gestation there is a decrease in circulating *N*-POMC(1-77) associated with a concomitant increase in *N*-POMC(50-74), and these authors speculated that these changes are a consequence of proteolytic cleavage of *N*-POMC(1-77) at the fetal adrenal ([377]Saphier, Glynn *et al.*, 1993). In the present study, however, infusion of the purified bovine glycosylated *N*-POMC(1-77) resulted in a significant increase in adrenal growth within 48 h, whereas the effects of *N*-POMC(1-49) on fetal adrenal growth were less consistent. It is possible that the glycosylation of bovine *N*-POMC(1-77) at the *N*-linked (Asparagine$_{65}$) and *O*-linked (Threonine$_{45}$) sites protect the *N*-POMC(1-77) peptide from proteolytic degradation in the fetal circulation, whereas the non-glycosylated *N*-POMC(1-49) peptide may be relatively labile, preventing the mitogenic effect of this peptide on the adrenal gland. It is also possible that the putative adrenal proteolytic enzyme, proposed by Bicknell and colleagues ([40]Bicknell, Hutchinson *et al.*, 1998), may cleave the full-length *N*-POMC(1-77) peptide to release the mitogenically active *N*-terminal fragment and γ_3-MSH ([148]Estivariz, Iturriza *et al.*, 1982). These authors have also proposed that this proteolytic enzyme is neurally activated. Thus, while both ACTH(1-39) and *N*-POMC(1-77) are co-secreted from the corticotrophs of the fetal pituitary ([406]Tanaka, Nomizu and Kurosumi, 1991), biological activity of the *N*-terminal fragment of POMC may be dependent on neural activation of the putative adrenal proteolytic enzyme ([257]Lowry, Silas *et al.*, 1983). Clearly, the results of the present study provide direct evidence that *N*-POMC(1-77) can stimulate fetal adrenal growth in late-gestation. It is possible that *N*-POMC(1-77) is directly mitogenic to the adrenal cells, or that this peptide induces the local production of growth factors which mediate adrenal growth. In addition, the growth promoting actions may be a result of either a direct action of intact *N*-POMC(1-77) at the fetal adrenal, or a consequence of proteolytic cleavage of *N*-POMC(1-77) at the adrenal and the subsequent indirect action of locally generated peptides such as *N*-POMC(1-49). While it appears that *N*-POMC(1-77) may have stimulated growth of both the fetal adrenal cortex and medulla, further studies are required to determine whether the growth promoting actions of this peptide are through induction of cellular hyperplasia or hypertrophy in the morphologically distinct *zonae* of the adrenal. Finally, it is possible that *N*-POMC(1-49) did stimulate cellular hyperplasia or

hypertrophy in a limited area of the adrenal cortex, which was not detectable as a change in total adrenal weight.

4.4.4   N-POMC peptides and fetal adrenal steroidogenesis

In the sheep fetus, γ_3-MSH has been shown to potentiate the steroidogenic action of ACTH on isolated adrenocortical cells ([137]Durand, Cathiard *et al.*, 1984b), without independently affecting steroid output. In the present study, while infusion of glycosylated bovine N-POMC(1-77) did not alter the fetal circulating levels of either ir-ACTH or cortisol, infusion of the full-length N-terminal peptide did result in a dramatic increase in adrenal mRNA expression of the CYP17 steroidogenic enzyme. The present study, therefore, is the first to describe a specific action of an N-POMC derived peptide on adrenal steroidogenic mRNA levels. It has been shown previously that the human glycopeptide N-POMC(1-76), N-glycosylated murine N-POMC(1-74) and γ_3-MSH, each increase the steroidogenic response of adult rat adrenal cells to ACTH *in vivo* and *in vitro* ([328]Pedersen and Brownie, 1980; [332]Pedersen, Brownie and Ling, 1980b; [9]Al-Dujaili, Hope *et al.*, 1981; [10]Al-Dujaili, Williams *et al.*, 1982; [150]Farese, Ling *et al.*, 1983; [387]Seger and Bennett, 1986). Synthetic bovine γ_3-MSH stimulates a rapid increase in the concentration of mitochondrial free cholesterol in adrenals of hypophysectomised rats, which coincides with the activation of cholesterol ester hydrolysis ([328]Pedersen and Brownie, 1980; [332]Pedersen, Brownie and Ling, 1980b; [330]Pedersen and Brownie, 1987a). In contrast, N-POMC(1-49) does not act to stimulate steroidogenesis either independently or in the presence of ACTH and it has therefore been proposed that the potentiation of ACTH stimulated steroidogenesis is dependent on the γ_3-MSH sequence contained within the N-terminal sequence of POMC ([387]Seger and Bennett, 1986).

In the present study, intra-fetal infusion of glycosylated bovine N-POMC(1-77), but not N-POMC(1-49), specifically stimulated CYP17 mRNA expression in the fetal adrenal. There was also a 3 fold increase in the ratio of adrenal CYP17 : 3βHSD mRNA expression in the N-POMC(1-77) infused fetal sheep. This ratio has been highlighted to be of particular significance in determining the steroid output of a range of steroidogenic tissues, including the developing adrenal ([105]Conley and Bird, 1997). One possibility is that the increase in CYP17 mRNA measured in the present study is, in part, a result of proteolytic cleavage of N-POMC(1-77) at the fetal adrenal to generate γ_3-MSH. I have shown, in Chapter 2, that while there is an increase in adrenal CYP11A1 and CYP21A1 at around 136 d of gestation in the ovine fetus, adrenal CYP17 mRNA levels do not increase until after 140 d of gestation. It may be that the late-gestation increase in adrenal CYP17 mRNA expression is also a result of the increase in circulating γ_3-MSH

concentrations which occurs after 138 d of gestation ([377]Saphier, Glynn *et al.*, 1993). The target site of action of *N*-POMC(1-77) in the fetal sheep adrenal may differ, however, from that reported in previous *in vitro* and *in vivo* studies in the rat adrenal, given that the rat adrenal lacks CYP17 activity and that the main corticosteroid secreted by the rat adrenal is therefore corticosterone rather than cortisol. The results of the present study suggest that the action of *N*-POMC derived peptides may be dependent on the relative role of CYP17 expression in adrenal steroidogenesis. While there was a significant relationship between adrenal CYP17 mRNA expression and plasma cortisol concentrations across the three treatment groups, the change in circulating cortisol in the *N*-POMC(1-77) infused animals did not reach statistical significance. Stimulation of an increase in fetal plasma cortisol concentrations may require a longer adrenal exposure to the *N*-POMC(1-77) peptide or a parallel increase in plasma ACTH(1-39) concentrations.

4.4.5   Speculation: Cortisol and N-terminal POMC peptides

In Chapter 2, I reported that exogenous cortisol administration does not stimulate adrenocortical growth in intact fetuses. This may be due to a pituitary response to cortisol that requires functional innervation of the fetal adrenal gland. Exogenous cortisol may stimulate the release of *N*-POMC peptides, whose action at the adrenal is dependent on a neurally mediated proteolytic cleavage at the adrenal. While the adrenal gland of the HPD fetus is structurally and functionally immature during late-gestation, due to the low circulating levels of ACTH(1-39) and cortisol post 130 d of gestation ([342]Phillips, Ross *et al.*, 1996), adrenal innervation may be sufficiently complete to mediate cleavage of *N*-POMC peptides released from the surgically disconnected fetal pituitary following cortisol infusion. It is possible that the pituitary of an intact fetal sheep at 109-116 d of gestation responds in the same fashion as the HPD fetus following cortisol administration from 135-140 d of gestation, but that innervation of the fetal adrenal at 109-116 d of gestation is insufficiently mature to cleave the *N*-POMC(1-77) precursor into its biologically active constituents. The circulating levels of the *N*-POMC peptides in the HPD and intact fetuses during infusion of cortisol or saline have yet to be determined.

In summary, peptides derived from the *N*-terminal region of POMC are present within the corticotrophic cells of the fetal pars distalis and circulate in high concentrations in the fetal sheep during late-gestation ([293]Mulvogue, McMillen *et al.*, 1986; [377]Saphier, Glynn *et al.*, 1993). The results of the present study suggest that, in addition to ACTH, the *N*-terminal derived POMC peptide, *N*-POMC(1-77), may also play a role in the increase in adrenal growth and steroidogenesis which occurs before birth. The decrease in plasma levels of *N*-POMC(1-77) after 138 d of gestation, which occurs concurrently with an increase in

N-POMC(50-74), may indicate the proteolytic cleavage of the former peptide to liberate the smaller, biologically active forms of N-POMC ([377]Saphier, Glynn *et al.*, 1993). Thus, a role for the N-terminal region of POMC in adrenal growth during fetal life is supported by the presence of this peptide in the fetal circulation during a period of rapid adrenocortical growth. Interestingly, it has recently been demonstrated that mice lacking the entire third exon of the POMC gene, and hence all POMC-derived peptides, have no macroscopically discernible adrenal glands ([444]Yaswen, Diehl *et al.*, 1999), highlighting the critical role which these peptides play in adrenal development. Further work is clearly required to determine the relative roles of the N-terminal POMC peptides and ACTH(1-39), and the nature of the interactions between these adreno-trophic peptides, which are critical in adrenal development and in the cascade of endocrine events which determines the normal timing of parturition and the successful transition from intra-uterine to extra-uterine life.

CHAPTER 5.
EFFECT OF INTRA-FETAL IGF-I INFUSION ON FETAL ADRENAL GROWTH AND FUNCTION

5.1 INTRODUCTION

Insulin-like growth factors (IGFs) are potent mitogenic and differentiating factors, and are implicated in the regulation of fetal adrenal growth ([123]D'Ercole, 1987). Fetal adrenal cells synthesise insulin-like growth factor-I (IGF-I) and IGF-II mRNAs and peptide ([178]Han, Lu *et al.*, 1992), possess IGF receptors ([333]Penhoat, Chatelain *et al.*, 1988) and respond to IGFs *in vitro* in a variety of ways ([299]Naaman, Chatelain *et al.*, 1989). IGFs stimulate adrenal cellular proliferation ([416]van Dijk, Tansswell and Challis, 1988; [299]Naaman, Chatelain *et al.*, 1989), activate second messenger systems ([32]Begeot, Langlois and Saez, 1989; [236]Langlois, Hinsch *et al.*, 1990), and promote adrenocortical cell differentiation either alone ([86]Chatelain, Penhoat *et al.*, 1988) or in combination with other factors ([336]Penhoat, Naville *et al.*, 1989; [302]Naseeruddin and Hornsby, 1990; [339]Pham-Huu-Trung and Binoux, 1990). These factors strongly support a role for IGFs as paracrine factors in the development of fetal adrenal glands.

Studies of the type 1 IGF receptor in adult rat ([20]Arafah, 1991) and human ([392]Shigematsu, Niwa *et al.*, 1989) adrenals have revealed a uniform distribution and receptor density throughout the medulla and cortex. The type-2 IGF receptor is ubiquitously expressed throughout the bovine adrenal, and in greater abundance than the type 1 IGF receptor ([426]Weber, Kiess *et al.*, 1994). While I have shown that adrenal IGF-II mRNA levels in the fetal sheep do not increase in the 15 d prior to delivery, I have demonstrated that there is a decrease in adrenal IGFBP-2 mRNA levels. Thus, there may be an increased exposure of fetal adrenocortical cells to locally synthesised IGFs as a consequence of the fall in IGFBP-2. In addition, IGF-I has been shown to enhance cAMP accumulation and steroidogenesis in ovine fetal adrenal cells in response to incubation with ACTH *in vitro* ([299]Naaman, Chatelain *et al.*, 1989), and IGF-I also increases the activity of 3βHSD in cultured bovine adrenocortical cells ([86]Chatelain, Penhoat *et al.*, 1988). While these studies have examined the effect of IGFs on adrenocortical cells *in vitro*, there have been no studies to date that have examined the impact of exogenous IGF-I on adrenal growth and function *in vivo*.

Administration of exogenous recombinant human IGF-I (*rh*IGF-I) increases the circulating levels of IGF-I and promotes the growth of major organs in the fetal sheep ([252]Lok, Owens *et al.*, 1996). These data are consistent with IGF-I modulating fetal growth. Thus, in the present study, *rh*IGF-I was infused intra-vascularly into fetal sheep to increase the circulating concentration of IGF-I in order to test the hypothesis that increased abundance of circulating IGF-I *in utero* will promote fetal adrenal growth and functional development.

5.2   MATERIALS AND METHODS

5.2.1   Animal protocols and surgery

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation. Twenty-two pregnant Border Leicester × Merino ewes with singleton fetuses were used in this study. The ewes were housed in individual pens in animal holding rooms, with a 12 h light / dark lighting regimen, and fed once daily with 1 kg of lucern chaff and 1 kg of Baramil joint stock rations between 0900 and 1300 h with water *ad libitum*. Surgery was carried out between 110 d and 118 d of gestation under general anaesthesia and using aseptic techniques, as described in Chapter 2 (*see section 2.2.3.1 on pg. 72*). Catheters were implanted, as described previously ([322]Owens, Kind *et al.*, 1994), into the fetal carotid artery, femoral artery, jugular vein, tarsal vein, umbilical vein and maternal utero-ovarian vein and filled with 50 $IU \cdot ml^{-1}$ heparinised saline. There was a recovery period of at least three days after surgery before fetal blood samples were collected.

5.2.2   Infusion regimen and blood sampling protocol

Infusate preparation. Recombinant human (*rh*) IGF-I (animal / media grade, catalogue number: IM 100; GroPep) was dissolved in 0.9 % $w \cdot v^{-1}$ saline solution containing 1 $mg \cdot ml^{-1}$ (0.1 % $w \cdot v^{-1}$) BSA to make a 1 $mg \cdot ml^{-1}$ *rh*IGF-I stock solution. BSA (1 $mg \cdot ml^{-1}$) in 0.9 % $w \cdot v^{-1}$ saline was prepared by dissolving 0.5 g of BSA in 6 ml of saline, removed from a sterile 500 ml saline bag. This BSA / saline solution was then filter sterilised using a 0.22 μm filter (Millex-GS 0.22 μm filter; Millipore Australia), by transferring the solution from one sterile syringe to another through a sterile three-way tap (Discofix; Braun Medical AG) attached to the 0.22 μm filter, before returning the filtered solution to the 500 ml saline bag. The BSA preparation used in this study has been shown to be free of IGFs and IGFBPs ([322]Owens, Kind *et al.*, 1994; [72]Carr, Owens *et al.*, 1995). 10 ml of the 1 $mg \cdot ml^{-1}$ BSA in 0.9 % $w \cdot v^{-1}$ saline solution was then transferred to a vial containing 10 mg of the *rh*IGF-I to dissolve the lyophilised peptide to a 1 $mg \cdot ml^{-1}$ *rh*IGF-I

stock solution. 5.91 ml of this 1 mg·ml^{-1} *rh*IGF-I stock solution was made up to 10 ml with 1 mg·ml^{-1} BSA in 0.9 % w·v^{-1} saline to make the 591 μg·ml^{-1} *rh*IGF-I infusate.

Infusion regimen. The *rh*IGF-I infusion rate of 30 μg·kg^{-1}·h^{-1} was based on the estimated weight of fetuses at 120 d of gestation, *i.e.* 2.7 kg, based on our previous studies. Thus, an infusion rate of 81 μg·h^{-1} of *rh*IGF-I was required. Infusion of 591 μg·ml^{-1} *rh*IGF-I at 3.288 ml·24 h^{-1}, using an SP200 series syringe pump (World Precision Instruments) ensured that a total of 1944 μg·24 h^{-1} of the *rh*IGF-I peptide was infused into each fetus in the *rh*IGF-I infused group (n=14 fetuses). Control fetuses received 3.288 ml·24 h^{-1} of saline (n=8 fetuses). The *rh*IGF-I or saline was infused into fetal sheep via the fetal tarsal vein for 10 d from 120-130 d of gestation, starting at 0900 h at 120 d of gestation (designated time 0).

Blood sampling protocol. Fetal arterial blood samples (2 ml) were collected into chilled collection tubes containing 125 IU lithium heparin every alternate day after surgery. Blood samples were centrifuged at 1800 g for 10 min at 4 °C before separation and storage of plasma at -20 °C for subsequent assay. Fetal arterial blood (0.5 ml) was collected on alternate days for measurement of whole blood p$_a$O$_2$, p$_a$CO$_2$, pH, O$_2$ saturation, and haemoglobin content using an ABL 550 acid base analyser and OSM2 haemoximeter.

5.2.3   *Tissue collection*

At 130 d of gestation, ewes were killed with an overdose of Lethabarb and fetal sheep were removed, weighed and killed by decapitation. The left adrenal gland from each fetus was quickly removed, weighed, snap frozen in liquid N$_2$ and stored at -80 °C until total RNA was extracted.

5.2.4   *Radioimmunoassays*

IGF-I radioimmunoassay. IGF-I was measured in plasma obtained from the femoral artery of *rh*IGF-I infused (n=5 fetuses; *n*=10 samples) and saline infused (n=10 fetuses; *n*=20 samples) fetuses by radioimmunoassay, after removing IGF binding proteins by acid chromatography of plasma ([322]Owens, Kind *et al.*, 1994). Plasma samples were collected at 120 d of gestation prior to the *rh*IGF-I infusion, and at 130 d of gestation prior to the end of the infusion. Briefly, the IGF-I radioimmunoassay used rabbit antiserum PM87 ([157]Francis, McNeil *et al.*, 1989) raised against human IGF-I. *rh*IGF-I was used as standard and radio-ligand. *rh*IGF-I and ovine IGF-I, purified from sheep plasma, are equipotent in this IGF-I radioimmunoassay ([157]Francis, McNeil *et al.*, 1987). The intra- and inter-assay COVs, determined by repeated analysis of reference adult sheep plasma

containing 330 ng·ml^{-1} IGF-I were 3.2 % and 12.4 % respectively ([322]Owens, Kind *et al.*, 1994).

Cortisol radioimmunoassay. Cortisol concentrations were measured in fetal plasma samples from the *rh*IGF-I infused group (n=5 fetuses; *n*=5 samples) and the saline infused group (n=10 fetuses; *n*=10 samples). Plasma samples were collected at 128 d or 130 d of gestation prior to the end of the infusion. Total cortisol concentrations in fetal sheep plasma were measured using a radioimmunoassay, validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 74*) The intra- and inter-assay COVs were <10 % and <20 % respectively.

5.2.5   *cDNA and antisense oligonucleotide probes and probe labelling*

cDNA probes. *h*CYP11A1 and *h*CYP17 cDNA probes were generously provided by Professor W Miller (Department of Pediatrics, UCSF, San Francisco, CA, USA) (*see section 2.2.3.1 on pg. 75 and Figures 2.1 and 2.2 on pp. 76 and 77*). A *h*3βHSD cDNA probe was donated by Dr. R Rodgers (Department of Medicine, Flinders University, SA, Australia) (*see section 2.2.3.1 on pg. 75 and Figure 2.3 on pg. 78*). An *o*IGF-II cDNA probe was a kind gift from Dr. R S Gilmour (Institute of Animal Physiology & Genetics, Cambridge, UK) (*see section 2.2.3.1 on pg. 79 and Figure 2.5 on pg. 81*). cDNAs were radiolabelled with α-[^{32}P] dCTP (3,000 Ci·mmol^{-1}) by the random priming oligomer method to a specific activity of 10^9 cpm·µg^{-1} or greater, as described in Chapter 2 (*see section 2.2.3.1 on pg. 83*).

Oligonucleotide probes. A 30 mer antisense oligonucleotide probe for rat 18S rRNA, complementary to nucleotides 151-180, was synthesised (GeneWorks) (*see section 2.2.3.1 on pg. 83 and Figure 2.7 on pg. 85*) and end-labelled using T4PNK and γ-[^{32}P] ATP (4,000 Ci·mmol^{-1}) as substrate, as described in Chapter 2 (*see section 2.2.3.1 on pg. 84*).

5.2.6   *Total RNA isolation*

Total RNA was extracted from one adrenal from each of eight *rh*IGF-I infused (n=8 fetuses) and five saline infused (n=5 fetuses) fetal sheep by homogenisation in 4 M guanidine hydrochloride solution and ultracentrifugation overnight at 36,000 rpm, through a cushion of 5.7 M CsCl in 100 mM EDTA ([90]Chirgwin, Przybyla *et al.*, 1979), as described in Chapter 2 (*see section 2.2.3.2 on pg. 84 and Figure 2.8 on pg. 86*).

5.2.7   *Northern blot analysis*

Total RNA samples (20 µg of adrenal RNA) were denatured by incubation in 2.2 M formaldehyde and 50 % v·v^{-1} formamide at 55 °C for 10 min, and separated by

electrophoresis in 1 % agarose gels containing 2.2 M formaldehyde, then transferred by gravity-feed blotting onto a Zetaprobe nitrocellulose membrane using $10\times$ SSC, as described in Chapter 4 (*see section 4.2.8 on pg. 139 and Figure 4.1 on pg. 140*). The Northern membrane was washed in $10\times$ SSC, 0.1 % SDS for 10 min at RT and exposed to UV light (12 sec at 120 J·cm^{-2}), prior to overnight incubation at 42 °C in 30 ml of either cDNA or antisense oligonucleotide hybridisation buffer (*see Appendix I*). The Northern membrane was then hybridised sequentially for 16 h (42 °C for cDNA probes or 50 °C for oligonucleotide probes) in 20-25 ml of fresh hybridisation buffer containing either $1-2\times10^6$ cpm·ml^{-1} of the cDNA probe or 5×10^5 cpm·ml^{-1} of the 30 mer antisense 18S rRNA oligonucleotide probe (*see Figure 4.1 on pg. 140*). The membrane was washed once (10 min) at RT in $1\times$ SSC, 0.1 % SDS; then twice (10 min each time) in $0.1\times$ SSC, 0.1 % SDS at 42 °C, then briefly air-dried and sealed in a plastic bag. The membrane was exposed to phosphorimage plates in BAS 2040 cassettes for 24-48 h and images were quantified on a Fuji-BAS 1000 phosphorimage scanner using Fuji MacBAS software (*see Figure 4.1 on pg. 140*). cDNA probes were stripped from membranes between hybridisations by washing in $0.01\times$ SSC, 0.5 % SDS for 10 min at 80 °C. A ratio of the density of each specific band with the density of the corresponding 18S rRNA band was calculated before comparisons were made.

The radiolabelled *h*CYP11A1, *h*CYP17 and *h*3βHSD cDNA probes and the 30 mer antisense 18S rRNA oligonucleotide probe were used to sequentially probe a single Northern blot of adrenal total RNA from *rh*IGF-I infused (n=8 fetuses) and saline infused (n=5 fetuses) fetal sheep.

5.2.8   *Statistical analysis*

Data are presented as the mean $\pm$ SEM. Total adrenal weight (the sum of the weights of the left and right adrenals), the ratio of adrenal : fetal body weight, and the ratio of adrenal steroidogenic enzyme mRNA : 18S rRNA were compared between groups using Student's *t*-test. Plasma concentrations of IGF-I and cortisol were also compared using Student's *t*-test. A probability of <5 % (p<0.05) was considered to be significant.

5.3   RESULTS

5.3.1   *Fetal plasma IGF-I and cortisol levels*

Fetal plasma IGF-I concentrations. *rh*IGF-I infusion (25 ± 1 µg·kg^{-1}·h^{-1}, using fetal weight at post-mortem at 130 d of gestation to estimate the infusion rate), from 120-130 d

of gestation, significantly increased the concentration of IGF-I in fetal plasma by 140 % compared with saline infused fetuses (rhIGF-I: 324 ± 35 ng·ml^{-1}; saline: 135 ± 17 ng·ml^{-1}).

Fetal plasma cortisol concentrations. Infusion of rhIGF-I into fetal sheep from 120-130 d of gestation did not alter fetal plasma cortisol concentrations throughout the infusion period when compared with the saline infused group (rhIGF-I: 7.0 ± 1.3 nmol·l^{-1}; saline: 9.2 ± 3.3 nmol·l^{-1}).

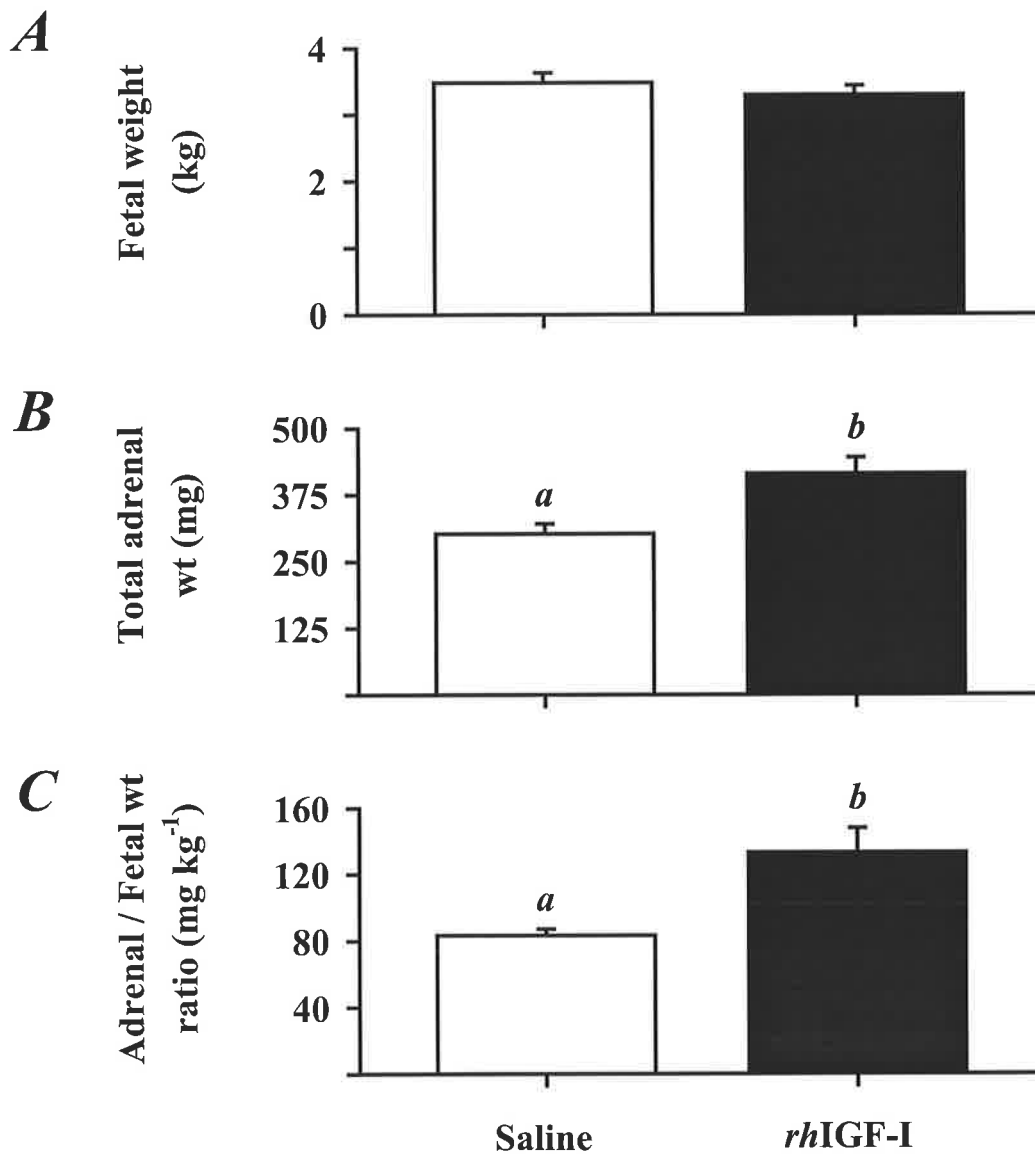### 5.3.2   Total fetal adrenal weight and fetal body weight

Adrenal weights and fetal body weights. Fetal body weights were not significantly different after intra-fetal infusion of rhIGF-I (3.29 ± 0.14 kg) or saline (3.48 ± 0.15 kg) (*Figure 5.1A*). rhIGF-I infusion did, however, result in a significant increase ($p<0.05$) in total adrenal weight (414 ± 30 mg) compared with the infusion of saline (301 ± 19 mg) (*Figure 5.1B*). The ratio of total adrenal : fetal body weight was also significantly greater ($p<0.05$) in the rhIGF-I infused group (131 ± 14 mg·kg^{-1}) compared with saline infused fetal sheep (87 ± 5 mg·kg^{-1}) (*Figure 5.1C*). Both absolute and relative adrenal weights in rhIGF-I infused fetuses lie substantially above the gestational profiles of adrenal weight and the ratio of adrenal : fetal body weight between 100 d and 147 d of gestation (*Figures 5.2A and 5.2B*).

5.3.3   Adrenal steroidogenic enzyme mRNA expression

There was no difference in the ratio of CYP11A1 mRNA (1.9 kb transcript), CYP17 mRNA (1.7 kb transcript) or 3βHSD mRNA (1.6 kb transcript) : 18S rRNA in fetal adrenals of rhIGF-I infused fetuses, compared with saline infused fetuses (*Figures 5.3 and 5.4*).

5.4   DISCUSSION

In this study, I have demonstrated a 33 % increase in total adrenal weight and a 50 % increase in the ratio of adrenal : fetal body weight in fetuses following a 10 d intra-fetal infusion of rhIGF-I (1944 μg·24 h^{-1}) from 120-130 d of gestation. Circulating IGF-I concentrations in the rhIGF-I infused fetuses were increased to levels similar to those achieved by chronic maternal, and hence fetal, hyperglycaemia in late-gestation ([323]Owens, Kind *et al.*, 1996). While intra-fetal infusion of rhIGF-I stimulated adrenal growth, steroidogenic enzyme gene expression and circulating cortisol levels were unchanged following the 10 d infusion period.

Figure 5.1   **Fetal weight, total adrenal weight and the ratio of adrenal : fetal body weight in saline and *rh*IGF-I infused fetal sheep at 130 d of gestation.**

(*A*) Fetal weight was not altered by the infusion of *rh*IGF-I into fetal sheep. (*B*) Total adrenal weight and (*C*) the ratio of adrenal : fetal body weight were significantly greater in the *rh*IGF-I infused group (black bar) when compared with the saline infused group (open bar). Significant differences between the *rh*IGF-I and saline infused groups are denoted by different superscripts, *e.g.* a < b (p<0.05).
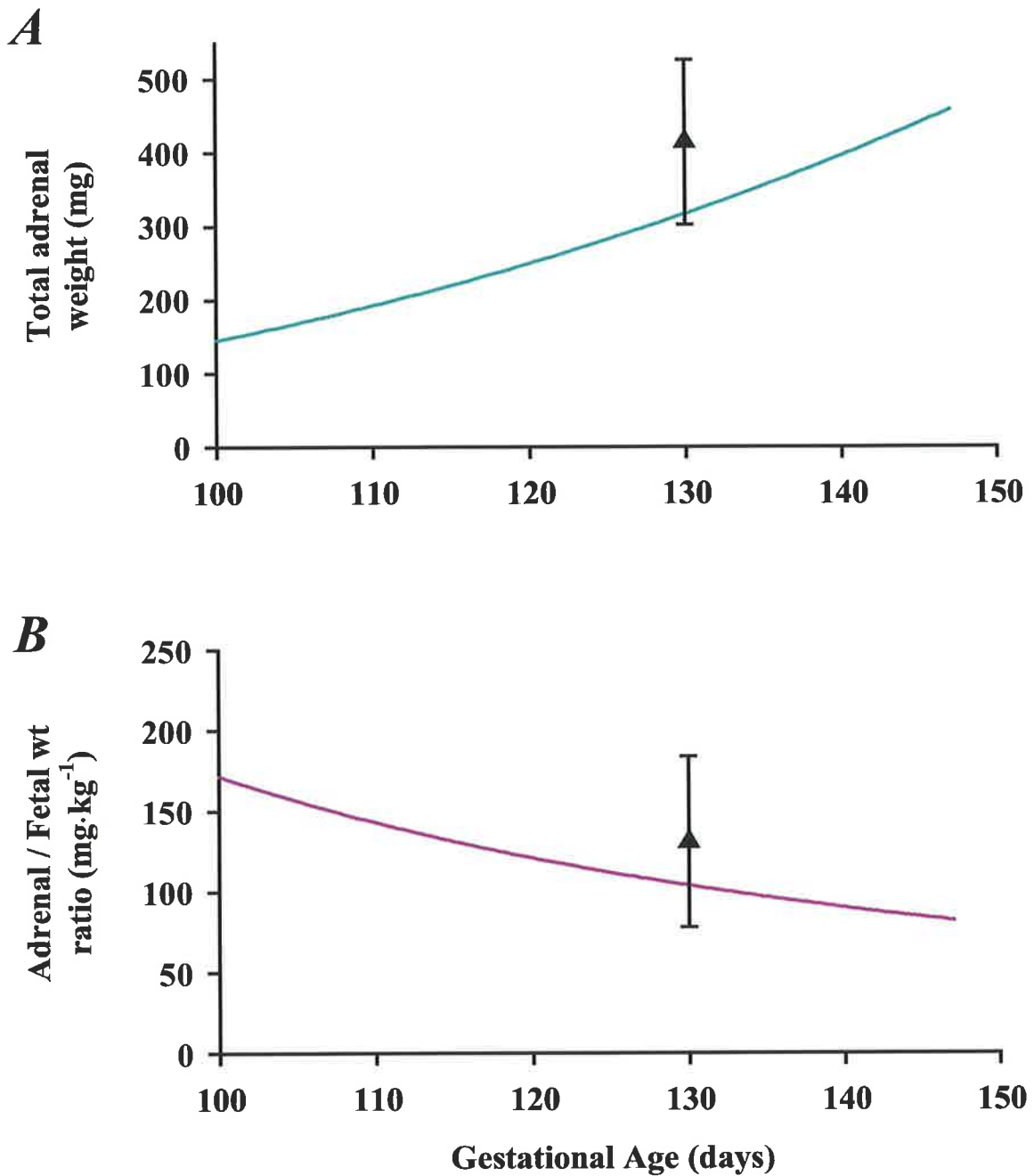
Figure 5.2   **Total adrenal weight and the ratio of adrenal : fetal body weight in *rh*IGF-I infused fetal sheep at 130 d of gestation compared with the respective gestational profiles between 100-147 d of gestation.**

(*A*) Total adrenal weight and (*B*) the ratio of adrenal : fetal body weight in the *rh*IGF-I infused (▲) group plotted against the gestational profile data (*from Study 1, Chapter 2*). The standard deviation of total adrenal weight of *rh*IGF-I infused fetuses is displayed for comparison with the gestational profile data using the power regression equation: **[Adrenal weight] = 10$^{-3.81341}$ × [Gestational age]$^{2.98733}$** (*equation 2.6 on pg. 95*).
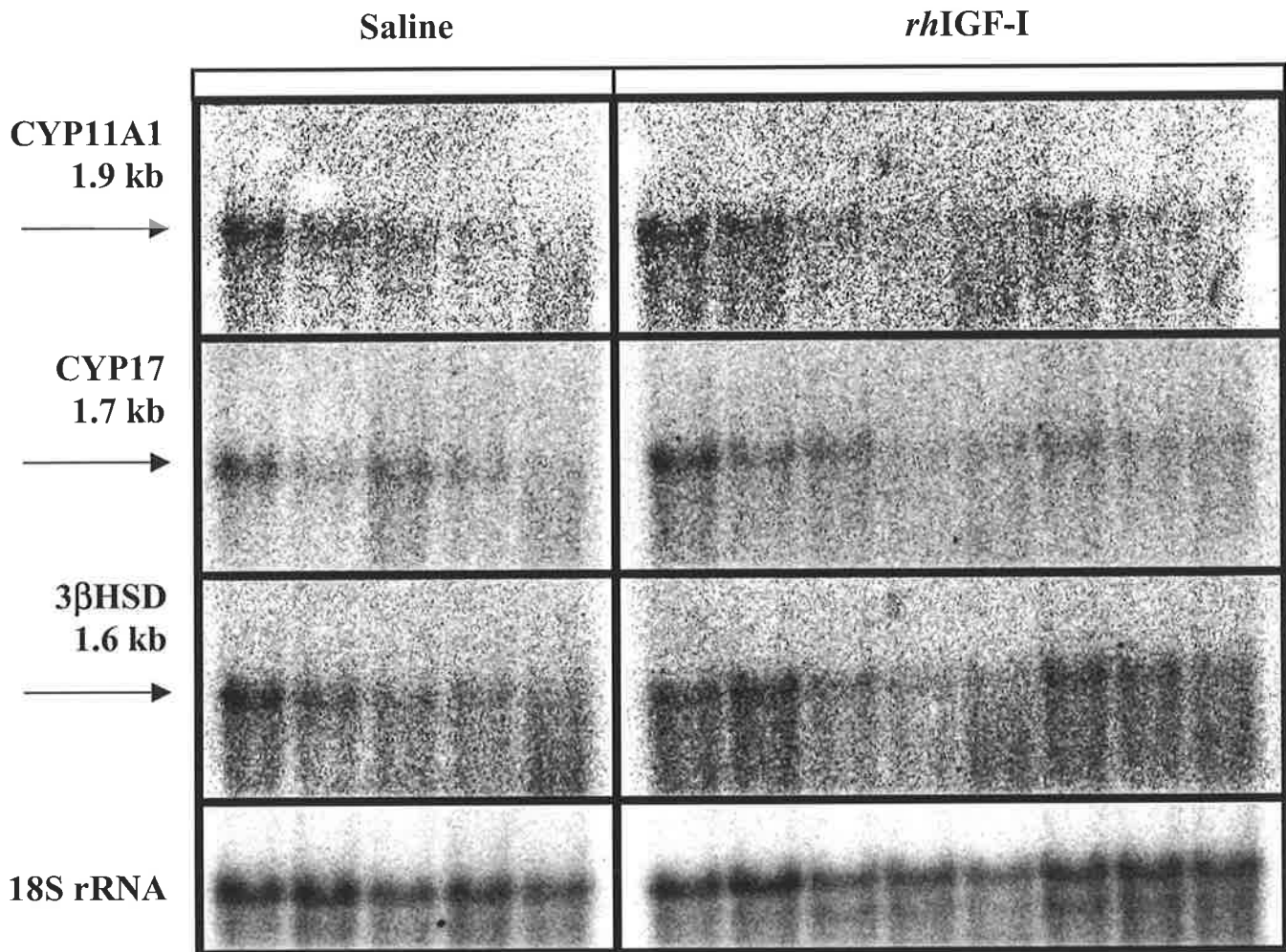
Figure 5.3   **Northern blot of adrenal steroidogenic enzyme mRNA expression from fetal sheep following a 10 d infusion of either saline or *rh*IGF-I from 120-130 d of gestation.**

Northern blot analyses of total RNA (20 µg·lane^{-1}) prepared from adrenal glands of fetal sheep following a 10 d infusion of either saline or *rh*IGF-I from 120-130 d of gestation. The Northern membrane was hybridised sequentially with the radiolabelled *h*CYP11A1, *h*CYP17 and *h*3βHSD cDNA and the 30 mer antisense 18S rRNA oligonucleotide probes.
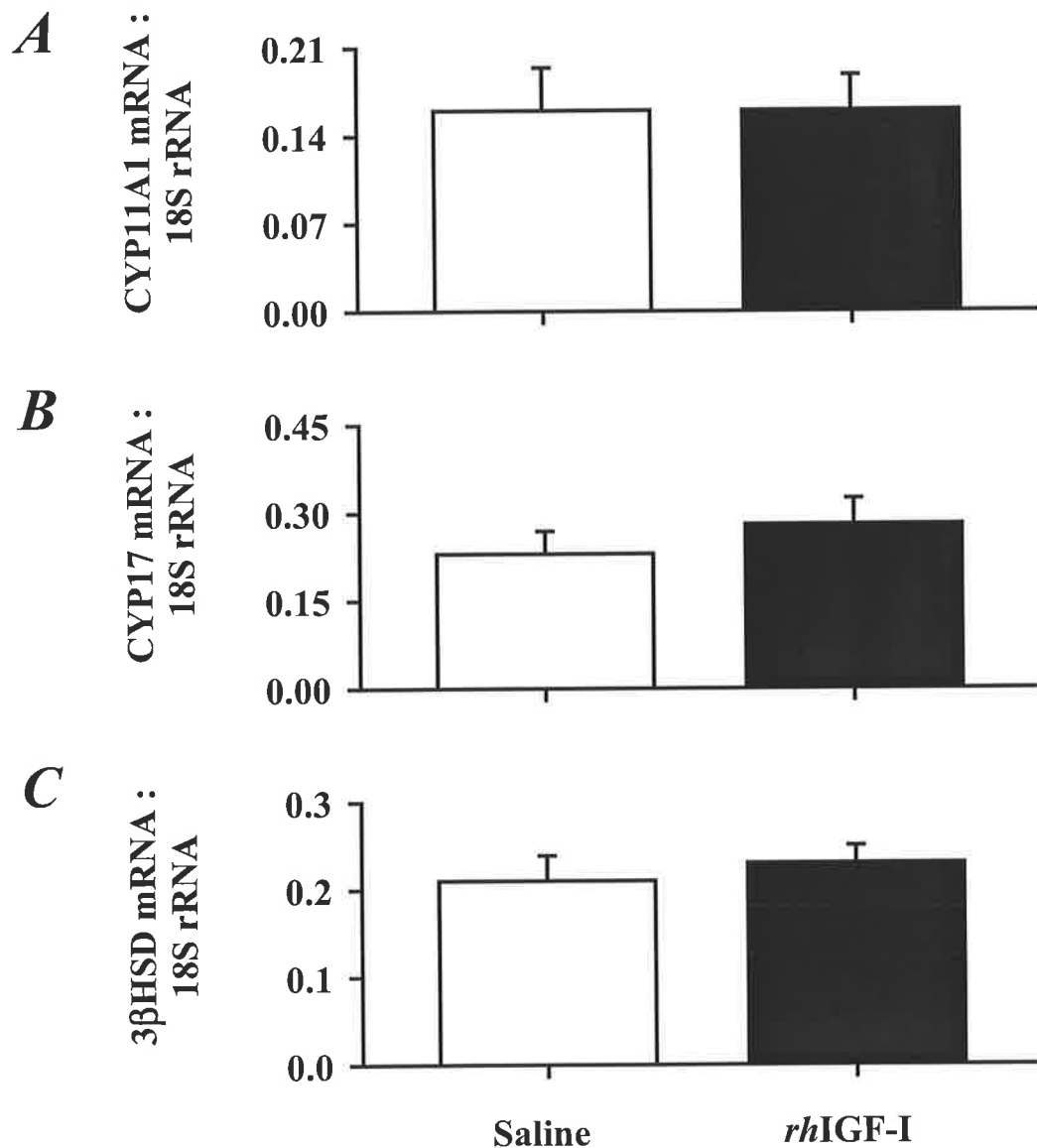
Figure 5.4   Steroidogenic enzyme mRNA expression in adrenals of fetal sheep following a 10 d infusion of either saline or *rh*IGF-I from 120-130 d of gestation.

The ratios of (*A*) CYP11A1, (*B*) CYP17 and (*C*) 3βHSD mRNA : 18S rRNA in fetal adrenals from saline infused (open bar) and *rh*IGF-I infused (black bar) fetal sheep at 130 d of gestation. There was no effect of a 10 d intra-fetal infusion of *rh*IGF-I on the adrenal mRNA expression of the steroidogenic enzymes.

5.4.1   IGF-I and fetal adrenal growth

Although chronic exposure to a physiological elevation of circulating IGF-I in late-gestation stimulated fetal adrenal growth, it remains unknown whether the increase in adrenal weight is due to cellular hypertrophy or hyperplasia. It is also unclear whether the increase in adrenal weight represents growth predominantly in the medullary area, and / or within the cortical *zonae*. Coulter and colleagues have demonstrated the presence of mRNA for the type 1 IGF receptor throughout the definitive, transitional and fetal *zonae* of the primate fetal adrenal cortex from mid- to late-gestation ([108]Coulter, Goldsmith *et al.*, 1996). This transcript was not detectable, however, within the adrenal medulla of the primate fetus ([108]Coulter, Goldsmith *et al.*, 1996). While previous studies have revealed that IGF-I induces cellular proliferation of fetal adrenocortical ([416]van Dijk, Tansswell and Challis, 1988; [299]Naaman, Chatelain *et al.*, 1989) and medullary chromaffin cells ([174]Hall and Ekanayake, 1991; [160]Frodin and Gammeltoft, 1994) *in vitro*, the lack of expression of the type 1 IGF receptor within the primate fetal adrenal medulla *in vivo* ([108]Coulter, Goldsmith *et al.*, 1996) suggests that IGFs may primarily modulate adrenocortical growth and / or function. While morphometric analyses of the relative areas of the adrenal cortex and medulla were not performed in the present study, growth of the *rh*IGF-I infused adrenals appeared, on coarse examination, to be comprised of both cortical and medullary components (unpublished observations). A detailed morphometric examination of the adrenal sections is required to quantitate the relative contributions of each zone to the increase in adrenal weight.

5.4.1.1   Modulation of IGF activity by adrenal IGFBPs

While IGF-I induces cellular proliferation of fetal adrenocortical ([416]van Dijk, Tansswell and Challis, 1988; [299]Naaman, Chatelain *et al.*, 1989) and medullary chromaffin cells ([174]Hall and Ekanayake, 1991; [160]Frodin and Gammeltoft, 1994) *in vitro*, comparisons between *in vitro* and *in vivo* studies must be treated with caution as the IGF-IGF receptor interactions may be modulated positively or negatively by circulating as well as tissue specific expression of IGFBPs. The expression of IGFBPs is developmentally regulated in a tissue specific manner ([261]Lund, Moats-Stoats *et al.*, 1986; [179]Han, Lund *et al.*, 1988; [61]Brice, Cheetham *et al.*, 1989; [51]Bondy, Werner *et al.*, 1990), with the mRNAs for IGFBP-1 to -6 being expressed in the human fetal adrenal at mid-gestation as determined by Northern blot or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis ([201]Ilvesmaki, Blum and Voutilainen, 1993a). In addition, Coulter and colleagues have shown that IGFBP-2 and IGFBP-6 mRNAs are expressed in all *zonae* of the primate fetal adrenal cortex, with the relative abundance of IGFBP-2 being greater than the other

IGFBPs ([108]Coulter, Goldsmith *et al.*, 1996). These data are consistent with observations from the mid-gestation human fetus ([201]Ilvesmaki, Blum and Voutilainen, 1993a) and sheep fetus ([121]Delhanty and Han, 1993), in which IGFBP-2 mRNA was detected by Northern blot analysis in ovine fetal adrenal glands. As the adrenal is not homogenous, however, this technique could not determine the specific cell types of the fetal ovine adrenal gland that express this binding protein. These IGF binding proteins may prevent access of the IGFs to some areas of adrenal tissue, but not others. Alternatively, locally produced IGFBPs may increase access of the exogenous *rh*IGF-I to the type 1 IGF receptor in some areas, by increasing the local IGF concentration. Finally, *rh*IGF-I infusion into the fetal sheep rapidly induced increases in total IGFBP activity (Dr. JA Owens, personal communication) which may themselves differentially target cells within the adrenal. Thus, determining the localisation of IGF receptors and the IGFBPs, and the access of IGFs associated with the IGFBPs to cells within the ovine fetal adrenal may also serve to elucidate the site of action of IGFs within the adrenal gland of the ovine fetus.

5.4.2   IGF-I and fetal adrenal steroidogenesis

Numerous *in vitro* studies have demonstrated that IGF-I enhances the steroidogenic response of adult and fetal adrenocortical cells to ACTH ([333]Penhoat, Chatelain *et al.*, 1988; [299]Naaman, Chatelain *et al.*, 1989), through mechanisms which involve an increase in ACTH receptor numbers ([333]Penhoat, Chatelain *et al.*, 1988; [32]Begeot, Langlois and Saez, 1989; [299]Naaman, Chatelain *et al.*, 1989; [334]Penhoat, Jaillard and Saez, 1989), the expression of stimulatory G protein subunits ([32]Begeot, Langlois and Saez, 1989; [236]Langlois, Hinsch *et al.*, 1990) and activity of specific steroidogenic enzymes ([86]Chatelain, Penhoat *et al.*, 1988; [333]Penhoat, Chatelain *et al.*, 1988; [299]Naaman, Chatelain *et al.*, 1989). It is interesting that while IGFs consistently enhance the steroidogenic responsiveness of adrenocortical cells to ACTH *in vitro*, infusion of *rh*IGF-I into the fetal circulation did not alter the expression of mRNA for the adrenal steroid-synthesising enzymes or the circulating level of cortisol. It is possible that the potentiating effects of IGF-I on ACTH induced adrenal steroidogenesis require elevated circulating levels of ACTH *in vivo*, which are normally only observed during episodes of acute fetal stress or during the final two weeks of gestation, *i.e.* after the period of *rh*IGF-I infusion in the present study. Indeed, *in situ* hybridisation revealed that removal of corticosteroid negative feedback at the pituitary by metyrapone infusion causes an increase in the relative abundance of mRNA for the type 1 IGF receptor ([108]Coulter, Goldsmith *et al.*, 1996). Thus, the adrenal expression of the type 1 IGF receptor may be modulated by the local intra-adrenal concentration of cortisol, or the fetal circulating level of ACTH.

In the present study, the 10 d intra-fetal infusion of *rh*IGF-I may have increased the adrenal responsiveness to ACTH via an increase in adrenal ACTH receptor abundance and an enhanced capacity for adenylate cyclase coupling, without an observable effect on steroidogenesis, due to the low circulating concentrations of ACTH from 120-130 d of gestation. Alternatively, IGFBPs may inhibit the steroid-potentiating effect of IGF-I on the fetal adrenal *in vivo*, while the disruption of tissue architecture and paracrine relationships following cell culture may remove this restriction *in vitro*. Finally, it is possible that locally produced IGF-II, which is abundant within the steroid-secreting cells of the fetal ovine adrenal cortex ([178]Han, Lu *et al.*, 1992), may have greater access to the type 1 IGF receptors than circulating IGF-I. Thus, it is possible that circulating IGF-I may predominantly stimulate adrenal growth in all cells expressing the type 1 IGF receptor, whereas locally synthesised IGF-II may specifically coordinate adrenocortical growth and steroidogenic responsiveness to ACTH, under the dual influence of both ACTH and cortisol. An examination and comparison of adrenal growth and steroidogenesis following IGF-I or IGF-II infusion from 120-130 d of gestation in the presence and absence of exogenous ACTH, and at a later stage of gestation, such as 130-145 d of gestation, would address these possibilities.

5.4.3 *Speculation: IGF-I and POMC derived peptides*

The absence of elevated circulating levels of ACTH during the infusion period did not prevent the trophic activity of IGF-I on fetal adrenal growth. Thus, while the potentiating action of IGF-I on ACTH induced steroidogenesis *in vitro* requires elevated levels of ACTH, the trophic effect of IGF-I on adrenal growth does not appear to operate via modulation of adrenal sensitivity to ACTH. It is possible that exogenous IGF-I enhances the responsiveness of the fetal adrenal to other trophic hormones that are at higher concentrations within the fetal circulation during the infusion period. Indeed, the circulating concentrations of the ACTH precursors, POMC and pro-ACTH are 10-20 fold higher than ACTH(1-39) during this period ([395]Silman, Holland *et al.*, 1979). While these peptides have been shown to be antagonistic to the steroidogenic actions of ACTH *in vitro* ([385]Schwartz, Kleftogiannis *et al.*, 1995), their effect on adrenal growth *in vivo* is unknown. In addition, the fetal circulating concentrations of *N*-POMC(1-77) are 20-50 fold higher than ACTH(1-39) during late-gestation ([377]Saphier, Glynn *et al.*, 1993), and biologically active peptides cleaved from this precursor are mitogenic for adrenal cells both *in vitro* ([256]Lowry, Estivariz *et al.*, 1985; [145]Estivariz, Carino *et al.*, 1988) and *in vivo* ([148]Estivariz, Iturriza *et al.*, 1982). Thus, IGF-I may enhance the adrenal responsiveness to growth promoting peptides that are at sufficient concentrations within the fetal circulation from

120-130 d of gestation to stimulate adrenal growth. It is also possible that IGF-I stimulates adrenal mitosis *in vivo* directly, without involving auxiliary endocrine pathways. Indeed, the mitogenic potential of IGF-I has been demonstrated on adrenocortical cells in culture, in the absence of co-incubation with other factors ([416]van Dijk, Tansswell and Challis, 1988; [299]Naaman, Chatelain *et al.*, 1989). A further possibility is that elevated circulating IGF-I may act indirectly, via the fetal pituitary, to stimulate the release of trophic peptides from the fetal corticotrophs which in turn stimulate adrenal growth. Measurement of the *N*-terminal POMC peptides and ACTH-containing peptides in fetal plasma during intra-fetal IGF-I infusion would help resolve these issues.

In summary, I have demonstrated that chronic exposure to a physiological elevation of circulating IGF-I in late-gestation promotes adrenal growth, but does not alter the expression of mRNA for the steroidogenic enzymes or circulating cortisol levels in the fetal sheep. It is possible that the low levels of ACTH within the fetal circulation during the infusion period were not sufficient to allow any augmentation of steroidogenic potential mediated by the infusion of *rh*IGF-I. It is also possible that the adrenal responsiveness to trophic hormones other than ACTH may have been enhanced by the intra-fetal infusion of *rh*IGF-I, resulting in the pronounced adrenal growth observed in the present study. Finally, the fetal pituitary output of adrenal mitogens may have been altered directly by the elevated circulating levels of IGF-I, or IGF-I may have acted simply as a direct mitogen *in vivo*, resulting in adrenal growth.

CHAPTER 6.
IMPACT OF CHRONIC FETAL GROWTH RESTRICTION ON FETAL ADRENAL GROWTH AND STEROIDOGENESIS

6.1 INTRODUCTION

It has been shown that exposure of the fetus to a range of acute or chronic stressful stimuli such as hypoxaemia, asphyxia or haemorrhage, leads to stimulation of the fetal HPA axis and an increase in fetal cortisol concentration ([47]Boddy, Jones *et al.*, 1974; [369]Rose, MacDonald *et al.*, 1978; [46]Bocking, McMillen *et al.*, 1986; [45]Bocking, Gagnon *et al.*, 1988; [6]Akagi and Challis, 1990; [192]Hooper, Coulter *et al.*, 1990; [327]Ozolins, Young and McMillen, 1992; [295]Murotsuki, Gagnon *et al.*, 1996). Transient or sustained increases in fetal plasma concentrations of cortisol result in a range of metabolic and physiological responses to intra-uterine stressors, which are important for fetal and neo-natal survival ([80]Challis and Brooks, 1989). While the steroidogenic responses of the fetal adrenal to acute (<4 h) ([47]Boddy, Jones *et al.*, 1974) or moderately prolonged (24-48 h) hypoxaemia ([58]Braems, Han and Challis, 1998; [192]Hooper, Coulter *et al.*, 1990) have been extensively investigated, there have been few detailed studies on the impact of chronically sustained hypoxaemia on fetal adrenal growth and steroidogenesis.

It has been shown that restriction of placental growth from conception, via maternal carunclectomy prior to pregnancy, results in (*1*) fetal hypoxaemia and hypoglycaemia throughout late-gestation, (*2*) an increase in fetal plasma cortisol during the last two weeks of gestation, (*3*) lower fetal body weight compared with age-matched control fetuses and (*4*) an increase in adrenal weight relative to fetal body weight ([364]Robinson, Kingston *et al.*, 1979; [343]Phillips, Simonetta *et al.*, 1996). Placental restriction does not, however, result in an increase in either immunoreactive or bioactive ACTH in the fetal circulation ([343]Phillips, Simonetta *et al.*, 1996). In addition, POMC mRNA levels in the pars distalis are lower in placentally restricted fetuses, when compared with normally grown fetal sheep ([343]Phillips, Simonetta *et al.*, 1996). The mechanisms underlying the enhanced fetal adrenal growth and steroidogenic responses to placental restriction are, therefore, unknown. In the present study, I have investigated the relationship between fetal adrenal growth and fetal body weight in a large cohort of placentally restricted and control fetal sheep. In addition, I have determined the impact of placental restriction on the mRNA

expression for the steroidogenic cytochrome P-450 enzymes: CYP11A1, CYP17, CYP21A1 and for 3βHSD in the fetal adrenal.

The elevation in circulating cortisol in placentally restricted fetal sheep, without a concomitant increase in ACTH, is similar to human fetal growth-restriction. Cordocentesis studies in small for gestational age human fetuses have also found that plasma cortisol concentrations are higher, and plasma ACTH concentrations lower than in normally grown fetuses at 18-38 weeks of gestation ([138]Economides, Nicolaides *et al.*, 1988). Interestingly in human fetal growth-restriction, brain growth is largely spared while liver and lymphoid tissues are disproportionately reduced in size ([64]Brooke, Wood and Butters, 1984). In the present study, therefore, I have investigated the relationship between fetal brain and liver weight, relative to fetal body weight, in placentally restricted and control fetal sheep, to characterise the phenotype of fetal growth restriction caused by placental restriction.

Previous studies have shown that there are high levels of expression of the insulin-like growth factor, IGF-II, in the steroidogenic cells of the fetal adrenal cortex during late-gestation ([178]Han, Lu *et al.*, 1992), and IGF-II is mitogenic when added to fetal rat adrenocortical cells in culture ([416]van Dijk, Tansswell and Challis, 1988). I have therefore investigated whether placental restriction stimulates IGF-II mRNA expression or, alternatively, alters the expression of the major IGF-II binding protein, IGFBP-2, which modulates IGF bioavailability in the fetal sheep adrenal during late-gestation. I have also measured the impact of restriction of placental growth and function on the expression of the adrenal steroidogenic factor-1 (SF-1). SF-1 is an orphan nuclear receptor and transcription factor which has been shown to be essential for the differentiation and normal function of steroidogenic cells within the adrenal ([434]Wong, Ikeda *et al.*, 1997). SF-1 consensus sequences are present in the 5' region of the genes encoding the P450 enzymes involved in steroidogenesis, and in the 5' region of the 3βHSD gene. Finally, I have determined the impact of placental restriction on the adrenal mRNA expression for the ACTH receptor (ACTH-R), in order to determine whether there is any evidence for an increased sensitivity of the fetal adrenal to the prevailing plasma levels of ACTH in placentally restricted fetuses.

6.2 MATERIALS AND METHODS

6.2.1 Animal protocols and surgery

Study 1. Placental restriction profile of fetal adrenal gland weight. All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal

Experimentation. During a four year period, data from fetal adrenal, brain, liver and fetal body weights were collected from a group of fetuses (n=237; 71 singletons, 166 twin fetuses) which had been used as control animals in a number of experimental studies, and from a second group (n=71; 54 singletons, 17 twin fetuses) in which restriction of placental growth was experimentally induced. The surgical procedure for placental restriction (carunclectomy) has previously been described ([364]Robinson, Kingston *et al.*, 1979; [343]Phillips, Simonetta *et al.*, 1996). Briefly, the majority of endometrial caruncles were removed from the uterus of non-pregnant Border Leicester × Merino ewes under general anaesthesia in order to reduce the number of placental cotyledons formed during pregnancy. Carunclectomy was performed under general anaesthesia induced by intra-venous injection of Pentothal (20 ml, 0.1 g·ml^{-1}) and maintained with 0.5-4.0 % fluothane inhalation anaesthetic in medical grade O_2. Ewes received a 2 ml intra-muscular injection of Ilium Penstrep prior to surgery. The uterus was exteriorised through a low abdominal mid-line incision and subsequently opened along the anti-mesometrial border from the level of the cervix to near the uterotubule junction in both horns. Most of the visible endometrial caruncles were then excised and the incisions in the uterus were sutured in a single layer using 2/0 chromic cat gut through the myometrium and serosa. The uterus was then replaced and the abdomen closed in two layers, the peritoneum and rectus sheet, followed by the skin and sub-cutaneous tissue, using 4/0 coated Vicryl (Johnson & Johnson). After a minimum of 10 weeks post-surgery, the ewes entered a mating programme and pregnancies were confirmed by ultrasound at approximately 60 d of gestation. The ewes were housed in individual pens in animal holding rooms, with a 12 h light / dark lighting regimen, and fed once daily with 1 kg of lucern chaff and 1 kg of Baramil joint stock rations between 0900 and 1300 h with water *ad libitum*.

Study 2. Placental restriction gene expression. Twelve pregnant ewes with singleton pregnancies were used in this study. The ewes were housed in individual pens in animal holding rooms, with a 12 h light / dark lighting regimen, and fed once daily between 0900 and 1300 h with water *ad libitum*. Catheterisation surgery was carried out on a group of carunclectomised (n=6 fetuses), and on a group of control fetuses (n=6 fetuses) between 102 d and 112 d of gestation, under general anaesthesia and using aseptic techniques, as described in Chapter 2 (*see section 2.2.1.3.1 on pg. 72*). There was a recovery period of at least three days after surgery before fetal blood samples were collected.

6.2.2 Tissue collection

Study 1. Placental restriction profile of fetal adrenal gland weight. Post-mortems were performed at 137-147 d of gestation on fetal lambs in the placental restriction (PR) group

(n=71 fetuses) and on the group of fetuses (n=237 fetuses) which had been used as control animals in a number of experimental studies. Fetal vascular catheters had been implanted in 61 fetuses from the PR group and in 106 fetuses from the control group, however, no blood samples were collected for the present study. At post-mortem, ewes were killed with an overdose of Lethabarb (25 ml at 325 mg·ml^{-1}) and fetal sheep were removed, weighed and killed by decapitation. Both adrenal glands, brain and liver of all fetuses were quickly removed and weighed.

Study 2. Placental restriction gene expression. At 140 d of gestation, ewes were killed with an overdose of Lethabarb (25 ml at 325 mg·ml^{-1}) and fetal sheep were removed, weighed and killed by decapitation. Both adrenal glands were quickly removed, weighed, snap frozen in liquid N$_2$ and stored at -80° C until total RNA was extracted.

6.2.3 Blood sampling protocol. Study 2. PR gene expression

Blood sampling protocol. Fetal arterial blood samples (2 ml) were collected into chilled tubes every 2-3 days between 130 d and 140 d of gestation for ir-ACTH and cortisol radioimmunoassays. Blood for cortisol assay was collected into tubes containing 125 IU lithium heparin. Blood for ir-ACTH assay was collected into tubes containing EDTA (18.6 g·l^{-1} of whole blood) and aprotinin (100 KIU in 100 µl·ml^{-1} of whole blood). Blood samples were centrifuged at 1800 g for 10 min at 4 °C before separation and storage of plasma at -20 °C for subsequent assay. Fetal arterial blood (0.5 ml) was collected on alternate days for measurement of whole blood p$_a$O$_2$, p$_a$CO$_2$, pH, O$_2$ saturation and haemoglobin content using an ABL 550 acid base analyser and OSM2 haemoximeter.

6.2.4 Radioimmunoassays. Study 2. PR gene expression

Cortisol radioimmunoassay. Cortisol concentrations were measured in fetal plasma samples from the control group (n=6 fetuses; *n*=14 samples) and PR group (n=6 fetuses; *n*=13 samples). Total cortisol concentrations in fetal sheep plasma were measured using a radioimmunoassay, validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 74*). The intra- and inter-assay COVs were <10 % and <20 % respectively.

ACTH radioimmunoassay. ir-ACTH concentrations were measured in fetal plasma samples collected between 130 and 140 d of gestation from the control group (n=6 fetal sheep; *n*=12 samples) and PR group (n=6 fetal sheep; *n*=8 samples). The concentrations of ir-ACTH were measured using a radioimmunoassay which has previously been validated for fetal sheep plasma, as described in Chapter 2 (*see section 2.2.2.2 on pg. 75*). The intra- and inter-assay COVs were <10 % and <20 % respectively.
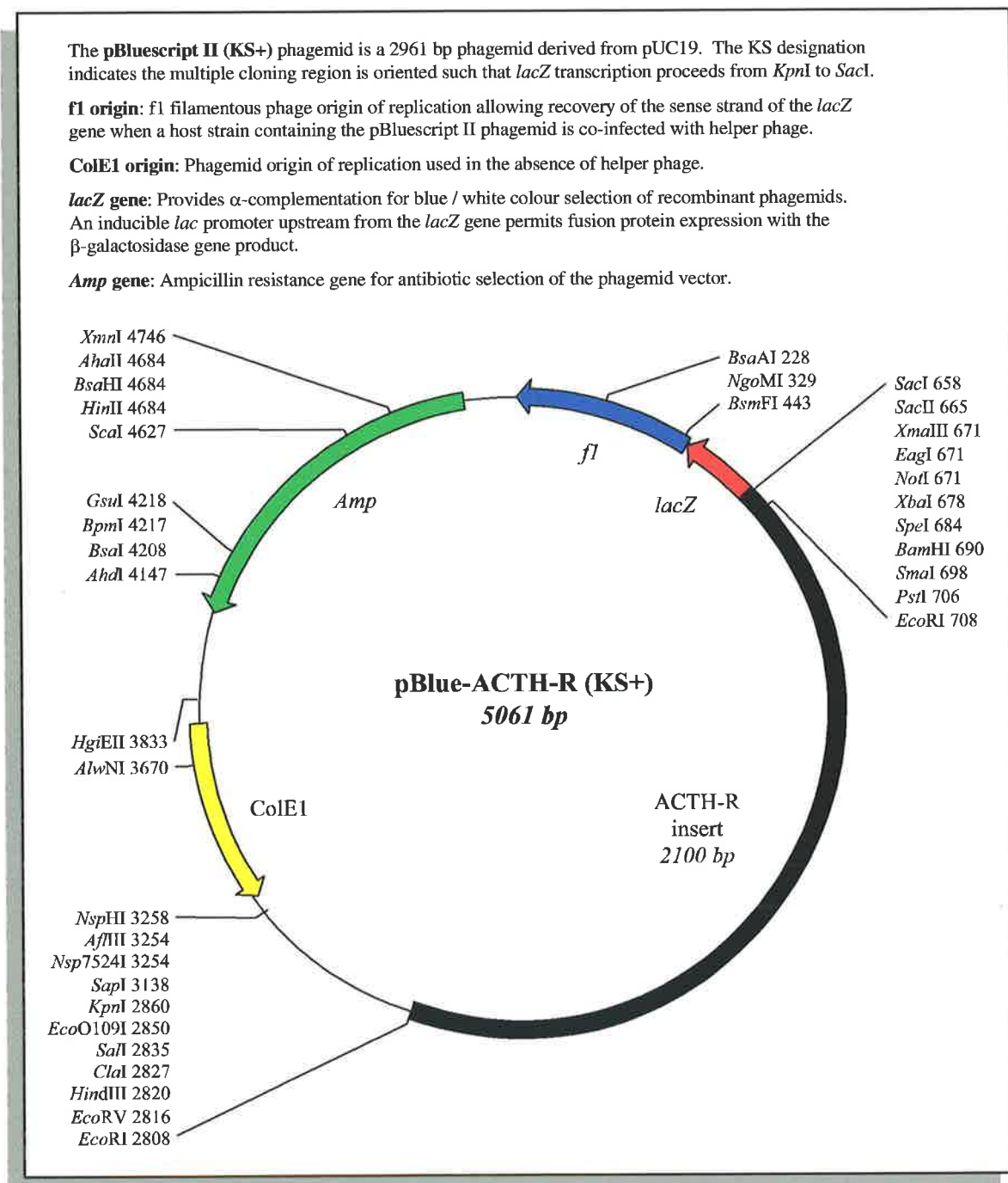
6.2.5 cDNA and antisense oligonucleotide probes and probe labelling

cDNA probes. *h*CYP11A1, *h*CYP17 and *h*CYP21A1 cDNA probes were generously provided by Professor W Miller (Department of Pediatrics, UCSF, San Francisco, CA, USA) (*see section 2.2.3.1 on pg. 75 and Figures 2.1 and 2.2 and 2.4 on pp. 76, 77 and 80 respectively*). A *h*3βHSD cDNA probe was donated by Dr. R Rodgers (Department of Medicine, Flinders University, SA, Australia) (*see section 2.2.3.1 on pg. 75 and Figure 2.3 on pg. 78*). An *o*IGF-II cDNA probe was a kind gift from Dr. R S Gilmour (Institute of Animal Physiology & Genetics, Cambridge, UK) (*see section 2.2.3.1 on pg. 79 and Figure 2.5 on pg. 81*). A *b*IGFBP-2 cDNA probe was generously provided by Dr. Jill Carr (Department of Biochemistry, The University of Adelaide, SA, Australia) (*see section 2.2.3.1 on pg. 79 and Figure 2.6 on pg. 82*). A 2100 bp *b*ACTH receptor cDNA probe including 200 bp of the 5' untranslated mRNA, 900 bp of the peptide coding region and 1 kb of the 3' untranslated region ([292]Mountjoy, Robbins *et al.*, 1992) and subcloned into the *Eco*R1 restriction site of the multiple cloning region within the pBluescript II (KS+) phagemid vector (*Figure 6.1*), was generously provided by Dr. Kathleen Mountjoy (Research Centre for Developmental Medicine and Biology, University of Auckland, New Zealand). A 1800 bp *b*SF-1 cDNA probe containing an open reading frame of 1383 bp encoding 461 amino acids ([191]Honda, Morohashi *et al.*, 1993) and subcloned into the multiple cloning region spanned by the *Bam*H1 and *Eco*R1 restriction sites within the pUC18 plasmid vector (*Figure 6.2*), was kindly donated by Dr. Ken-ichirou Morohashi (Department of Molecular Biology, Graduate School of Medicine Science, Kyushu University, Higashi-ku Fukuoka, Japan). cDNAs were radiolabelled with α-[^{32}P] dCTP (3,000 Ci·mmol^{-1}) by the random priming oligomer method to a specific activity of 10^9 cpm·µg^{-1} or greater, as described in Chapter 2 (*see section 2.2.3.1 on pg. 83*).

Oligonucleotide probes. A 30 mer antisense oligonucleotide probe for rat 18S rRNA, complementary to nucleotides 151-180, was synthesised (GeneWorks) (*see section 2.2.3.1 on pg. 83 and Figure 2.7 on pg. 85*) and end-labelled using T4PNK and γ-[^{32}P] ATP (4,000 Ci·mmol^{-1}) as substrate, as described in Chapter 2 (*see section 2.2.3.1 on pg. 84*).

6.2.6 Total RNA isolation. Study 2. PR gene expression

Total RNA was extracted from one adrenal from each fetus in the control (n=6 fetal sheep) and PR (n=6 fetal sheep) groups by homogenisation in 4 M guanidine hydrochloride solution and ultracentrifugation overnight at 36,000 rpm, through a cushion of 5.7 M CsCl in 100 mM EDTA ([90]Chirgwin, Przybyla *et al.*, 1979), as described in Chapter 2 (*see section 2.2.3.2 on pg. 84 and Figure 2.8 on pg. 86*).

The **pBluescript II (KS+)** phagemid is a 2961 bp phagemid derived from pUC19. The KS designation indicates the multiple cloning region is oriented such that *lacZ* transcription proceeds from *Kpn*I to *Sac*I.

f1 origin: f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

ColE1 origin: Phagemid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant phagemids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the phagemid vector.

*Xmn*I 4746
*Aha*II 4684
*Bsa*HI 4684
*Hin*II 4684
*Sca*I 4627

*Gsu*I 4218
*Bpm*I 4217
*Bsa*I 4208
*Ahd*I 4147

*Hgi*EII 3833
*Alw*NI 3670

*Nsp*HI 3258
*Afl*III 3254
*Nsp*7524I 3254
*Sap*I 3138
*Kpn*I 2860
*Eco*O109I 2850
*Sal*I 2835
*Cla*I 2827
*Hin*dIII 2820
*Eco*RV 2816
*Eco*RI 2808

*Bsa*AI 228
*Ngo*MI 329
*Bsm*FI 443

*Sac*I 658
*Sac*II 665
*Xma*III 671
*Eag*I 671
*Not*I 671
*Xba*I 678
*Spe*I 684
*Bam*HI 690
*Sma*I 698
*Pst*I 706
*Eco*RI 708

f1

Amp

lacZ

pBlue-ACTH-R (KS+)
5061 bp

ColE1

ACTH-R
insert
2100 bp

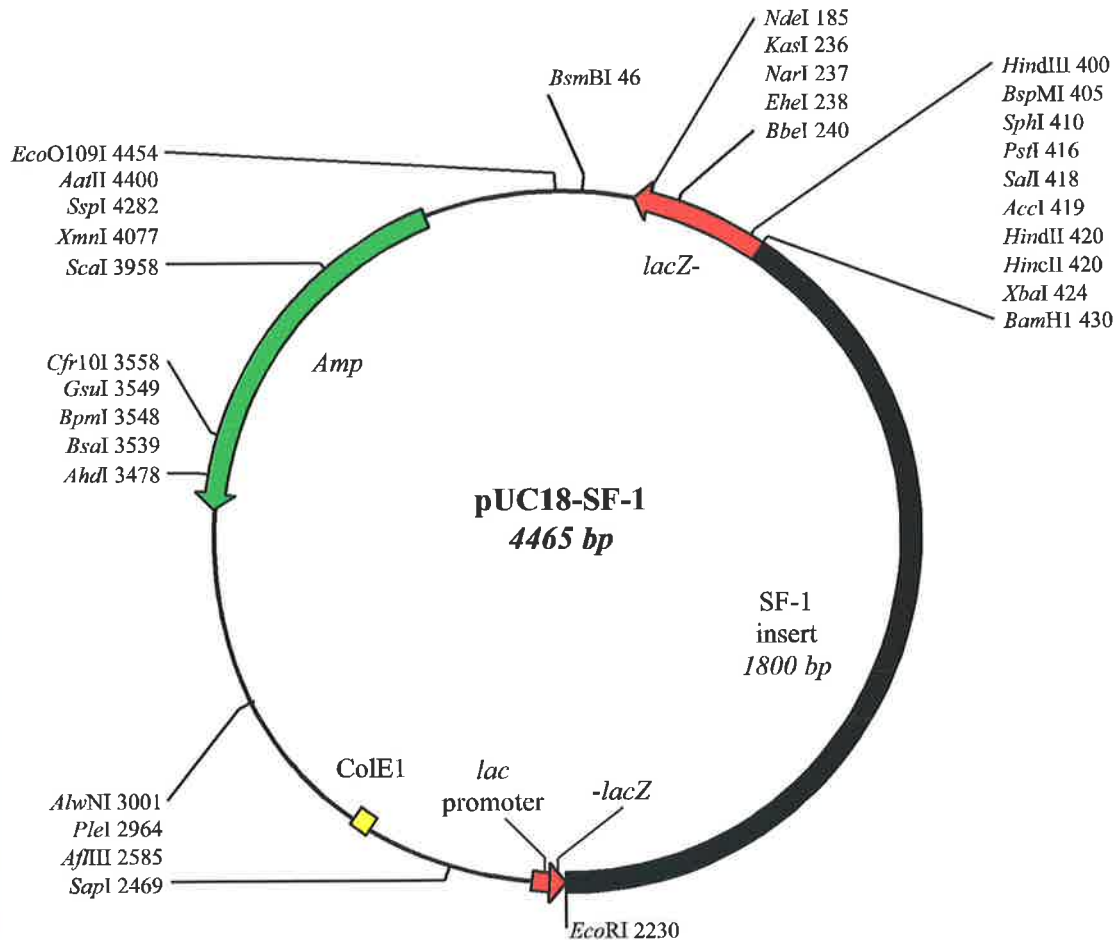**Figure 6.1**   **Plasmid map of the pBluescript II (KS+) phagemid vector containing the 2100 bp *b*ACTH-R cDNA insert.**

E. coli DH5α was transformed with the pBluescript II (KS+) phagemid containing the 2100 bp *b*ACTH-R cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Eco*R1 restriction enzyme digestion.

The **pUC18** plasmid is a 2686 bp plasmid derived from pBR322, M13mp18 and pUC12.

ColE1 origin: Plasmid origin of replication used in the absence of helper phage.

lacZ **gene**: Provides α-complementation for blue / white colour selection of recombinant plasmids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

Amp **gene**: Ampicillin resistance gene for antibiotic selection of the plasmid vector.

BsmBI 46

NdeI 185
KasI 236
NarI 237
EheI 238
BbeI 240

HindIII 400
BspMI 405
SphI 410
PstI 416
SalI 418
AccI 419
HindII 420
HincII 420
XbaI 424
BamH1 430

EcoO109I 4454
AatII 4400
SspI 4282
XmnI 4077
ScaI 3958

lacZ-

Cfr10I 3558
GsuI 3549
BpmI 3548
BsaI 3539
AhdI 3478

Amp

pUC18-SF-1
4465 bp

SF-1
insert
1800 bp

AlwNI 3001
PleI 2964
AflIII 2585
SapI 2469

ColE1

lac
promoter

-lacZ

EcoRI 2230

Figure 6.2   **Plasmid map of the pUC18 plasmid vector containing the 1800 bp *b*SF-1 cDNA insert.**

E. coli DH5α was transformed with the pUC18 plasmid containing the 1800 bp *b*SF-1 cDNA insert. Bacterial colonies were selected on the basis of ampicillin resistance and the insert was recovered from the plasmid via *Bam*H1 / *Eco*R1 restriction enzyme digestion.

6.2.7 Northern blot analysis. Study 2. PR gene expression

Total RNA samples (20 µg of adrenal RNA) were denatured by incubation in 2.2 M formaldehyde and 50 % v·v^{-1} formamide at 55 °C for 10 min and separated by electrophoresis in 1 % agarose gels containing 2.2 M formaldehyde, then transferred by gravity-feed blotting onto Zetaprobe nitrocellulose membranes using 10× SSC, as described in Chapter 4 (*see section 4.2.8 on pg. 139 and Figure 4.1 on pg. 140*). Membranes were washed in 10× SSC, 0.1 % SDS for 10 min at RT and exposed to UV light (12 sec at 120 J·cm^{-2}), prior to overnight incubation at 42 °C in 30 ml of either cDNA or antisense oligonucleotide hybridisation buffer (*see Appendix I*). Membranes were hybridised sequentially for 20 h (42 °C for cDNA probes or 50 °C for oligonucleotide probes) in 30 ml of fresh hybridisation buffer, containing either 1-2×10^6 cpm·ml^{-1} of the cDNA probe or 5×10^5 cpm·ml^{-1} of the 30 mer antisense 18S rRNA oligonucleotide probe (*see Figure 4.1 on pg. 140*). Membranes were washed once (10 min) at RT in 1× SSC, 0.1 % SDS; then twice (10 min each time) in 0.1× SSC, 0.1 % SDS at 42 °C, then briefly air-dried and sealed in a plastic bag. Membranes were exposed to phosphorimage plates in BAS 2040 cassettes for 24-48 h and images were quantified on a Fuji-BAS 1000 phosphorimage scanner using Fuji MacBAS software. cDNA probes were stripped from membranes between hybridisations by washing in 0.01× SSC, 0.5 % SDS for 10 min at 80 °C. A ratio of the density of each specific band with the density of the corresponding 18S rRNA band was calculated before comparisons were made.

The radiolabelled *h*CYP11A1, *h*CYP17, *h*CYP21A1, *h*3βHSD *o*IGF-II, *b*IGFBP-2, *b*ACTH-R and *b*SF-1 cDNA probes were used to probe two Northern blots of fetal adrenal total RNA from the control (n=6 fetuses) and PR (n=6 fetuses) groups. One Northern membrane was hybridised sequentially with the *h*CYP11A1, *h*CYP17, *h*3βHSD and *h*CYP21A1 cDNAs, while the second membrane was hybridised sequentially with the *b*SF-1, *b*ACTH-R, *o*IGF-II, *b*IGFBP-2 cDNA probes.

6.2.8 Statistical analysis

Study 1. Placental restriction profile of fetal adrenal gland weight. Data are presented as the mean ± SEM. Fetal weight, the ratio of fetal brain : liver weight and the ratio of total adrenal : fetal body weight were compared in the large cohort of 308 control and PR fetuses using a two-way ANOVA, with treatment group (control and PR) and parity (singleton and twin) as the specified factors. Two separate two-way ANOVAs were also used to compare the ratio of total adrenal : fetal body weight, using treatment group (control and PR) and gender (male and female) as the specified factors, in (*1*) singleton and

(*2*) twin fetuses. If a significant interaction between two major factors was identified in an ANOVA, then the data were split on the basis of the interacting factor and re-analysed. Data from the 308 control and PR fetuses between 137-147 d of gestation were used to calculate regression equations of (*1*) the ratio of brain : fetal body weight (g·kg^{-1}) vs. fetal body weight (kg), (*2*) the ratio of liver : fetal body weight (g·kg^{-1}) vs. fetal body weight (kg) and (*3*) the ratio of total adrenal (the sum of the weights of the left and right adrenals) : fetal body weight (mg·kg^{-1}) vs. fetal body weight (kg).

Study 2. Placental restriction gene expression. Data are presented as the mean $\pm$ SEM. Mean gestational plasma ir-ACTH and cortisol concentrations were calculated for each fetus and the hormonal concentrations were compared between the control and PR groups using an unpaired Student's *t*-test. The ratio of total adrenal : fetal body weight and the ratios of adrenal steroidogenic enzymes and IGF-II, IGFBP-2, ACTH-R and SF-1 mRNA : 18S rRNA were compared in the control and PR groups using an unpaired Student's *t*-test. A probability of <5 % ($p<0.05$) was considered to be significant.

6.3   RESULTS

1.1.1   *Study 1. Placental restriction profile of fetal adrenal gland weight*

6.3.1.1   *Fetal outcome and phenotype characteristics*

Fetal weight. In the large cohort of control and PR fetuses, fetal body weight was significantly lower ($p<0.0001$) in the PR group when compared with control animals, for both singleton (PR: 3.49 ± 0.16 kg; control: 4.69 ± 0.08 kg) and twin fetal sheep (PR: 3.10 ± 0.23 kg; control: 4.20 ± 0.05 kg) (*Figure 6.3A*).
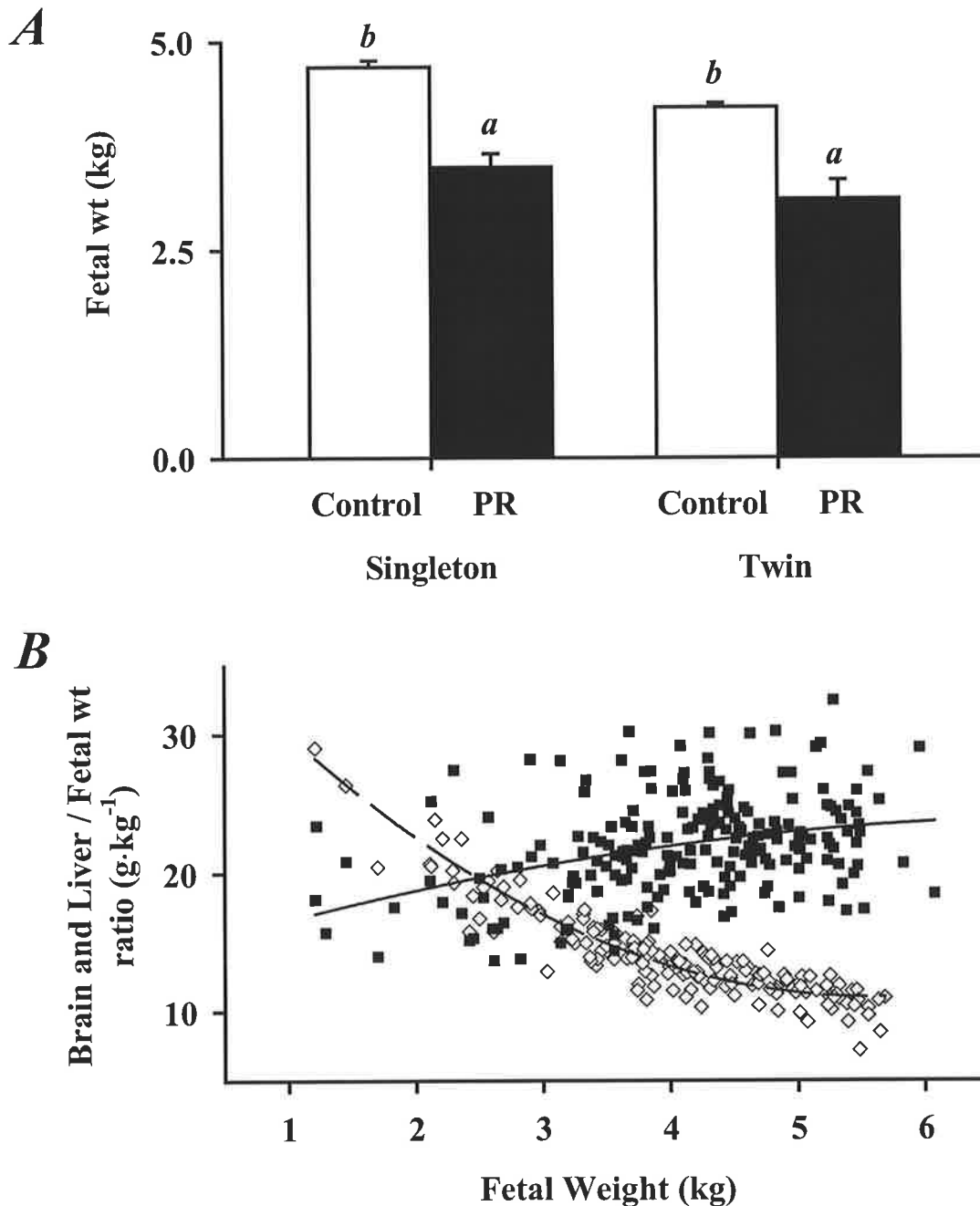
The ratios of brain and liver weight : fetal body weight. The ratio of brain : fetal body weight (g·kg^{-1}) increased as a function of decreasing fetal body weight (kg), while the ratio of liver : fetal body weight (g·kg^{-1}) decreased as a function of decreasing fetal body weight (kg), when control and PR fetuses were treated as a single group (*Figure 6.3B*). The increase in the ratio of brain : fetal body weight and the decrease in the ratio of liver : fetal body weight with decreasing fetal body weight is described by equations *6.1 and 6.2*:

[Brain : Fetal weight] $= 0.91$**[Fetal weight]**$^2 - 10.1$**[Fetal weight]** $+$ **39.2**
(*Equation 6.1*, $R^2=0.85$, $p<0.0001$).

[Liver : Fetal weight] $= -0.18$**[Fetal weight]**$^2 + 2.7$**[Fetal weight]** $+$ **14.1**
(*Equation 6.2*, $R^2=0.12$, $p<0.0001$).

Figure 6.3 Fetal weight and the ratio of brain and liver weight to fetal body weight in control and PR singleton and twin fetal sheep between 137 d and 147 d of gestation.

(*A*) Fetal weight was significantly lower in singleton and twin fetuses in the 71 PR fetuses (solid bar) between 137 d and 147 d of gestation when compared with the 237 control fetuses (open bar) in this gestational age range. (*B*) The ratios of brain : fetal body weight (◇, — — —) and liver : fetal body weight (■, ————) in control and PR fetuses between 137-147 d of gestation. The relative brain weight increased while relative liver weight decreased, with decreasing fetal body weight, in control and PR fetuses which were small for gestational age, according to the equations *6.1 and 6.2*:

[Brain : Fetal weight] $= 0.91[\text{Fetal weight}]^2 - 10.1[\text{Fetal weight}] + 39.2 \ (R^2=0.85)$
[Liver : Fetal weight] $= -0.18[\text{Fetal weight}]^2 + 2.7[\text{Fetal weight}] + 14.1 \ (R^2=0.12)$

Finally, the ratio of fetal brain : liver weight was significantly higher ($p<0.001$) in the PR group than in the control group for both singleton (PR: 0.79 ± 0.05 g·g^{-1}; control: 0.53 ± 0.03 g·g^{-1}) and twin fetal sheep (PR: 1.06 ± 0.17 g·g^{-1}; control: 0.63 ± 0.03 g·g^{-1}). In addition, the ratio of fetal brain : liver weight was significantly higher ($p<0.001$) in twin fetuses from the control and PR groups, when compared with singleton fetuses.

6.3.1.2   Ratio of total adrenal : fetal body weight

Ratio of total adrenal : fetal body weight. In the large cohort of control and PR fetuses, the ratio of total adrenal : fetal body weight (mg·kg^{-1}) increased as a function of decreasing fetal body weight (kg), when all 308 fetuses were treated as a single group (*Figure 6.4A*). The increase in the ratio of total adrenal : fetal body weight with decreasing fetal body weight in the 237 fetal sheep in the control group and in the 71 fetal sheep in the PR group is described by equation *6.3*:
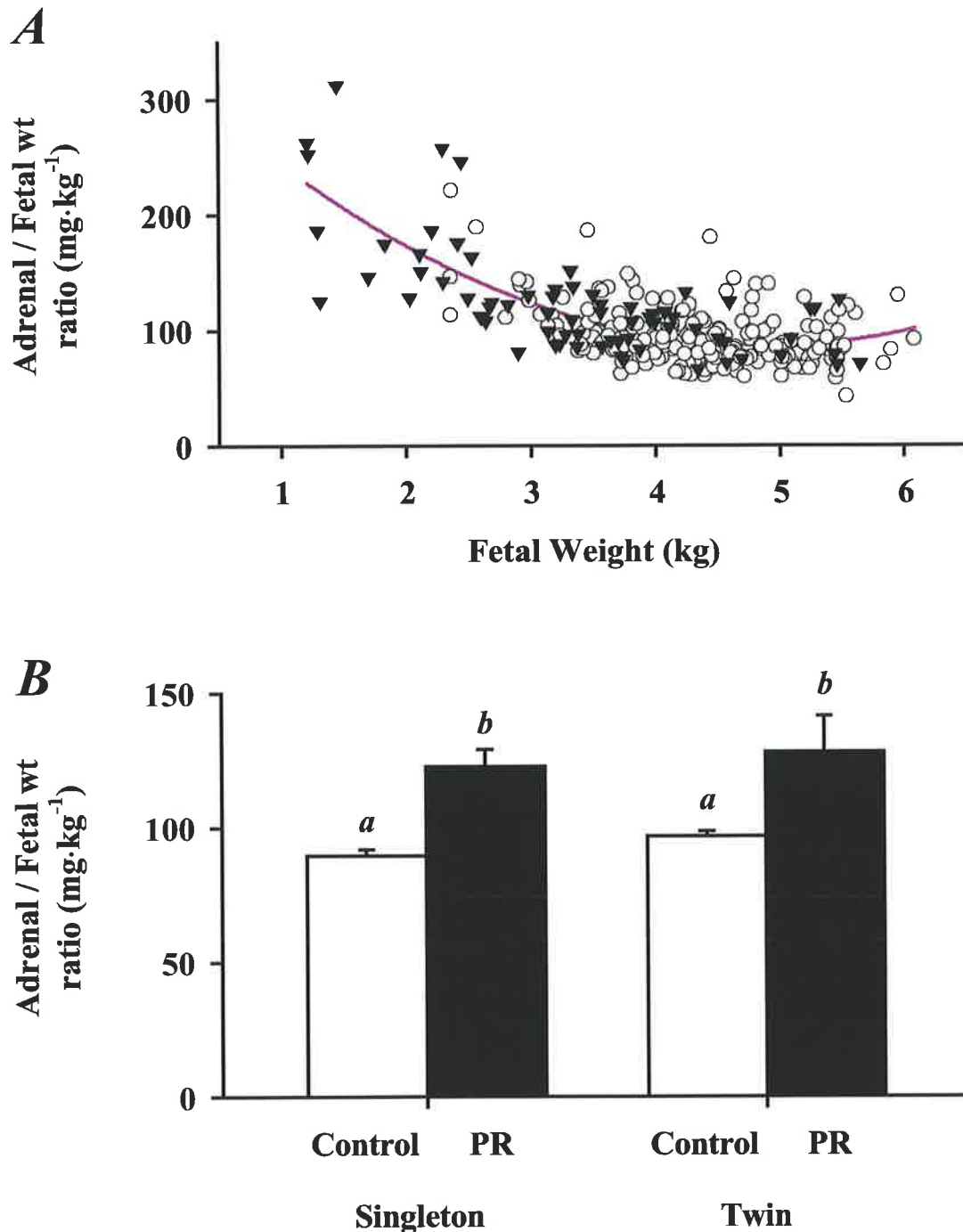
[Adrenal : Fetal weight] = 10.3[Fetal weight]^2 − 101.3[Fetal weight] + 334.8
(*Equation 6.3*, $R^2=0.50$, $p<0.0001$).

The ratio of total adrenal : fetal body weight was significantly higher ($p<0.001$) in the PR group than in the control group, for both singleton (PR: 122.8 ± 6.4 mg·kg^{-1}; control: 89.6 ± 2.3 mg·kg^{-1}) and twin fetal sheep (PR: 128.0 ± 13.5 mg·kg^{-1}; control: 96.7 ± 2.0 mg·kg^{-1}) (*Figure 6.4B*). There were no differences, however, in the ratio of total adrenal : fetal body weight between male and female fetuses in either singleton (control male: 92 ± 3 mg·kg^{-1} and control female: 86 ± 3 mg·kg^{-1}; PR male: 120 ± 8 mg·kg^{-1} and PR female 125 ± 12 mg·kg^{-1}) or twin pregnancies (control male: 94 ± 3 mg·kg^{-1} and control female 97 ± 2 mg·kg^{-1}; PR male: 111 ± 11 mg·kg^{-1} and PR female 148 ± 25 mg·kg^{-1}).

6.3.2   Study 2. Placental restriction and adrenal mRNA expression

Fetal outcome, blood gas and blood glucose status. Fetal body weights and placental weights were significantly lower ($p<0.05$) in this subset of PR fetuses (3.31 ± 0.23 kg and 264.4 ± 29.3 g respectively; n=6 fetuses) when compared with controls (4.30 ± 0.29 kg and 573.3 ± 64.9 g respectively; n=6 fetuses). Fetal p_aO_2 was also significantly lower ($p<0.05$) in the PR group (14.2 ± 0.8 mmHg; n=6 fetuses) when compared with the control group (21.8 ± 1.9 mmHg, n=6 fetuses) from 130-140 d of gestation. There was no difference, however, in the fetal plasma glucose concentrations between the two groups (PR: 1.12 ± 0.10 mmol·l^{-1}; control: 1.31 ± 0.11 mmol·l^{-1}).

A

B

Figure 6.4   **Profile of the ratio of adrenal : fetal body weight in control and PR singleton and twin fetuses between 137-147 d of gestation.**

(*A*) The ratio of adrenal : fetal body weight of control (O) and PR (▼) singleton and twin fetuses increased with decreasing fetal weight in fetuses below ~3.5 kg, according to the equation: **[Adrenal : Fetal weight] = 10.3[Fetal weight]^2 – 101.3[Fetal weight] + 334.8** (R^2=0.50), (*see equation 6.3 on pg. 182*). (*B*) The ratio of adrenal : fetal weight was significantly greater in PR fetuses (solid bar) when compared with control fetuses (open bar) in both singletons and twins. Significant differences between the control and PR groups are denoted by different superscripts, *e.g.* a < b (p<0.05).

Fetal adrenal weights, cortisol and ir-ACTH concentrations. The ratio of total adrenal : fetal body weight was significantly higher (p<0.05) in the PR group (113.1 ± 7.8 mg·kg^{-1}, n=6 fetuses) when compared with control fetuses (86.9 ± 5.6 mg·kg^{-1}, n=6 fetuses). Plasma cortisol concentrations between 130 d and 140 d of gestation were also significantly higher (p<0.05) in the PR fetuses (3.7 ± 0.9 nmol·l^{-1}) when compared with control fetuses (1.7 ± 0.2 nmol·l^{-1}). There was no difference, however, in the plasma concentrations of ir-ACTH between the PR (86.6 ± 17.6 pg·ml^{-1}) and control (84.7 ± 16.7 pg·ml^{-1}) fetuses across this gestational age range.
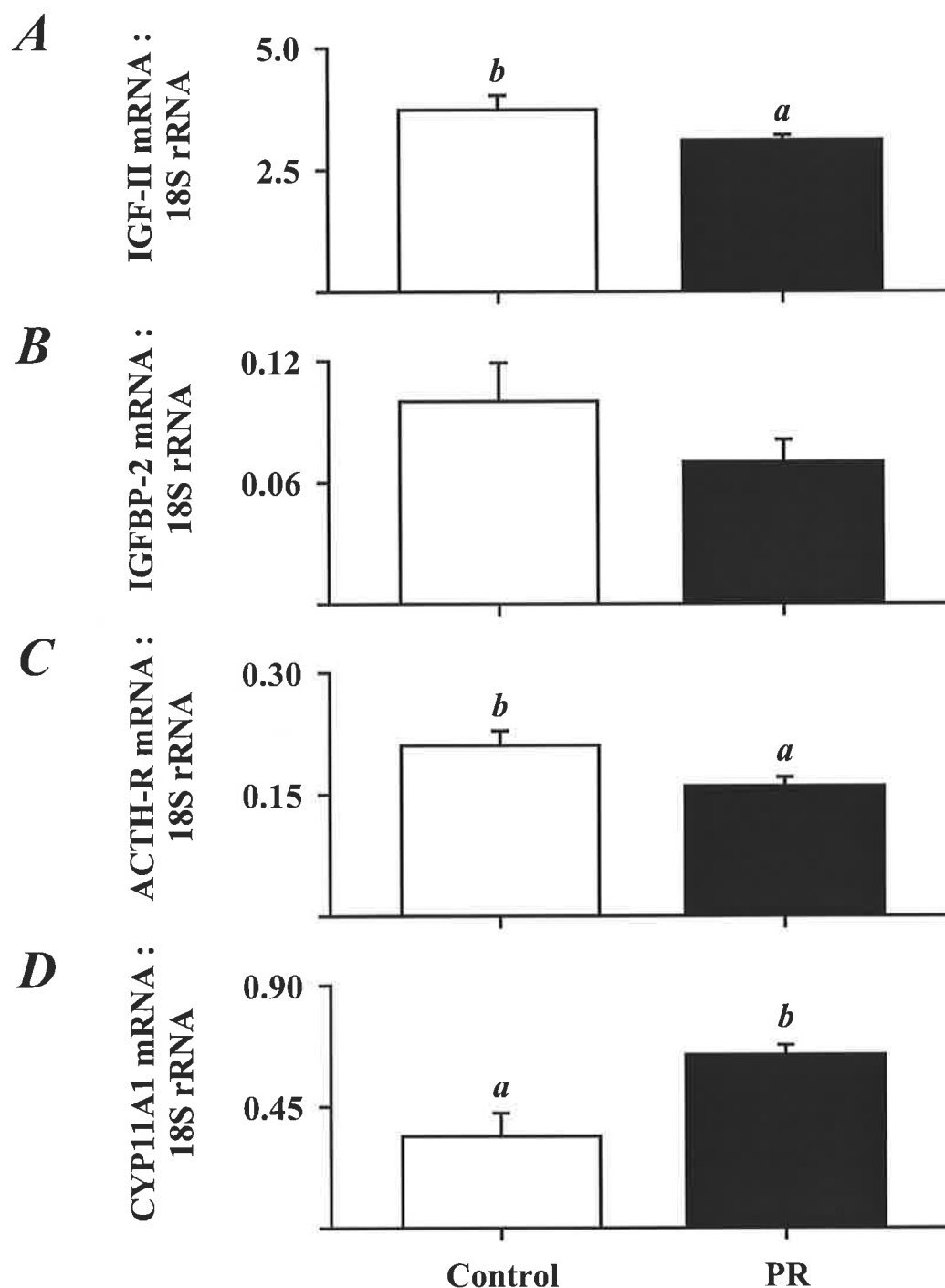
Adrenal IGF-II and IGFBP-2 mRNA expression. The oIGF-II cDNA identified six transcripts in the size range 1.8-6.0 kb by Northern blot analysis of total RNA from the fetal adrenals. The ratio of total IGF-II mRNA : 18S rRNA was lower (p<0.05) in the PR group (3.10 ± 0.10) when compared with the control group (3.73 ± 0.29) (*Figure 6.5A*). There was no difference, however, in the abundance of adrenal IGFBP-2 mRNA between the PR and control groups (PR: 0.07 ± 0.01; control: 0.10 ± 0.02) (*Figure 6.5B*).

Adrenal ACTH receptor and SF-1 mRNA expression. The ratio of ACTH-R (3.6 kb transcript) mRNA : 18S rRNA was significantly lower (p<0.05) in adrenals from PR fetuses (0.16 ± 0.01) when compared with adrenals from control fetuses (0.21 ± 0.02) (*Figure 6.5C*). There was no difference, however, in the ratio of adrenal SF-1 (3.5 kb transcript) mRNA : 18S rRNA between the PR (0.31 ± 0.02) and control (0.37 ± 0.05) groups.

Adrenal steroidogenic enzyme mRNA expression. The ratio of CYP11A1 (1.9 kb transcript) mRNA : 18S rRNA was significantly greater (p<0.005) in adrenals from the PR group when compared with the control group (*Figures 6.5D, 6.6 and Table 6.1*). There was no difference, however, in the ratio of adrenal CYP17 (1.7 kb transcript) mRNA : 18S rRNA, or in the ratios of 3βHSD (1.6 kb transcript) and CYP21A1 (2 transcripts; 2.2 and 1.8 kb) mRNA : 18S rRNA between the PR and control fetuses (*Figure 6.6 and Table 6.1*).

6.4   DISCUSSION

I have demonstrated that fetal weight was lower and the ratio of fetal brain : liver weight was higher in placentally restricted fetuses, in a large cohort of growth restricted and normally grown fetal sheep after 137 d of gestation. When the control and placental restriction groups were combined, there was a consistent relationship between the relative brain and liver weights with decreasing fetal body weight. These progressive

Figure 6.5   Adrenal IGF-II, IGFBP-2, ACTH-R and CYP11A1 mRNA expression in control and PR fetal sheep at 140 d of gestation.

The ratios of (*A*) IGF-II, (*B*) IGFBP-2, (*C*) ACTH-R and (*D*) CYP11A1 mRNA : 18S rRNA in fetal adrenals from control (open bar) and PR (solid bar) fetal sheep at 140 d of gestation. Significant differences ($p<0.05$) in the ratios between the control and PR groups are denoted by different superscripts.
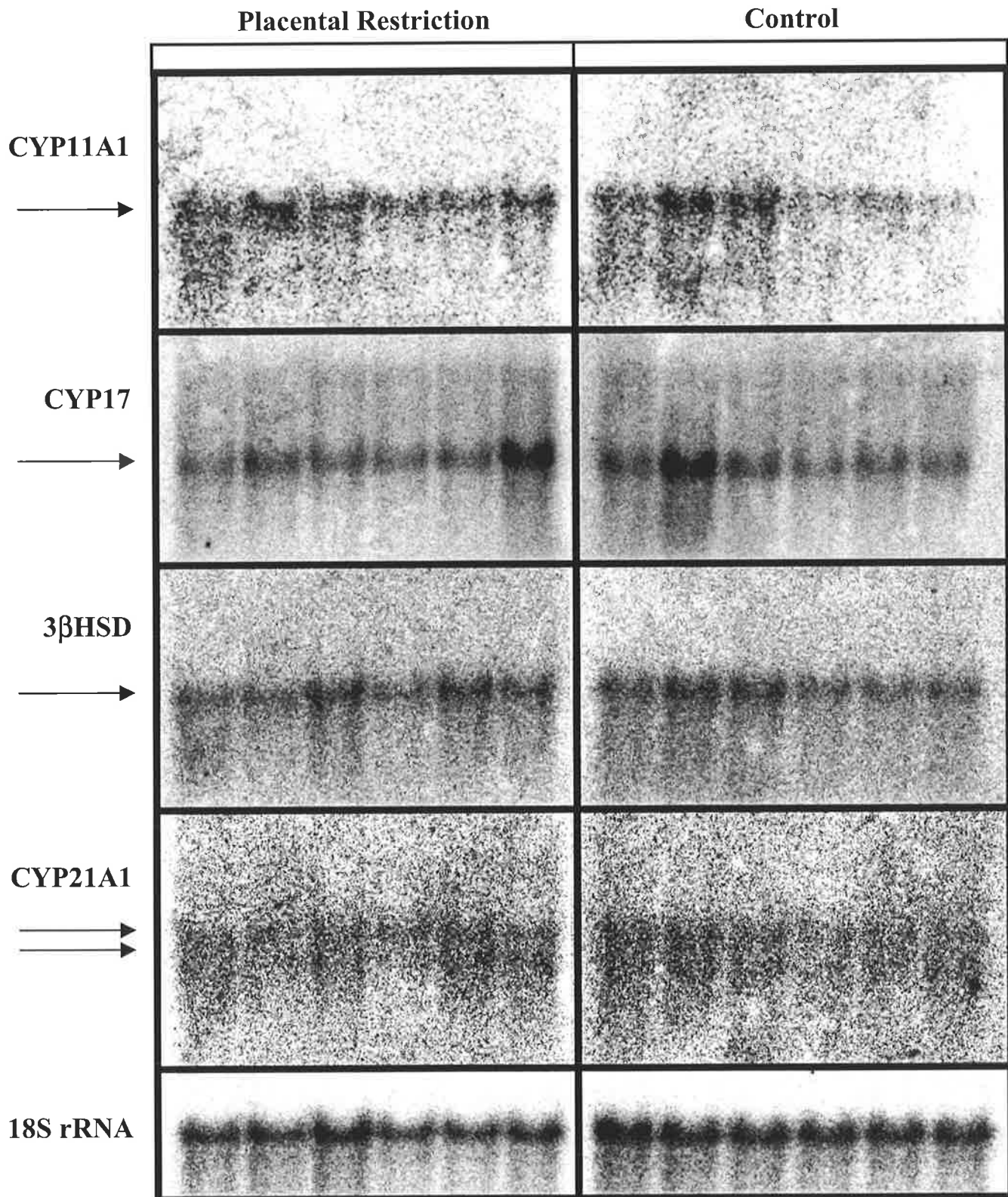
Figure 6.6   **Northern blot of adrenal steroidogenic enzyme mRNA expression from control and PR fetal sheep.**

Northern blot analyses of total RNA (20 µg·lane^{-1}) prepared from adrenal glands from control and PR fetal sheep at 140 d of gestation, and hybridised sequentially with the radiolabelled hCYP11A1, hCYP17, h3βHSD and hCYP21A1 cDNA and the 30 mer antisense 18S rRNA oligonucleotide probes.

Table 6.1   **Adrenal steroidogenic enzyme mRNA : 18S rRNA ratios in control and PR fetal sheep.**

Treatment group	Adrenal Steroidogenic Enzyme mRNA : 18S rRNA			
	CYP11A1 : 18S	CYP17 : 18S	3βHSD : 18S	CYP21A1 : 18S
Control	0.34 ± 0.09	0.34 ± 0.06	0.70 ± 0.07	0.48 ± 0.03
PR	0.64 ± 0.04 *	0.41 ± 0.07	0.78 ± 0.09	0.60 ± 0.07

The mean ratios of CYP11A1, CYP17, 3βHSD and CYP21A1 mRNA : 18S rRNA in fetal adrenals from control and PR fetal sheep at 140 d of gestation. Significant differences ($p < 0.05$) in the mean ratios between the control and PR groups are denoted by an asterix.

changes in organ growth were particularly marked when fetal body weight fell below ~3.5 kg. The small size of the liver in the IUGR fetus is intriguing. The fetal liver is perfused by umbilical venous return, and thus is presented with higher concentrations of O_2 and substrate than, for example, the brain. Yet liver growth clearly fails to keep pace with brain growth in small for gestational age human fetuses ([64]Brooke, Wood and Butters, 1984), and in experimental animal models of fetal IUGR ([235]Lafeber, Rolph and Jones, 1984). There is a delay in the normal development of hepatocyte function in the growth-restricted fetal guinea pig, and in the maturation of hepatic metabolic processes, which at 60-63 d of gestation (term≈70 days of gestation) is comparable to the liver 5-10 d earlier in normal gestation ([235]Lafeber, Rolph and Jones, 1984). Adrenal growth was also affected by surgical restriction of placental growth in the present study. Total adrenal weight as a proportion of fetal body weight was greater in placentally restricted than control fetuses. In addition, the changes in adrenal growth relative to fetal body weight appear to be quantitatively related to the degree of fetal growth restriction. Thus, the present study confirms earlier reports of increased adrenal growth in placentally restricted fetal sheep ([364]Robinson, Kingston *et al.*, 1979; [343]Phillips, Simonetta *et al.*, 1996) and is consistent with the brain and adrenal 'sparing' phenotype observed in asymmetric human fetal growth restriction ([64]Brooke, Wood and Butters, 1984). Importantly, it appears that the same relationship between adrenal growth and the degree of fetal growth restriction exists in both the placentally restricted fetal sheep and in 'spontaneously' growth restricted fetuses, *i.e.* those control singleton and twin fetuses which were <3.5 kg.

One potential stimulus for adrenal growth is the decrease in p_aO_2 associated with moderate and severe placental and fetal growth restriction ([364]Robinson, Kingston *et al.*, 1979; [343]Phillips, Simonetta *et al.*, 1996). It is well established that blood flow to the brain, heart and adrenals is increased during acute hypoxaemia while blood flow to the gastrointestinal, renal and peripheral vascular beds decreases ([210]Jensen, Hohmann and Kunzel, 1987; [212]Jansen, Belik *et al.*, 1989; [75]Carter, Homan *et al.*, 1995). This redistribution of fetal cardiac output is maintained during episodes of prolonged hypoxaemia ([45]Bocking, Gagnon *et al.*, 1988; [373]Rurak, Richardson *et al.*, 1990) and we have also shown that there is an increase in the vascularity of adrenals in placentally restricted fetal sheep ([1]Adams, Phillips *et al.*, 1998). While it is established that ACTH acts to increase blood flow and decrease vascular resistance in the fetal adrenal ([75]Carter, Homan *et al.*, 1995), we have found no increase in circulating ACTH in the placentally restricted sheep fetus ([343]Phillips, Simonetta *et al.*, 1996) and there is no evidence for an altered responsiveness of adrenal

blood flow to ACTH after embolisation of the fetal placental circulation for 8 days ([295]Murotsuki, Gagnon *et al.*, 1996).

6.4.1   *Adrenal IGF-II and IGFBP-2 mRNA expression*

While adrenal growth was promoted as a consequence of placental restriction, the expression of adrenal IGF-II mRNA was suppressed in this group. I have previously shown, in Chapter 2, that there is no relationship between fetal adrenal IGF-II mRNA expression and adrenal growth during the last 15 d of gestation. Furthermore, adrenal IGF-II mRNA expression is maintained while adrenal growth decreases in late-gestation after surgical disconnection of the fetal hypothalamo-pituitary axis (*see Chapter 2*). Interestingly, Lu and co-workers have found that intra-fetal infusion of ACTH (n=4 fetuses) or cortisol (n=3 fetuses) for 84 h, beginning at 120-125 d of gestation, resulted in a decrease in fetal adrenal IGF-II mRNA levels ([258]Lu, Han *et al.*, 1994). It has also been reported that cortisol infusion results in a decrease in IGF-II mRNA expression in other fetal tissues including the liver ([258]Lu, Han *et al.*, 1994). It may be that the increase in adrenal cortisol output which occurs in the hypoxaemic growth restricted sheep fetus after 125 d of gestation ([343]Phillips, Simonetta *et al.*, 1996) acts to decrease the expression of adrenal IGF-II mRNA. Given that there is no change in the expression of the IGF-II binding protein, IGFBP-2, this could result in a decrease in IGF-II protein within the adrenal. Interestingly, Braems and colleagues ([58]Braems, Han and Challis, 1998) have recently reported that reducing the maternal fraction of inspired O_2 for 48 h also resulted in a significant decrease in the expression of adrenal IGF-II mRNA in fetuses after 134 d of gestation. These authors speculated that the decrease in IGF-II expression may shift the metabolic energy of the adrenocortical cells away from growth and towards steroidogenesis. In the present study, however, adrenal growth was increased in the chronically hypoxaemic fetuses, despite the decrease in adrenal IGF-II mRNA levels and the lack of an increase in the immunoreactive or bioactive concentrations of ACTH in the fetal circulation ([343]Phillips, Simonetta *et al.*, 1996). This suggests that both adrenal growth and steroidogenesis may be maintained by other pituitary derived or non-pituitary derived hormones and intra-adrenal growth factors during chronic hypoxaemia. It has been suggested that the *N*-terminal POMC peptides, pro-γ-MSH and γ₃-MSH may have a role in adrenal mitosis and hyperplasia in the late-gestation sheep fetus ([377]Saphier, Glynn *et al.*, 1993) and other hormones, such as AII and prostaglandin E₂ (PGE₂) are also potential candidates for the stimulation of adrenal growth in spontaneously and experimentally growth restricted fetal sheep ([294]Murotsuki, Challis *et al.*, 1995).

6.4.2 Adrenal ACTH receptor mRNA expression

In the present study, I have also found a decrease in adrenal mRNA expression for the ACTH-R in growth restricted fetal sheep. It has recently been demonstrated that steroidogenic factor-1 (SF-1), is essential for basal expression of ACTH-R in the human and mouse adrenal ([104]Commas, Pullinger *et al.*, 1997; [269]Marchal, Naville *et al.*, 1998). I found no effect, however, of placental restriction on SF-1 expression in the fetal sheep adrenal. Other factors which stimulate ACTH-R expression in ovine adrenocortical cells include ACTH and glucocorticoids and it has also been shown that IGF-I and -II increase the expression of ACTH-R in human adrenocortical cells ([13]l'Allemand, Penhoat *et al.*, 1996; [345]Picard-Hagen, Penhoat *et al.*, 1997). The decrease in adrenal ACTH-R expression in the growth restricted fetal sheep, therefore, may be a consequence of the fall in adrenal IGF-II expression. Alternatively, hypoxaemia may induce expression of inhibitory factors, such as transforming growth factor β (TGFβ), which are known to decrease ACTH-R expression within the adrenal ([352]Rainey, Viard and Saez, 1989). Importantly, our results suggest that the increase in circulating cortisol that occurs in the absence of an increase in ACTH in the growth restricted fetus, is not due to an increase in adrenal ACTH-R mRNA expression.

6.4.3 Prolonged hypoxaemia and fetal ir-ACTH and cortisol concentrations

It is clear from a number of studies that an increase in fetal cortisol is a consistent response to prolonged or repeated fetal hypoxaemia in late-gestation. Furthermore, this increase is not always associated with an increase in fetal ACTH ([192]Hooper, Coulter *et al.*, 1990; [59]Braems, Matthews and Challis, 1996; [343]Phillips, Simonetta *et al.*, 1996). In experiments in which hypoxaemia was induced in fetal sheep by a reduction in utero-placental blood flow for 24 h, fetal ACTH concentrations were increased only at 2 h after the onset of hypoxaemia and then returned to baseline values ([192]Hooper, Coulter *et al.*, 1990). In contrast, fetal cortisol concentrations were elevated by 2 h after the onset of hypoxaemia and remained elevated throughout the 24 h period ([192]Hooper, Coulter *et al.*, 1990). Similarly, when hypoxaemia was induced in fetal sheep for 48 h through a reduction in the maternal fraction of inspired O_2, fetal cortisol concentrations were increased in the absence of a sustained fetal ACTH response ([59]Braems, Matthews and Challis, 1996). In the present study, restriction of placental growth by maternal carunclectomy induced a marked increase in fetal circulating cortisol levels, in the absence of elevated plasma ir-ACTH concentrations. Finally, cordocentesis studies in human fetuses that were small for gestational age have also found that plasma cortisol concentrations are higher and plasma ACTH concentrations are lower than in normally grown fetuses at 18-38 weeks of

gestation ([138]Economides, Nicolaides *et al.*, 1988). In contrast to these studies, fetal ACTH concentrations remained elevated during a 21 day period in which the placentae of fetal sheep were repeatedly embolised ([303]Naville, Rainey and Mason, 1991). It is possible that the fetal hypothalamo-pituitary axis adapts to the effects of prolonged, sustained hypoxaemia induced through either maternal hypoxaemia, reduction of utero-placental blood flow or placental restriction, whereas repeated hypoxaemic episodes, such as those experienced during placental embolisation studies, may stimulate pituitary ACTH secretion.

6.4.4   *Adrenal steroidogenic enzyme mRNA expression*

I reported, in Chapter 2, that adrenal CYP11A1 and CYP21A1 mRNA levels increase after 135 d of gestation, whereas adrenal CYP17 mRNA increase later, after 140 d of gestation. In contrast, the expression of adrenal 3βHSD did not change between 130 and 145 d of gestation ([342]Phillips, Ross *et al.*, 1996). It is possible, therefore, that ACTH acts differentially on the expression of the adrenal steroidogenic enzymes during late-gestation. Previous *in vivo* studies have demonstrated that intra-fetal ACTH infusion specifically increases CYP11A1 and CYP17 mRNA levels ([408]Tangalakis, Coghlan *et al.*, 1990), although it appears from *in vitro* studies that the *CYP17* gene is more sensitive to regulation by ACTH than the other steroidogenic enzymes ([43]Bird, Pasquarette *et al.*, 1996). In the current study, I have also found a differential response of adrenal steroidogenic enzyme expression to fetal growth restriction. Placental restriction resulted in a relative increase in adrenal CYP11A1 mRNA in the absence of any change in CYP17, 3βHSD and CYP21A1 mRNA levels. Braems and co-workers have also recently reported that there was an increase in the adrenal mRNA levels of CYP11A1, 3βHSD and CYP21A1 but not CYP17 after 48 h fetal hypoxaemia ([58]Braems, Han and Challis, 1998). These data suggest that factors other than ACTH may stimulate an increase in adrenal steroidogenesis in the chronically hypoxaemic sheep fetus. One potential candidate is the placental hormone, PGE_2. Fetal PGE_2 concentrations are increased during periods of hypoxaemia induced by reduction of uterine blood flow and placental embolisation ([192]Hooper, Coulter *et al.*, 1990; [294]Murotsuki, Challis *et al.*, 1995). It has been demonstrated, however, that the actions of PGE_2 on adrenocortical cells *in vitro* are similar to those of ACTH, *i.e.* PGE_2 acts to stimulate cAMP production and CYP17 and 3βHSD mRNA expression ([350]Rainey, Naville *et al.*, 1991). Another possibility is that placental restriction results in an increase in the action of factors such as AII or TGFβ, which are known to antagonise the actions of ACTH on adrenal CYP17 mRNA expression ([303]Naville, Rainey and Mason, 1991; [42]Bird, Magness *et al.*, 1992).

In summary, I have demonstrated that placentally restricted fetal sheep exhibit the adrenal and brain 'sparing' phenotype, and disproportionately small liver size, characteristic of human fetuses which are small for gestational age. In addition, I have demonstrated that the quantitative relationship between adrenal growth and the degree of fetal growth restriction (as determined by fetal body weight) is continuous amongst fetuses in which growth was restricted experimentally by surgical restriction of placental growth, and in which fetal growth restriction occurred spontaneously *in utero*. These findings suggest that placental restriction is an appropriate model in which to study the impact of spontaneous fetal growth restriction on the fetal HPA axis. While adrenal growth was promoted in the placentally restricted group, the expression of adrenal IGF-II mRNA was suppressed, in the absence of any changes in the adrenal mRNA expression for IGFBP-2. There was a decrease in the expression of ACTH-R mRNA and a specific increase in the expression of CYP11A1 mRNA in the adrenals of placentally restricted fetal sheep. This suggests that factors other than ACTH may also play an important role in the stimulation of adrenal growth and steroidogenesis during chronic fetal hypoxaemia. Factors other than ACTH may be particularly important when the fetus is exposed to chronic, sustained hypoxaemia as distinct from repeated dynamic changes in its oxygenation.

CHAPTER 7.   GENERAL DISCUSSION AND CONCLUSIONS

7.1   *THE ROLE OF CORTISOL IN FETAL ADRENAL DEVELOPMENT*

In this thesis, I have characterised the gestational profile of the weight of adrenal glands from approximately 400 fetal sheep between 42 d and 147 d of gestation (*see equation 2.6 on pg. 95*). I have used this gestational profile to compare adrenal weight, following disconnection of the fetal hypothalamus and pituitary at around 110 d of gestation, with adrenal weight in fetuses with an intact HP axis (*see Figure 2.10 on pg. 92*). I reported, in Chapter 2, that fetal adrenal weight at 139-141 d of gestation was significantly lower after fetal HPD compared with age-matched control fetuses, and was consistent with adrenal weight in intact fetuses at approximately 114-128 d of gestation (*see Figure 2.17 on pg. 102*). Interestingly, cortisol infusion in HPD fetuses for 5 d during late-gestation restored adrenal weight to values comparable to intact fetuses at 140 d of gestation. In contrast, adrenal weight remained unchanged when cortisol was infused for 7 d into fetuses with an intact HP axis, in mid-gestation, prior to the pre-partum cortisol surge.

I also demonstrated, in Chapter 2, that there is a differential pattern of expression of mRNA for the adrenal steroidogenic enzymes during the 15 d preceding delivery in the sheep fetus. There was an increase in adrenal CYP11A1 and CYP21A1 mRNA abundance between 130 d and 135 d of gestation, and an increase in adrenal CYP17 mRNA levels after 141 d of gestation. It would appear from these and other ([407]Tangalakis, Coghlan *et al.*, 1989; [298]Myers, McDonald and Nathanielsz, 1992b) studies that there is a substantial increase in the expression of mRNA for CYP11A1, CYP21A1 and CYP17 within the fetal adrenal gland at the time of the pre-partum cortisol surge. This is the first study, however, in which a differential time-course of adrenal steroid hydroxylase gene expression has been demonstrated during the final 15 d of gestation.

Adrenal levels of CYP11A1, CYP21A1 and 3βHSD mRNA were all significantly lower at 139-141 d of gestation after disconnection of the fetal hypothalamus and pituitary at around 110 d of gestation, and I have shown in a previous study that the abundance of adrenal CYP17 mRNA is also significantly lower following fetal HPD ([342]Phillips, Ross *et al.*, 1996). These data are consistent with the lack of a pre-partum cortisol surge in the HPD sheep fetus ([342]Phillips, Ross *et al.*, 1996). It is interesting, in the present study, that while there is no change in the adrenal level of 3βHSD mRNA in intact fetal sheep

between 130 d and 145 d of gestation, the level of adrenal 3βHSD mRNA was significantly lower in the HPD than in the intact group at 139-141 d of gestation. This implies that there are factors present in the circulation of the HPD sheep fetus which exert an inhibitory effect on the expression of this steroidogenic enzyme within the fetal adrenal, or that fetal HPD may have removed a pituitary factor which is required for the maintenance of adrenal 3βHSD gene expression throughout late-gestation. Finally, intra-fetal cortisol infusion in both HPD fetuses in late-gestation, and intact fetuses prior to the normal pre-partum cortisol surge, had no effect on adrenal steroidogenic enzyme mRNA levels. In this thesis, I have therefore shown that:

- *There is a differential time-course of adrenal steroid hydroxylase gene expression during the final 15 d of gestation, concomitant with the rapid adrenocortical growth and hypercortisolism required for parturition in the sheep.*

- *An intact hypothalamo-pituitary axis is essential for the increase in adrenal growth and steroidogenesis preceding delivery in the fetal sheep.*

- *There is a differential effect of exogenous cortisol infusion in HPD fetal sheep on adrenal growth and steroid synthetic capacity, such that adrenal growth is stimulated while expression of mRNA for the adrenal steroidogenic enzymes remains unchanged.*

- *Intra-fetal infusion of cortisol into fetuses with an intact hypothalamo-pituitary axis, prior to the ontogenic increase in circulating cortisol levels, does not affect adrenal growth or expression of mRNA for the adrenal steroidogenic enzymes.*

The ACTH sequence is present in a range of molecular weight forms within the fetal circulation, including bioactive ACTH(1-39) and the larger molecular weight ACTH precursors (POMC and pro-ACTH) ([217]Jones, 1980; [342]Phillips, Ross *et al.*, 1996). After 135 d of gestation, the relative increase in the levels of ACTH(1-39) in the fetal circulation ([279]McMillen, Merei *et al.*, 1995), may represent changes in the post-translational processing of ACTH in a single corticotrophic cell type, or a change in the predominant type of corticotrophic cell in the fetal pituitary. Antolovich and colleagues demonstrated that cortisol mediates the morphological maturation of the corticotrophs within the fetal pars distalis, however there was no significant change in the ratio of 'adult' : 'fetal' type corticotrophs in the pars distalis of HPD fetuses infused with cortisol ([19]Antolovich, Perry *et al.*, 1989). These findings suggest that an intact HP axis may be essential for cortisol to promote the full morphological maturation of the pars distalis corticotrophs in the fetal sheep. In this thesis, I found no evidence to suggest that cortisol can act at the fetal

pituitary *in vivo*, following surgical disconnection of the pituitary from the hypothalamus, to alter post-translational processing of POMC to ACTH(1-39). Circulating ir-ACTH and ACTH(1-39) concentrations were unaltered following a 5 d infusion of cortisol into HPD fetal sheep (*Chapter 2, Study 3*). In addition, a 7 d infusion of cortisol into intact fetal sheep from 109-116 d of gestation did not alter circulating ir-ACTH concentrations, compared with control fetuses (*Chapter 3*). In this thesis, I have therefore shown that:

- *Infusion of cortisol into fetuses following disconnection of the fetal hypothalamus and pituitary does not affect circulating ir-ACTH or ACTH(1-39) concentrations.*

- *Infusion of cortisol into fetuses with an intact hypothalamo-pituitary axis, prior to the ontogenic increase in circulating cortisol levels, does not affect circulating ir-ACTH concentrations.*

From these studies, it appears that cortisol does not alter the basal circulating levels of ACTH when administered to fetuses with an intact hypothalamo-pituitary axis in mid-gestation, or in fetuses with a disconnected hypothalamo-pituitary axis in late-gestation. Intra-fetal cortisol infusion, however, restored the adrenal weight of HPD fetuses to values comparable to age-matched intact fetuses. Thus, it is possible that cortisol may act directly at the adrenal of the HPD fetus to mediate adrenal growth in late-gestation. Alternatively, cortisol may act at the fetal pituitary to stimulate the release of adrenal growth promoting peptides, derived from POMC, which do not contain the ACTH peptide sequence. Fetal HPD abolishes the normal ontogenic increase in circulating ACTH(1-39) after 136 d of gestation ([342]Phillips, Ross *et al.*, 1996). Thus, it may be that while adrenal growth and steroidogenic function are maintained by the actions of ACTH(1-39) and / or other POMC related peptides until ~130 d of gestation, the late-gestation increase in ACTH(1-39) which stimulates the rapid adrenocortical growth and hypercortisolism required for parturition in the sheep is absent in the HPD fetus. In this thesis, I demonstrated that fetal plasma ir-ACTH concentrations were not altered by the infusion of cortisol in either the HPD fetal sheep from 135-140 d of gestation, or the intact fetuses from 109-116 d of gestation, with respect to their control groups. There was a 3 fold difference, however, between the fetal ir-ACTH levels in the 116 d cortisol infused intact fetuses (30.8 ± 3.1 pg·ml^{-1}) and the 140 d cortisol infused HPD fetuses (93 ± 10 pg·ml^{-1}). Thus, while fetal plasma ir-ACTH concentrations were not altered by the infusion of cortisol in either the HPD fetuses or the intact fetuses, with respect to their control groups, the endocrine environment in which cortisol was administered differed considerably with respect to each other. It is possible that the level of ACTH within the

fetal circulation, and / or the duration of adrenal exposure to this peptide *in vivo*, determines the effectiveness of cortisol in stimulating adrenal growth.

7.1.1   *Model 1 – Direct role of cortisol in adrenal development*

Cortisol may act directly at the adrenal to modulate the activity of locally produced growth factors and / or their binding proteins to mediate adrenal growth in late-gestation

In this model, the direct action of cortisol at the adrenal requires the presence of the fetal pituitary. Cortisol would be unable to stimulate adrenal growth during mid-gestation, in fetuses with an intact HP axis (*as demonstrated in Chapter 3*), due either to the presence of a pituitary derived 'inhibitory' factor, which interferes with the autocrine / paracrine effect of cortisol at the fetal adrenal, or to an inadequate duration of exposure to trophic 'permissive / priming' factors secreted from the fetal pituitary. Cortisol would also be unable to stimulate adrenal growth in hypophysectomised fetuses ([56]Boshier, Holloway and Liggins, 1981), due to the absence of the putative 'permissive / priming' factors derived from the fetal pituitary, which are present in the HPD fetus. Disconnection of the fetal hypothalamus and pituitary, however, may result in lower circulating levels of the putative 'inhibitory' factor, while levels of the 'permissive / priming' factor remain unchanged, thus enabling the direct trophic effect of cortisol at the fetal adrenal. Alternatively, the later cortisol infusion period in the HPD fetuses (135-140 d of gestation) compared with the intact fetuses (109-116 d of gestation) may have afforded the adrenal a longer duration of exposure to the putative 'permissive / priming' factors from the fetal pituitary, such as ACTH(1-39) or other POMC derived peptides, enhancing the local effects of cortisol (*Figure 7.1*).

7.1.1.1   *11βHSD-2 and intra-adrenal exposure to cortisol*

The tissue-specific functions of glucocorticoids during fetal life may be regulated via expression of the intra-cellular microsomal enzyme, 11βHSD-2 ([431]Whorwood, Franklyn *et al.*, 1992). It is possible that 11βHSD-2 within the fetal sheep adrenal limits the activity of locally produced glucocorticoids on adrenocortical cells up until around 125 d of gestation. The pre-partum increase in adrenocortical growth and steroid output in late-gestation is coincident with a decrease in adrenal 11βHSD-2 mRNA expression ([281]McMillen, Warnes *et al.*, 1999). In addition, in Chapter 3, I demonstrated a decrease in the expression of mRNA for 11βHSD-2 within the fetal adrenal following a 7 d intra-fetal infusion of cortisol from 109-116 d of gestation (*Figure 7.1, step 1*).
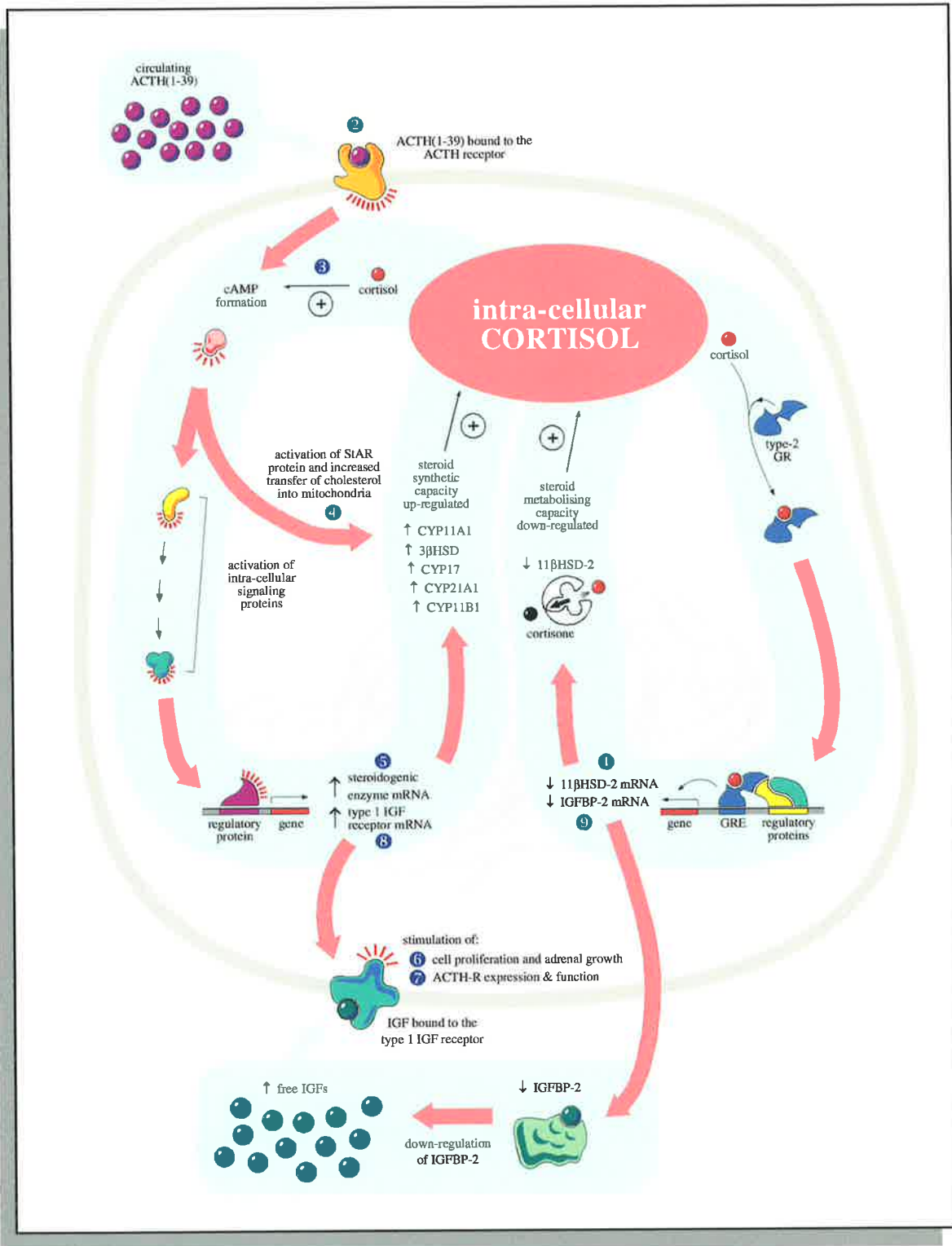
Figure 7.1   Speculation: Cortisol may act directly at the fetal adrenal gland to
promote adrenal development *in utero.*

Descriptions of steps ❶ through ❾ are provided in the text.

In this thesis, I have therefore shown that:

- *Cortisol infusion, prior to the ontogenic increase in circulating cortisol levels, inhibits the expression of mRNA for the steroid metabolising enzyme 11βHSD-2 within the fetal adrenal gland.*

It is intriguing to suggest that inhibition of the expression of mRNA for this steroid metabolising enzyme, by endogenous cortisol, may enhance the intra-adrenal exposure to glucocorticoids in late-gestation. In contrast to the effect of cortisol on adrenal growth in HPD fetuses, I measured no change in adrenal weight when cortisol was infused into fetuses with an intact HP axis from 109-116 d of gestation, despite the fall in 11βHSD-2 mRNA expression. These results confirm that intra-adrenal exposure to cortisol at this stage of gestation is not sufficient to promote adrenal growth.

In vitro studies have shown that dexamethasone-treated ovine adrenocortical cells contain more cholesterol and produce more pregnenolone than control cells ([344]Picard-Hagen, Darbeida and Durand, 1995). Thus, glucocorticoids may increase the pool of intra-mitochondrial cholesterol as well as mitochondrial ability to synthesise pregnenolone. In addition, adrenal cells from 120-138 d ovine fetuses, cultured in the presence of ACTH(1-24) plus metyrapone or aminoglutethimide (inhibitors of steroid synthesis) produce less cAMP in response to ACTH(1-24) stimulation than cells cultured in the presence of ACTH(1-24) alone ([117]Darbeida, Naaman and Durand, 1987). Conversely, these authors demonstrated that cells cultured for 48 h in the presence of dexamethasone or cortisol release more cAMP than control cells when stimulated by ACTH(1-24). Thus, when the fetal adrenal is stimulated by increased circulating levels of ACTH (*Figure 7.1, step 2*), such as *in vitro* experiments, or in the week before birth ([80]Challis and Brooks, 1989), and during chronic intra-uterine stress ([343]Phillips, Simonetta *et al.*, 1996), a fall in adrenal 11βHSD-2 mRNA expression and increased intra-adrenal exposure to glucocorticoids may be important in enhancing the cAMP response to the prevailing high ACTH concentrations (*Figure 7.1, step 3*). The action of cAMP in mediating ACTH induced steroidogenesis is two fold. cAMP directly stimulates phosphorylation of existing StAR protein, and the rapid synthesis of new StAR protein, increasing the flow of cholesterol into mitochondria and thus increasing substrate availability to steroid hydroxylase enzymes ([287]Miller and Strauss, 1999) (*Figure 7.1, step 4*). In addition, chronic stimulation of cAMP synthesis by ACTH induces an increase in transcription of the steroid hydroxylase genes ([408]Tangalakis, Coghlan *et al.*, 1990) (*Figure 7.1, step 5*).

Thus, endogenous cortisol synthesis may inhibit intra-cellular glucocorticoid degradation via 11βHSD-2, and enhance ACTH induced steroidogenesis via increased cAMP synthesis.

7.1.1.2 The fetal adrenal IGF axis

In Chapter 2, I demonstrated that there was no change in adrenal IGF-II mRNA levels throughout the last 15 d of gestation, when the fetal adrenal is growing rapidly. IGF-II mRNA levels were also maintained in the fetal adrenal following surgical disconnection of the fetal hypothalamus and pituitary, which inhibits adrenal growth in late-gestation, and following subsequent intra-fetal cortisol infusion, which restores adrenal growth to levels comparable to age-matched control fetuses. In Chapter 5, fetal circulating IGF-I concentrations were chronically elevated via intra-fetal infusion of *rh*IGF-I. Fetal circulating IGF-I levels in this study were similar to levels achieved in hyperglycaemic fetuses, induced via maternal glucose infusion in late-gestation ([323]Owens, Kind *et al.*, 1996). Chronic exposure to a physiological elevation of circulating IGF-I between 120 d and 130 d of gestation resulted in a 33 % increase in total adrenal weight and a 50 % increase in the ratio of total adrenal : fetal body weight. In this thesis, I have therefore shown that:

- *Adrenal IGF-II mRNA levels are not directly related to either adrenal growth or steroidogenic capacity, remaining unchanged throughout the last 15 d of gestation, and after intra-fetal infusion of cortisol following disconnection of the fetal hypothalamus and pituitary (i.e. during periods of rapid adrenal growth).*

- *Chronic stimulation of the type 1 IGF receptor via intra-fetal infusion of rhIGF-I stimulates fetal adrenal growth in utero.*

It remains unknown, however, whether the increase in adrenal weight is due to cellular hypertrophy or hyperplasia, or whether the increase in adrenal weight represents growth predominantly in the medullary area, and / or within the cortical *zonae*. While previous studies have revealed that IGF-I induces cellular proliferation of fetal adrenocortical ([416]van Dijk, Tansswell and Challis, 1988; [299]Naaman, Chatelain *et al.*, 1989) (*Figure 7.1, step 6*) and medullary chromaffin cells ([174]Hall and Ekanayake, 1991; [160]Frodin and Gammeltoft, 1994) *in vitro*, the lack of expression of the type 1 IGF receptor within the primate fetal adrenal medulla *in vivo* ([108]Coulter, Goldsmith *et al.*, 1996) suggests that IGFs may primarily modulate adrenocortical growth.

It is interesting that while IGFs enhance the steroidogenic responsiveness of adrenocortical cells to ACTH *in vitro* ([86]Chatelain, Penhoat *et al.*, 1988; [333]Penhoat, Chatelain *et al.*, 1988; [299]Naaman, Chatelain *et al.*, 1989) and adrenal growth *in vivo* (*as demonstrated in Chapter 5*), infusion of *rh*IGF-I into the fetal circulation did not alter the expression of mRNA for the adrenal steroid-synthesising enzymes, or the circulating level of cortisol. In this thesis, I have therefore shown that:

- *Chronic stimulation of the type 1 IGF receptor via intra-fetal infusion of rhIGF-I does not affect adrenal expression of mRNA for the steroid synthesising enzymes, or fetal circulating levels of cortisol.*

The 10 d intra-fetal infusion of *rh*IGF-I may have increased the adrenal responsiveness to ACTH via an increase in adrenal ACTH receptor abundance ([333]Penhoat, Chatelain *et al.*, 1988; [334]Penhoat, Jaillard and Saez, 1989) and an enhanced capacity for adenylate cyclase coupling ([299]Naaman, Chatelain *et al.*, 1989) (*Figure 7.1, step 7*). The absence of an observable effect of intra-fetal *rh*IGF-I infusion on adrenal steroidogenesis, however, may be due to the low circulating concentrations of bioactive ACTH present between 120 d and 130 d of gestation. *In vitro*, IGF-I has been shown to enhance cAMP accumulation and steroidogenesis in adult bovine adrenocortical cells in response to ACTH ([333]Penhoat, Chatelain *et al.*, 1988), in part, via an up-regulation of receptors for ACTH and AII ([333]Penhoat, Chatelain *et al.*, 1988; [334]Penhoat, Jaillard and Saez, 1989) (*Figure 7.1, step 7*). IGF-I also enhances cAMP accumulation and steroidogenesis in response to incubation with ACTH in fetal ovine adrenal cells *in vitro* ([299]Naaman, Chatelain *et al.*, 1989) (*Figure 7.1, step 7*). It is possible that the potentiating effects of IGF-I on ACTH induced adrenal steroidogenesis require elevated circulating levels of bioactive ACTH *in vivo*, which are normally only observed during episodes of acute fetal stress or during the final two weeks of gestation, *i.e.* after the period of *rh*IGF-I administered in Chapter 5.

In cultured adult bovine adrenocortical cells, treatment with AII or ACTH for 48 h increases the abundance of receptors for IGF-I, which can be partially reproduced by the activation of either the cAMP or the phosphatidyl inositide pathways ([255]Louveau, Penhoat and Saez, 1989). Thus, it appears that receptors for IGF-I are positively regulated by peptide hormones specific for steroidogenic cells (*Figure 7.1, step 8*). Indeed, *in situ* hybridisation revealed that removal of corticosteroid negative feedback at the pituitary by metyrapone infusion, resulting in elevated fetal circulating levels of ACTH, causes an increase in the relative abundance of mRNA for the type 1 IGF receptor ([108]Coulter, Goldsmith *et al.*, 1996). Thus, the adrenal expression of the type 1 IGF receptor, and

hence the adrenal responsiveness to IGFs, may be modulated by the local intra-adrenal concentration of cortisol, or the fetal circulating level of ACTH (*Figure 7.1, step 8*).

Alternatively, IGFBPs may inhibit the steroid-potentiating effect of IGF-I on the fetal adrenal *in vivo*, while the disruption of tissue architecture and paracrine relationships following cell culture removes this restriction *in vitro*. In addition, it is possible that locally produced IGF-II, which is abundant within the steroid-secreting cells of the fetal ovine adrenal cortex ([178]Han, Lu *et al.*, 1992), may have greater access to the type 1 IGF receptors than circulating IGF-I. Thus, it is possible that circulating IGF-I may predominantly stimulate adrenal growth in all cells expressing the type 1 IGF receptor whereas locally synthesised IGF-II, expressed predominantly within the cortex of the ovine fetal adrenal ([178]Han, Lu *et al.*, 1992), may specifically coordinate adrenocortical growth and steroidogenic responsiveness to ACTH, under the dual influence of both ACTH and cortisol. An examination and comparison of adrenal growth and steroidogenesis following IGF-I or IGF-II infusion between 120 d and 130 d of gestation in the presence and absence of exogenous ACTH, and at a later stage of gestation, such as 130-145 d of gestation, would address these possibilities.

The absence of elevated circulating levels of ACTH during the infusion period did not prevent the trophic activity of IGF-I on fetal adrenal growth. It is possible that exogenous IGF-I enhances the responsiveness of the fetal adrenal to other trophic hormones that are at higher concentrations within the fetal circulation during the infusion period. Indeed, the circulating concentrations of the ACTH precursors, POMC and pro-ACTH are 10-20 fold higher than ACTH(1-39) during this period ([395]Silman, Holland *et al.*, 1979). While these peptides are antagonistic to the steroidogenic actions of ACTH *in vitro* ([385]Schwartz, Kleftogiannis *et al.*, 1995), their effect on adrenal growth *in vivo* is unknown. In addition, the fetal circulating concentrations of *N*-POMC(1-77) are 20-50 fold higher than ACTH(1-39) during late-gestation ([377]Saphier, Glynn *et al.*, 1993), and biologically active peptides cleaved from this precursor are mitogenic for adrenal cells both *in vitro* ([256]Lowry, Estivariz *et al.*, 1985; [145]Estivariz, Carino *et al.*, 1988) and *in vivo* ([148]Estivariz, Iturriza *et al.*, 1982). Thus, IGF-I may enhance the adrenal responsiveness to growth promoting peptides that are at sufficient concentrations within the fetal circulation from 120-130 d of gestation to stimulate adrenal growth.

A further possibility is that elevated circulating IGF-I may act indirectly, via the fetal pituitary, to stimulate the release of trophic peptides from the fetal corticotrophs which in turn stimulate adrenal growth. Measurement of the circulating levels of the ACTH

precursors, ACTH(1-39) and the *N*-POMC derived peptides, during the IGF-I infusion period, would help resolve these issues. Finally, it is possible that IGF-I stimulates adrenal mitosis *in vivo* directly, without involving auxiliary endocrine pathways. Indeed, the mitogenic potential of IGF-I has been demonstrated on adrenocortical cells in culture, in the absence of co-incubation with other factors ([416]van Dijk, Tansswell and Challis, 1988; [299]Naaman, Chatelain *et al.*, 1989) (*Figure 7.1, step 6*).

7.1.1.3   *Cortisol and fetal adrenal IGF binding protein-2*

The distribution, peptide level and actions of IGF-I and IGF-II within the fetal adrenal may be determined by the location and abundance of IGF binding proteins. In Chapter 2, I demonstrated that the ontogenic expression of mRNA for IGFBP-2 decreased by more than 50 % in the fetal adrenal between 130 d and 145 d of gestation. In Chapter 3, I demonstrated a reduction in the adrenal expression of mRNA for IGFBP-2 following a 7 d intra-fetal cortisol infusion, prior to the normal pre-partum cortisol surge (*Figure 7.1, step 9*). In this thesis, I have therefore shown that:

- *There is a late-gestation decrease in the ontogenic expression of mRNA for IGFBP-2 within the ovine fetal adrenal.*

- *Cortisol infusion, prior to the ontogenic increase in circulating cortisol levels, inhibits the expression of mRNA for IGFBP-2 within the fetal adrenal gland.*

One possibility is that the late-gestation increase in circulating cortisol acts to inhibit the expression of IGFBP-2 mRNA in tissues that are sensitive to glucocorticoids at this time (*Figure 7.1, step 9*). In this way, IGF-II peptide may have an enhanced autocrine / paracrine effect on the adrenal gland, mediating the rapid growth that occurs in this gland prior to birth. While the circulating levels of cortisol were sufficient to inhibit the adrenal mRNA expression of IGFBP-2, there may have been an insufficient duration of exposure to the permissive / priming effects of ACTH(1-39), or other trophic POMC derived peptides, by 109 d of gestation to allow the changes in IGFBP-2 to exert an effect on adrenal growth or function. Thus, while some elements of the intra-adrenal growth factor axis were modulated by the mid-gestation cortisol infusion, these changes appear to have been insufficient to stimulate growth within the fetal adrenal gland. It is interesting to speculate that while cortisol and ACTH may regulate the mRNA and peptide expression of IGF-II ([421]Voutilainen and Miller, 1987; [258]Lu, Han *et al.*, 1994) and the type 1 IGF receptor ([108]Coulter, Goldsmith *et al.*, 1996) within the fetal adrenal, endogenous cortisol also regulates the tissue delivery and localisation of IGF-II through modulation of the gene

expression of its major binding protein, IGFBP-2 (*Figure 7.1, step 9*). The mRNA expression of IGFBP-2 was not measured in the adrenal glands of HPD fetuses, in which adrenal growth was restored by intra-fetal cortisol infusion, and therefore remains as an area for further study.

The IGF binding proteins may prevent access of the IGFs to some areas of adrenal tissue, but not others. Alternatively, locally produced IGFBPs may increase access of the exogenous *rh*IGF-I to the type 1 IGF receptor in some areas, by increasing the local IGF concentration. Thus, determining the localisation of IGF receptors and the IGFBPs, and the access of IGFs associated with the IGFBPs to cells within the fetal adrenal may also serve to elucidate the site of action of IGFs within the adrenal gland of the ovine fetus.

7.1.2   Model 2 – Indirect role of cortisol in adrenal development

Cortisol may act indirectly, via the fetal pituitary, to stimulate the release of pituitary derived peptides that do not contain the ACTH sequence, which are trophic for the fetal adrenal.

While cortisol infusion into hypophysectomised fetuses does not stimulate adrenal growth ([56]Boshier, Holloway and Liggins, 1981), I have demonstrated that cortisol infusion into fetuses following disconnection of the fetal hypothalamus and pituitary stimulated adrenal growth without altering the circulating levels of ir-ACTH or ACTH(1-39). A second possibility, therefore, is that the effect of exogenous cortisol on fetal adrenal growth may be mediated through the release of POMC-derived peptides that do not contain the ACTH(1-39) sequence. In this model, while ACTH(1-39) is important in inducing the adrenal cytological maturation leading to the enhanced adrenal steroidogenesis observed during late-gestation ([56]Boshier, Holloway and Liggins, 1981; [80]Challis and Brooks, 1989), cortisol may stimulate growth within the adrenal indirectly by stimulating the release of non ACTH-containing pituitary derived peptides, which are trophic for the fetal adrenal.

7.2   THE ROLE OF *N-POMC* PEPTIDES IN FETAL ADRENAL DEVELOPMENT

Changes in the post-translational processing of POMC in the fetal pituitary in late-gestation may coordinate the regulation of fetal adrenal growth, steroidogenesis and cortisol output.

The *N*-terminal region of POMC contains peptides that are involved in the rapid adrenal regeneration following bilateral adrenal enucleation in adult rats ([145]Estivariz, Carino *et al.*, 1988). In addition, administration of trypsinised *N*-POMC(1-77) to seven week old rats significantly increases adrenocortical mitosis, and *N*-POMC(1-28) and *N*-POMC(2-59)

stimulate adrenal growth *in vivo* and DNA synthesis in adrenocortical cells *in vitro* ([148]Estivariz, Iturriza *et al.*, 1982). γ₃-MSH [*N*-POMC(51-77)] or *N*-POMC(1-77), however, are inactive in this *in vitro* model, suggesting that adrenal growth and mitosis are a consequence of post-secretional cleavage of *N*-POMC(1-77) to release mitogenically active *N*-terminal fragments and γ₃-MSH ([148]Estivariz, Iturriza *et al.*, 1982) (*Figure 7.2, step 1*). In Chapter 4, intra-fetal infusion of the purified bovine glycopeptide *N*-POMC(1-77) from 136-138 d of gestation resulted in a significant increase in adrenal growth. In contrast, infusion of the *N*-POMC(1-77) cleavage product, *N*-POMC(1-49), had no effect on fetal adrenal growth. In this thesis, I have therefore shown that:

- *Intra-fetal infusion of bovine N-POMC(1-77) peptide, but not N-POMC(1-49), induces adrenal growth in the late-gestation ovine fetus.*

It is possible that the glycosylated bovine *N*-POMC(1-77) is directly mitogenic to the adrenal cells, or that this peptide induces the local production of growth factors which mediate adrenal growth in these fetuses. Indeed, tissue levels of IGF-I and IGF-II peptide are increased in adult rat adrenal glands undergoing either adrenal regeneration following adrenal enucleation, or compensatory adrenal growth following unilateral adrenalectomy ([412]Townsend, Dallman and Miller, 1990). Since no increase in circulating IGF levels was detected in these models of adrenal growth, the local rise in IGF peptides likely reflects an increase in the local production of these growth factors; and may also represent a response to elevated circulating *N*-POMC peptides, which have been implicated in the adrenal mitogenic response in these perturbations ([257]Lowry, Silas *et al.*, 1983; [145]Estivariz, Carino *et al.*, 1988; [149]Estivariz, Morano *et al.*, 1988) (*Figure 7.2, step 2*).

The intact glycosylated *N*-POMC(1-77) sequence may be stable within the fetal circulation, while the *N*-POMC(1-49) sequence is labile, preventing the mitogenic effect of this peptide on the adrenal gland. It has also been proposed that, through a neurally mediated mechanism, an adrenal cortical protease is activated that cleaves the *N*-terminal fragment of POMC to generate one peptide [*N*-POMC(1-49)] which serves as a locally acting mitogen, and a second peptide (γ₃-MSH) which potentiates the action of ACTH via enhanced CEH enzyme activity ([328]Pedersen and Brownie, 1980) (*Figure 7.2, step 3*).

It may be that fetal HPD results in lower circulating levels of these *N*-POMC peptides, resulting in smaller adrenals and altered steroidogenic function due to the changes in the ratio of *N*-POMC-related : ACTH-related peptides. It is tempting to speculate that in the previously described Model 2, exogenous cortisol may alter the processing or secretion of *N*-POMC peptides from the pituitary of the HPD fetus, which then mediate the increase
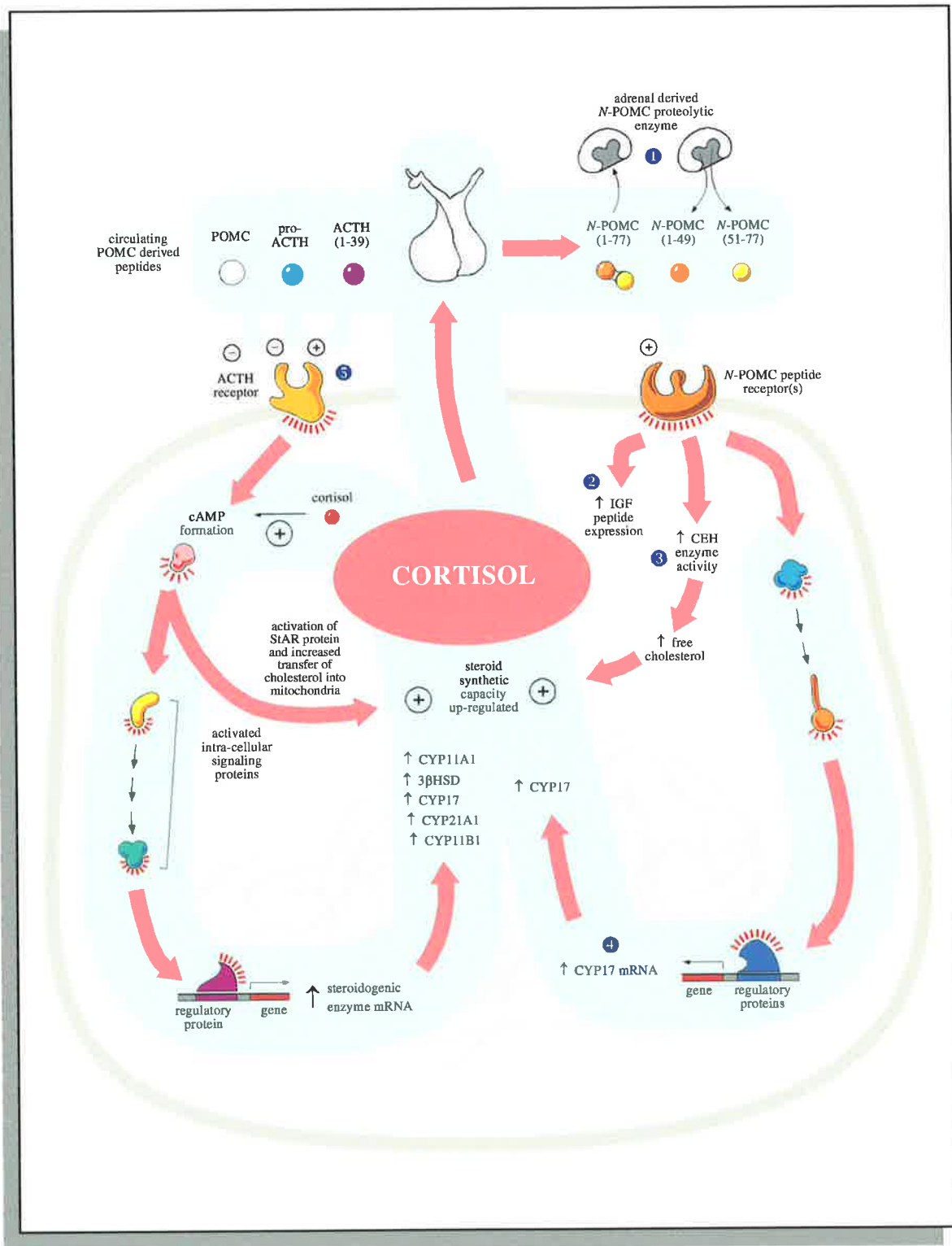
Figure 7.2   Speculation: Indirect   role   of   cortisol   in   adrenocortical   development and actions of POMC derived peptides.

Descriptions of steps ❶ through ❺ are provided in the text.

in adrenal growth observed in HPD fetuses infused with cortisol. In Chapter 2, I reported that exogenous cortisol administration does not stimulate adrenocortical growth in intact fetuses. This may be due to a pituitary response to cortisol that requires functional innervation of the fetal adrenal gland. Exogenous cortisol may stimulate the release of *N*-POMC peptides, whose action is dependent on neurally mediated proteolytic cleavage at the adrenal ([40]Bicknell, Hutchinson *et al.*, 1998). While the adrenal gland of the HPD fetus is structurally and functionally immature during late-gestation, due to the low circulating levels of ACTH(1-39) and cortisol post 130 d of gestation ([342]Phillips, Ross *et al.*, 1996), adrenal innervation may be sufficiently complete to mediate cleavage of *N*-POMC peptides released from the surgically disconnected fetal pituitary in response to intra-fetal cortisol infusion. It is possible that the pituitary of an intact fetal sheep at 109-116 d of gestation responds in the same fashion as the HPD fetus, following cortisol administration, but that innervation of the fetal adrenal at 109-116 d of gestation is insufficiently mature to cleave the *N*-POMC(1-77) precursor into its biologically active constituents. The circulating levels of the *N*-POMC peptides in the HPD and intact fetuses during infusion of cortisol or saline have yet to be determined.

Pro-γ-MSH [*N*-POMC(1-77)] has been proposed as a potential mediator of steroid synthesis within the fetal adrenal gland ([328]Pedersen and Brownie, 1980). These authors demonstrated that treatment of isolated rat adrenocortical cells with pro-γ-MSH significantly potentiates the steroidogenic effect of ACTH(1-24). In addition, prior treatment of the 16 kDa fragment of POMC with trypsin for 30 sec dramatically increases the dose dependent synergism with ACTH(1-24) ([328]Pedersen and Brownie, 1980). The trypsinised 16 kDa fragment of POMC and γ₃-MSH each stimulate cholesterol ester hydrolase activity in the adrenal gland of hypophysectomised female rats ([328]Pedersen and Brownie, 1980) (*Figure 7.2, step 3*). I have demonstrated, in Chapter 4, that intra-fetal infusion of glycosylated bovine *N*-POMC(1-77), but not *N*-POMC(1-49), specifically stimulated CYP17 mRNA expression in the fetal adrenal (*Figure 7.2, step 4*). There was also a 3 fold increase in the ratio of adrenal CYP17 : 3βHSD mRNA expression in the *N*-POMC(1-77) infused fetal sheep. This ratio has been highlighted to be of particular significance in determining the steroid output of a range of steroidogenic tissues, including the developing adrenal ([105]Conley and Bird, 1997). The adrenal mRNA expression of CYP11A1, 3βHSD and CYP21A1 remained unchanged following the intra-fetal infusion of either *N*-POMC(1-77) or *N*-POMC(1-49), compared with saline infusion. The target site of action of *N*-POMC(1-77) in the fetal sheep adrenal may differ, however, from that reported in previous *in vitro* and *in vivo* studies in the rat adrenal, given that the rat adrenal

lacks CYP17 enzyme activity and that the main corticosteroid secreted by the rat adrenal is therefore corticosterone rather than cortisol. The results of the present study suggest that the action of *N*-POMC derived peptides may be dependent on the relative role of CYP17 expression in adrenal steroidogenesis. Finally, despite the significant increase in adrenal CYP17 mRNA expression measured in the *N*-POMC(1-77) infused group, neither of the *N*-POMC peptide infusions altered the fetal circulating levels of cortisol or ir-ACTH, compared with saline infused fetuses. In this thesis, I have therefore shown that:

- *Intra-fetal infusion of bovine N-POMC(1-77) peptide, but not N-POMC(1-49), induces expression of mRNA for the adrenal steroidogenic enzyme CYP17.*

- *Intra-fetal infusion of either bovine N-POMC(1-77) peptide or N-POMC(1-49) did not alter the fetal circulating levels of cortisol or ir-ACTH.*

It is possible that the amount of *N*-POMC(1-77) infused in the present study was sufficient to cause some changes in the steroidogenic elements within the adrenocortical cells without altering the level of glucocorticoids secreted from the adrenal gland. While γ_3-MSH markedly potentiates the steroidogenic effect of ACTH in adult rat adrenal cells *in vitro*, when administered alone this peptide is only weakly steroidogenic ([332]Pedersen, Brownie and Ling, 1980b; [150]Farese, Ling *et al.*, 1983). Similarly, Durand and co-workers demonstrated that it is necessary to culture ovine fetal adrenal cells for at least 4 d in the presence of γ_3-MSH before corticosteroid production is stimulated in the absence of ACTH ([137]Durand, Cathiard *et al.*, 1984b), whereas when co-incubated with ACTH, γ_3-MSH potentiates the steroidogenic response to acute stimulation by ACTH after only 3 d in culture. The fetal adrenal, therefore, may require elevated ACTH concentrations before γ_3-MSH stimulates an increase in steroidogenesis *in vivo*. It is also possible that following surgical disconnection of the fetal pituitary from the hypothalamus, cortisol may stimulate the release of adrenal growth promoting peptides derived from the *N*-terminal region of POMC which are, in the absence of elevated circulating levels of ACTH(1-39), unable to stimulate adrenal steroidogenesis.

Saphier and colleagues recently reported that the molar ratio of *N*-POMC(1-77) : ACTH(1-39) is 20-50 in the fetal sheep in late-gestation, whereas this ratio is 2 in the adult ewe ([377]Saphier, Glynn *et al.*, 1993). The finding by these investigators of a decrease in the plasma levels of *N*-POMC(1-77) concurrent with an increase in *N*-POMC(50-74) after 138 d of gestation, may indicate the proteolytic cleavage of the former peptide to liberate the smaller, biologically active forms of *N*-POMC ([328]Pedersen and Brownie, 1980). Thus, a role for the *N*-terminal region of POMC in adrenal growth during fetal life is supported

by the presence of this peptide in the fetal circulation during a period of rapid adrenocortical growth. It may be that the late-gestation increase in adrenal CYP17 mRNA expression, demonstrated in Chapter 2, is a result of the increase in circulating γ$_3$-MSH concentrations which occurs after 138 d of gestation ([377]Saphier, Glynn *et al.*, 1993). The decrease in circulating levels of the full-length *N*-POMC(1-77) peptide, concomitant with an increase in the circulating levels of the cleavage product γ$_3$-MSH, indicates that proteolytic cleavage may be active at this time, to liberate the biologically active peptides within *N*-POMC(1-77).

7.3   *IMPACT OF PLACENTAL RESTRICTION ON ADRENOCORTICAL DEVELOPMENT*

In Chapter 6, I demonstrated that fetal weight was lower and the ratio of fetal brain : liver weight was higher in placentally restricted fetuses, in a large cohort of growth restricted and normally grown fetal sheep after 137 d of gestation. When the control and placental restriction groups were combined, there was a consistent relationship between the relative brain and liver weights with decreasing fetal body weight. These progressive changes in organ growth were particularly marked when fetal body weight fell below ~3.5 kg. In Chapter 6, I also demonstrated that the ratio of total adrenal : fetal body weight increases as fetal body weight decreases in the large cohort of growth restricted and normally grown fetal sheep. In the cohort, adrenal weight as a proportion of body weight increased progressively as fetal body weight decreased. This increase was particularly marked when fetal body weight fell below ~3.5 kg, irrespective of whether fetal growth was restricted experimentally by restriction of placental growth, or whether it occurred spontaneously, *i.e.* in those control singleton and twin fetuses which were <3.5 kg. These results confirm earlier reports of increased adrenal growth in placentally restricted fetal sheep ([364]Robinson, Kingston *et al.*, 1979; [343]Phillips, Simonetta *et al.*, 1996) and are consistent with the brain and adrenal 'sparing' phenotype observed in asymmetric human fetal growth restriction ([64]Brooke, Wood and Butters, 1984). This suggests that placental restriction is an appropriate model in which to study the impact of spontaneous growth restriction on the fetal HPA axis. In this thesis, I have therefore shown that:

- *Surgical restriction of placental growth and function induces fetal growth restriction in late-gestation which is characterised by the brain and adrenal 'sparing' phenotype observed in asymmetric human fetal growth restriction.*

- *There is a quantitative relationship between adrenal growth and the degree of fetal growth restriction, which exists in both placentally restricted fetal sheep and in 'spontaneously' growth restricted fetuses.*

While adrenal growth was promoted as a consequence of placental restriction, the expression of mRNA for adrenal IGF-II was suppressed in this group, in the absence of any changes in adrenal IGFBP-2 mRNA expression. In this thesis, I have therefore shown that:

- *Placental restriction results in a decrease in the adrenal expression of mRNA for IGF-II, while adrenal mRNA expression for IGFBP-2 remains unchanged.*

Interestingly, Lu and co-workers have found that intra-fetal infusion of ACTH (n=4 fetuses) or cortisol (n=3 fetuses) for 84 h, beginning at 120-125 d of gestation, resulted in a decrease in fetal adrenal IGF-II mRNA levels ([258]Lu, Han *et al.*, 1994). It has also been reported that cortisol infusion results in a decrease in IGF-II mRNA expression in other fetal tissues including the liver ([258]Lu, Han *et al.*, 1994). It may be that the increase in adrenal cortisol output, which occurs in the hypoxaemic growth restricted sheep fetus after 125 d of gestation ([343]Phillips, Simonetta *et al.*, 1996), acts to decrease the expression of adrenal IGF-II mRNA. Given that there is no change in the expression of the IGF-II binding protein, IGFBP-2, this could result in a decrease in IGF-II protein within the adrenal.

In addition to the pre-partum increase in circulating levels of cortisol within the ovine fetus, the fetal HPA axis can generate a marked cortisol response to either acute ([47]Boddy, Jones *et al.*, 1974; [6]Akagi and Challis, 1990; [327]Ozolins, Young and McMillen, 1992) or chronic ([364]Robinson, Kingston *et al.*, 1979; [45]Bocking, Gagnon *et al.*, 1988; [295]Murotsuki, Gagnon *et al.*, 1996; [343]Phillips, Simonetta *et al.*, 1996) intra-uterine stress. In this thesis, I have demonstrated that placental restriction, via maternal carunclectomy, results in a premature increase in fetal circulating cortisol, and an increase in the adrenal expression of mRNA for the steroidogenic enzyme CYP11A1 in the ovine fetus in late-gestation. In this thesis, I have therefore shown that:

- *Restriction of placental growth results in an increased adrenal expression of the steroid synthesising enzyme CYP11A1 and a premature increase in fetal circulating cortisol in late-gestation.*

Intra-fetal ACTH infusion specifically increases CYP11A1 and CYP17 mRNA levels ([408]Tangalakis, Coghlan *et al.*, 1990), although it appears from *in vitro* studies that the *CYP17* gene is more sensitive to regulation by ACTH than the other steroidogenic enzymes ([43]Bird, Pasquarette *et al.*, 1996). In contrast, placental restriction resulted in a relative increase in adrenal CYP11A1 mRNA in the absence of any change in CYP17,

3βHSD and CYP21A1 mRNA levels. These data suggest that factors other than ACTH may stimulate an increase in adrenal steroidogenesis in the chronically hypoxaemic sheep fetus. One potential candidate is the placental hormone, PGE_2. Fetal PGE_2 concentrations are increased during periods of hypoxaemia induced by reduction of uterine blood flow and placental embolisation ([192]Hooper, Coulter *et al.*, 1990; [294]Murotsuki, Challis *et al.*, 1995). It has been shown *in vitro*, however that the actions of PGE_2 on adrenocortical cells are similar to those of ACTH, *i.e.* PGE_2 acts to stimulate cAMP production and CYP17 and 3βHSD mRNA expression ([350]Rainey, Naville *et al.*, 1991). Thus, other factors may be responsible for the specific increase in the adrenal expression of mRNA for CYP11A1, observed in placentally restricted fetal sheep

It is interesting that while an increase in fetal cortisol is a consistent response to prolonged or repeated fetal hypoxaemia in late-gestation, this increase is not always associated with an increase in fetal circulating ACTH ([192]Hooper, Coulter *et al.*, 1990; [59]Braems, Matthews and Challis, 1996; [343]Phillips, Simonetta *et al.*, 1996). Indeed, cordocentesis studies in human fetuses that were small for gestational age have found that plasma cortisol concentrations are higher and plasma ACTH concentrations are lower than in normally grown fetuses at 18-38 weeks of gestation ([138]Economides, Nicolaides *et al.*, 1988). In Chapter 6, placental restriction of the ovine fetus was also associated with elevated fetal circulating cortisol, while fetal ir-ACTH levels were unchanged in fetuses of carunclectomised ewes. In Chapter 6, I demonstrated that placental restriction resulted in a decrease in the expression of mRNA for the ACTH receptor. In addition, the orphan nuclear receptor, steroidogenic factor-1, which is essential for the basal expression of the ACTH receptor in human and mouse adrenals ([104]Commas, Pullinger *et al.*, 1997; [269]Marchal, Naville *et al.*, 1998), was unaffected by placental restriction and chronic fetal growth restriction. In this thesis, I have therefore shown that:

- *While restriction of placental growth via maternal carunclectomy results in a premature increase in fetal circulating cortisol in late-gestation, the concentration of ir-ACTH within the fetal circulation and the adrenal expression of mRNA for the ACTH receptor and SF-1 remain unchanged.*

Factors which stimulate ACTH receptor expression in ovine adrenocortical cells include ACTH and glucocorticoids, and it has also been shown that IGF-I and -II increase the expression of the ACTH receptor in human adrenocortical cells ([131]l'Allemand, Penhoat *et al.*, 1996; [345]Picard-Hagen, Penhoat *et al.*, 1997). Thus, the decrease in adrenal ACTH receptor mRNA expression in the growth restricted fetal sheep may be a

consequence of the fall in adrenal IGF-II mRNA expression. Importantly, our results suggest that the increase in circulating cortisol that occurs in the absence of an increase in ACTH, is not due to an increase in adrenal sensitivity to ACTH via enhanced ACTH receptor mRNA expression in the growth restricted fetus.

In Chapter 6, I demonstrated that adrenal growth was increased in the placentally restricted fetuses, despite the decrease in adrenal IGF-II mRNA levels and the lack of an increase in the immunoreactive or bioactive concentrations of ACTH in the fetal circulation ([343]Phillips, Simonetta *et al.*, 1996). This suggests that both adrenal growth and steroidogenesis may be maintained by other pituitary derived or non-pituitary derived hormones and intra-adrenal growth factors during chronic hypoxaemia. As previously described in Model 2, it has been suggested that the *N*-terminal POMC peptides, pro-γ-MSH and γ3-MSH may play a role in mediating adrenal growth and steroidogenic function in the late-gestation sheep fetus ([377]Saphier, Glynn *et al.*, 1993), while other hormones, such as AII and PGE2 are also potential candidates for the stimulation of adrenal growth and function in spontaneously and experimentally growth restricted fetal sheep ([294]Murotsuki, Challis *et al.*, 1995).

7.4   *INTERACTIONS BETWEEN HYPOPHYSIAL AND LOCAL MEDIATORS OF ADRENO-CORTICAL GROWTH AND FUNCTION BEFORE BIRTH*

The functional changes in the pituitary-adrenal axis in late-gestation may be, in part, dependent on the interactions between ACTH and other POMC derived peptides, and the pre-partum increase in cortisol, both within the fetal adrenal and the fetal pituitary.

ACTH is present in the fetal sheep circulation in a variety of molecular weight forms throughout gestation. The ratio of the HMW forms of ACTH (POMC and pro-ACTH) to ACTH(1-39) is higher in the fetal than the adult pituitary ([395]Silman, Holland *et al.*, 1979), and plasma ACTH(1-39) levels increase during the last two weeks of gestation ([224]Jones and Roebuck, 1980; [70]Carr, Jacobs *et al.*, 1995; [342]Phillips, Ross *et al.*, 1996). Castro and colleagues have demonstrated that the ratio of bioactive : ir-ACTH from near-term fetal sheep is higher than that found in plasma from immature fetal lambs ([77]Castro, Valego *et al.*, 1992), and it has been demonstrated that the HMW ACTH-containing peptides can inhibit the cortisol response of fetal ovine adrenocortical cells to ACTH(1-24) ([368]Roebuck, Jones *et al.*, 1980; [385]Schwartz, Kleftogiannis *et al.*, 1995) (*Figure 7.2, step 5*). Thus, the change in the ratio of HMW ACTH-containing peptides : ACTH(1-39) throughout gestation has the potential to: (*1*) activate StAR protein and stimulate mRNA transcription of the adrenal steroidogenic enzymes, particularly CYP11A1 and CYP17, (*2*) synergise

with the steroid potentiating effects of γ_3-MSH on increased CEH enzyme activity, thereby increasing intra-cellular free cholesterol, (*3*) potentiate the cortisol induced inhibition of adrenal 11βHSD-2 mRNA expression, thereby increasing intra-adrenal exposure to cortisol, (*4*) potentiate the cortisol induced inhibition of adrenal IGFBP-2 mRNA expression, and thus (*5*) indirectly enhance adrenal exposure to both circulating and intra-adrenal IGFs.

The autocrine / paracrine effects of cortisol may occur only after prolonged adrenal exposure to ACTH(1-39) and *N*-POMC derived peptides. Thus, the high circulating levels of ACTH(1-39) and *N*-POMC(1-77) in the fetal sheep during late-gestation may serve to stimulate cytological maturation, expression of the type 1 IGF receptor and the synthesis of glucocorticoids. These steroids, in turn, mediate the local milieu of growth factors, via alterations in the gene expression of the IGF cognate binding proteins, to bring about the rapid hyperplasia characteristic of adrenocortical growth prior to birth. The IGF axis may, therefore, be modulated within the adrenal gland, by the combined actions of ACTH containing peptides, fragments of the *N*-terminal region of POMC and cortisol. The data presented in this thesis provide evidence that an intact hypothalamo-pituitary axis and cortisol each play important separate roles in the stimulation of adrenal growth and steroidogenesis that occurs before delivery in the sheep.

In summary, exposure to cortisol at specific times in fetal and neo-natal life can irreversibly trigger the programmed development of a range of fetal tissues including the brain ([414]Uno, Tarara *et al.*, 1989; [378]Sapolsky, Uno *et al.*, 1990), lung ([244]Liggins, 1976), liver ([154]Fowden, Coulson and Silver, 1990), and cardiovascular system ([34]Benediktsson, Lindsay *et al.*, 1993; [139]Edwards, Benediktsson *et al.*, 1993). Thus, it is critical that during fetal life, adrenal growth and steroidogenesis are tightly regulated to ensure the appropriate exposure of developing tissues to cortisol. This thesis has examined the role of a number of key fetal factors that modulate the growth and development of the fetal sheep adrenal during gestation. Interactions among pituitary-derived peptides, intra-adrenal exposure to glucocorticoids and the local adrenal and endocrine IGF axes in the growth and functional activation of the ovine fetal adrenal gland before birth have been examined. The involvement of these systems in the fetal response to chronic stress and intra-uterine growth restriction has also been considered.

REFERENCES

1. **Adams MB, Phillips ID, Simonetta G, McMillen IC.** Differential effects of increasing gestational age and placental restriction on tyrosine hydroxylase, Phenylethanolamine N-Methyltransferase and Proenkephalin A mRNA levels in the fetal sheep adrenal. *Journal of Neurochemistry.* 71: 394-401, 1998.

2. **Adashi EY, Resnick CE, Hernandez ER, Svoboda ME, VanWyk JJ.** Characterization and regulation of a specific cell membrane receptor for somatomedin-C/insulin-like growth factor-I in cultured rat granulosa cells. *Endocrinology.* 122: 194-201, 1988.

3. **Adashi EY, Resnick CE, Svoboda ME.** Follicle-stimulating hormone enhances somatomedin C binding to cultured rat granulosa cells. *Journal of Biological Chemistry.* 261: 3923-3926, 1986.

4. **Adashi EY, Resnick CE, Svoboda ME, VanWyk JJ.** A novel role for somatomedin C in the cytodifferentiation of the ovarian granulosa cell. *Endocrinology.* 115: 1227-1231, 1984.

5. **Adashi EY, Resnick CE, Svoboda ME, VanWyk JJ.** Somatomedin-C enhances induction of luteinizing hormone receptors by follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology.* 116(6): 2369-2375, 1985.

6. **Akagi K, Challis JRG.** Threshold of hormonal and biophysical responses to acute hypoxemia in fetal sheep at different gestational ages. *Canadian Journal of Physiology and Pharmacology.* 68(5): 549-555, 1990.

7. **Akana SF, Dallman MF, Bradbury MJ, Scribner KA, Strack AM, Walker CD.** Feedback and facilitation in the adrenocortical system: unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress. *Endocrinology.* 131(1): 57-68, 1992.

8. **Akana SF, Scribner KA, Bradbury MJ, Strack AM, Walker CD, Dallman MF.** Feedback sensitivity of the rat hypothalamo-pituitary-adrenal axis and its capacity to adjust to exogenous corticosterone. *Endocrinology.* 131(2): 585-594, 1992.

9. **Al-Dujaili EAS, Hope AS, Estivariz FE, Lowry PJ, Edwards CRW.** Circulating human pituitary pro-gamma-melanotropin enhances the adrenal response to ACTH. *Nature.* 291: 156-159, 1981.

10. **Al-Dujaili EAS, Williams BC, Edwards CRW, Salacinski P, Lowry PJ.** Human γ-melanotropin precursor potentiates corticotropin-induced adrenal steroidogenesis by stimulating RNA synthesis. *Biochemical Journal.* 204(1): 301-305, 1982.

11. **Alexander G.** Studies on the placenta of the sheep (*Ovis aries L.*): Effect of surgical reduction in the number of caruncles. *Journal of Reproduction and Fertility.* 7: 307-322, 1964.

12. **Alexander DP, Britton VHT, Nixon J, Parker RA.** Steroid secretion by the adrenal gland of fetal and neonatal sheep. *Journal of Endocrinology.* 40: 1-13, 1968.

13. **l'Allemand D, Penhoat A, Lebrethon MC, Ardevol R, Baehr V, Oelkers W, Saez JM.** Insulin-like growth factors enhance steroidogenic enzyme and corticotropin receptor messenger ribonucleic acid levels and corticotropin steroidogenic responsiveness in cultured human adrenocortical cells. *Journal of Clinical Endocrinology and Metabolism.* 81: 3892-3897, 1996.

14. **Allen MC.** Developmental outcome and follow-up of the small for gestational age infant. *Seminars in Perinatology.* 8(2): 123-156, 1984.

15. **Anderson ABM, Pierrepoint CG, Griffiths K, Turnbull AC.** Steroid metabolism in the adrenals of fetal sheep in relation to natural and corticotrophin-induced parturition. *Journal of Reproduction and Fertility (Supplemental).* 16: 25-37, 1972.

16. **Antolovich GC, Clarke IJ, McMillen IC, Perry RA, Robinson PM, Silver M, Young IR.** Hypothalamo-pituitary disconnection in the fetal sheep. *Neuroendocrinology.* 51: 1-9, 1990.

17. **Antolovich GC, McMillen IC, Robinson PM, Silver M, Young IR, Perry RA.** The effect of hypothalamo-pituitary disconnection on the functional and morphologic development of the pituitary-adrenal axis in the fetal sheep, in the last third of gestation. *Neuroendocrinology.* 54: 254-261, 1991.

18. **Antolovich GC, McMillen IC, Robinson PM, Silver M, Young IR, Perry RA.** Effect of cortisol infusion on the pituitary-adrenal axis of the hypothalamo-pituitary-disconnected fetal sheep. *Neuroendocrinology.* 56: 312-319, 1992.

19. **Antolovich GC, Perry RA, Trahair JF, Silver M, Robinson PM.** The development of the corticotrophs in the fetal sheep pars distalis: the effect of adrenalectomy or cortisol infusion. *Endocrinology.* 124: 1333-1339, 1989.

20. **Arafah BM.** Insulin-like growth factor-I receptors in the rat adrenals. *Journal of Laboratory and Clinical Medicine.* 118: 531-537, 1991.

21. **Ariyoshi N, Kim YC, Artemenko I, Bhattacharyya KK, Jefcoate CR.** Characterization of the rat Star gene that encodes the predominant 3.5-kilobase pair mRNA. ACTH stimulation of adrenal steroids in vivo precedes elevation of Star mRNA and protein. *Journal of Biological Chemistry.* 273(13): 7610-7619, 1998.

22. **Baker J, Liu JP, Robertson EJ, Efstratiadis A.** Role of insulin-like growth factors in embryonic and postnatal growth. *Cell.* 75: 73-82, 1993.

23. **Bark TH, McNurlan MA, Lang CH, Garlick PJ.** Increased protein synthesis after acute IGF-I or insulin infusion is localized to muscle in mice. *American Journal of Physiology.* 275(1): E118-E123, 1998.

24. **Barker DJP.** Mothers, babies and disease in later life. Barker DJP (editor). BMJ Publishing Group, London, pp. 37-93, 1994.

25. **Barker DJP, Bull AR, Osmond C, Simmonds SJ.** Fetal and placental size and risk of hypertension in adult life. *British Medical Journal.* 301: 259-262, 1990.

26. **Barker DJP, Osmond C, Winter PD, Margretts B**. Weight in infancy and death from ischaemic heart disease. *Lancet*. 2: 577-580, 1989.

27. **Barlow DP, Stoger R, Herrmann BG, Saito K, Schweifer N**. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature*. 349: 84-87, 1991.

28. **Barnes RJ, Comline RS, Silver M**. The effects of bilateral adrenalectomy or hypophysectomy of the foetal lamb in utero. *Journal of Physiology*. 264(2): 429-477, 1977.

29. **Baserga R**. IGF-1 receptor as the restriction point of the cell cycle. *Annals of the New York Academy of Sciences*. 663: 154-157, 1992.

30. **Baserga R, Porcu P, Rubini M, Sell C**. Cell cycle control by the IGF-1 receptor and its ligands. *Advances in Experimental Medicine and Biology*. 343: 105-112, 1993.

31. **Bassett JM, Thorburn GD**. Foetal plasma corticosteroids and the initiation of parturition in sheep. *Journal of Endocrinology*. 44: 285-286, 1969.

32. **Begeot M, Langlois D, Saez JM**. Insulin-like growth factor-I and insulin increase the stimulatory guanine nucleotide binding protein (Gs) in cultured bovine adrenal cells. *Molecular and Cellular Endocrinology*. 66: 53-57, 1989.

33. **Bell ME, Myers TR, Myers DA**. Expression of proopiomelanocortin and prohormone convertase-1 and -2 in the late gestation fetal sheep pituitary. *Endocrinology*. 139(12): 5135-5143, 1998.

34. **Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CRW**. Glucocorticoid exposure in utero: a new model for adult hypertension. *Lancet*. 341: 339-341, 1993.

35. **Bennett HPJ**. Isolation of pituitary peptides by reversed-phase high performance liquid chromatography. Expansion of the resolving power of reversed-phase columns by manipulating pH and the nature of the ion-pairing reagent. *Journal of Chromatography*. 266: 501-510, 1983.

36. **Bennett HPJ**. Biosynthetic fate of the amino-terminal fragment of pro-opiomelanocortin within the intermediate lobe of the mouse pituitary. *Peptides*. 7(4): 615-622, 1986.

37. **Bernier M, Chatelain P, Mather JP, Saez JM**. Regulation of gonadotropin receptors, gonadotropin responsiveness, and cell multiplication by somatomedin-C and insulin in cultured pig Leydig cells. *Journal of Cellular Physiology*. 129(2): 257-263, 1986.

38. **Bertagna X, Girard F, Seurin D, Luton JP, Bricaire H, Mains RE, Eipper BA**. Evidence for a peptide similar to 16K fragment in man. Its relationship to ACTH. *Journal of Clinical Endocrinology and Metabolism*. 51(1): 182-184, 1980.

39. **Bertagna X, Seurin D, Pique L, Luton J, Bricaire H, Girard F**. Peptides related to the NH_2-terminal end of pro-opiocortin in man. *Journal of Clinical Endocrinology and Metabolism*. 56(3): 489-495, 1983.

40. **Bicknell AB, Hutchinson EG, Lomthaisong K, Gladwell RT, Lowry PJ**. Three dimensional structure of an adrenal protease predicts substrate specificity and retention at site of secretion in compensatory growth. (Abstract #OC31). Proceedings of the 189[th] Annual Conference of the Society for Endocrinology, UK. November 23[rd]-24[th], 1998.

41. **Birch NP, Estivariz FE, Bennett HPJ, Loh YP**. Differential glycosylation of N-POMC1-77 regulates the production of γ_3-MSH by purified pro-opiomelanocortin converting enzyme. A possible mechanism for tissue-specific processing. *FEBS Letters*. 290(1-2): 191-194, 1991.

42. **Bird IM, Magness RR, Mason JI, Rainey WE**. Angiotensin-II acts via the type 1 receptor to inhibit 17α hydroxylase cytochrome P450 expression in ovine adrenocortical cells. *Endocrinology*. 130: 3112-3121, 1992.

43. **Bird IM, Pasquarette MM, Rainey WE, Mason IR**. Differential control of 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. *Journal of Clinical Endocrinology and Metabolism*. 81: 2171-2178, 1996.

44. **Bird IM, Zheng J, Corbin CJ, Magness RR, Conley AJ**. Immunohistochemical analysis of AT1 receptor versus P450c17 and 3β HSD expression in ovine adrenals. *Endocrine Research*. 22(4): 349-353, 1996.

45. **Bocking AD, Gagnon R, White SE, Homan J, Milne KM, Richardson B**. Circulatory responses to prolonged hypoxemia in fetal sheep. *American Journal of Obstetrics and Gynecology*. 159: 1418-1424, 1988.

46. **Bocking AD, McMillen IC, Harding R, Thorburn GD**. Effect of reduced uterine blood flow on fetal and maternal cortisol. *Journal of Developmental Physiology*. 8: 237-245, 1986.

47. **Boddy K, Jones CT, Mantell C, Ratcliffe JG, Robinson JS**. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology*. 94(2): 588-591, 1974.

48. **Boggaram V, John ME, Simpson ER, Waterman MR**. Effect of ACTH on the stability of mRNAs encoding bovine adrenocortical P-450_{scc}, $\text{P-450}_{11\beta}$, $\text{P-450}_{17\alpha}$, P-450_{C21}, and adrenodoxin. *Biochemical and Biophysical Research Communications*. 160: 1227-1232, 1989.

49. **Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW**. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene*. 2(2): 95-113, 1977.

50. **Bonds DR, Gabbe SG, Kuman SW, Taylor T**. Fetal weight/placental weight ratio and perinatal outcome. *American Journal of Obstetrics and Gynecology*. 149: 195-200, 1984.

51. **Bondy CA, Werner H, Roberts CT Jr, LeRoith D**. Cellular pattern of insulin-like growth factor-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. *Molecular Endocrinology*. 4: 1386-1388, 1990.

52. **Boshier DP, Gavin CB, Holloway H.** Morphometric analyses of adrenal gland growth in fetal and neonatal sheep II. The adrenal medulla, with some observations on its ultrastructure. *Journal of Anatomy.* 167: 15-30, 1989.

53. **Boshier DP, Holloway H.** Morphometric analyses of adrenal gland growth in fetal and neonatal sheep I. The adrenal cortex. *Journal of Anatomy.* 167: 1-14, 1989.

54. **Boshier DP, Holloway H.** Morphometric analyses of adrenal gland growth in fetal and neonatal sheep. III. Volumes of the major organelles within zona fasciculata steroidogenic cells. *Journal of Anatomy.* 178: 175-187, 1991.

55. **Boshier DP, Holloway H, Liggins GC.** Growth and cytodifferentiation of the lamb adrenal cortex prior to parturition. *Journal of Anatomy.* 130: 97-111, 1980.

56. **Boshier DP, Holloway H, Liggins GC.** Effects of cortisol and ACTH on adrenocortical growth and cytodifferentiation in the hypophysectomized fetal sheep. *Journal of Developmental Physiology.* 3: 355-373, 1981.

57. **Boshier DP, Holloway H, Liggins GC, Marshall RJ.** Morphometric analyses of the effects of thyrotrophin releasing hormone and cortisol on the lungs of fetal sheep. *Journal of Developmental Physiology.* 12(1): 49-54, 1989.

58. **Braems GA, Han VKM, Challis JRG.** Gestational age-dependent changes in the levels of mRNAs encoding cortisol biosynthetic enzymes and IGF-II in the adrenal gland of fetal sheep during prolonged hypoxemia. *Journal of Endocrinology.* 159: 257-264, 1998.

59. **Braems GA, Matthews SG, Challis JRG.** Differential regulation of proopiomelanocortin messenger ribonucleic acid in the pars distalis and pars intermedia of the pituitary gland after prolonged hypoxemia in fetal sheep. *Endocrinology.* 137: 2731-2738, 1996.

60. **Bransome ED.** Regulation of adrenal growth. Differences in the effects of ACTH in normal and dexamethasone-suppressed guinea pigs. *Endocrinology.* 83: 956-964, 1968.

61. **Brice AL, Cheetham JE, Bolton VN, Hill NC, Schofield PN.** Temporal changes in the expression of the insulin-like growth factor II gene associated with tissue maturation in the human fetus. *Development.* 106: 543-554, 1989.

62. **Brieu, V, Durand P.** Changes in the ratio of bioactive and immunoreactive adrenocorticotropin-like activity released by pituitary cells from ovine fetuses and lambs. *Endocrinology.* 120: 936-940, 1987.

63. **Brieu, V, Durand P.** ACTH released by pituitary cells from ovine fetuses and lambs: polymorphism and biological activity. *Neuroendocrinology.* 49: 300-308, 1989.

64. **Brooke OG, Wood C, Butters F.** The body proportions for small-for-dates infants. *Early Human Development.* 10(1-2): 85-94, 1984.

65. **Brown EH, Coghlan JP, Hardy KJ, Wintour EM.** Aldosterone, corticosterone, cortisol, 11-deoxycortisol and 11-deoxy-corticosterone concentrations in the blood of chronically cannulated ovine fetuses. Effect of ACTH. *Acta Endocrinologica.* 8: 364-374, 1978.

66. **Browne CA, Bennett HPJ, Solomon S.** Isolation and characterization of corticotropin- and melanotropin-related peptides from the neurointermediate lobe of the rat pituitary by reversed-phase liquid chromatography. *Biochemistry.* 20: 4538-4546, 1981.

67. **Butler TG, Schwartz J, McMillen IC.** Functional heterogeneity of corticotrophs in the anterior pituitary of the sheep fetus. *Journal of Physiology.* 516(3): 907-913, 1999.

68. **Campbell LE, Yu M, Yang K.** Ovine 11β-hydroxysteroid dehydrogenase type 2 gene predicts a protein distinct from that deduced by the cloned kidney cDNA at the C-terminus. *Molecular and Cellular Endocrinology.* 119: 113-118, 1996.

69. **Carr BR, Simpson ER.** Lipoprotein utilization and cholesterol synthesis by the human fetal adrenal gland. *Endocrine Reviews.* 2(3): 306-326, 1981.

70. **Carr GA, Jacobs RA, Young IR, Schwartz J, White A, Crosby S, Thorburn GD.** Development of ACTH1-39 and precursor peptide secretory responses in the fetal sheep during the last third of gestation. *Endocrinology.* 136: 5020-5027, 1995.

71. **Carr JM.** Insulin-like growth factor binding proteins (IGFBPs) in growth and development of the ovine fetus. PhD thesis, Department of Biochemistry, The University of Adelaide, Adelaide, South Australia, Australia, 1994.

72. **Carr JM, Owens JA, Grant PA, Walton PE, Owens PC, Wallace JC.** Circulating insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and tissue mRNA levels of IGFBP-2 and IGFBP-4 in the ovine fetus. *Journal of Endocrinology.* 145(3): 545-557, 1995.

73. **Carrascosa A, Audi L.** Human studies on the biological actions of IGF-1. Evidence suggesting that human fetal and postnatal epiphyseal cartilage is a target for IGF-1 action. *Journal of Pediatric Endocrinology.* 6(3-4): 257-261, 1993.

74. **Carter AM, Detmer A.** Blood flow to the placenta and lower body in the growth-retarded guinea pig fetus. *Journal of Developmental Physiology.* 13: 261-269, 1990.

75. **Carter AM, Homan J, Fraser M, Richardson BS, Challis JRG.** Inhibition of ACTH secretion blocks hypoxia induced increase of adrenal cortical blood flow in fetal sheep. *American Journal of Physiology.* 269: E598-E604, 1995.

76. **Carter AM, Richardson BS, Homan J, Towstoless M, Challis JRG.** Regional adrenal blood flow responses to adrenocorticotrophic hormone in fetal sheep. *American Journal of Physiology.* 264: E264-E269, 1993.

77. **Castro MI, Valego NK, Zehnder TJ, Rose JC.** The ratio of plasma bioactive to immunoreactive ACTH-like activity increases with gestational age in the fetal lamb. *Journal of Developmental Physiology.* 18: 193-201, 1992.

78. **Cathiard A-M, Crozat A, Durand P, Saez JM.** In vitro spontaneous and adrenocorticotropin-dependent maturation of the steroidogenic pathway of the ovine fetal cells. *Endocrinology.* 116: 585-590, 1985.

79. **Cavaliere H, Knobel M, Medeiros-Neto G.** Effect of thyroid hormone therapy on plasma insulin-like growth factor 1 levels in normal subjects, hypothyroid patients and endemic cretins. *Hormone Research.* 25: 132-139, 1987.

80. **Challis JRG, Brooks AN.** Maturation and activation of hypothalamic-pituitary-adrenal function in fetal sheep. *Endocrine Reviews*: 182-210, 1989.

81. **Challis JRG, Lye SJ.** Parturition. In: The physiology of reproduction, 2nd edition. Knobil E, Neill JD (editors). Raven Press, New York, pp. 985-1031, 1994.

82. **Chan YL, Gutell R, Noller HF, Wool IG.** The nucleotide sequence of a rat 18S ribosomal nucleic acid gene and a proposal for the secondary structure of 18S ribosomal ribonucleic acid. *Journal of Biological Chemistry.* 259: 224-230, 1984.

83. **Chang ACY, Cochet M, Cohen SN.** Structural organisation of human genomic DNA encoding the pro-opiomelanocortin gene. *Proceedings of the National Academy of Sciences of the United States of America.* 77: 4890-4894, 1980.

84. **Chang CW, Naseeruddin SA, Hornsby PJ.** The response of 21-hydroxylase messenger ribonucleic acid levels to adenosine 3',5'-monophosphate and 12-O-tetradecanoylphorbol-13-acetate in bovine adrenocortical cells is dependent on culture conditions. *Endocrinology.* 128(1): 604-610, 1991.

85. **Charlton V, Johengen M.** Fetal intravenous nutritional supplementation ameliorates the development of embolization-induced growth retardation in sheep. *Pediatric Research.* 22: 55-61, 1987.

86. **Chatelain P, Penhoat A, Perrard-Sapori MH, Jaillard C, Naville D, Saez JM.** Maturation of steroidogenic cells: a target for IGF-I. *Acta Paediatrica Scandinavica (Supplemental).* 347: 104-109, 1988.

87. **Cheng CY, Hornsby PJ.** Expression of 11β-hydroxylase and 21-hydroxylase in long-term cultures of bovine adrenocortical cells requires extracellular matrix factors. *Endocrinology.* 130(5): 2883-2889, 1992.

88. **Cherradi N, Defaye G, Chambaz EM.** Characterization of the 3β-hydroxysteroid dehydrogenase activity associated with bovine adrenocortical mitochondria. *Endocrinology.* 134: 1358-1364, 1994.

89. **Cheung CY.** Enhancement of adrenomedullary catecholamine release by adrenal cortex in fetus. *American Journal of Physiology.* 247: E693-E697, 1984.

90. **Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ.** Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry.* 18: 5294-5299, 1979.

91. **Chomczynski P.** A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques.* 15(3): 532-537, 1993.

92. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* 162: 156-159, 1987.

93. **Chretien M, Boileau G, Lazure C, Seidah NG.** Processing of peptide hormone and neuropeptide precursors. In: Cell biology and the secretory process. Cantin M (editor). Basel, Karger, pp. 214-238, 1984.

94. **Chua SC, Szabo P, Vitek A, Grzeschik KH, John M, White PC.** Cloning of cDNA encoding steroid 11β-hydroxylase (p450c11). *Proceedings of the National Academy of Sciences of the United States of America.* 84: 7193-7197, 1987.

95. **Chung B-C, Matteson KJ, Voutilainen R, Mohandas TK, Miller WL.** Human cholesterol side chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. *Proceedings of the National Academy of Sciences of the United States of America.* 83: 8962-8966, 1986.

96. **Chung B-C, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE, Miller WL.** Cytochrome P450c17 (steroid 17α-hydroxylase/17,20 lyase): Cloning of human adrenal and testis cDNA indicates the same gene is expressed in both tissues. *Proceedings of the National Academy of Sciences of the United States of America.* 84: 407-411, 1987.

97. **Clapp JF, McLaughlin MK, Gellis J, Hemphill C, Catalano PM.** Regional distribution of cerebral blood flow in experimental intrauterine growth retardation. *American Journal of Obstetrics and Gynecology.* 150(7): 843-846, 1984.

98. **Clapp JF, Szeto HH, Larrow RW, Hewitt J, Mann LI.** Umbilical blood flow response to embolization of the uterine circulation. *American Journal of Obstetrics and Gynecology.* 138: 60-67, 1980.

99. **Clark KE, Durnwald M, Austin JE.** A model for studying chronic reduction in uterine blood flow in pregnant sheep. *American Journal of Physiology.* 242: H297-H301, 1982.

100. **Clemmons DR.** IGF binding proteins and their functions. *Molecular and Reproductive Development.* 35: 368-374, 1993.

101. **Clemmons DR, Klibanski A, Underwood LE, McArthur JW, Ridgeway EC, Beitins IZ, Van Wyk JJ.** Reduction of plasma immunoreactive somatomedin C during fasting in humans. *Journal of Clinical Endocrinology and Metabolism.* 53: 1247-1250, 1981.

102. **Cohen SN, Chang ACY.** Studies of cloned DNA encoding the structure for the bovine corticotropin-β–lipotropin precursor protein. *Annals of the New York Academy of Sciences.* 343: 415-424, 1980.

103. **Cohen SN, Chang ACY, Hsu L.** Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Sciences of the United States of America.* 69: 2110-2114, 1972.

104. **Commas FM, Pullinger GD, Barker S, Clark AJ.** The mouse adrenocorticotropin receptor gene: cloning and characterization of its promoter and evidence for a role for the orphan nuclear receptor steroidogenic factor-1. *Molecular Endocrinology.* 11: 867-876, 1997.

105. **Conley AJ, Bird IM.** The role of cytochrome P450 17α hydroxylase and 3β hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals. *Biology of Reproduction.* 56: 789-799, 1997.

106. **Conley AJ, Head JR, Stirling DT, Mason IJ.** Expression of steroidogenic enzymes in the bovine placenta and fetal adrenal glands throughout gestation. *Endocrinology.* 130(5): 2641-2650, 1992.

107. **Coulter CL, Adams MB, Warnes KE, McMillen IC**. Effect of cortisol on the expression of 11βHSD-1 and 11βHSD-2 mRNA in the sheep fetus. (Abstract #91). Proceedings of the 42nd Annual Conference of the Endocrine Society of Australia, Melbourne, Victoria, Australia, September 26th-29th, 1999.

108. **Coulter CL, Goldsmith PC, Mesiano S, Voytek CC, Martin MC, Han VKM, Jaffe RB**. Functional maturation of the primate fetal adrenal *in vivo*: I. Role of insulin-like growth factors (IGFs), IGF-I receptor, and IGF binding proteins in growth regulation. *Endocrinology*. 137(10): 4487-4498, 1996.

109. **Coulter CL, Young IR, Browne CA, McMillen IC**. Effect of hypophysectomy on the growth and development of the sheep adrenal gland: a morphometric study. *Acta Endocrinologica (Copenhagen)*. 120: 301-307, 1989.

110. **Creasy RK, Barrett CT, de Swiet M, Kahanpaa KV, Rudolph AM**. Experimental intrauterine growth retardation in the sheep. *American Journal of Obstetrics and Gynecology*. 112(4): 566-573, 1972.

111. **Crosby SR, Stewart MF, Ratcliffe JG, White A**. Direct measurement of the precursors of adrenocorticotrophin in human plasma by two-site immunoradiometric assay. *Journal of Clinical Endocrinology and Metabolism*. 67: 1272-1277, 1988.

112. **Dallman MF**. Control of adrenocortical growth in vivo. *Endocrine Research*. 10(3-4): 213-242, 1985.

113. **Dallman MF, Engeland WC, Holzwarth MA, Scholz PM**. Adrenocorticotropin inhibits compensatory adrenal growth after unilateral adrenalectomy. *Endocrinology*. 107: 1397-1404, 1980.

114. **Dallman MF, Engeland WC, Shinsako J**. Compensatory adrenal growth: a neurally mediated reflex. *American Journal of Physiology*. 231: 408-414, 1976.

115. **Darbeida H, Durand P**. Glucocorticoid enhancement of adrenocorticotropin-induced 3',5'-cyclic adenosine monophosphate production by cultured ovine adrenocortical cells. *Endocrinology*. 121(3): 1051-1055, 1987.

116. **Darbeida H, Durand P**. Mechanism of glucocorticoid enhancement of the responsiveness of ovine adrenocortical cells to adrenocorticotropin. *Biochemical and Biophysical Research Communications*. 166(3): 1183-1191, 1990.

117. **Darbeida H, Naaman E, Durand P**. Glucocorticoid induction of the maturation of ovine fetal adrenocorticoidal cells. *Biochemical and Biophysical Research Communications*. 145: 999-1005, 1987.

118. **Davorean JB, Hsueh AJW, Li CH**. Somatomedin C augments FSH-induced differentiation of cultured rat granulosa cells. *American Journal of Physiology*. 249: E26-E31, 1985.

119. **Dawes GS, Fox HE, Leduc BM, Liggins GC, Richards RT**. Respiratory movements and rapid eye movement sleep in the foetal lamb. *Journal of Physiology*. 220: 119-143, 1972.

120. **DeChiara, T, Efstratiadis A, Robertson EJ**. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*. 345: 78-80, 1990.

121. **Delhanty PJD, Han VKM.** The expression of insulin-like growth factor binding protein-2 (IGFBP-2) and insulin-like growth factor-II (IGF-II) genes in the tissues of the developing ovine fetus. *Endocrinology.* 132(1): 41-52, 1993.

122. **Delhanty PJD, Hill DJ, Shimasaki S, Han VKM.** Insulin-like growth factor binding protein-4, -5 and -6 mRNAs in the human fetus: Localization to sites of growth and differentiation? *Growth Regulation.* 3: 8-11, 1993.

123. **D'Ercole AJ.** Somatomedins/insulin-like growth factors and fetal growth. *Journal of Developmental Physiology.* 9: 481-495, 1987.

124. **D'Ercole AJ, Applewhite GT, Underwood LE.** Evidence that somatomedin is synthesized by multiple tissues in the fetus. *Developmental Biology.* 75: 315-328, 1980.

125. **D'Ercole AJ, Hill DJ, Strain AJ, Underwood LE.** Tissue and plasma somatomedin-C/insulin-like growth factor I concentrations in the human fetus during the first half of gestation. *Pediatric Research.* 20: 253-255, 1986.

126. **Dodic M, May CN, Wintour EM, Coghlan JP.** An early prenatal exposure to excess glucocorticoid leads to hypertensive offspring in sheep. *Clinical Science.* 94: 149-155, 1998.

127. **Doody KM, Carr BR, Rainey WE, Byrd W, Murry BA, Strickler RC, Thomas JL, Mason IJ.** 3β-hydroxysteroid dehydrogenase/isomerase in the fetal zone and neocortex of the human fetal adrenal gland. *Endocrinology.* 126(5): 2487-2492, 1990.

128. **Drost M, Holm LW.** Prolonged gestation in ewes after foetal adrenalectomy. *Journal of Endocrinology.* 40(3): 293-296, 1968.

129. **Durand P.** ACTH receptor levels in lamb adrenals at late gestation and early neonatal stages. *Biology of Reproduction.* 20: 837-845, 1979.

130. **Durand P, Cathiard A-M, Dacheux F, Naaman E, Saez JM.** In vitro stimulation and inhibition of adrenocorticotropin release by pituitary cells from ovine fetuses and lambs. *Endocrinology.* 118: 1387-1394, 1986.

131. **Durand P, Cathiard A-M, Locatelli A, Saez JM.** Modifications of the steroidogenic pathway during spontaneous and adrenocorticotrophin-induced maturation of ovine fetal adrenal. *Endocrinology.* 110: 500-505, 1982.

132. **Durand P, Cathiard A-M, Locatelli A, Saez JM.** Effects of fetal hypophysectomy and the in vitro treatment of pituitary extracts on the maturation of cultured ovine fetal adrenal cells. *Endocrinology.* 116: 578-584, 1985.

133. **Durand P, Cathiard A-M, Morea A-M, Dazord A, Saez JM.** Maturation of adrenocorticotropin-sensitive adenylate cyclase of ovine fetal adrenal during late pregnancy. *Endocrinology.* 108: 2114-2118, 1981.

134. **Durand P, Cathiard A-M, Naaman E, Brieu V, Saez JM.** The influence of plasma lipoproteins on steroidogenesis of cultured ovine fetal and neonatal adrenal cells. *Journal of Steroid Biochemistry.* 26(4): 425-431, 1987.

135. **Durand P, Cathiard A-M, Naaman E, Saez JM**. De novo synthesis of cholesterol by ovine fetal and neonatal adrenocortical cells. *Molecular and Cellular Endocrinology*. 57: 215-223, 1988.

136. **Durand P, Cathiard A-M, Saez JM**. In vitro maturation of steroidogenic capacity of ovine fetal and neonatal adrenal cells. *Endocrinology*. 114: 1128-1134, 1984a.

137. **Durand P, Cathiard A-M, Seidah NG, Chretien M, Saez JM**. Effects of proopiomelanocortin-derived peptides, methionine-enkephalin and forskolin on the maturation of ovine fetal adrenal cells in culture. *Biology of Reproduction*. 31(4): 694-704, 1984b.

138. **Economides DL, Nicolaides KH, Linton EA, Perry LA, Chard T**. Plasma cortisol and adrenocorticotropin in appropriate and small for gestational age fetuses. *Fetal Therapy*. 3: 158-164, 1988.

139. **Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR**. Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet*. 341: 355-357, 1993.

140. **Eipper BA, Mains RE**. Analysis of the common precursor to corticotropin and endorphin. *Journal of Biological Chemistry*. 253(16): 5732-5744, 1978.

141. **Ekman R, Hakanson R, Larsson I, Sundler F, Thorell JI**. Radioimmunoassay of pro-gamma-melanotropin, the amino-terminal fragment of proopiolipomelanocortin. *Endocrinology*. 111(2): 578-583, 1982.

142. **Emmanoulides GC, Townsend DE, Bauer RA**. Effects of single umbilical artery ligation in the lamb fetus. *Pediatrics*. 42: 919-927, 1968.

143. **Engeland WC, Dallman MF**. Compensatory adrenal growth is neurally mediated. *Neuroendocrinology*. 19: 352-362, 1975.

144. **Engeland WC, Dallman MF**. Neural mediation of compensatory adrenal growth. *Endocrinology*. 99(6): 1659-1662, 1976.

145. **Estivariz FE, Carino M, Lowry PJ, Jackson S**. Further evidence that N-terminal pro-opiomelanocortin peptides are involved in adrenal mitogenesis. *Journal of Endocrinology*. 116: 201-206, 1988.

146. **Estivariz FE, Gillies G, Lowry PJ**. Biosynthesis and control of secretion of corticotropic, lipotropic and melanotropic peptides. *Pharmacology and Therapeutics*. 13: 61-67, 1981.

147. **Estivariz FE, Hope J, McLean C, Lowry PJ**. Purification and characterisation of a gamma-melanotropin precursor from frozen human pituitary glands. *Biochemical Journal*. 191(1): 125-132, 1980.

148. **Estivariz FE, Iturriza FC, McLean C, Hope J, Lowry PJ**. Stimulation of adrenal mitogenesis by N-terminal pro-opiomelanocortin peptides. *Nature*. 297: 419-422, 1982.

149. **Estivariz FE, Morano MI, Carino M, Jackson S, Lowry PJ**. Adrenal regeneration in the rat is mediated by mitogenic N-terminal pro-opiomelanocortin peptides generated by changes in precursor processing in the anterior pituitary. *Journal of Endocrinology*. 116: 207-216, 1988.

150. **Farese RV, Ling N, Sabir MA, Larson RE, Trudeau WC**. Comparison of effects of adrenocorticotropin and lys-γ₃ melanocyte-stimulating hormone on steroidogenesis, adenosine 3',5' monophosphate production and phospholipid metabolism in rat adrenal fasciculata-reticularis cells in vitro. *Endocrinology.* 112: 129-132, 1983.

151. **Fay RA, Ellwood DA**. Categories of intrauterine growth retardation. *Fetal and Maternal Medical Review.* 5: 203-212, 1993.

152. **Fora MA, Butler TG, Rose JC, Schwartz J**. Adrenocorticotropin secretion by fetal sheep anterior and intermediate lobe pituitary cells in vitro: Effects of gestation and adrenalectomy. *Endocrinology.* 137: 3394-3400, 1996.

153. **Fowden AL**. Fetal metabolism and energy balance. In: Textbook of fetal physiology. Thorburn GD, Harding R (editors). Oxford University Press, Oxford, pp. 70-82, 1994.

154. **Fowden AL, Coulson RL, Silver M**. Endocrine regulation of tissue glucose-6-phosphatase activity in the fetal sheep during late gestation. *Endocrinology.* 126: 2823-2830, 1990.

155. **Fowden AL, Mundy L, Silver M**. Developmental regulation of glucogenesis in the sheep fetus during late gestation. *Journal of Physiology.* 508(3): 937-947, 1998.

156. **Fowden AL, Szemere J, Hughes P, Gilmour RS, Forhead AJ**. The effects of cortisol on the growth rate of the sheep fetus during late gestation. *Journal of Endocrinology.* 151(1): 97-101, 1996.

157. **Francis GL, McNeil KA, Wallace JC, Ballard FJ, Owens PC**. Sheep insulin-like growth factors I and II: sequences, activities and assays. *Endocrinology.* 124(3): 1173-1183, 1989.

158. **Fraser M, Jeffray TM, Challis JRG**. Developmental regulation of corticotrophin receptor (ACTH-R) gene expression in the adrenal gland of the ovine fetus and newborn lamb: Effects of cortisol infusion during late pregnancy. (Session 6). Proceedings of The International Fetal Physiology Symposium, The Given Institute of the University of Colorado, Aspen, Colorado, USA. June 26[th]-30[th], 1999.

159. **Freemark M, Driscoll P, Maaskant R, Petryk A, Kelly P**. Ontogenesis of prolactin receptors in the human fetus in early gestation. The implications for tissue differentiation and development. *Journal of Clinical Investigation.* 99: 1107-1117, 1997.

160. **Frodin M, Gammeltoft S**. Insulin-like growth factors act synergistically with fibroblast growth factor and nerve growth factor to promote chromaffin cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America.* 91: 1771-1775, 1994.

161. **Fuller RS, Brake AJ, Thorner J**. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca^{2+}-dependent serine protease. *Proceedings of the National Academy of Sciences of the United States of America.* 86: 1434-1438, 1989.

162. **Fuller RS, Brake AJ, Thorner J**. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science.* 246: 482-486, 1989.

163. **Funder JW, Pearce PT, Smith R, Smith AI.** Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science.* 242: 583-585, 1988.

164. **Gagnon R, Challis JRG, Johnston J, Fraher L.** Fetal endocrine responses to chronic placental embolization in the late-gestation ovine fetus. *American Journal of Obstetrics and Gynecology.* 170: 929-938, 1994.

165. **Gasson JC.** Steroidogenic activity of high molecular weight forms of corticotropin. *Biochemistry.* 18: 4215-4224, 1979.

166. **Gillies G, Estivariz FE, Lowry PJ.** Investigations on the nature of corticotrophin releasing factor using the perfused isolated rat anterior pituitary cell column. *Journal of Endocrinology.* 80(2): 56P-57P, 1979.

167. **Gilmour RS, Prosser CG, Fleet IR, Cocco L, Saunders JC, Brown KD, Corps AN.** From animal to molecule: aspects of the biology of insulin-like growth factors. *British Journal of Cancer. Supplement.* 9: 23-30, 1988.

168. **Glickman JA, Challis JRG.** The changing response pattern of sheep fetal adrenal cells throughout the course of gestation. *Endocrinology.* 106: 1371-1376, 1980.

169. **Glover DM.** DNA cloning: a practical approach. Glover DM (editor). IRL Press, Washington DC, USA, 1985.

170. **Gluckman PD.** Functional maturation of the neuroendocrine system in the perinatal period: studies of the somatotropic axis in the ovine fetus. *Journal of Developmental Physiology.* 6(3): 301-312, 1984.

171. **Gluckman PD, Butler JH.** Parturition-related changes in IGF-I and II in the perinatal lamb. *Journal of Endocrinology.* 99: 223-232, 1983.

172. **Gluckman PD, Holdaway IM.** Prolactin and somatomedin studies in the syndrome of growth hormone-independent growth. *Clinical Endocrinology.* 5(5): 545-549, 1976.

173. **Halasz B, Szentagothai J.** Histologischer beweis einer nervosen signalubermittlung von der nebennierenrinde zum hypothalamus. *Zeitschrift fur Zellforschung und Mikroskopische Anatomie.* 50: 297-306, 1959.

174. **Hall BK, Ekanayake S.** Effects of growth factors on the differentiation of neural crest cells and neural crest cell-derivatives. *International Journal of Developmental Biology.* 35: 367-387, 1991.

175. **Han VKM, Asano H, Matsell DG, Delhanty PJD, Hayatsu J, Bassett N, Phillips ID, Nygard K, Stepaniuk O.** Insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) in fetal development: physiology and pathophysiology. In: The insulin-like growth factors and their regulatory proteins. Baxter RC, Gluckman PD, Rosenfeld RG (editors). Excerpta Medica, Amsterdam, pp. 263-273, 1994.

176. **Han VKM, D'Ercole AJ, Lund PK.** Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science.* 236: 193-197, 1987.

177. **Han VKM, Hill DJ, Strain AJ, Towle AC, Lauder JM, Underwood LE, D'Ercole AJ.** Identification of somatomedin/insulin-like growth factor immunoreactive cells in the human fetus. *Pediatric Research.* 22: 245-249, 1987.

178. **Han VKM, Lu F, Bassett N, Yang K, Delhanty PJD, Challis JRG**. Insulin-like growth factor-II messenger ribonucleic acid is expressed in steroidogenic cells of the developing ovine adrenal gland: evidence of an autocrine/paracrine role for IGF-II. *Endocrinology*. 131: 3100-3109, 1992.

179. **Han VKM, Lund PK, Lee DC, D'Ercole AJ**. Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization and tissue distribution. *Journal of Clinical Endocrinology and Metabolism*. 66: 422-429, 1988.

180. **Harding J, Jones CT, Robinson JS**. Studies on experimental growth retardation in sheep. The effects of a small placenta in restricting transport to and growth of the fetus. *Journal of Developmental Physiology*. 7: 427-442, 1985.

181. **Hay WW Jr**. Energy and substrate requirements of placenta and fetus. *Proceedings of the Nutrition Society*. 50: 321-324, 1991.

182. **Hay WW Jr, Wilkening RB**. Metabolic activity of the placenta. In: Textbook of fetal physiology. Thorburn GD, Harding R (editors). Oxford University Press, Oxford, pp. 30-47, 1994.

183. **Hennessy DP, Coghlan JP, Hardy KJ, Scoggins BA, Wintour EM**. The origin of cortisol in the blood of fetal sheep. *Journal of Endocrinology*. 95: 71-79, 1982.

184. **Hill DJ**. Peptide growth factor interactions in embryonic and fetal growth. *Hormone Research*. 38(5-6): 197-202, 1992.

185. **Hill DJ**. Hormonal control of fetal growth. In: Fetus and noenate: physiology and clinical applications, volume 3, Growth. Hanson MA, Spencer JA, Rodeck CH (editors). Cambridge University Press, Cambridge, pp. 57-95, 1995.

186. **Hill DJ, Clemmons DR**. Similar immunological distribution of insulin-like growth factor binding proteins -1, -2, and -3 in human fetal tissues. *Growth Factors*. 6: 315-326, 1992.

187. **Hill DJ, Clemmons DR, Wilson S, Han VKM, Strain AJ, Milner RDG**. Immunological distribution of one form of insulin-like growth factor (IGF) binding protein and IGF peptides in human fetal tissues. *Journal of Molecular Endocrinology*. 2: 31-38, 1989.

188. **Hill DJ, Hogg J**. Growth factors and the regulation of pre- and postnatal growth. In: Clinical endocrinology and metabolism, perinatal endocrinology, volume 3, number 3. Jones CT (editor). Bailliere Tindall, London, pp. 579-625, 1989.

189. **Hillier K, Wallis R**. Prostaglandins, steroids and the human cervix. In: The cervix in pregnancy and labour. Ellwood DA, Anderson AG (editors). Churchill Livingstone, Edinburgh, p. 34, 1981.

190. **Holzwarth MA, Shinsako J, Dallman MF**. Adrenal regeneration. Time course, effect of hypothalamic hemi-islands and response to unilateral adrenalectomy. *Neuroendocrinology*. 31: 168-176, 1980.

191. **Honda S, Morohashi K, Nomura M, Takeya H, Kitajima M, Omura T**. Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *Journal of Biological Chemistry*. 268: 7494-7502, 1993.

192. **Hooper SB, Coulter CL, Deayton JM, Harding R, Thorburn GD.** Fetal endocrine responses to prolonged hypoxemia in sheep. *American Journal of Physiology.* 259: R703-R708, 1990.

193. **Hope J, Ratter SJ, Estivariz FE, McLoughlin L, Lowry PJ.** Development of a radioimmunoassay for an amino-terminal peptide of pro-opiocortin containing the γ-MSH region: measurement and characterization in human plasma. *Clinical Endocrinology.* 15(3): 221-227, 1981.

194. **Horiba N, Nomura K, Hizuka N, Takaro K, Demura H, Shizume K.** Effects of IGF-I on proliferation and steroidogenesis of cultured adrenal zone glomerulosa cells. *Endocrinologica Japonica.* 34: 611-614, 1987.

195. **Hornsby PJ.** Regulation of adrenocortical cell proliferation in culture. *Endocrine Research.* 10(3-4): 259-281, 1985a.

196. **Hornsby PJ.** The regulation of adrenocortical function by control of growth and structure. In: Adrenal Cortex. Anderson DC, Winter JSD (editors). Butterworth, London, pp. 1-31, 1985b.

197. **Hornsby PJ, Crivello JF.** The role of lipid peroxidation and biological antioxidants in the function of the adrenal cortex. Part 2. *Molecular and Cellular Endocrinology.* 30: 123-147, 1983.

198. **Hotta M, Baird A.** Differential effects of transforming growth factor type β on the growth and function of adrenocortical cells in vitro. *Proceedings of the National Academy of Sciences of the United States of America.* 83: 7795-7799, 1986.

199. **Houghton DC, Young IR, McMillen IC.** Evidence for hypothalamic control of the diurnal rhythms in prolactin and melatonin in the fetal sheep during late gestation. *Endocrinology.* 136: 218-223, 1995a.

200. **Houghton DC, Young IR, McMillen IC.** Response of prolactin to different photoperiods after surgical disconnection of the hypothalamus and pituitary in sheep fetuses. *Journal of Reproduction and Fertility.* 104: 199-206, 1995b.

201. **Ilvesmaki V, Blum WF, Voutilainen R.** Insulin-like growth factor binding proteins in the human adrenal gland. *Molecular and Cellular Endocrinology.* 97: 71-79, 1993a.

202. **Ilvesmaki V, Blum WF, Voutilainen R.** Insulin-like growth factor-II in human fetal adrenal glands: regulation by ACTH, protein kinase C and growth factors. *Journal of Endocrinology.* 137: 533-542, 1993b.

203. **Isgaard J, Nilsson A, Lindahl A, Jansson JO, Isaksson OG.** Effects of local administration of GH and IGF-1 on longitudinal bone growth in rats. *American Journal of Physiology.* 250(4): E367-E372, 1986.

204. **Jackson S, Hodgkinson S, Estivariz FE, Lowry PJ.** IGF 1 and 2 in two models of adrenal growth. *Journal of Steroid Biochemistry and Molecular Biology.* 40: 399-404, 1991.

205. **Jacob R, Hu X, Niederstock D, Hasan S, McNulty PH, Sherwin RS, Young LH.** IGF-I stimulation of muscle protein synthesis in the awake rat: permissive role of insulin and amino acids. *American Journal of Physiology.* 270(1): E60-E66, 1996.

206. **Jacobs R, Robinson JS, Owens JA, Falconer J, Webster MED**. The effect of prolonged hypobaric hypoxia on growth of fetal sheep. *Journal of Developmental Physiology*. 10: 97-112, 1988.

207. **Jansson T, Persson E**. Placental transfer of glucose and amino acids in intrauterine growth retardation: Studies with substrate analogs in the awake guinea pig. *Pediatric Research*. 28: 203-208, 1990.

208. **Jansson T, Thordstein M, Kjellmer I**. Placental blood flow and fetal weight following uterine artery ligation. Temporal aspects of intrauterine growth retardation in the guinea pig. *Biology of the Neonate*. 49: 172-180, 1986.

209. **Jefcoate CR, McNamara BC, Artemenko I, Yamazaki T**. Regulation of cholesterol movement to mitochondrial cytochrome P450scc in steroid hormone synthesis. *Journal of Steroid Biochemistry and Molecular Biology*. 43: 751-767, 1992.

210. **Jensen A, Hohmann M, Kunzel W**. Dynamic changes in organ blood flow and oxygen consumption during acute asphyxia in fetal sheep. *Journal of Developmental Physiology*. 9: 325-326, 1987.

211. **Jia L-G, Canny BJ, Orth DN, Leong DA**. Distinct classes of corticotropes mediate corticotropin-releasing hormone and arginine vasopressin-stimulated adrenocorticotropin release. *Endocrinology*. 128: 197-203, 1991.

212. **Jiang W, Uht R, Bohn MC**. Regulation of phenylethanolamine N-methyltransferase (PNMT) mRNA in the rat adrenal medulla by corticosterone. *International Journal of Developmental Neuroscience*. 7: 513-520, 1989.

213. **John ME, John MC, Ashley P, MacDonald RJ, Simpson ER, Waterman MR**. Identification and characterization of cDNA clones specific for cholesterol side-chain cleavage cytochrome P-450. *Proceedings of the National Academy of Sciences of the United States of America*. 81: 5628-5632, 1984.

214. **John ME, John MC, Boggaram V, Simpson ER, Waterman MR**. Transcriptional regulation of steroid hydroxylase genes by corticotropin. *Proceedings of the National Academy of Sciences of the United States of America*. 83: 4715-4719, 1986.

215. **John ME, Okamura T, Dee A, Adler B, John MC, White PC, Simpson ER, Waterman MR**. Bovine steroid-hydroxylase: Regulation of biosynthesis. *Biochemistry*. 25: 2846-2853, 1986.

216. **John ME, Simpson ER, Carr BR, Magness RR, Rosenfeld R, Waterman MR, Mason JI**. Ontogeny of adrenal steroid hydroxylases: evidence for cAMP-independent gene expression. *Molecular and Cellular Endocrinology*. 50: 263-268, 1987.

217. **Jones CT**. Age-dependent changes in the processing of corticotropin-containing peptides in pituitary cells of the foetal sheep. *Biochemical Society Transactions*. 8(5): 585-586, 1980.

218. **Jones CT.** Observations on the control of prenatal growth in the guinea pig. In: Advances in fetal physiology, research in perinatal medicine, volume 8. Gluckman PD, Johnston BM, Nathanielsz PW (editors). Perinatology Press, Ithaca, New York, pp. 287-302, 1989.

219. **Jones CT, Boddy K, Robinson JS.** Changes in the concentration of adrenocorticotrophin and corticosteroid in the plasma of fetal sheep in the latter half of pregnancy and during labour. *Journal of Endocrinology.* 72: 293-300, 1977.

220. **Jones CT, Edwards AV, Tindell D.** Inhibitory effects of proopiomelanocortin on cortical and medullary activity in the calf adrenal. *Journal of Developmental Physiology.* 17: 69-73, 1992.

221. **Jones CT, Harding JE, Gu W, Lafeber HN.** Placental metabolism and endocrine effects in relation to the control of fetal and placental growth. In: The endocrine control of the fetus. Kunzel W, Jensen A (editors). Springer-Verlag, Heidelberg, pp. 213-222, 1988.

222. **Jones CT, Lafeber HN, Price DA, Parer JT.** Studies on the growth of the fetal guinea pig. Effects of reduction in uterine blood flow on the plasma sulphation-promoting activity and on the concentrations of insulin-like growth factors I and II. *Journal of Developmental Physiology.* 9(2): 181-201, 1987.

223. **Jones CT, Parer JT.** The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea-pig. *Journal of Physiology.* 343: 525-537, 1983.

224. **Jones CT, Roebuck MM.** ACTH peptides and the development of the fetal adrenal. *Journal of Steroid Biochemistry.* 12: 77-82, 1980.

225. **Kallen CB, Arakane F, Christenson LK, Watari H, Devoto L, Strauss JF 3rd.** Unveiling the mechanism of action and regulation of the steroidogenic acute regulatory protein. *Molecular and Cellular Endocrinology.* 145(1-2): 39-45, 1998.

226. **Keutmann HT, Eipper BA, Mains RE.** Partial characterization of a glycoprotein comprising the NH_2-terminal region of mouse tumor cell pro-adrenocorticotropic hormone / endorphin. *Journal of Biological Chemistry.* 254(18): 9204-9208, 1979.

227. **Kiess W, Yang Y, Kessler U, Hoeflich A.** Insulin-like growth factor II (IGF-II) and the IGF-II/mannose-6-phosphate receptor: the myth continues. *Hormone Research.* 41(2): 66-73, 1994.

228. **Kind KL, Owens JA, Robinson JS, Quinn KJ, Grant PA, Walton PE, Gilmour RS, Owens PC.** Effect of restriction of placental growth on expression of IGFs in fetal sheep: relationship to fetal growth, circulating IGFs and binding proteins. *Journal of Endocrinology.* 146(1): 23-34, 1995.

229. **King SR, Ronen-Fuhrmann T, Timberg R, Clark BJ, Orly J, Stocco DM.** Steroid production after in vitro transcription, translation, and mitochondrial processing of protein products of complementary deoxyribonucleic acid for steroidogenic acute regulatory protein. *Endocrinology.* 136: 5165-5176, 1995.

230. **Klempt M, Hutchins AM, Gluckman PD, Skinner SJ.** IGF binding protein-2 gene expression and the location of IGF-I and IGF-II in fetal rat lung. *Development.* 115: 765-772, 1992.

231. **de Kloet ER, Reul JM, Sutanto W**. Corticosteroids and the brain. *Journal of Steroid Biochemistry and Molecular Biology*. 37(3): 387-394, 1990.

232. **Koster A, Saftig P, Matzner U, von Figura K, Peters C**. Targeted disruption of the M(r) 46,000 mannose-6-phosphate receptor gene in mice results in misrouting of lysosomal proteins. *EMBO Journal*. 12(13): 5219-5223, 1993.

233. **Kramer MS, Olivier M, McLean FH, Dougherty GE, Willis DM, Usher RD**. Determinants of fetal growth and body proportionality. *Pediatrics*. 86: 18-26, 1990.

234. **Lachance Y, Luu-The V, Labrie C, Simard J, Dumont M, de Launoit Y, Guerin S, Leblanc G, Labrie F**. Characterization of human 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase gene and its expression in mammalian cells. *Journal of Biological Chemistry*. 265(33): 20469-20475, 1990.

235. **Lafeber HN, Rolph TP, Jones CT**. Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *Journal of Developmental Physiology*. 6: 441-459, 1984.

236. **Langlois D, Hinsch KD, Saez JM, Begeot M**. Stimulatory effect of insulin and insulin-like growth factor I on G1 proteins and angiotensin-II-induced phosphoinositide breakdown in cultured bovine adrenal cells. *Endocrinology*. 126: 1867-1872, 1990.

237. **Langlois DA, Matthews SG, Yu M, Yang K**. Differential expression of 11β-hydroxysteroid dehydrogenase 1 and 2 in the developing ovine fetal liver and kidney. *Journal of Endocrinology*. 147(3): 405-411, 1995.

238. **Lassarre C, Hardouin S, Daffos F, Forestier F, Frankenne F, Binoux M**. Serum insulin-like growth factor binding proteins in the human fetus: Relationships with growth in normal subjects and in subjects with intrauterine growth retardation. *Pediatric Research*. 9: 219-221, 1991.

239. **Lehoux JG, Fleury A, Ducharme L**. The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal in vivo. *Endocrinology*. 139(9): 3913-3922, 1998.

240. **Levidiotis ML, Wintour EM, McKinley MJ, Oldfield BJ**. Hypothalamic-hypophyseal vascular connections in the fetal sheep. *Neuroendocrinology*. 49(1): 47-50, 1989.

241. **Li J, Saunders JC, Gilmour RS, Silver M, Fowden AL**. Insulin-like growth factor II messenger ribonucleic acid expression in fetal tissues of the sheep during late gestation. *Endocrinology*. 132: 2083-2089, 1993.

242. **Lighten AD, Hardy K, Winston RM, Moore GE**. Expression of mRNA for the insulin-like growth factors and their receptors in human preimplantation embryos. *Molecular Reproduction and Development*. 47(2): 134-139, 1997.

243. **Liggins GC**. Premature parturition after infusion of corticotrophin or cortisol into foetal lambs. *Journal of Endocrinology*. 42: 323-329, 1968.

244. **Liggins GC**. Adrenocortical-linked maturational events in the fetus. *American Journal of Obstetrics and Gynecology*. 126: 931-938, 1976.

245. **Liggins GC, Fairclough RL, Grieves SA, Kendall JZ, Knox BS.** The mechanism of initiation of parturition in the ewe. *Recent Progress in Hormone Research.* 29: 111-150, 1973.

246. **Liggins GC, Kennedy PC.** Effects of electrocoagulation of the foetal lamb hypophysis on growth and development. *Journal of Endocrinology.* 40: 371-381, 1968.

247. **Liggins GC, Kennedy PC, Holm LW.** Failure of initiation of parturition after electrocoagulation of the pituitary of the fetal lamb. *American Journal of Obstetrics and Gynecology.* 98: 1080-1086, 1967.

248. **Liggins GC, Schellenberg JC, Finberg K, Kitterman JA, Lee CH.** The effects of ACTH1-24 or cortisol on pulmonary maturation in the adrenalectomized ovine fetus. *Journal of Developmental Physiology.* 7(2): 105-111, 1985.

249. **Lin T, Blaisdell J, Haskell JF.** Hormonal regulation of type I insulin-like growth factor receptor of Leydig cells in hypophysectomised rats. *Endocrinology.* 123: 134-139, 1988.

250. **Lin T, Haskell J, Vinson N, Terracio L.** Direct stimulatory effects of insulin-like growth factor-I on Leydig cell steroidogenesis in primary culture. *Biochemical and Biophysical Research Communications.* 137: 950-956, 1986.

251. **Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A.** Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf-r). *Cell.* 75(1): 59-72, 1993.

252. **Lok F, Owens JA, Mundy L, Robinson JS, Owens PC.** Insulin-like growth factor I promotes growth selectively in fetal sheep in late gestation. *American Journal of Physiology.* 270 (*Regulatory Integrative and Comparative Physiology. 39*): R1148-R1155, 1996.

253. **Loose DS, Do YS, Chen TL, Feldman D.** Demonstration of glucocorticoid receptors in the adrenal cortex: evidence for a direct dexamethasone suppressive effect on the rat adrenal gland. *Endocrinology.* 107: 137-146, 1980.

254. **Lorence MC, Murray BA, Trant JM, Mason JI.** Human 3β-hydroxysteroid dehydrogenase / Δ^5-Δ^4 isomerase from human placenta: expression in non-steroidogenic cell of a protein that catalyzes dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology.* 126(5): 2493-2498, 1990.

255. **Louveau I, Penhoat A, Saez JM.** Regulation of IGF-I receptors by corticotropin and angiotensin-II in cultured bovine adrenocortical cells. *Biochemical and Biophysical Research Communications.* 163: 32-36, 1989.

256. **Lowry PJ, Estivariz FE, Silas L, Linton EA, McLean C, Crocombe K.** The case for pro-γ-MSH as the adrenal growth factor. *Endocrine Research.* 10(3-4): 243-258, 1985.

257. **Lowry PJ, Silas L, McLean C, Linton EA, Estivariz FE.** Pro-γ-melanocyte-stimulating hormone cleavage in adrenal gland undergoing compensatory growth. *Nature.* 306: 70-73, 1983.

258. **Lu F, Han VKM, Milne WK, Fraser M, Carter AM, Berdusco ETM, Challis JRG**. Regulation of insulin-like growth factor-II gene expression in the ovine fetal adrenal gland by adrenocorticotropic hormone and cortisol. *Endocrinology.* 134: 2628-2635, 1994.

259. **Lu F, Yang K, Challis JRG**. Regulation of ovine fetal pituitary function by corticotrophin-releasing hormone, arginine vasopressin and cortisol in vitro. *Journal of Endocrinology.* 143: 199-208, 1994.

260. **Lund J, Faucher DJ, Ford SP, Porter JC, Waterman MR, Mason JI**. Developmental expression of bovine adrenocortical steroid hydroxylases. *Journal of Biological Chemistry.* 263(31): 16195-16201, 1988.

261. **Lund PK, Moats-Stoats BM, Hynes MA, Simmons JG, Jansen M, D'Ercole AJ, Van Wyk JJ**. Somatomedin C/insulin-like growth factor-1 mRNA in rat fetal and adult tissues. *Journal of Biological Chemistry.* 261(31): 14539-14544, 1986.

262. **Lye SJ, Challis JRG**. In vivo adrenocorticotropin (1-24)-induced accumulation of cyclic adenosine monophosphate by ovine fetal adrenal cells is inhibited by concomitant infusion of metopirone. *Endocrinology.* 115: 1584-1587, 1984.

263. **Lye SJ, Sprague CL, Mitchell BF, Challis JRG**. Activation of ovine fetal adrenal function by pulsatile or continuous administration of $ACTH_{1-24}$. I. Effects on fetal plasma corticosteroids. *Endocrinology.* 113: 770-776, 1983.

264. **Madill D, Bassett JM**. Corticosteroid release by adrenal tissue from foetal and newborn lambs in response to corticotrophin stimulation in a perifusion system in vitro. *Journal of Endocrinology.* 58: 78-87, 1973.

265. **Mains RE, Berard CA, Denault J-B, Zhou A, Johnson RC, Leduc R**. PACE4: a subtilisin-like endoprotease with unique properties. *Biochemical Journal.* 321: 587-593, 1997.

266. **Mains RE, Eipper BA**. Synthesis and secretion of corticotrophins, melanotrophins and endorphins by rat intermediate pituitary cells. *Journal of Biological Chemistry.* 254(16): 7885-7894, 1979.

267. **Manchester L, Challis JRG**. The effects of adrenocorticotropin, guanylylimidodiphosphate, dibutyryl adenosine 3',5'-monophosphate, and exogenous substrates on corticosteroid output by ovine fetal adrenal cells at different times in pregnancy. *Endocrinology.* 111: 889-895, 1982.

268. **Manchester L, Lye SJ, Challis JRG**. Activation of ovine fetal adrenal function by pulsatile or continuous administration of ACTH1-24. II. Effects on adrenal cell responses in vitro. *Endocrinology.* 113: 777-782, 1983.

269. **Marchal R, Naville D, Durand P, Begeot M, Penhoat A**. A steroidogenic factor-1 binding element is essential for basal human ACTH receptor gene transcription. *Biochemical and Biophysical Research Communications.* 247: 28-32, 1998.

270. **Mason JI, Hemsell PG, Korte K**. Steroidogenesis in dispersed cells of human fetal adrenal. *Journal of Clinical Endocrinology and Metabolism.* 56(5): 1057-1062, 1983.

271. **Matsui M, Takahashi Y, Hishinuma M, Kanagawa H**. Insulin and insulin-like growth factor-I (IGF-I) stimulate the development of bovine embryos fertilized in vitro. *Journal of Veterinary Medical Science.* 57(6): 1109-1111, 1995.

272. **Matteson KJ, Chung BC, Miller WL**. Molecular cloning of DNA complementary to bovine adrenal P450scc mRNA. *Biochemical and Biophysical Research Communications.* 120: 264-270, 1984.

273. **Matteson KJ, Chung BC, Urdea MS, Miller WL**. Study of cholesterol side-chain cleavage (20, 22-desmolase) deficiency causing congenital lipoid adrenal hyperplasia using bovine-sequence P450scc oligodeoxyribonucleotide probes. *Endocrinology.* 118: 1296-1305, 1986.

274. **Matteson KJ, Phillips III JA, Miller WL, Chung B-C, Orlando PJ, Frisch H, Ferrandez A, Burr IM**. P450 XXI (steroid 21 Hydroxylase) gene deletions are not found in family studies of congenital adrenal hyperplasia. *Proceedings of the National Academy of Sciences of the United States of America.* 84: 5858-5862, 1987.

275. **Matteson KJ, Picado Leonard J, Chung BC, Mohandas TK, Miller WL**. Assignment of the gene for adrenal P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase) to human chromosome 10. *Journal of Clinical Endocrinology and Metabolism.* 63: 789-791, 1986.

276. **McCusker RH, Clemmons DR**. The insulin-like growth factor binding proteins: structure and biological functions. In: The insulin-like growth factors, structure and biological functions. Schofield PN (editor). Oxford University Press, Oxford, pp. 110-150, 1992.

277. **McDonald TJ, Nathanielsz PW**. Bilateral destruction of fetal paraventricular nuclei prolongs gestation in sheep. *American Journal of Obstetrics and Gynecology.* 165: 764-770, 1991.

278. **McMillen IC, Antolovich GC, Mercer JE, Perry RA, Silver M**. Proopiomelanocortin messenger RNA levels are increased in the anterior pituitary of the sheep fetus after adrenalectomy in late gestation. *Neuroendocrinology.* 52: 297-302, 1990.

279. **McMillen IC, Merei JJ, White A, Crosby S, Schwartz J**. Increasing gestational age and cortisol alter the ratio of ACTH precursors: ACTH secreted from the anterior pituitary of the fetal sheep. *Journal of Endocrinology.* 144: 569-576, 1995.

280. **McMillen IC, Phillips ID, Ross JT, Robinson JS, Owens JA**. Chronic stress – the key to parturition? *Journal of Reproduction, Fertility and Development.* 7: 499-507, 1995.

281. **McMillen IC, Warnes KE, Adams MB, Robinson JS, Owens JA, Coulter CL**. Impact of placental restriction on expression of 11βHSD-1 and 11βHSD-2 mRNA in placenta and fetal tissues in the late gestation sheep fetus. (Abstract #131). Proceedings of the 42nd Annual Conference of the Endocrine Society of Australia, Melbourne, Victoria, Australia, September 26th-29th, 1999.

282. **Meaney MJ, Aitkin DH, van Berkel C, Bhatnagar S, Sapolsky M**. Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science.* 239: 766-768, 1988.

283. **Mellor DJ**. Nutritional and placental determination of foetal growth and consequences for the newborn lamb. *British Veterinary Journal*. 139: 307-324, 1983.

284. **Mesiano S, Young IR, Hey AW, Browne CA, Thorburn GD**. Hypophysectomy of the fetal lamb leads to a fall in the plasma concentration of insulin-like growth factor I (IGF-I) but not IGF-II. *Endocrinology*. 124: 1485-1491, 1989.

285. **Messing J**. New M13 vectors for cloning. *Methods in Enzymology*. 101: 20-78, 1983.

286. **Miller WL**. Molecular biology of steroid hormone synthesis. *Endocrine Reviews*. 9: 295-318, 1988.

287. **Miller WL, Strauss JF 3ʳᵈ**. Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. *Journal of Steroid Biochemistry and Molecular Biology*. 69(1-6): 131-141, 1999.

288. **Monder C, Shackleton CHL**. 11β-hydroxysteroid dehydrogenase: fact or fancy? *Steroids*. 44: 383-417, 1984.

289. **Morohashi K, Fujii-Kuriyama Y, Okada Y, Sogawa Y, Hirose T, Inayama S, Omura T**. Molecular cloning and nucleotide sequence of cDNA for mRNA or mitochondrial cytochrome P-450 (SCC) of bovine adrenal cortex. *Proceedings of the National Academy of Sciences of the United States of America*. 81: 4647-4651, 1984.

290. **Morohashi K, Sogawa K, Omura T, Fujii-Kuriyama Y**. Gene structure of human cytochrome P-450(scc), cholesterol desmolase. *Journal of Biochemistry*. 101: 879-887, 1987.

291. **Morohashi K, Yoshioka H, Gotoh O, Okada Y, Yamamoto K, Miyata T, Sogawa K, Fujii-Kuriyama Y, Omura T**. Molecular cloning and nucleotide sequence of DNA of mitochondrial cytochrome P-450(11β) of bovine adrenal cortex. *Journal of Biochemistry*. 102: 559-568, 1987.

292. **Mountjoy KG, Robbins LS, Mortrud MT, Cone RD**. The cloning of a family of genes that encode the melanocortin receptors. *Science*. 257: 1248-1251, 1992.

293. **Mulvogue HM, McMillen IC, Robinson PM, Perry RA**. Immunocytochemical localisation of pro-γ-MSH, γ-MSH, ACTH and β-endorphin/β-lipotrophin in fetal sheep pituitary: an ontogenetic study. *Journal of Developmental Physiology*. 8: 355-368, 1986.

294. **Murotsuki J, Challis JRG, Johnston L, Gagnon R**. Increased fetal plasma prostaglandin E2 concentrations during fetal placental embolization in pregnant sheep. *American Journal of Obstetrics and Gynecology*. 173: 30-35, 1995.

295. **Murotsuki J, Gagnon R, Matthews SG, Challis JRG**. Effects of long-term hypoxemia on pituitary-adrenal function in fetal sheep. *American Journal of Physiology*. 271: E678-E685, 1996.

296. **Myers DA, Ding XY, Nathanielsz PW**. Effect of fetal adrenalectomy on messenger ribonucleic acid for proopiomelanocortin in the anterior pituitary and for corticotrophin releasing hormone in the paraventricular nucleus of the ovine fetus. *Endocrinology*. 128: 2985-2991, 1991.

297. **Myers DA, McDonald TJ, Nathanielsz PW.** Effect of bilateral lesions of the ovine fetal hypothalamic paraventricular nuclei at 118-122 days of gestation on subsequent adrenocortical steroidogenic enzyme gene expression. *Endocrinology.* 131(1): 305-310, 1992a.

298. **Myers DA, McDonald TJ, Nathanielsz PW.** Effect of placement of dexamethasone adjacent to the ovine fetal paraventricular nucleus on adrenocortical steroid hydroxylase messenger ribonucleic acid. *Endocrinology.* 131(3): 1329-1335, 1992b.

299. **Naaman E, Chatelain PG, Saez JM, Duand P.** In vitro effect of insulin-like growth factor-I on cell multiplication and adrenocorticotropin responsiveness of fetal adrenal cells. *Biology of Reproduction.* 40: 570-577, 1989.

300. **Naaman-Reperant E, Cathiard AM, Durand PH.** Ontogenesis of cholesterol side-chain cleavage activity in the ovine adrenal gland. *Journal of Steroid Biochemistry and Molecular Biology.* 43: 301-310, 1992.

301. **Nakanishi S, Inoue A, Kita T, Nakamura M, Chang ACY, Cohen SN, Numa S.** Nucleotide sequence of cloned cDNA for bovine corticotropin-β-lipotropin precursor. *Nature.* 278: 423-427, 1979.

302. **Naseeruddin SA, Hornsby PJ.** Regulation of 11β- and 17α-hydroxylases in cultured bovine adrenocortical cells: 3', 5'-cyclic adenosine monophosphate, insulin-like growth factor-I, and activators of protein kinase C. *Endocrinology.* 127: 1673-1680, 1990.

303. **Naville D, Rainey WE, Mason JI.** Corticotropin regulation of 3β-hydroxysteroid dehydrogenase in ovine adrenocortical cells; inhibition by transforming growth factor-β. *Molecular and Cellular Endocrinology.* 75: 257-263, 1991.

304. **Neill JD, Smith PF, Luque EH, Munzo deToro M, Nagy G, Mulchahey JJ.** Detection and measurement of hormone secretion from individual pituitary cells. *Recent Progress in Hormone Research.* 43: 175-229, 1987.

305. **Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW.** P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics.* 6(1): 1-42, 1996.

306. **Nishikawa T, Sasano H, Omura M, Suematsu S.** Regulation of expression of the steroidogenic acute regulatory (StAR) protein by ACTH in bovine adrenal fasciculata cells. *Biochemical and Biophysical Research Communications.* 223: 12-18, 1996.

307. **Nissley SP, Haskell JF, Sasaki N, De Vroede MA, Rechler MM.** Insulin-like growth factor receptors. *Journal of Cell Science. Supplement.* 3: 39-51, 1985.

308. **Nissley SP, Rechler MM.** Somatomedin/insulin-like growth factor tissue receptors. *Clinics in Endocrinology and Metabolism.* 13(1): 43-67, 1984.

309. **Nonaka Y, Matsukawa N, Morohashi K, Omura T, Ogihara T, Teraska H, Okamoto M.** Molecular cloning and sequence analysis of cDNA encoding rat adrenal cytochrome P450$_{11\beta}$. *FEBS Letters.* 255: 21-26, 1989.

310. **Norman AW, Litwack G.** Adrenal corticoids. In: Hormones, 2nd edition. Norman AW, Litwack G (editors). Academic Press, Orlando, Florida, pp. 281-317, 1997.

311. **Norman LJ, Challis JRG.** Synergism between systemic corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin release in vivo varies as a function of gestational age in the ovine fetus. *Endocrinology.* 120(3): 1052-1058, 1987.

312. **Norman LJ, LyeSJ, Wlodek ME, Challis JRG.** Changes in pituitary responses to synthetic ovine corticotrophin releasing factor in fetal sheep. *Canadian Journal of Physiology and Pharmacology.* 63: 1398-1403, 1985.

313. **Nussdorfer GG.** Cytophysiology of the adrenal cortex. *International Review of Cytology.* 98: 1-405, 1986.

314. **Nylund L, Lunell N-O, Lewander R, Sarby B.** Uteroplacental blood flow index in intrauterine growth retardation of fetal or maternal origin. *British Journal of Obstetrics and Gynaecology.* 90: 16-20, 1983.

315. **Olson DM, Lye SJ, Challis JRG.** Prostaglandin concentrations in ovine maternal and fetal tissues at late gestation. *Pediatric Research.* 20: 83-86, 1986.

316. **Orchinik M, Murray TF, Moore FL.** A corticosteroid receptor in neural membranes. *Science.* 252: 1848-1851, 1991.

317. **Owens JA.** Endocrine and substrate control of fetal growth: placental and maternal influences and insulin-like growth factors. *Reproduction, Fertility and Development.* 3: 501-517, 1991.

318. **Owens JA, Falconer J, Robinson JS.** Effect of restriction of placental growth on umbilical and uterine blood flows. *American Journal of Physiology.* 250: R427-R434, 1986.

319. **Owens JA, Falconer J, Robinson JS.** Effect of restriction of placental growth on oxygen delivery to and consumption by the pregnant uterus and fetus. *Journal of Developmental Physiology.* 9: 137-150, 1987a.

320. **Owens JA, Falconer J, Robinson JS.** Effect of restriction of placental growth on fetal and utero-placental metabolism. *Journal of Developmental Physiology.* 9: 225-238, 1987b.

321. **Owens JA, Falconer J, Robinson JS.** Glucose metabolism in pregnant sheep when placental growth is restricted. *American Journal of Physiology.* 257: R350-R357, 1989.

322. **Owens JA, Kind KL, Carbone F, Robinson JS, Owens PC.** Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology.* 140: 5-13, 1994.

323. **Owens JA, Kind K, Smeaton TC, Lord APD, Robinson JS, Owens PC.** Circulating insulin-like growth factors (IGF) and IGF binding protein-1 are acutely regulated by glucose in fetal sheep in late gestation. (Abstract P1-542). Proceedings of the 10th International Congress of Endocrinology, San Francisco, USA. June 12th-15th, 1996.

324. **Owens JA, Owens PC, Robinson JS**.   Experimental fetal growth retardation: metabolic and endocrine aspects.   In: Advances in fetal physiology, research in perinatology, volume 8.   Gluckman PD, Johnston BM, Nathanielsz PW (editors). Perinatology Press, Ithaca, New York, pp. 263-286, 1989.

325. **Ozolins IZ, Antolovich GC, Browne CA, Perry RA, Robinson PM, Silver M, McMillen IC**. Effect of adrenalectomy or long term cortisol or CRF infusion on the concentration and molecular weight distribution of ACTH in fetal sheep plasma. *Endocrinology*. 129: 1942-1950, 1991.

326. **Ozolins IZ, Young IR, McMillen IC**.   Effect of cortisol infusion on basal and corticotropin-releasing factor (CRF)-stimulated plasma ACTH concentrations in the sheep fetus after surgical isolation of the pituitary. *Endocrinology*. 127: 1833-1840, 1990.

327. **Ozolins IZ, Young IR, McMillen IC**.   Surgical disconnection of the hypothalamus from the fetal pituitary abolishes the corticotrophic response to intrauterine hypoglycemia or hypoxemia in the sheep during late gestation. *Endocrinology*. 130(5): 2438-2445, 1992.

328. **Pedersen RC, Brownie AC**. Adrenocortical response to corticotropin is potentiated by part of the amino-terminal region of pro-corticotropin/endorphin. *Proceedings of the National Academy of Sciences of the United States of America*. 77: 2239-2243, 1980.

329. **Pedersen RC, Brownie AC**. Lys-γ_3-melanotropin binds with high affinity to the rat adrenal cortex. *Endocrinology*. 112(4): 1279-1287, 1983.

330. **Pedersen RC, Brownie AC**. γ_3-melanotropin promotes mitochondrial cholesterol accumulation in the rat adrenal cortex. *Molecular and Cellular Endocrinology*. 50: 149-156, 1987a.

331. **Pedersen RC, Brownie AC**. High density lipoprotein influences the response of rat adrenocortical cells to γ_3-melanotropin. *Molecular and Cellular Endocrinology*. 50: 157-164, 1987b.

332. **Pedersen RC, Brownie AC, Ling N**.   Pro-adrenocorticotropin/endorphin-derived peptides: Coordinate action on adrenal steroidogenesis. *Science*. 208: 1044-1046, 1980.

333. **Penhoat A, Chatelain PG, Jaillard C, Saez JM**.   Characterization of insulin-like growth factor I and insulin receptors on cultured bovine adrenal fasciculata cells. Role of these peptides on adrenal cell function. *Endocrinology*. 122: 2518-2526, 1988.

334. **Penhoat A, Jaillard C, Saez JM**. Synergistic effect of corticotropin and insulin-like growth factor I on corticotropin receptors and corticotropin responsiveness in cultured bovine adrenocortical cells. *Biochemical and Biophysical Research Communications*. 165: 355-359, 1989.

335. **Penhoat A, Leduque P, Jaillard C, Chatelain PG, Dubois PM, Saez JM**. ACTH and angiotensin II regulation of IGF-I and its binding proteins in cultured bovine adrenal cells. *Journal of Molecular Endocrinology*. 7: 223-232, 1991.

336. **Penhoat A, Naville D, Jaillard C, Chatelain PG, Saez JM**. Hormonal regulation of insulin-like growth factor I secretion by bovine adrenal cells. *Journal of Biological Chemistry*. 264: 6858-6862, 1989.

337. **Perrard-Sapori MH, Chatelain PG, Jaillard C, Saez JM**. Characterization and regulation of somatomedin-C/insulin-like growth factor I (Sm-C/IGF-I) receptors on cultured pig Leydig cells. Effects of Sm-C/IGF-I on luteotropin receptors and steroidogenesis. *European Journal of Biochemistry*. 165(1): 209-214, 1987.

338. **Perry RA, Mulvogue HM, McMillen IC, Robinson PM**. Immunohistochemical localization of ACTH in the adult and fetal sheep pituitary. *Journal of Developmental Physiology*. 7: 397-404, 1985.

339. **Pham-Huu-Trung MT, Binoux M**. Insulin-like growth factor I (IGF-I) induces cortisol production in bovine adrenocortical cells in primary culture. *Journal of Steroid Biochemistry*. 36: 583-588, 1990.

340. **Philipps AF, Persson B, Hall K, Lake M, Skottner A, Sanengen T, Sara VR**. The effects of biosynthetic insulin-like growth factor/supplementation on somatic growth, maturation and erythropoiesis on the neonatal rat. *Pediatric Research*. 23(3): 298-305, 1988.

341. **Phillips ID, Fielke SL, Young IR, McMillen IC**. The relative roles of the hypothalamus and cortisol in the control of prolactin gene expression in the anterior pituitary of the sheep fetus. *Journal of Neuroendocrinology*. 8: 929-933, 1996.

342. **Phillips ID, Ross JT, Owens JA, Young IR, McMillen IC**. The peptide ACTH(1-39), adrenal growth and steroidogenesis in the sheep fetus after disconnection of the hypothalamus and pituitary. *Journal of Physiology*. 491: 871-879, 1996.

343. **Phillips ID, Simonetta G, Owens JA, Robinson JS, Clarke IJ, McMillen IC**. Placental restriction alters the functional development of the pituitary-adrenal axis in the sheep fetus during late gestation. *Pediatric Research*. 40: 1-6, 1996.

344. **Picard-Hagen N, Darbeida H, Durand PH**. Glucocorticoids enhance the cholesterol side-chain cleavage activity of ovine adrenocortical mitochondria. *Journal of Steroid Biochemistry and Molecular Biology*. 55(1): 57-65, 1995.

345. **Picard-Hagen N, Penhoat A, Hue D, Jaillard C, Durand P**. Glucocorticoids enhance corticotropin receptor mRNA levels in ovine adrenocortical cells. *Journal of Molecular Endocrinology*. 19(1): 29-36, 1997.

346. **Pillion DJ, Arnold P, Yand M, Stockard CR, Grizzle WE**. Receptors for insulin and insulin-like growth factor I in human adrenal gland. *Biochemical and Biophysical Research Communications*. 165: 204-211, 1989.

347. **Pillion DJ, Yang M, Grizzle W**. Distribution of receptors for insulin and insulin-like growth factor I (somatomedin C) in the adrenal gland. *Biochemical and Biophysical Research Communications*. 154: 138-145, 1988.

348. **Poore KR, Young IR, Canny BJ, Thorburn GD**. Studies on the role of ACTH in the regulation of adrenal responsiveness and the timing of parturition in the ovine fetus. *Journal of Endocrinology*. 158: 161-171, 1998.

349. **Power SGA, Challis JRG.** Tissue specific concentration changes in estrone and estradiol during spontaneous and ACTH-induced parturition in sheep. *Canadian Journal of Physiology and Pharmacology.* 65: 130-135, 1987.

350. **Rainey WE, Naville D, Cline N, Mason JI.** Prostaglandin E2 is a positive regulator of adrenocorticotopin receptors, 3β hydroxysteroid dehydrogenase and 17α hydroxylase expression in bovine adrenocortical cells. *Endocrinology.* 129: 1333-1339, 1991.

351. **Rainey WE, Oka K, Magness RR, Mason JI.** Ovine fetal adrenal synthesis of cortisol: regulation by adrenocorticotropin, angiotensin II and transforming growth factor-β. *Endocrinology.* 129: 1784-1790, 1991.

352. **Rainey WE, Viard I, Saez JM.** Transforming growth factor β treatment decreases ACTH receptors on ovine adrenocortical cells. *Journal of Biological Chemistry.* 264: 21474-21477, 1989.

353. **Ramachandran J, Suyama AT.** Inhibition of replication of normal adrenocortical cells in culture by adrenocorticotropin. *Proceedings of the National Academy of Sciences of the United States of America.* 72: 113-117, 1975.

354. **Rao AJ, Long JA, Ramachandran J.** Effects of antiserum to adrenocorticotropin on adrenal growth and function. *Endocrinology.* 102: 371-378, 1978.

355. **Rechler MM, Nissley SP.** The nature and regulation of the receptors for insulin-like growth factors. *Annual Review of Physiology.* 47: 425-442, 1985.

356. **Rechler MM, Nissley SP.** Insulin-like growth factor (IGF)/somatomedin receptor subtypes: structure, function, and relationships to insulin receptors and IGF carrier proteins. *Hormone Research.* 24(2-3): 152-159, 1986.

357. **Rieger D, Luciano AM, Modina S, Pocar P, Lauria A, Gandolfi F.** The effects of epidermal growth factor and insulin-like growth factor I on the metabolic activity, nuclear maturation and subsequent development of cattle oocytes in vitro. *Journal of Reproduction and Fertility.* 112(1): 123-130, 1998.

358. **Riley SC, Boshier DP, Luu-The V, Labrie F, Challis JRG.** Immunohistochemical localization of 3β-hydroxysteroid / Δ^5-Δ^4-isomerase, tyrosine hydroxylase and phenylethanolamine *N*-methyl transferase in adrenal glands of sheep fetuses throughout gestation and in neonates. *Journal of Reproduction and Fertility.* 96: 127-134, 1992.

359. **Roberts JL, Herbert E.** Characterization of a common precursor to corticotropin and β-lipotropin: identification of β-lipotropin peptides and their arrangement relative to corticotropin in the precursor synthesised in the cell-free system. *Proceedings of the National Academy of Sciences of the United States of America.* 74: 5300-5304, 1977.

360. **Robinson JS.** Fetal growth. In: Obstetrics. Chamberlain GVP, Turnbull AC (editors). Churchill Livingstone, London, pp. 141-150, 1989.

361. **Robinson JS, Falconer J, Owens JA.** Intrauterine growth retardation: clinical and experimental. *Acta Paediatrica Scandinavica (Supplemental).* 319: 135-142, 1985.

362. **Robinson JS, Hart IC, Kingston EJ, Jones CT, Thorburn GD**. Studies on the growth of the fetal sheep. The effects of reduction of placental size on hormone concentration in fetal plasma. *Journal of Developmental Physiology*. 2: 239-248, 1980.

363. **Robinson JS, Jones CT, Kingston EJ**. Studies on experimental growth retardation in the sheep. The effects of maternal hypoxaemia. *Journal of Developmental Physiology*. 5(2): 89-100, 1983.

364. **Robinson JS, Kingston EJ, Jones CT, Thorburn GD**. Studies on experimental growth retardation in sheep. The effect of removal of endometrial caruncles on fetal size and metabolism. *Journal of Developmental Physiology*. 1: 379-398, 1979.

365. **Robinson JS, Owens JA, Owens PC**. Fetal growth and growth retardation. In: Textbook of fetal physiology. Thorburn GD, Harding R (editors). Oxford University Press, Oxford, UK, pp. 83-94, 1994.

366. **Robinson PM, Comline RS, Fowden AL, Silver M**. Adrenal cortex of fetal lamb: changes after hypophysectomy and effects of synacthen on cytoarchitecture and secretory activity. *Quarterly Journal of Experimental Physiology*. 68: 15-27, 1983.

367. **Robinson PM, Rowe EJ, Wintour EM**. The histogenesis of the adrenal cortex in the foetal sheep. *Acta Endocrinologica*. 91: 134-149, 1979.

368. **Roebuck MM, Jones CT, Holland D, Silman R**. In vitro effects of high molecular weight forms of ACTH on the fetal sheep. *Nature*. 284: 616-618, 1980.

369. **Rose JC, MacDonald AA, Heymann MA, Rudolph AM**. Developmental aspects of the pituitary-adrenal axis response to hemorrhagic stress in lamb fetuses in utero. *Journal of Clinical Investigation*. 61(2): 424-432, 1978.

370. **Rose JC, Meis PJ, Morris M**. Ontogeny of endocrine (ACTH, vasopressin, cortisol) responses to hypotension in lamb fetuses. *American Journal of Physiology*. 240: E656-E661, 1981.

371. **Rose JC, Meis PJ, Urban RB, Greiss FC**. In vivo evidence for increased adrenal sensitivity to adrenocorticotropin-(1-24) in the lamb fetus in late gestation. *Endocrinology*. 111: 80-85, 1982.

372. **Ross JT, Phillips ID, Owens JA, McMillen IC**. Cortisol differentially regulates pituitary-adrenal function in the sheep fetus after disconnection of the hypothalamus and pituitary. *Journal of Neuroendocrinology*. 9: 663-668, 1997.

373. **Rurak DW, Richardson BS, Patrick JE, Carmichael L, Homan J**. Blood flow and oxygen delivery to fetal organs and tissues during sustained hypoxemia. *American Journal of Physiology*. 258: R1116-R1122, 1990.

374. **Saez JM, Durand P, Cathiard AM**. Ontogeny of the ACTH receptor, adenylate cyclase and steroidogenesis in adrenal. *Molecular and Cellular Endocrinology*. 38: 93-102, 1984.

375. **Saez JM, Morera AM, Gallet D**. Opposite effects of ACTH and glucocorticoids on adrenal DNA synthesis in vivo. *Endocrinology*. 100: 1268-1275, 1977.

376. **Sambrook K, Fritsch EF, Maniatis T**. Molecular cloning, a laboratory manual, volume 1, 2nd edition. Cold Spring Harbor Laboratory Press, New York, USA, pp. 1.82-1.84, 1989.

377. **Saphier PW, Glynn BP, Woods RJ, Shepherd DAL, Jeacock MK, Lowry PJ**. Elevated levels of N-terminal pro-opiomelanocortin peptides in fetal sheep plasma may contribute to fetal adrenal gland development and the pre-parturient cortisol surge. *Endocrinology*. 133: 1459-1461, 1993.

378. **Sapolsky RM, Uno H, Rebert CS, Finch CE**. Hippocampal damage associated with prolonged glucocorticoid exposure in primates. *Journal of Neuroscience*. 10: 2897-2902, 1990.

379. **Sara VR, Hall K**. Insulin-like growth factors and their binding proteins. *Physiological Reviews*. 707: 591-614, 1990.

380. **Sasano H, Mason JI, Sasano N**. Immunohistochemical study of cytochrome P-450 17α in human adrenocortical disorders. *Human Pathology*. 20: 113-117, 1989.

381. **Sasano H, Sasano N, Okamoto M**. Immunohistochemical demonstration of cholesterol side-chain cleavage cytochrome P-450 in bovine and human adrenals. *Pathology, Research and Practice*. 184(5): 337-342, 1989.

382. **Sato SM, Mains RE**. Plasticity in the adrenocorticotropin-related peptides produced by primary cultures of rat neonatal pituitary. *Endocrinology*. 122: 68-77, 1988.

383. **Schmand B, Neuvel J, Haas DeSmolders H, Hoeks J, Treffers PE, Koppe JG**. Psychological development of children who were treated antenatally with corticosteroids to prevent respiratory distress syndrome. *Pediatrics*. 86: 58-64, 1990.

384. **Schwartz J, Ash P, Ford V, Raff H, Crosby S, White A**. Secretion of adrenocorticotrophin (ACTH) and ACTH precursors in ovine pituitary cells: actions of CRF, AVP and glucocorticoids. *Journal of Endocrinology*. 140: 189-195, 1994.

385. **Schwartz J, Kleftogiannis F, Jacobs R, Thorburn GD, Crosby SR, White A**. Biological activity of adrenocorticotropic hormone precursors on ovine adrenal cells. *American Journal of Physiology (Endocrinology and Metabolism)*. 268(31): E623-E629, 1995.

386. **Schwartz J, Vale W**. Dissociation of the adrenocorticotropin secretory responses to corticotropin-releasing factor (CRF) and vasopressin or oxytocin by using a specific cytotoxic analog of CRF. *Endocrinology*. 122: 1695-1700, 1988.

387. **Seger MA, Bennett HPJ**. Structure and bioactivity of the amino-terminal fragment of pro-opiomelanocortin. *Journal of Steroid Biochemistry*. 25: 703-710, 1986.

388. **Seidah NG, Chretien M, Day R**. The family of subtilisin/kexin like pro-protein and pro-hormone convertases: Divergent or shared functions. *Biochimie*. 76: 197-209, 1994.

389. **Seidah NG, Marcinkiewicz M, Benjannet S, Gaspar L, Beaubien G, Mattei MG, Lazure C, Mbikay M, Chretien M**. Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. *Molecular Endocrinology*. 5: 111-122, 1991.

390. **Shibasaki T, Ling N, Guillemin R**. Pituitary immunoreactive γ-melanotropins are glycosylated oligopeptides. *Nature.* 285(5764): 416-417, 1980.

391. **Shibasaki T, Masui H, Sato G, Ling, Guillemin R**. Secretion of pro-opiomelanocortin-derived peptides by a pituitary adenoma from a patient with Cushing's disease. *Journal of Clinical Endocrinology and Metabolism.* 52(2): 350-353, 1981.

392. **Shigematsu K, Niwa M, Kurihara M, Yamashita K, Kawai K, Tsuchiyama H**. Receptor autoradiographic localization of insulin-like growth factor-I (IGF-I) binding sites in human fetal and adult adrenal glands. *Life Sciences.* 45: 383-389, 1989.

393. **Short JM, Fernandez JM, Sorge JA, Huse WD**. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Research.* 16(15): 7583-7600, 1988.

394. **Silman RE, Chard T, Lowry PJ, Smith I, Young IM**. Human foetal pituitary peptides and parturition. *Nature.* 260: 716-718, 1976.

395. **Silman RE, Holland D, Chard T, Lowry PJ, Hope J, Rees LH, Thomas A, Nathanielsz PW**. ACTH related peptides in adult and fetal sheep pituitary glands. *Journal of Endocrinology.* 123: 371-377, 1979.

396. **Silman RE, Holland D, Chard T, Lowry PJ, Hope J, Robinson JS, Thorburn GD**. The ACTH 'family tree' of the rhesus monkey changes with development. *Nature.* 276: 526-528, 1978.

397. **Simonetta G, Rourke AK, Owens JA, Robinson JS, McMillen IC**. Impact of placental restriction on the development of the sympathoadrenal system. *Pediatric Research.* 42(6): 805-811, 1997.

398. **Simonetta G, Walker DW, McMillen IC**. Effect of feeding on the diurnal rhythm of plasma cortisol and adrenocorticotrophic hormone concentrations in the pregnant ewe and sheep fetus. *Experimental Physiology.* 76(2): 219-229, 1991.

399. **Simonian MH, Gill GN**. Regulation of the fetal human adrenal cortex: effects of adrenocorticotrophin on growth and function of monolayer cultures of fetal and definitive zone cells. *Endocrinology.* 108: 1769-1775, 1981.

400. **Simpson ER, Waterman MR**. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annual Review of Physiology.* 50: 427-440, 1988.

401. **Smolders-de Haas H, Neuvel J, Schmand B, Treffers PE, Koppe JG, Hoeks J**. Physical development and medical history of children who were treated antenatally with corticosteroids to prevent respiratory distress syndrome: a 10 to 12 year follow-up. *Pediatrics.* 86(1): 65-70, 1990.

402. **Stocco DM, Clark BJ**. Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews.* 17(3): 221-244, 1996.

403. **Sutcliffe JG**. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harbor Symposia on Quantitative Biology.* 43(1): 77-90, 1979.

404. **Szabo L, Mottershead DG, Ballard FJ, Wallace JC**. The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-I for binding. *Biochemical and Biophysical Research Communications*. 151: 207-214, 1988.

405. **Tanaka I, Nakai Y, Jingami H, Fukata J, Nakao K, Oki S, Nakanishi S, Numa S, Imura H**. Existence of γ-melanotropin (γ-MSH)-like immunoreactivity in bovine and human pituitary gland. *Biochemical and Biophysical Research Communications*. 94(1): 211-217, 1980.

406. **Tanaka S, Nomizu M, Kurosumi K**. Intracellular sites of proteolytic processing of pro-opiomelanocortin in melanotrophs and corticotrophs in the rat pituitary. *Journal of Histochemistry and Cytochemistry*. 39(6): 809-821, 1991.

407. **Tangalakis K, Coghlan JP, Connell J, Crawford R, Darling P, Hammond VE, Haralambidis J, Penshow J, Wintour EM**. Tissue distribution and levels of gene expression of three steroid hydroxylases in ovine fetal adrenal glands. *Acta Endocrinologica*. 120: 225-232, 1989.

408. **Tangalakis K, Coghlan JP, Crawford R, Hammond VE, Wintour EM**. Steroid hydroxylase gene expression in the ovine adrenal gland following ACTH infusion. *Acta Endocrinologica*. 123: 371-377, 1990.

409. **Tangalakis K, Lumbers ER, Moritz KM, Towstoless MK, Wintour EM**. Effect of cortisol on blood pressure and vascular reactivity in the ovine fetus. *Experimental Physiology*. 77: 709-717, 1992.

410. **Tapanainen PJ, Bang P, Wilson K, Unterman TG, Vreman HJ, Rosenfeld RG**. Maternal hypoxia as a model for intrauterine growth retardation: effects on insulin-like growth factors and their binding proteins. *Pediatric Research*. 36: 152-158, 1994.

411. **Toney K, Bateman A, Gagnon C, Bennett HPJ**. Aspartimide formation in the joining peptide sequence of porcine and mouse pro-opiomelanocortin. *Journal of Biological Chemistry*. 268: 1024-1031, 1993.

412. **Townsend SF, Dallman MF, Miller WL**. Rat insulin-like growth factor-I and -II mRNAs are unchanged during compensatory adrenal growth but decrease during ACTH-induced adrenal growth. *Journal of Biological Chemistry*. 265: 22117-22122, 1990.

413. **Uno H, Lohmiller L, Thieme C, Kemnitz JW, Engle MJ, Roecker EB, Farrel PM**. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. *Developmental Brain Research*. 53: 157-167, 1990.

414. **Uno H, Tarara R, Else JG, Suleman MA, Sapolsky RM**. Hippocampal damage associated with prolonged and fatal stress in primates. *Journal of Neuroscience*. 9: 1705-1711, 1989.

415. **Upton FZ, Szabo L, Wallace JC, Ballard FJ**. Characterization and cloning of a bovine insulin-like growth factor binding protein. *Journal of Molecular Endocrinology*. 5: 77-84, 1990.

416. **van Dijk JP, Tansswell AK, Challis JRG**. Insulin-like growth factor (IGF)-II and insulin, but not IGF-I are mitogenic for fetal rat adrenal cells in vitro. *Journal of Endocrinology*. 119: 509-516, 1988.

417. **Veldhuis JD, Rodgers RJ**. Mechanisms subserving the steroidogenic synergism between follicle-stimulating hormone and insulin-like growth factor I (somatomedin C). Alterations in cellular sterol metabolism in swine granulosa cells. *Journal of Biological Chemistry*. 262(16): 7658-7664, 1987.

418. **Veldhuis JD, Rodgers RJ, Dee A, Simpson ER**. The insulin-like growth factor, somatomedin C, induces the synthesis of cholesterol side-chain cleavage cytochrome P-450 and adrenodoxin in ovarian cells. *Journal of Biological Chemistry*. 261: 2499-2502, 1986.

419. **Vileisis RA, D'Ercole**. Tissue and serum concentrations of somatomedin-C / insulin-like growth factor 1 in fetal rats made growth retarded by uterine artery ligation. *Pediatric Research*. 20: 126-130, 1986.

420. **Vinson GP, Pudney JA, Whitehouse BJ**. The mammalian adrenal circulation and the relationship between adrenal blood flow and steroidogenesis. *Journal of Endocrinology*. 105: 285-294, 1985.

421. **Voutilainen R, Miller WL**. Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, $P450_{scc}$, in human steroidogenic tissues. *Proceedings of the National Academy of Sciences of the United States of America*. 84: 1590-1594, 1987.

422. **Voutilainen R, Miller WL**. Developmental and hormonal regulation of mRNAs for insulin-like growth factor II and steroidogenic enzymes in human fetal adrenals and gonads. *DNA*. 7: 9-15, 1988.

423. **Walker BR, Williams BC**. Corticosteroids and vascular tone: mapping the messenger maze. *Clinical Science*. 82: 597-605, 1992.

424. **Wang HS, Lim J, English J, Irvine L, Chard T**. The concentration of insulin-like growth factor-I and insulin-like growth factor binding protein-1 in human umbilical cord serum at delivery: relationship to birth weight. *Journal of Endocrinology*. 129: 459-464, 1991.

425. **Webb PD**. Development of the adrenal cortex in the fetal sheep: an ultrastructural study. *Journal of Developmental Physiology*. 2: 161-181, 1980.

426. **Weber MM, Kiess W, Beikler T, Simmler P, Reichel M, Adelmann B, Kessler U, Engelhardt D**. Identification and characterization of insulin-like growth factor I (IGF-I) and IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors in bovine adrenal cells. *European Journal of Endocrinology*. 130: 265-270, 1994.

427. **White PC**. Genetic diseases of steroid metabolism. *Vitamins and Hormones*. 49: 131-195, 1994.

428. **White PC, Dupont B, New MI, Leiberman E, Hochberg Z, Rosler A**. A mutation in *CYP11B1* (Arg[488]-His) associated with steroid 11β-hydroxylase deficiencies in Jews of Moroccan origin. *Journal of Clinical Investigation*. 87: 1664-1667, 1991.

429. **White PC, New MI, DuPont B**. Cloning and expression of cDNA encoding a bovine adrenal cytochrome P-450 specific for steroid 21-hydroxylation. *Proceedings of the National Academy of Sciences of the United States of America.* 81: 1986-1990, 1984.

430. **White PC, New MI, DuPont B**. Structure of human steroid 21-hydroxylase genes. *Proceedings of the National Academy of Sciences of the United States of America.* 83: 5111-5115, 1986.

431. **Whorwood CB, Franklyn JA, Sheppard MC, Stewart PM**. Tissue localization of 11β-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. *Journal of Steroid Biochemistry and Molecular Biology.* 41: 21-28, 1992.

432. **Wintour EM**. Developmental aspects of the hypothalamic-pituitary-adrenal axis. *Journal of Developmental Physiology.* 6: 291-299, 1984.

433. **Wintour EM, Brown EH, Denton DA, Hardy KJ, McDougall JG, Oddie CJ, Whipp GT**. The ontogeny and regulation of corticosteroid secretion by the ovine foetal adrenal. *Acta Endocrinologica.* 79: 301-316, 1975.

434. **Wong, M, Ikeda Y, Luo X, Caron KM, Weber TJ, Swain A, Schimmer BP, Parker KL**. Steroidogenic factor 1 plays multiple roles in endocrine development and function. *Recent Progress in Hormone Research.* 52: 167-182, 1997.

435. **Wood CE, Cheung CY, Brace RA**. Fetal heart rate, arterial pressure, and blood volume responses to cortisol infusion. *American Journal of Physiology.* 253: R904-R909, 1987.

436. **Wright NA, Voncina D**. Studies on the postnatal growth of the rat adrenal cortex. *Journal of Anatomy.* 123: 147-156, 1977.

437. **Wright NA, Voncina D, Morley AR**. An attempt to demonstrate cell migration from the zona glomerulosa in the prepubertal male rat adrenal cortex. *Journal of Endocrinology.* 59: 451-459, 1973.

438. **Wylie AH, Kerr JFR, Currie AR**. Cell death in the normal neonatal rat adrenal cortex. *Journal of Pathology.* 111: 255-261, 1973.

439. **Xia P, Tekpetey FR, Armstrong DT**. Effect of IGF-I on pig oocyte maturation, fertilization, and early embryonic development in vitro, and on granulosa and cumulus cell biosynthetic activity. *Molecular Reproduction and Development.* 38(4): 373-379, 1994.

440. **Yang K, Berdusco ETM, Challis JRG**. Opposite effects of glucocorticoid on hepatic 11β-hydroxysteroid dehydrogenase mRNA activity in fetal and adult sheep. *Journal of Endocrinology.* 143: 121-126, 1994.

441. **Yang K, Challis JRG**. Fetal and adult sheep adrenal cortical cells contain glucocorticoid receptors. *Biochemical and Biophysical Research Communications.* 162(2): 604-611, 1989.

442. **Yang K, Matthews SG**. Cellular localization of 11β hydroxysteroid dehydrogenase 2 gene expression in the ovine adrenal gland. *Molecular and Cellular Endocrinology.* 111: R19-R23, 1995.

443. **Yang K, Smith CL, Dales D, Hammond GL, Challis JRG.** Cloning of an ovine 11 β-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid: tissue and temporal distribution of its messenger ribonucleic acid during fetal and neonatal development. *Endocrinology.* 131: 2120-2126, 1992.

444. **Yaswen L, Diehl N, Brennan MB, Hochgeschwender U.** Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nature Medicine.* 9: 1066-1070, 1999.

445. **Yoshioka H, Morohashi K, Sogawa K, Yamane M, Kominiami S, Takemori S, Okada Y, Omura T, Fujii-Kuriyama T.** Structural analysis of cloned cDNA for mRNA of microsomal cytochrome P-450(C21) which catalyses steroid 21-hydroxylation in bovine adrenal cortex. *Journal of Biological Chemistry.* 261: 4106-4109, 1986.

446. **Zajicek G, Ariel I, Arber N.** The streaming adrenal cortex: Direct evidence of centripetal migration of adrenocytes by estimation of cell turnover rate. *Journal of Endocrinology.* 111: 477-482, 1986.

447. **Zuber MX, John ME, Okamura T, Simpson ER, Waterman MR.** Bovine adrenocortical Cytochrome P-450$_{17\alpha}$: regulation of gene expression by ACTH and elucidation of primary sequence. *Journal of Biological Chemistry.* 261: 2475-2482, 1986.

APPENDICES

APPENDIX I   *SOLUTIONS AND REAGENTS*

SOLUTION   *SUPPLIER*

1 % agarose denaturing gel (100 ml)

1 g molecular biology grade agarose	BDH Laboratory Supplies
79 ml sterile ddH$_2$O	
5 ml 20× Northern running buffer	*(see below)*
16 ml deionised formaldehyde	APS Ajax Finechem
(add formaldehyde after molten agarose solution has cooled to ~55° C)	

1× cDNA hybridisation buffer

7 % w·v^{-1} SDS	BDH Laboratory Supplies
50 % v·v^{-1} deionised formamide	BDH Laboratory Supplies
5× SSPE	*(see below)*
100 μg·ml^{-1} of heat denatured salmon sperm DNA	Boehringer Mannheim Australia

50× Denhart's

10 g·l^{-1} Ficol type 400	Pharmacia
10 g·l^{-1} polyvinylpyrolidone	BDH Laboratory Supplies
10 g·l^{-1} BSA fraction V	Sigma-Aldrich

4 M guanidine hydrochloride solution

4 M guanidinium thiocyanate	Merck
25 mM sodium citrate	APS Ajax Finechem
0.5 % w·v^{-1} sodium laurylsarcosine	Sigma-Aldrich
3.3 μl·ml^{-1} Sigma antifoam A	Sigma-Aldrich
1 μl·ml^{-1} β-mercaptoethanol	BDH Laboratory Supplies

Luria-Bertani (LB) agar plates (pH 7.0)

10 g·l^{-1} bacto-tryptone	Difco Laboratories
5 g·l^{-1} bacto-yeast extract	Difco Laboratories
10 g·l^{-1} NaCl	BDH Laboratory Supplies
1.5 % w·v^{-1} bacto-agar	Difco Laboratories

Luria-Bertani (LB) broth (pH 7.0)

10 g·l^{-1} bacto-tryptone	Difco Laboratories
5 g·l^{-1} bacto-yeast extract	Difco Laboratories
10 g·l^{-1} NaCl	BDH Laboratory Supplies

20× Northern running buffer (pH 7.0)

400 mM MOPS	BDH Laboratory Supplies
(3-(N-morpholino) propane sulphonic acid)	
100 mM sodium acetate	Ajax Chemicals
10 mM EDTA	BDH Laboratory Supplies

1× oligonucleotide hybridisation buffer

7 % w·v^{-1} SDS	BDH Laboratory Supplies
5× SSC	(*see below*)
20 mM sodium dihydrogen orthophosphate monohydrate	BDH Laboratory Supplies
(NaH$_2$PO$_4$·H$_2$O)	
5× Denhart's	(*see above*)
100 μg·ml^{-1} of heat denatured salmon sperm DNA	Boehringer Mannheim Australia

70 mM phosphate buffered, 4 % paraformaldehyde solution (pH 7.0)

4 % w·v^{-1} paraformaldehyde	Sigma-Aldrich
45 mM disodium hydrogen orthophosphate anhydrous	BDH Laboratory Supplies
(Na$_2$HPO$_4$)	
25 mM sodium dihydrogen orthophosphate dihydrate	BDH Laboratory Supplies
(NaH$_2$PO$_4$·2H$_2$O)	
0.2 % v·v^{-1} glutaraldehyde	BDH Laboratory Supplies

100 mM phosphate buffered saline (pH 7.4)

(*PBS tablets*)	Sigma-Aldrich
1 tablets in 200 ml of sterile ddH$_2$O makes:	
100 mM phosphate buffer	
2.7 mM KCl	
137 mM NaCl	

1× RNA loading buffer

50 % v·v^{-1} glycerol	BDH Laboratory Supplies
1 mM EDTA	BDH Laboratory Supplies
4 mg·ml^{-1} bromophenol blue	BDH Laboratory Supplies
4 mg·ml^{-1} xylene cyanole	Sigma-Aldrich

SOC medium (pH 7.0)

20 g·l⁻¹ bacto-tryptone	Difco Laboratories
5 g·l⁻¹ bacto-yeast extract	Difco Laboratories
0.5 g·l⁻¹ NaCl	BDH Laboratory Supplies
10 mM MgCl$_2$	BDH Laboratory Supplies
20 mM glucose	BDH Laboratory Supplies

10× SSC (pH 7.0)

1.5 M NaCl	BDH Laboratory Supplies
150 mM sodium citrate	APS Ajax Finechem

5× SSPE (pH 7.4)

50 mM sodium dihydrogen orthophosphate monohydrate (NaH$_2$PO$_4$·H$_2$O)	BDH Laboratory Supplies
750 mM NaCl	BDH Laboratory Supplies
5 mM EDTA	BDH Laboratory Supplies

1× tris-acetate EDTA (TAE) (pH 8.0)

40 mM tris-acetate	BDH Laboratory Supplies
1 mM EDTA	BDH Laboratory Supplies

10× tris borate electrophoresis buffer (TBE) (pH 8.0)

900 mM tris base	Sigma-Aldrich
900 mM boric acid	BDH Laboratory Supplies
20 mM EDTA	BDH Laboratory Supplies

10 mM tris-EDTA (TE) buffer (pH 8.0)

10 mM tris HCl	Sigma-Aldrich
1 mM EDTA	BDH Laboratory Supplies

Ajax Chemicals
9 Short Rd., Auburn
New South Wales 2114, Australia

Ambion
2130 Woodward St., Austin
Texas 78744-1832, USA

Amersham International
White Lion Rd., Amersham
Buckinghamshire HP7 9LL
United Kingdom

Apple
1 Infinite Loop, Cupertino
California 95014, USA

APS Ajax Finechem
9 Short St., Auburn
New South Wales 2144, Australia

Baxter Healthcare
1 Baxter Dr., Old Toongabbie
New South Wales 2146, Australia

BDH Laboratory Supplies
Poole, Dorset BH15 1TD
United Kingdom

Beckman Coulter Australia
24 College St., Gladesville
New South Wales 2111, Australia

Berthold Australia
36b Clements Ave., Bundoora
Victoria 3083, Australia

Biorad Laboratories
(Headquarters)
1000 Alfred Nobel Dr., Hercules
California 94547, USA

Boehringer Mannheim Australia
31 Victoria Ave., Castle Hill
New South Wales 2154, Australia

Braun Medical AG
CH-6021 Emmenbrucke, Germany

Chem-Supply
62 Bedford St., Gillman
South Australia 5013, Australia

Clontech Laboratories
1020 East Meadow Circle, Palo Alto
California 94303, USA

Commonwealth Serum Laboratories
45 Poplar Rd., Parkville
Victoria 3052, Australia

Critchley Electrical Products
101 Deakin St., Auburn
New South Wales 2144, Australia

Crown Scientific
107 Ledger Rd., Beverley
South Australia 5009, Australia

David Bull Laboratories
7-23 Lexia Pl., Mulgrave
Victoria 3170, Australia

Difco Laboratories
PO Box 331058, Detroit
Michigan, USA

Eppendorf
Netheler-Hinz
Hamburg 22331, Germany

GeneWorks
39 Winwood St., Thebarton
South Australia 5031, Australia

Gilson
72 Rue Gambetta
95400 Villiers Le Bel
France

GroPep
Gate 11, Victoria Dr., Adelaide
South Australia 5000, Australia

Hawksley and Sons
Marlborough Rd., Lancing
West Sussex BN15 8TN
United Kingdom

Hoefer Scientific
654 Minnesota St., San Francisco
California 94107, USA

Hybaid
111-113 Waldegrave Rd.
Teddington, Middlesex TW11 8LL
United Kingdom

ICI
Australia Operations
Pharmaceuticals Division
1 Nicholson St., Melbourne
Victoria 3000, Australia

ICN Biomedicals Australasia
PO Box 187, 167 Prospect Hwy.
Unit 12 Seven Hills
New South Wales 2147, Australia

Johnson & Johnson
1 Khartoum Rd., North Ryde
New South Wales 2113, Australia

Kimberly-Clark
52 Alfred St., Milson Point
New South Wales 2061, Australia

Kinematica AG
Luzernerst 147a, CH-6014
Littau/Lucerne, Switzerland

Kodak Australasia
171 Elizabeth St., Coburg
Victoria 3058, Australia

Linde Gas
Head Office
66 Loftus Rd., Pinkeba
Queensland 4008, Australia

MBI Fermentas
Graiciuno 8
Vilnius 2028, Lithuania

Merck
207 Colchester Rd., Kilsyth
Victoria 3137, Australia

Millipore Australia
Private Bag 18, Lane Cove
New South Wales 2066, Australia

Molecular Dynamics
928 East Arques Ave., Sunnyvale
California 94086-4520, USA

National Diagnostics
305 Patton Dr., Atlanta
Georgia 30336, USA

National Institutes of Health
Bethesda
Maryland 20892, USA

Olympus Optical Company
Technology Research Institute
2951 Ishikawa-cho, Hachiojo-shi
Tokyo 192, Japan

Orion Diagnostica
PO Box 83
Espoo FIN-02101, Finland

Owl Scientific Plastics
PO Box 566, Cambridge
Massachusetts 02139, USA

Parke-Davis
32 Cawarra Rd., Caringbah
New South Wales 2229, Australia

Pel-Freez Biologicals
PO Box 68, Rogers
Arkansas 72757, USA

Pharmacia
59 Kirby St., Rydalmere
New South Wales 2116, Australia

Qiagen
17 Austin St., Fairfield
Victoria 3078, Australia

Radiometer Pacific
9B Crittenden Rd., Findon
South Australia 5023, Australia

Rhone Merieux Australia
261 Tingira St., Pinkeba
Queensland 4008, Australia

Rhone-Poulent Rorer Australia
7 Maitland Pl., NorWest Business Park
Baulkham Hills
New South Wales 2153, Australia

Ridley Agri Products
PO Box 596, Murray Bridge
South Australia 5253, Australia

Rye and Grains
53 Oaklands Rd., Summerton Park
South Australia 5044, Australia

Sarstedt Australia
PO Box 90, Inglefarm
South Australia 5098, Australia

Savant Instruments
100 Colin Dr., Holbrook
New York 11741-4306, USA

Sigma-Aldrich
PO Box 14508, St. Louis
Missouri 63176, USA

Sony
14 Lum St., Export Park
South Australia 5950, Australia

Stratagene
11011 North Torrey Rd., La Jolla
California 92037, USA

Troy Laboratories
98 Long St., Smithfield
New South Wales 2164, Australia

UpJohn
59 Kirby St., Rydalmere
New South Wales 2116, Australia

Vector Laboratories
30 Ingold Rd., Burlingame
California 94010, USA

Virbac Australia
15 Pritchard Pl., Peakhurst
New South Wales 2210, Australia

WDT
Siemensstrabe
14 D-30827 Garbsen, Germany

World Precision Instruments
175 Sarasota Center Boulevard, Sarasota
Florida 34240-9258, USA

APPENDIX III — INDEX OF REAGENTS, EQUIPMENT AND SUPPLIERS

APPENDIX IV — INTERNET RESOURCES

DESCRIPTION	INTERNET SITE
DNA sequence database	http://www.ncbi.nlm.nih.gov/
Journal literature database	http://igm.nlm.nih.gov/ http://www.ncbi.nlm.nih.gov/PubMed/
Meta-search engines	http://hotbot.lycos.com/ http://www.ncbi.nlm.nih.gov/ http://www.alexa.com/ http://www.altavista.com/ http://www.askjeeves.com/ http://www.metacrawler.com/ http://www.northernlight.com/

REFERENCE   *PAGE*

[1] Adams, Phillips *et al.*, 1998 188

[2] Adashi, Resnick *et al.*, 1988 ....50, 52

[3] Adashi, Resnick and Svoboda, 1986..52

[4] Adashi, Resnick *et al.*, 1984 50

[5] Adashi, Resnick *et al.*, 1985 54

[6] Akagi and Challis, 1990...4, 172, 209

[7] Akana, Dallman *et al.*, 1992..4

[8] Akana, Scribner *et al.*, 1992..4

[9] Al-Dujaili, Hope *et al.*, 1981...44, 155

[10] Al-Dujaili, Williams *et al.*, 1982..45, 155

[11] Alexander, 1964...59

[12] Alexander, 1968...22

[13] l'Allemand, Penhoat *et al.*, 1996...64, 190, 210

[14] Allen, 1984..57

[15] Anderson, Pierrepoint *et al.*, 1972 22

[16] Antolovich, Clarke *et al.*, 1990..28, 34, 73

[17] Antolovich, McMillen *et al.*, 1991...108

[18] Antolovich, McMillen *et al.*, 1992...110, 194

[19] Antolovich, Perry *et al.*, 1989...30, 35, 194

[20] Arafah, 1991..51, 158

[21] Ariyoshi, Kim *et al.*, 1998 11

[22] Baker, Liu *et al.*, 1993 ....46, 49, 50

[23] Bark, McNurlan *et al.*, 1998 46

[24] Barker, 1994..58

[25] Barker, Bull *et al.*, 1990 ....5, 6

[26] Barker, Osmond *et al.*, 1989 ....5, 6

[27] Barlow, Stoger *et al.*, 1991 47

[28] Barnes, Comline and Silver, 1977 ....35, 69

[29] Baserga, 1992 46

[30] Baserga, Porcu *et al.*, 1993 46

[31] Bassett and Thorburn, 1969 28

[32] Begeot, Langlois and Saez, 1989 ....53, 158, 169

[33] Bell, Myers and Myers, 1998 30

[34] Benediktsson, Lindsay *et al.*, 1993 ....5, 6, 212

[35] Bennett, 1983 136

[36] Bennett, 1986 30

[37] Bernier, Chatelain *et al.*, 1986 52

[38] Bertagna, Girard *et al.*, 1980 ....31, 40

[39] Bertagna, Seurin *et al.*, 1983 ....40, 45

[40] Bicknell, Hutchinson *et al.*, 1998 ....44, 151, 152, 154, 206

[41] Birch, Estivariz *et al.*, 1991 30

[42] Bird, Magness *et al.*, 1992 191

[43] Bird, Pasquarette *et al.*, 1996 ....191, 209

[44] Bird, Zheng *et al.*, 1996 141

[45] Bocking, Gagnon *et al.*, 1988 ....172, 188, 209

[46] Bocking, McMillen *et al.*, 1986 ....74, 172

[47] Boddy, Jones *et al.*, 1974 ....172, 209

[48] Boggaram, John *et al.*, 1989 27

[49] Bolivar, Rodriguez *et al.*, 1977 79

[50] Bonds, Gabbe *et al.*, 1984 57

[51] Bondy, Werner *et al.*, 1990 ....49, 168

[52] Boshier, Gavin and Holloway, 1989 105

[53] Boshier and Holloway, 1989 ....19, 20, 68, 104, 105

[54] Boshier and Holloway, 1991 ....18, 20

[55] Boshier, Holloway and Liggins, 1980 105

[56] Boshier, Holloway and Liggins, 1981 ....36, 37, 105, 113, 126, 132, 196, 203

[57] Boshier, Holloway *et al.*, 1989 3

[58] Braems, Han and Challis, 1998 ....65, 172, 189, 191

[59] Braems, Matthews and Challis, 1996 ....63, 64, 190, 210

[60] Bransome, 1968 18

[61] Brice, Cheetham *et al.*, 1989 ....49, 168

[62] Brieu and Durand, 1987 ....25, 31, 35

[63] Brieu and Durand, 1989 ....25, 31, 33, 35

[64] Brooke, Wood and Butters, 1984 ....58, 173, 188, 208

[65] Brown, Coghlan *et al.*, 1978 22

[66] Browne, Bennett and Solomon, 1981 136

[67] Butler, Schwartz and McMillen, 1999 35

[68] Campbell, Yu and Yang, 1996 ....38, 116

[69] Carr and Simpson, 1981 9

[70] Carr, Jacobs *et al.*, 1995 ....33, 211

[71] Carr, 1994 ....46, 69

[72] Carr, Owens *et al.*, 1995 ....108, 159

[73] Carrascosa and Audi, 1993 46

[74] Carter and Detmer, 1990 59

[75] Carter, Homan *et al.*, 1995 188

[76] Carter, Richardson *et al.*, 1993 39

[77] Castro, Valego *et al.*, 1992 ....33, 211

[78] Cathiard, Crozat *et al.*, 1985 ....22, 27

[79] Cavaliere, Knobel and Medeiros-Neto, 1987 48

[80] Challis and Brooks, 1989 ....3, 4, 17, 68, 71, 113, 132, 172, 198, 203

[81] Challis and Lye, 1994 3

[82] Chan, Gutell *et al.*, 1984 83

[83] Chang, Cochet and Cohen, 1980 28

[84] Chang, Naseeruddin and Hornsby, 1991 54

[85] Charlton and Johengen, 1987 ....59, 60

[86] Chatelain, Penhoat *et al.*, 1988 ....54, 158, 169, 200

[87] Cheng and Hornsby, 1992 54

[88] Cherradi, Defaye and Chambaz, 1994 12

[89] Cheung, 1984 3

[90] Chirgwin, Przybyla *et al.*, 1979 ....84, 116, 161, 176

[91] Chomczynski, 1993 138

[92] Chomczynski and Sacchi, 1987 138

[93] Chretien, Boileau *et al.*, 1984 30

[94] Chua, Szabo *et al.*, 1987 14

[95] Chung, Matteson *et al.*, 1986 ....12, 14, 75

[96] Chung, Picado-Leonard *et al.*, 1987 ....12, 14, 75

[97] Clapp, McLaughlin *et al.*, 1984 59

[98] Clapp, Szeto *et al.*, 1980 59

[99] Clark, Durnwald and Austin, 1982 60

[100] Clemmons, 1993 47

[101] Clemmons, Klibanski *et al.*, 1981 48

[102] Cohen and Chang, 1980 29

[139] Edwards, Benediktsson *et al.*, 1993 ....5, 6, 38, 212

[140] Eipper and Mains, 1978 28

[141] Ekman, Hakanson *et al.*, 1982 40

[142] Emmanoulides, Townsend and Bauer, 1968 60

[143] Engeland and Dallman, 1975 ....43, 152

[144] Engeland and Dallman, 1976 151

[145] Estivariz, Carino *et al.*, 1988 .... 40, 42, 43, 54, 111, 150, 151, 170, 201, 203, 204

[146] Estivariz, Gillies and Lowry, 1981 40

[147] Estivariz, Hope *et al.*, 1980 40

[148] Estivariz, Iturriza *et al.*, 1982 .... 39, 42, 135, 150, 151, 154, 170, 201, 204

[149] Estivariz, Morano *et al.*, 1988 ....43, 54, 150, 151, 204

[150] Farese, Ling *et al.*, 1983 ....44, 135, 155, 207

[151] Fay and Ellewood, 1993 58

[152] Fora, Butler *et al.*, 1996 ....32, 33

[153] Fowden, 1994 57

[154] Fowden, Coulson and Silver, 1990 ....3, 6, 212

[155] Fowden, Mundy and Silver, 1998 3

[156] Fowden, Szemere *et al.*, 1996 4

[157] Francis, McNeil *et al.*, 1989 160

[158] Fraser, Jeffray and Challis, 1999 ....36, 131

[159] Freemark, Driscoll *et al.*, 1997 ....129, 130

[160] Frodin and Gammeltoft, 1994 ....51, 168, 199

[161] Fuller, Brake and Thorner, 1989 30

[162] Fuller *et al.*, 1989b 30

[163] Funder, Pearce *et al.*, 1988 114

[164] Gagnon, Challis *et al.*, 1994 60

[165] Gasson, 1979 128

[166] Gillies, Estivariz and Lowry, 1979 31

[167] Gilmour, Prosser *et al.*, 1988 47

[168] Glickman and Challis, 1980 ....21, 23, 25, 27

[169] Glover, 1985 79

[170] Gluckman, 1984 48

[171] Gluckman and Butler, 1983 48

[172] Gluckman and Holdaway, 1976 48

[173] Halasz and Szentagothai, 1959 152

[174] Hall and Ekanayake, 1991 ....51, 168, 199

[211] Jia, Canny *et al.*, 1991 32

[212] Jansen, Belik *et al.*, 1989 188

[213] John, John *et al.*, 1984 26

[214] John, John *et al.*, 1986 26

[215] John, Okamura *et al.*, 1986 26

[216] John, Simpson *et al.*, 1987 ....25, 26, 68, 105

[217] Jones, 1980 ....70, 110, 194

[218] Jones, 1989 62

[219] Jones, Boddy and Robinson, 1977 20

[220] Jones, Edwards and Tindell, 1992 128

[221] Jones, Harding *et al.*, 1988 60

[222] Jones, Lafeber *et al.*, 1987 58

[223] Jones and Parer, 1983 60

[224] Jones and Roebuck, 1980 ....33, 128, 211

[225] Kallen, Arakane *et al.*, 1998 ....10, 11

[226] Keutmann, Eipper and Mains, 1979 40

[227] Kiess, Yang *et al.*, 1994 47

[228] Kind, Owens *et al.*, 1995 48

[229] King, Ronen-Fuhrmann *et al.*, 1996 11

[230] Klempt, Hutchins *et al.*, 1992 49

[231] de Kloet, Reul and Sutanto, 1990 2

[232] Koster, Saftig *et al.*, 1993 47

[233] Kramer, Olivier *et al.*, 1990 57

[234] Lachance, Luu-The *et al.*, 1990 14

[235] Lafeber, Rolph and Jones, 1984 ....59, 60, 188

[236] Langlois, Hinsch *et al.*, 1990 ....53, 158, 169

[237] Langlois, Matthews *et al.*, 1995 39

[238] Lassarre, Hardouin *et al.*, 1991 58

[239] Lehoux, Fleury and Ducharme, 1998 ....11, 107

[240] Levidiotis, Wintour *et al.*, 1989 31

[241] Li, Saunders *et al.*, 1993 ....55, 71, 79

[242] Lighten, Hardy *et al.*, 1997 46

[243] Liggins, 1968 ....36, 70, 113

[244] Liggins, 1976 ....3, 6, 212

[245] Liggins, Fairclough *et al.*, 1973 ....3, 21, 28

[246] Liggins and Kennedy, 1968 ....3, 28

[354] Rao, Long and Ramachandran, 1978 42

[355] Rechler and Nissley, 1985 ....47, 48, 51

[356] Rechler and Nissley, 1986 ....47, 48

[357] Rieger, Luciano *et al.*, 1998 46

[358] Riley, Boshier *et al.*, 1992 106

[359] Roberts and Herbert, 1977 28

[360] Robinson, 1989 57

[361] Robinson, Falconer and Owens, 1985 ....57, 59, 63

[362] Robinson, Hart *et al.*, 1980 ....62, 63

[363] Robinson, Jones and Kingston, 1983 63

[364] Robinson, Kingston *et al.*, 1979 ....60, 62, 172, 174, 188, 208, 209

[365] Robinson, Owens and Owens, 1994 59

[366] Robinson, Comline *et al.*, 1983 ....20, 69

[367] Robinson, Rowe and Wintour, 1979 ....19, 21

[368] Roebuck, Jones *et al.*, 1980 ....33, 211

[369] Rose, MacDonald *et al.*, 1978 ....4, 172

[370] Rose, Meis and Morris, 1981 ....4, 21, 25

[371] Rose, Meis *et al.*, 1982 27

[372] Ross, Phillips *et al.*, 1997 ....35, 113, 126, 129

[373] Rurak, Richardson *et al.*, 1990 188

[374] Saez, Durand and Cathiard, 1984 ....27, 36

[375] Saez, Morera and Gallet, 1977 ....36, 70

[376] Sambrook, Fritsch and Maniatis, 1989 79

[377] Saphier, Glynn *et al.*, 1993 ....45, 135, 154, 156, 157, 170, 189, 201, 207, 208, 211

[378] Sapolsky, Uno *et al.*, 1990 ....6, 212

[379] Sara and Hall, 1990 46

[380] Sasano, Mason and Sasano, 1989 15

[381] Sasano, Sasano and Okamoto, 1989 15

[382] Sato and Mains, 1988 40

[383] Schmand, Neuvel *et al.*, 1990 5

[384] Schwartz, Ash *et al.*, 1994 32

[385] Schwartz, Kleftogiannis *et al.*, 1995 ....33, 70, 107, 128, 170, 201, 211

[386] Schwartz and Vale, 1988 32

[387] Seger and Bennett, 1986 ....29, 30, 152, 153, 155

[388] Seidah, Chretien and Day, 1994 30

[389] Seidah, Marcinkiewicz *et al.*, 1991 30

[426] Weber, Kiess *et al.*, 1994 ....51, 158

[427] White, 1994 ....10, 15

[428] White, DuPont *et al.*, 1991 14

[429] White, New and DuPont, 1984 14

[430] White, New and DuPont, 1986 ....14, 75

[431] Whorwood, Franklyn *et al.*, 1992 ....38, 196

[432] Wintour, 1984 20

[433] Wintour, Brown *et al.*, 1975 .... 19, 21, 22, 23, 27, 68, 104

[434] Wong, Ikeda *et al.*, 1997 ....64, 173

[435] Wood, Cheung and Brace, 1987 5

[436] Wright and Voncina, 1977 18

[437] Wright, Voncina and Morley, 1973 18

[438] Wylie, Kerr and Currie, 1973 20

[439] Xia, Tekpetey and Armstrong, 1994 46

[440] Yang, Berdusco and Challis, 1994 39

[441] Yang and Challis, 1989 70

[442] Yang and Matthews, 1995 124

[443] Yang, Smith *et al.*, 1992 38

[444] Yaswen, Diehl *et al.*, 1999 157

[445] Yoshioka, Morohashi *et al.*, 1986 14

[446] Zajicek, Ariel and Arber, 1986 18

[447] Zuber, John *et al.*, 1986 ....14, 26

NOTES

HYPOPHYSIAL AND LOCAL MEDIATORS OF
ADRENOCORTICAL GROWTH AND FUNCTION BEFORE BIRTH
Jacob Tavern Ross

Department of Physiology
The University of Adelaide
South Australia

Submitted February 29, 2000

The following errors are acknowledged and corrected in this PhD thesis.

1. Reference 416 should be cited as ([416]van Dijk, Tanswell and Challis, 1988). This reference appears on Page (P) 54 Line (L) 17, P54 L21, P158 L10, P168 L11, P168 L23, P171 L4, P173 L16, P199 L28, P202 L5, P244 L1 and PA22 L27.

2. P72 L2-3 should read: "Fetuses were selected from one of three control protocols for the purposes of other studies previously carried out by other researchers in the laboratory which included:"

3. P159 sentence 1 should read: "Administration of IGF-I increases the circulating levels of IGF-I and promotes the growth of major organs - including the adrenal, although this is in absolute, rather than in relative terms ([252]Lok, Owens *et al.*, 1996)."

4. P160 section 5.2.2 Infusion regimen and blood sampling protocol. An additional sentence following L11 should read: "3 of the 14 IGF-I infused and 3 of the 8 saline infused fetuses were used from a previous study ([252]Lok, Owens *et al.*, 1996) for some of the measurements in this thesis."

5. P160 L25-26. The number of fetuses and plasma samples reported for the IGF-I radioimmunoassay should read: "*rh*IGF-I infused (n=10 fetuses, n=20 samples) and saline infused (n=5 fetuses, n=10 samples)."

6. P161 L4-5: The number of fetuses and plasma samples reported for the cortisol radioimmunoassay should read: "*rh*IGF-I infused group (n=10 fetuses, n=10 samples) and the saline infused group (n=5 fetuses, n=5 samples)."

7. P163 L2: The fetal plasma IGF-I values for the recombinant human IGF-I infused and saline infused groups should be reported as: "(*rh*IGF-I: 352 ± 26 ng ml^{-1}; saline: 138 ± 11ng ml^{-1})."

8. P174 sentence 1 should read: "Data were collected from a group of fetuses (n=237; 71 singletons,166 twin fetuses) and from a second group (n=71; 54 singletons, 17 twin fetuses) in which placental restriction was experimentally induced in experiments carried out by other researchers in the laboratory during a four year period."