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Pancreatic and adrenal development and function in an ovine model of polycystic ovary syndrome

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Declaration

I hereby declare that the work in this thesis was carried out by the author, and if others contributed they are acknowledged. This thesis is submitted for the fulfillment of the degree of Doctor of Philosophy, and not for any other degree or qualification.

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Abstract

Polycystic Ovary Syndrome (PCOS) is a complex disorder encompassing reproductive and metabolic dysfunction. Ovarian hyperandrogenism is an endocrine hallmark of human PCOS. In animal models, PCOS-like abnormalities can be recreated by *in utero* over-exposure to androgenic steroid hormones.

This thesis investigated pancreatic and adrenal development and function in a unique model of PCOS. Fetal sheep were directly exposed (day 62 and day 82 of gestation) to steroidal excesses - androgen excess (testosterone propionate - TP), estrogen excess (diethylstilbestrol - DES) or glucocorticoid excess (dexamethasone - DEX).

At d90 gestation there was elevated expression of genes involved in β-cell development and function: *PDX-1 (P<0.001),* and *INS (P<0.05), INSR* (*P*<0.05) driven by androgenic excess only in the female fetal pancreas. β-cell numbers (*P*<0.001) and *in vitro* insulin secretion (*P*<0.05) were also elevated in androgen exposed female fetuses. There was a significant increase in insulin secreting β-cell numbers (*P*<0.001) and *in vivo* insulin secretion (glucose stimulated) (*P*<0.01) in adult female offspring, specifically associated with prenatal androgen excess.

At d90 gestation, female fetal adrenal gene expression was perturbed by fetal estrogenic exposure. Male fetal adrenal gene expression was altered more dramatically by fetal glucocorticoid exposure. In female adult offspring from androgen exposed pregnancies there was increased adrenal steroidogenic gene expression and *in vivo* testosterone secretion (*P*<0.01). This highlights that the adrenal glands may contribute towards excess androgen secretion in PCOS, but such effects might be secondary to other metabolic alterations driven by prenatal androgen exposure, such as excess insulin secretion Thus there may be dialogue between the pancreas and adrenal gland, programmed during early life, with implications for adult health

Given both hyperinsulinaemia and hyperandrogenism are common features in PCOS, we suggest that their origins may be at least partially due to altered fetal steroidal environments, specifically excess androgenic stimulation.

Presentations and publications relating to this thesis

Chapter 3: The effects of prenatal steroids on fetal pancreatic development and function

Paper

"The Pancreas Is Altered by Implications for Clinical Conditions Such as Polycystic *In Utero* Androgen Exposure: Ovary Syndrome (PCOS)"

Rae MT, Grace C, Hogg K, Wilson LM, McHaffie SL, **Ramaswamy S**, MacCallum J, Connolly F, McNeilly AS, Duncan C. PLoS One. Feb 2013;8(2)

Oral presentation

"The effects of direct fetal exposure of excess steroids on ovine fetal pancreas"

Ramaswamy S, Grace C, Duncan WC, Rae MT

Post Graduate Research Conference, Edinburgh Napier University, Edinburgh 2012

Chapter 4: The effects of prenatal steroids on adult pancreatic structure and function

Oral presentation

"*In utero* steroid exposure alters adult pancreas function"

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Post Graduate Research Conference, Edinburgh Napier University, Edinburgh. April 2014

Poster presentation

"In utero steroid exposure permanently alters adult pancreatic function in an ovine model of polycystic ovary syndrome (PCOS)"

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Society for Reproduction and Fertility, Edinburgh, 2014

Chapter 5: The effects of prenatal steroids on fetal adrenal development

Poster presentation

"*In utero* steroid exposure alters adrenal steroidogenesis in an ovine model of polycystic ovary syndrome (PCOS)"

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Chapter 6: The effects of prenatal steroids on postnatal adrenal development and function

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"*In utero* steroid exposure alters adult adrenal steroidogenic gene expression and function"

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"*In utero* steroid exposure alters ovine adrenal steroidogenic gene expression and function in an ovine model of Polycystic ovary syndrome (PCOS)"

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Abbreviations

ABC	Avidin Biotin Complex
aCoA	Acetyl CoA carboxylase
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen response elements
ATP	Adenosine triphosphate
B2M	Beta-2 microglobulin
β-actin	Beta actin
BSA	Bovine serum albumin
cAMP	Cyclic ammonium monophosphate
cDNA	Complimentary deoxyribonucleic acid
CAH	Congenital adrenal hyperplasia
CREB	Cyclic ammonium monophosphate response element
CRH	Corticotrophic releasing hormone
Cyp1A1	Cytochrome P4501A1
DAB	3'3'-diaminobenzidine
DES	Diethylstilbestrol
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DNA	Deoxyribo nucleic acid
DNMT	DNA methyltrasnferase
DI	Disposition Index
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FFA	Free fatty acids

FOAD	Fetal origins of adult diseases
FSH	Follicle stimulating hormone
GK	Glucokinase
GLUT-1	Glucose transporter-1
GLUT-2	Glucose transporter-2
GLUT-4	Glucose transporter-4
GnRH	Gonadotrophic releasing hormone
G3PD	Glyceraldehyde 3-phosphate dehydrogenase
G6P	Glucose 6 phosphate
G6Pase	Glucose 6 phosphatase
GR	Glucocorticoid receptor
GSIS	Glucose stimulated Insulin synthesis
GTT	Glucose tolerance test
HPG	Hypothalamic pituitary gonadal
HPA	Hypothalamic pituitary adrenal
HRP	Horse radish peroxidase
HSD3β	Hydroxysteroid dehydrogenase 3β
HSD11β	Hydroxysteroid dehydrogenase 11β
HSD17β	Hydroxysteroid dehydrogenase 17β
hCG	Human chorionic gonadotrophic
IGF-I	Insulin growth factor type 1
IGF-II	Insulin growth factor type 2
IGFR-I	Insulin growth factor receptor type 1
IGFR-II	Insulin growth factor receptor type 2
IGT	Impaired glucose tolerance
IHC	Immunohistochemsitry
IMGD	Insulin mediated glucose disposal
INS	Insulin gene
INSR	Insulin receptor
IR	Insulin resistance
IRS	Insulin receptor substrate
i.v	Intravenous
LDL	Low density lipoprotein

LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MAPK	Mitogen activated protein kinase
MDH1	Malate dehydrogenase 1
mRNA	Messenger Ribonucleic acid
MODY	Maturity onset diabetes in young
MR	Mineralocorticoid receptor
MS	Metabolic syndrome
NADH	Nicotinamide adenine dinucleotide
NIDDM	Non insulin dependent diabetes mellitus
NIH	National Institute of Health
PA	Prenatal androgen
PCOS	Polycystic ovary syndrome
PEPCK	Phosphoenolpyruvate carboxykinase
PDK-1	Phosphoinositide dependant kinase-1
PDK-2	Phosphoinositide dependant kinase-2
PDX-1	Pancreatic duodenal homeobox-1
P450scc	P450 side chain cleavage
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PVN	Para ventricular nucleus
qRTPCR	Quantitative real time polymerase chain reaction
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPL19	Ribosomal protein L19 gene
RPS2	Ribosomal protein S2
RPS26	Ribosomal protein S26
SEM	Standard error of the mean
SSH	Sex steroid hormones
StAR	Steroid acute regulatory protein
STZ	Steptozotocin
TBS	Tris buffered saline
TBST	Tris buffered saline with tween

T2DM	Type 2 diabetes mellitus
TEMED	Tetramethylethylenediamine
TMB	Tetramethylbenzidine
TP	Testosterone propionate
VNTR	Variable number of tandem repeats
YWHAZ	Tyrosine 3-monooxygenase mRNA
ZDF	Zucker diabetic fatty
18s	18S ribosomal R

Chapter 1 Literature Review

1.0 Introduction

Endocrine regulation involves the release of hormones from an endocrine gland into the circulatory system, eliciting changes only in the target organ(s) expressing its cognate receptors, transducing the chemical message into cellular responses, as regulated by the specific hormone-receptor interaction. The endocrine glands and their target organs are controlled by tightly regulated endocrine axes, which function on the basis of either positive or negative feedback loops. Homeostatic regulation favours negative feedback, whereby the endocrine gland delivering stimulatory effects (to either another endocrine gland or non-endocrine tissue) is negatively regulated by the outcome of its effects upon the target tissue. Examples of endocrine glands are thyroid, ovary, testis and adrenal, which operate within feedback circuits involving the hypothalamus and pituitary gland, with major homeostatic outcomes such as the hypothalamic-pituitary-gonadal (HPG) axis, which regulate reproductive (ovary and testis) functions. In comparison, pancreatic function is responsive to circulating glucose concentrations, which determine which islet hormone is released to ensure tight regulation of blood glucose levels. Altered endocrine gland function can lead to eventual overwhelming of the 'buffering capacity' and inbuilt plasticity leading to endocrine axis dysfunction with potential for progression into a disease state. Variability in the function, and also perhaps resilience, of a particular endocrine axis is dependant not only upon the individuals genetics, but also epigenetics and, through both direct contemporary, and perhaps also through epigenetic manipulation at an earlier stage of life, environmental (exogenous) factors. This latter point of early-life effects with health legacies onwards into adulthood is a central tenant of the Barker hypothesis (Barker, 2004). The Barker hypothesis centres upon the association between low birth weight and adult onset of diabetes, where the associations are thought to be consequences of developmental plasticity, a phenomenon which most organs or cells are known to express (Bateson *et al*., 2004). This developmental plasticity and *in utero* pressures upon it can give rise to a range of different

physiological or morphological states in response to different environmental factors during development (Barker, 2004).

Hormones are known to act as epigenetic signals *in utero* playing a major role in developmental programming (Fowden and Forhead, 2009). Altered function of any endocrine component, whether driven by endogenous maternal or placental factors during key developmental periods can lead to altered risk of developing altered function and potentially disease later in adulthood. Polycystic Ovary Syndrome (PCOS), which is associated with reproductive, metabolic and endocrine disturbances in women later in adult life, may have origins stemming back to fetal life, as evidenced by numerous studies in animal models of this condition (Abbott and Bird, 2009; Bruns *et al*., 2004a; Hogg *et al*., 2011; Padmanabhan *et al*., 2006; Rae *et al*., 2013; Roland *et al*., 2010). The focus of this thesis is one such ovine model of PCOS, honing in upon the pancreas and adrenal glands as potential foci of the effects of *in utero* steroid hormone excesses during development, and onwards into adult life, with respect to organ structure, function and mechanistic underpinnings of alterations investigated.

1.0.1 Polycystic Ovary Syndrome (PCOS)

Polycystic Ovary syndrome (PCOS) is a combination of both reproductive and metabolic disorders found in women, and characterized by hypersecretion of luteinizing hormone (LH), hyperinsulinemia, ovarian hyperandrogenism (Dumesic *et al*., 2007) and polycystic ovaries on ultrasonography (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group., 2004). PCOS has been a scientific challenge for researchers (Hsu, 2013), and is a complex condition for clinicians and a frustrating experience for women. In women of reproductive age, PCOS is the most common endocrine abnormality, affecting about 6-8% of the population (Diamanti-Kandarakis & Piperi, 2005). Clinical conditions such as Cushing's syndrome, androgen-secreting tumors and congenital adrenal hyperplasia (CAH) mimic some features of this PCOS, but are excluded prior to diagnosis (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group., 2004).

Evidence from PCOS research has been translated to healthcare professionals leading to (Teede *et al*, 2010) use of metformin (an insulin sensitizing agent) (Diamanti-Kandarakis *et al*., 2010). Although a clear origin of PCOS in women is poorly understood, it appears likely that origins involve early life interactions between genetics and environment (Deligeoroglou *et al,* 2009) with epigenetic modifications (Xu *et al*, 2010; Xu *et al*., 2011) as a contributing factor influencing the pathophysiology of PCOS.

1.0.1.1 Criteria for clinical diagnosis of PCOS

It is estimated that about 6.6% of women in the United States suffer from PCOS during their reproductive years, leading to an economic burden of about $4.4 billion to treat associated type 2 diabetes mellitus, menstrual dysfunction and infertility (Azziz *et al*, 2005). In Australia AU$400 million healthcare spend occurs on PCOS, breaking down into 40% for PCOS associated diabetes, 12% infertility and 31% of menstrual dysfunction of total costs (Azziz *et al*, 2005). Given such high healthcare spend it has been critical to define strict criteria for the diagnosis and management of this condition. National Institute of Health (NIH) meetings held in 1990 initially defined PCOS as a combination oligomenorrhoea or chronic anovulation and hyperandrogenism (biochemical or clinical) (Zawadski and Dunaif, 1992). Although the extra ovarian factors e.g. insulin resistance, obesity and metabolic syndrome likely account for this syndrome, ovarian dysfunction remains the intrinsic disturbance of PCOS that is clinically recognized and acted upon.

A redefined definition of PCOS was given at the joint consensus meeting held at Rotterdam in 2003 between the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM). According to this, PCOS was defined, excluding all the other etiologies as presence of two among the following three criteria: (a) hyperandrogenism (clinical or biochemical); (b) oligo and/or anovulation; (c) polycystic ovaries (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group., 2004). Regardless of the criteria used, recently, the

Androgen Excess Society (AES) has emphasized that androgen excess remains the central feature of PCOS combined with ovarian dysfunction (oligo-ovulation and/or polycystic ovaries) (Azziz et al., 2009).

On the other hand hyperandrogenism, hyperinsulinaemia (excess insulin secretion) and obesity, which are the common features of PCOS, are also the factors that confer the increased risk of type-2 diabetes and cardiovascular diseases (Rajkhowa *et al*, 2000). However in about 75% of women with PCOS, the polycystic ovarian morphology has become of pathophysiological significance (Azziz *et al*., 2006). Furthermore, according to the recent Rotterdam consensus, less severe metabolic derangements such as obesity, insulin resistance and hyperglycemia seen in PCOS women in addition to anovulatory dysfunction will be included in the broad spectrum of the PCOS diagnosis criteria (Broekmans *et al*., 2006).

1.0.2 Proposed Origins of PCOS

1.0.2.1 Genetic linkage

PCOS shows some genetic basis in the development of the syndrome (Carey *et al*., 1993; Xita *et al*., 2002), however the literature is conflicting in a number of cases. Cytochrome P450 side chain cleavage (P450scc) encoded by *CYP11A1* gene has a polymorphic pentanucleotide repeat $(tttta)_n$ in its promoter region, which is present in four-, six-, eight- and nine-repeat units in the normal population and is also associated with serum testosterone levels, however, absence of the four-repeat-units allele is seen in Greek women suffering from PCOS (Diamanti-Kandarakis *et al*., 2000). However, a Spanish study suggested that this polymorphism had no influence on hyperandrogenism in women with PCOS (San Millá *et al*., 2001). A more recent study has revealed the presence of >8 pentanucleotide repeat from the same allele in a South Indian population of women suffering from PCOS (Reddy *et al*., 2014), so ethnic and/or racial difference could be one possible reason behind the differences between these results. Certainly, a study in Singapore confirmed that mutations in the LH β- subunit are associated with

higher concentrations of serum testosterone in women with PCOS (Ramanujam *et al*., 1999). Decreased levels of polymorphic CAG repeats in the androgen receptor gene are associated with increased hirsutism with normal testosterone levels in Hispanic women (Legro *et al*., 1994) and low serum androgen levels in a subset population of women suffering from anovulatory infertility and polycystic ovaries (Mifsud *et al*., 2000). Genetic studies also link PCOS with disordered insulin metabolism (Franks *et al*., 2001). For example, in women suffering from PCOS, the *insulin gene* (INS) variable number of tandem repeat (VNTR), which lies in the 5' regulatory region of the genes has been identified as the major susceptibility locus (Waterworth *et al*., 1997) linking to hyperinsulinaemia/insulin resistance in PCOS (Waterworth *et al*., 1997).

Insulin receptor (*INSR*) is an another gene that has been investigated in PCOS, where in one study, 22 patients with PCOS were screened and no abnormalities in the tyrosine kinase domain of the insulin receptor were detected (Conway *et al*., 1994). This result was confirmed in a second study, where again no mutations were detected when the entire coding region of *INSR* was examined (Talbot *et al*., 1996). This clearly suggests that women with insulin resistance in PCOS are unlikely to have *INSR* mutations. Although the above studies suggest a genetic link to PCOS, the mode of inheritance and genetic aetiology is still unclear and requires further investigation.

1.0.2.2 Epigenetics and PCOS traits

Epigenetic modifications of DNA are mechanisms involved in the inheritance of the gene expression patterns in cells without any alterations in the DNA sequences (Bjornsson *et al*., 2004; Chong and Whitelaw, 2004). Such modifications are known to occur via at least two distinct epigenetic mechanisms, histone modification and DNA methylation.

Nucleosomes, the basic building block of the chromosome in eukaryotes comprise of DNA wrapped around an octameric complex of two molecules of each of the four histones, which are the proteins that aid in packaging of the DNA into nucleosomes. Amino acid termini of each of the histones such as, H2A, H2B, H3 and H4 can be modified via several post transcriptional mechanisms such as methylation, acetylation, phosphorylation, glycosylation and ADP ribosylation at specific residues in histones N-terminal tails (Zhang and Ho, 2010).

DNA methylation is a second class of epigenetic modification, where DNA methyltransferase (DNMTs) enzymes modify the cytosine base at the C5 position of cytosine (Jones and Takai, 2001). These epigenetic modifications are essential for mammalian development; however, they can be modified by environmental factors, which can alter gene expression, thus organ function, and hence further lead to potential development of diseases. The *in utero* environment is susceptible to such environment mediated epigenetic modifications leading to permanent alteration of the phenotype in adulthood (Bjornsson *et al*., 2004). It is evident from emerging data that such abnormal epigenetic regulation of gene expression plays a major role in the developmental origins of adult diseases (Pinney and Simmons, 2010; Waterland and Jirtle, 2004). Such epigenetic links have been made between growth retardation at birth and development of type 2- diabetes in adulthood (Martin-Gronet and Ozanne, 2005). For example, prenatal glucocorticoid exposure in rats (F0) leads to altered glucose homeostasis in the F1 offspring and without any further exposure this can be passed onto F2 generation (Drake *et al*., 2005). Moreover, in pregnant rats exposed to endocrine disruptors, reproductive abnormalities such as spermatogenic cell defects and sub-fertility can be observed in F1-F4 generations (Anway *et al*., 2005). Therefore, epigenetic reprogramming of the germ-line is possible, so it may play a major role in disease inheritance (Anway *et al*., 2005). As altered epigenetic modifications are associated with development of type-2-diabetes (Pinney and Simmons, 2010) and reproductive disorders (Anway *et al*., 2005), it is possible that, such modifications contribute to effects of prenatal steroid exposure, which in turn may underpin some aspects of multifaceted

conditions/syndromes such as PCOS (Figure 1.1) (Piltonen *et al*., 2002). A recent study suggests a negative correlation between global DNA methylation and women with PCOS, however the study was based upon a small sample population (Xu *et al*., 2010). Therefore, large sample size, and tissue specific DNA methylation study (Xu Ning *et al*., 2010) is where currently there is significant focus, and these will aid understanding the relation between epigenetics and *in utero* androgen overexposure origins of PCOS in adulthood.

1.0.2.3 Fetal Origins of PCOS

The nutritional and hormonal environment affect the developing fetus *in utero* which may further lead to altered risk/likelihood of development of metabolic syndrome and other pathophysiological conditions later in adult life (Barker, 2004). In a similar fashion, excess androgens during fetal life induce polycystic ovary syndrome (PCOS) like phenotypes later in adulthood in animal models derived from numerous species such as monkeys, sheep and rats (Abbott *et al*., 2005).

Androgen excess during fetal life leads to PCOS-like phenotype developing later in adulthood by inducing changes in tissue differentiation (Abbott *et al*., 2005). Androgens for example, can program the hypothalamus in terms of the release pattern of gonadotrophic hormones in rats (Sokka *et al*.,1996). Increased LH secretion and aberrant ovarian follicular development due to reduced sensitivity of hypothalamic GnRH secretory system to steroidal negative feedback was also observed in sheep exposed *in utero* to excess androgens (Robinson *et al*., 1999) (Figure 1.1) and prenatal androgen exposure in sheep led to intrauterine growth retardation, postnatal catch-up growth and multi-follicular ovarian development (Manikkam *et al*., 2004). Ovulatory dysfunction was also observed in prenatally androgenized female rhesus monkey which exhibit 40-50% fewer menstrual cycles when compared to normal females (Abbott *et al*., 1998). These same rhesus monkeys also exhibit enlarged polycystic ovaries similar to the ovarian morphology seen in

case of women with PCOS (Abbott *et al*., 1998) and exhibited LH hypersecretion in adult life when exposed to excess androgens *in utero* (Dumesic *et al*., 1997).

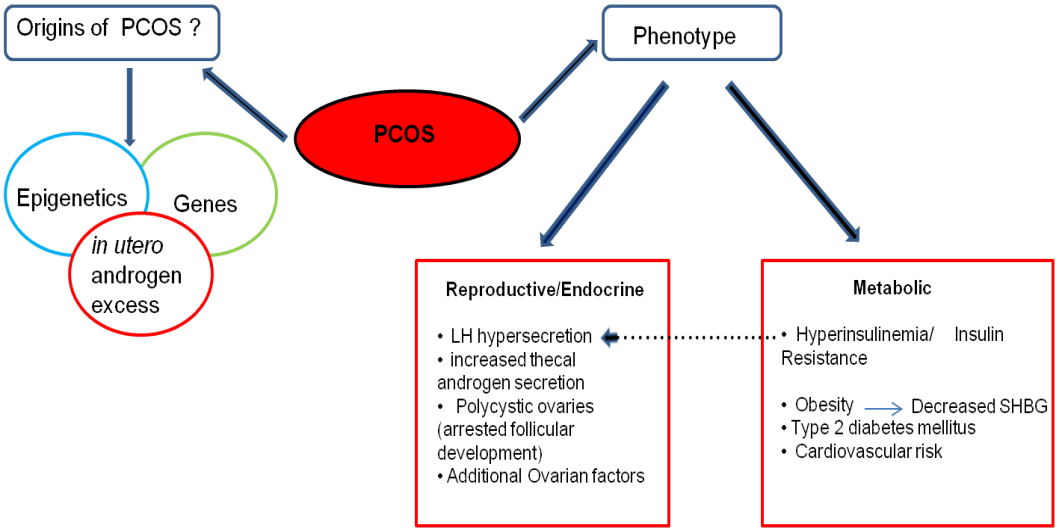


Figure 1.1 Overview of the possible origins of PCOS and its related phenotype.

In addition to reproductive disorders, fetal androgen excess also leads to metabolic disorders that characterize PCOS, such as pancreatic β-cell dysfunction, insulin secretion and altered adipose tissue distribution (Dunaif and Finegood, 1996; Hogg et al, 2011; Roland et al., 2010). For example, impaired insulin sensitivity during early postnatal life is seen in case of prenatally androgenized sheep (Recabarren et al., 2005), together with elevated cholesterol and hypertension after puberty (King et al., 2007) and impaired β-cell function (Eisner et al., 2000), irregular anovulatory cycles, adrenal and ovarian androgen excess and LH hypersecretion is also observed in adult female rhesus monkeys exposed in utero to excess testosterone (Abbott et al., 1998). The coupling of insulin resistance and impaired β-cell response to glucose then predisposes development of adult type 2 diabetes mellitus (Bruns et al., 2004a).

As a contributing factor for polycystic ovaries, prenatally androgenized sheep exhibit persistent follicular cysts implying impaired follicular growth (Manikkam *et al*., 2006) (Figure 1.1). A polyfollicular phenotype of increased primary follicles and decreased primordial follicles is observed in this case

when androgen treatment covers the period of days 30-90 of gestation (term is ~147days) (Steckler *et al.*, 2005), whereas such a phenotype is less evident when exposure occurs after d60 of gestation (Hogg *et al*., 2012). So, whilst this latter study did identify gene expression changes in ovarian cells predictive of future excess androgen secretion (Hogg *et al*., 2012), it nonetheless demonstrates that timing/duration of exposure is critical in terms of phenotypic outcomes. In humans, excess adrenal androgen production during intrauterine life due to congenital adrenal hyperplasia from 21-hydroxylase deficiency in women is associated with a PCOS phenotype including polycystic ovaries, insulin resistance, central adiposity, LH hyper secretion, anovulatory cycles and ovarian hyperandrogenism (Hague et al., 1990), further cementing the relationship between excess *in utero* androgen exposure and development of PCOS.

This thesis will focus upon on the above fetal origins of adult disease (FOAD) hypothesis, with respect to *in utero* excess steroids as seen in PCOS women, in terms of PCOS associated metabolic and endocrine aberrations.

1.0.3 Reproductive Phenotype of PCOS

1.0.3.1 Abnormal Follicle development

Polycystic ovaries are a cardinal feature of PCOS. Independent of follicular atresia and ovulatory status, increased proportion of primary follicles reciprocal to the proportion of primordial follicles is seen in women with polycystic ovary morphology (Maciel *et al*., 2004; Webber *et al*., 2003) and according to the Rotterdam criteria, 90-100% of women with polycystic ovary syndrome have polycystic ovaries (Welt *et al*., 2006).

1.0.3.2 The ovarian legacy of prenatal excess androgen exposure

The experimental evidence of increased recruitment of ovarian follicles in adult hyperandrogenism comes from testosterone administration to adult

female rhesus monkeys, which is associated with increased numbers of primary follicles (Weil *et al*., 1998). This androgen exposure in the rhesus monkey increased mRNA expression of follicle stimulating hormone (FSH) in the pituitary and both insulin-like growth factor (IGF-1) and insulin like growth factor receptor (IGF-1R) in the granulosa cells (Vendola *et al*., 1999; Weil *et al*., 1998). The same polyfollicular phenotype induced in sheep *in utero* from day 30-90 of gestation (total gestation-147 days) decreased the number of primordial follicles but increased the number of growing (primary, preantral and antral) follicles (Steckler *et al*., 2005). However, maternal administration of prenatal testosterone at d60 gestation in pregnant sheep resulted in no structural changes in the fetal ovarian morphology at d90 gestation (Hogg *et al*., 2011), again highlighting subtle differences in outcome dependent upon the window of gestation the exposure occurred in. Prenatally androgenized female rhesus monkeys demonstrated increased testosterone and 17α-hydroxyprogesterone during adulthood in response to recombinant human chorionic gonadotrophic (hCG) hormone, again strongly suggestive of ovarian hyperandrogenism (Eisner *et al*., 2002). Underpinning such ovarian hyperandrogenism, even in the absence of altered follicle recruitment, in a sheep model of maternal androgenization, there was increased expression of key genes coding for steroidogenic enzymes in adult offspring (11 months old), such as *StAR, CYP11A1, CYP17* and also the LH receptor (*LHR),* suggesting a potential contribution to adult ovarian phenotype of PCOS (Hogg *et al*., 2012).

1.0.4 Effect of prenatal steroidal environment on intraovarian factors

1.0.4.1 Ovarian Hyperandrogenism

Ovarian hyperandrogenism is observed in 70-80% PCOS patients (Diamanti-Kandarakis *et al*., 2007). This has been ascribed to augmented expression of genes coding for steroidogenic enzymes such as P450side chain cleavage, 17α-hydroxylase/17α-lyase ($P450_{c17}$/*CYP17*) and 3β- hydroxysteroid dehydrogenase (*3β-HSD*) and consequently excess biosynthesis of androgen

in ovarian follicular theca cells (J. R. Wood et al., 2003). Thus it appears evident that thecal steroidogenic defect remains an intrinsic factor for increased androgen production in PCOS women (Nelson *et al*., 2001).

1.0.4.2 Luteinizing hormone (LH) hypersecretion

The enhanced gonadotrophin- releasing hormone (GnRH) pulsatility leading to enhanced LH hypersecretion is a neuroendocrine hallmark of PCOS (Birch *et al*., 2003). Impaired negative feedback underpins high LH concentrations in anovulatory PCOS women (Dumesic *et al*., 2007) via excessive action of androgen on hypothalamic-pituitary axis leading to elevated levels of LH (Jonard and Dewailly, 2004), although increased LH secretion in adolescent PCOS patients, is at least partially a result of reduced progesterone (P4) negative feedback activity (Chhabra *et al*., 2005). Similarly, female rhesus monkeys exposed to fetal male testosterone levels *in utero* have shown clinical manifestation of PCOS like LH hypersecretion during adult life (Dumesic *et al*., 1997), again adding weight to the association of *in utero* excess androgens with postnatal development of PCOS.

1.0.5 PCOS associated Metabolic Phenotype

The prevalence of metabolic syndrome is increased in PCOS women as compared to weight and age matched non-PCOS patients (Ramos and Olden, 2008). This metabolic syndrome (MS) associated with PCOS is a combination of central adiposity, increased fasting glucose and dyslipidemia (Grundy *et al*., 2005), these coexisting abnormalities sharing a common link, insulin resistance (IR) (Grundy, 2007). As regards evidence from animals models, adult female rats exposed to androgens prenatally develop increased visceral adiposity, increased fasting glucose and impaired glucose tolerance suggesting of impaired insulin action (Roland *et al*., 2010). Midgestational (d60-90) androgen exposure and postnatal overfeeding in sheep resulted in reduced insulin sensitivity (insulin resistance) and altered insulin dynamics in response to glucose challenge in the female offspring, which clearly suggests the prenatal androgenic effects leading to insulin resistance worsen with

postnatal weight gain (Padmanabhan *et al*., 2010), thus suggesting interactions between pre- and postnatal environments as regards severity of metabolic dysfunction.

1.0.5.1 Glucose intolerance

Hyperglycemia/dysglycemia or excess circulating glucose concentrations is a condition related to insulin resistance, in terms of being the end result of increased hepatic glucose production and decreased peripheral glucose uptake, which if unchecked may eventually lead to type 2 diabetes mellitus development (T2DM) (Guillausseau and Laloi-Michelin, 2003). Pancreatic β-cells secret excess insulin in order to compensate for peripheral insulin resistance, since IR effectively means that increased insulin is required to achieve the necessary insulin-mediated glucose disposal into peripheral tissues (Bergman *et al*., 2002). So, when insulin secretion by pancreatic β-cell is no longer able to meet the demands of increased glucose, dysglycemia results (Bergman, 2007). Dysglycemia with elevated triglycerides and low concentrations of high-density lipoprotein (LDL) is common in women suffering from PCOS (Wild *et al*., 1985). According to the National Institutes of Child Health and Human Development (NICHD) (Zawadzki and Dunaif, 1992), 20% of obese PCOS patients meet the criteria for impaired glucose tolerance (IGT) (Dunaif *et al*.,1987). In contrast, lean women with PCOS show no significant glucose tolerance differences when compared to age and weight matched controls (Dunaif *et al*., 1987), suggesting that metabolic features are varied within PCOS phenotypes, in accordance with the Rotterdam criteria (Moran and Teede, 2009). It is clear, however, that preconception adiposity and maternal body weight in both women and ewes can impair glucoregulation during pregnancy causing altered glycemic control and increased fetal growth (Clausen *et al.,* 2009; Ford *et al*., 2009). Moreover, prenatal maternal androgen exposure from gestational day 40-80 in addition to gestational glucose challenge resulted in altered glucoregulation in terms of increased insulin secretion in response to glucose challenge in female monkey offspring at ~1.5 months postnatal age, suggestive of experimentally induced fetal androgen excess leading to altered pancreatic

function (Abbott *et al*., 2010) and female rat offspring exposed prenatally to dihydrotestosterone (DHT) at gestational day 16-18, exhibited increased circulating glucose concentrations, suggesting that *in utero* effects of androgens can have long-term metabolic defects during postnatal life similar to those seen in women with PCOS (Roland *et al*., 2010).

1.0.5.2 Insulin Signaling

Insulin acts through several mechanisms to regulate glucose homeostasis, by stimulating insulin mediated glucose uptake in skeletal muscle, cardiac muscle, adipose tissue, and in addition, suppressing hepatic glucose synthesis (Bergman, 2007) and lipolysis (Groop *et al*., 1992), resulting in decreased circulating free fatty acids, further mediating its effects on hepatic glucose production (Bergman and Mittelman, 1998; Rebrin *et al*., 1995).

Insulin signals its actions in target tissues by binding to the insulin receptor located on the plasma membrane of target cells (Cheatham and Kahn, 1995; Kahn, 1994). This receptor is a heterotetramer consisting of two α, β dimers and bridged by disulphide bonds (Kasuga., 1982). The α subunit is located extracellular and performs a dual role, firstly containing the ligand-binding domain and secondly inhibition of the intrinsic kinase activity of β-subunit (Cheatham and Kahn, 1995; Saltiel and Kahn, 2001). The β-subunits' cytoplasmic portion possesses intrinsic protein tyrosine kinase activity, which in turn gets activated by ligand auto phosphorylation (Kasuga *et al*.,1982). Once insulin is bound to its receptor, it then triggers downstream signaling by activating tyrosine-kinases, which then phosphorylates intracellular substrates such as insulin receptor substrates (IRS) 1-4, for further signal transduction (Cheatham and Kahn, 1995; Myers *et al*, 1994). IRS are phosphorylated on specific motifs, and these phosphorylated sites bind to the SH2 domain of phosphatidylinositol 3-kinase (PI3-K) signalling molecule (Cheatham and Kahn, 1995; Saltiel and Kahn, 2001; Sun *et al.*, 1991) initiating downstream signaling pathways. Translocation of the glucose transporter (GLUT-4) from intracellular vesicles to the cell surface for glucose uptake (Cheatham and Kahn, 1995; Choi and Kim, 2010) is an important step functionally in terms of

insulin mediated glucose uptake, and this action occurs via, two pathways : firstly the activation of PI3K (phosphorylation) by kinases helps in conversion of membrane phospholipids and phosphatidylinositol 4,5-biphosphate, leading to 3-phosphoinositide-dependant protein kinase (PDK-1 and PDK-2) activation. Secondly, these protein kinases activate the serine/threonine kinases such as Akt/protein Kinase B (PKB), which in turn transmits the signal by phosphorylating ASI 160, a 160kDa substrate (Choi and Kim, 2010; Saltiel and Kahn, 2001) initiating the translocation. Apart from glucose transportation, AKT/PKB pathway activation via insulin is also known to play a key role in glycogen synthesis (Choi and Kim, 2010; Saltiel and Kahn, 2001) and gluconeogenesis (Logie *et al*., 2007), which highlights the importance of this pathway for metabolism. Insulin activated Akt pathway further activates downstream glucose transporters such as GLUT1, GLUT2 and GLUT-4 (Barthel *et al*., 1999; Eguez *et al*., 2005; Jiang *et al*., 2008). GLUT-1 is expressed ubiquitously (Manel *et al*., 2003), GLUT-2 is involved in hepatic glucose uptake and absorption of sugars by the intestine and liver (Pilkis and Granner, 1992), whilst GLUT-4 is mainly expressed in muscle and adipose tissue (Jiang *et al*., 2008). Insulin signalling is also involved in stimulation of cell growth and differentiation via the mitogen activated protein kinase (MAPK ERK) pathway (McKay and Morrison, 2007). MAPK acts via IRS resulting in activation of *Ras* (Saltiel and Kahn, 2001), which further results in stimulation of a series of serine/threonine kinase pathways such as MAPK-ERK1/2, which is then translocated into the nucleus initiating cell growth and differentiation (Saltiel and Kahn, 2001).

Insulin resistance (IR) can be defined as the decreased response to insulin in terms of insulin mediated glucose disposal into target tissues (Ben-Haroush *et al*., 2004) and 50% to 80% of women with PCOS are insulin resistant (Legro *et al*., 2004). In the pathophysiology of PCOS, insulin resistance interacts to cause both hyperandrogenism and anovulation, hence, it plays an integral role in PCOS (Poretsky *et al*., 1999). A synergistic activity of insulin with LH in the synthesis of androgen occurs in ovarian theca cells (Poretsky *et al*., 1999), which leads to arrest follicular development and aberrant follicular recruitment (Willis *et al*., 1998). Insulin acts through its receptors in

the theca cells by stimulating ovarian P450c17 mRNA expression and its enzyme activity via the phosphoinositide 3-kinase (PI3K) pathway (Baillargeon and Carpentier, 2007). In PCOS patients, increased serine phosphorylation of the insulin receptor may be a cause for decreased insulin sensitivity and compensatory hyperinsulinemia, which may then be the underlying stimulation for increased activity of the P450c17 enzyme system (Zhang *et al*.,1995). This combined effect in PCOS women may explain the relation between insulin resistance and hyperandrogenism (Dunaif, 1997).

A number of abnormalities with insulin action have been identified in women with PCOS, including impaired hepatic gluconeogenesis suppression (Dunaif,, 1989), and abnormalities in insulin receptor signaling (Dunaif, 1997). The excessive phosphorylation of serine residues of the insulin receptor is though to underlie insulin resistance in approximately 50% of women with PCOS (Dunaif, 1997). As evidence for this, cultured skin fibroblasts from women with PCOS display selective insulin resistance, where both insulin and IGF-1 stimulated glycogen synthesis is observed to be significantly decreased as compared to fibroblasts from non-PCOS women (Book and Dunaif, 1999) suggesting that the metabolic arm of insulin signaling can be altered without any alteration in the mitogenic pathway (Saltiel and Kahn, 2001). Basal auto-phosphorylation of the insulin receptor was significantly increased in the cultured skin fibroblasts and skeletal muscle from PCOS women, suggesting a potential mechanism for PCOS related insulin resistance (Dunaif *et al*.,1995) and also, recently, Rajkhowa *et al* (2009), observed decreased ERK activity in response to insulin in skeletal muscle biopsies from women with PCOS, suggestive of abnormalities in the mitogenic pathway in addition to the metabolic pathway. However, the exact relationship between altered insulin signaling and PCOS, at mechanistic levels remains poorly understood. Although, intriguingly, there have been recent studies indicating that maternal prenatal androgen exposure in an ovine model of PCOS leads to increased insulin secretion in absence of insulin resistance, suggesting an altered adult PCOS metabolic phenotype due to prenatal androgen exposure whereby hyperinsulinemia may in fact be

a primary defect as opposed to simply a secondary, compensatory effort by the pancreas to compensate for IR (Hogg *et al.*, 2011; Rae *et al*., 2013).

1.0.5.3 Hyperinsulinemia

Decreased insulin clearance or increased insulin secretion can lead to a hyperinsulinemic condition (Hücking *et al*., 2008). The first clinical evidence of β-cell dysfunction in PCOS women was demonstrated by Ehrmann *et al* (1995), where decreased post-prandial insulin secretory response and β-cell dysfunction to an oscillatory glucose infusion were observed. Compared to weight and aged matched controls, disposition index (DI) of insulin was significantly decreased in both lean and obese PCOS women (Dunaif and Finegood, 1996). Furthermore, obese adolescent girls with PCOS demonstrated β-cell dysfunction in terms of glucose intolerance (Arslanian *et al*., 2001). Taken together, these findings suggest that, independent of obesity; defects are evident in women with PCOS in terms of glucose stimulated insulin secretion. This backed up by evidence from animal studies, for example, female mice exposed to testosterone and streptozotocin (STZ) induced β-cell stress, demonstrated systemic oxidative stress and predisposition to β-cell failure (Liu *et al*., 2010), suggesting a role of excess androgens, in leading to β-cell stress and predisposition to β-cell failure in hyperandrogenic conditions. In addition, prenatal androgen exposure to pregnant female rhesus monkeys (term~165 days) during both early (gestational day 40) and late gestation (gestational day 100-115) demonstrated diminished β-cell function and female adult offspring exhibited impaired β-cell function (Eisner *et al*., 2000). This suggests that prenatal androgen exposure alters insulin:glucose homeostasis in the female offspring irrespective of the timing of gestational exposure and has implications in terms of understanding the metabolic abnormalities associated with PCOS. Hyperinsulinemia associated with PCOS is usually considered a consequence of insulin resistance (IR) (Goodarzi *et al*., 2005). However, prenatal androgen exposure in mice resulted in altered pancreatic β-cell glucose sensitivity (Roland *et al*., 2010).

Mice exposed to excess prenatal androgens showed increased glucose concentrations in absence of insulin resistance (normal peripheral insulin sensitivity) in response to glucose challenge in the female offspring (Roland *et al*., 2010) and these offspring also exhibited normal body weights compared to control animals, supporting the absence of insulin resistance (Roland *et al*., 2010) and corresponding to some aspects observed in adolescent PCOS girls (McCartney *et al*., 2006). This impaired pancreatic islet function might further lead to type 2 diabetes in the presence of other metabolic derangements such as obesity, since any dysfunction in β-cells may become clinically relevant only when the pancreas is placed under some form of 'stress' due to increased demand to compensate for underlying IR. Indeed, women with PCOS are at increased risk for diabetes development, likely due to impaired β-cell function causing lesser compensatory ability where IR is introduced to the overall equation (Legro *et al*., 1999).

There remains the question of whether hyperinsulinemia is completely consequential of insulin resistance. Given evidence of β-cell dysfunction, it is a possibility that at very least a sub-component of hyperinsulinemia could be attributable to a pancreatic β-cell defect as a primary cause. Tentative evidence for this can be seen in the studies of Roland *et al*., (2010), where increased insulin secretion was observed in animals prenatally exposed to excess androgens during early life. A recent study, involving ovine exposure to midgestational (d60-d102) androgens, resulted in increased insulin secretion in adult offspring with no changes in glucose tolerance, suggesting absence of insulin resistance in these animals (Hogg *et al*, 2011). This led to the hypothesis that an underlying primary pancreatic alteration might precede pancreatic functional changes in terms of compensatory hyperinsulinaemia to IR (Rae *et al*., 2013).

Maternal prenatal androgen exposure in sheep altered expression of key pancreatic developmental genes *in vivo* such as pancreatic duodenal homeobox-1 (*Pdx-1*), insulin receptor (*INSR*), insulin like growth factor-1 (*IGFR-1*), which were significantly elevated in the female fetal pancreas; and

in vitro glucose challenge of fetal pancreatic tissue from androgen exposed female fetuses resulted in excess insulin secretion compared to female controls (Rae *et al*., 2013). Moreover, during postnatal life, prenatal androgen exposure increased insulin secreting β-cells in the female adult pancreas, and caused increased insulin response to *in vivo* glucose load, in the absence of altered peripheral insulin signaling again suggestive of a primary pancreatic alteration (Rae *et al*., 2013). In addition to female pancreas, the latter study also investigated the male fetal pancreas to address the possibility of phenotypically male pancreas developing in females due to excess androgens, but found no changes in male fetal pancreas with respect to pancreatic gene expression or function (Rae *et al*., 2013). However, this may be due to altered endogenous male fetal androgen synthesis in response to exogenous androgen load (Connolly *et al*., 2013). Overall, this study suggests that there could be a primary β-cell dysfunction due to prenatal overexposure to androgens, with implications for metabolic abnormalities associated in women with PCOS.

1.0.5.4 Obesity and molecular dysregulation associated with PCOS

While not an essential feature of PCOS in terms of diagnosis, obesity is common in PCOS (Gambineri *et al*., 2002), and in the USA, among women diagnosed with PCOS, 42% were obese and 24% were overweight (Azziz *et al*., 2004). Certainly. PCOS has been associated with development of abdominal adiposity (Holte *et al*., 1994) as when compared to the general population, there is a fourfold increased prevalence rate of PCOS among obese and overweight premenopausal women (Alvarez-Blasco *et al*., 2006). This enlargement of adipose tissue mass and accumulation of free fatty acids (FFA) in non adipose tissue is associated with lipotoxicity and insulin resistance (Carpentier, 2008). Similarly, postnatal overfeeding in the female sheep offspring from prenatally androgenized pregnancies (d60-90) resulted in reduced insulin sensitivity (insulin resistance) and increased glucose concentrations during *in vivo* glucose challenge, respectively, which clearly suggests the prenatal androgenic effects leading to insulin resistance are

exacerbated by postnatal weight gain (Padmanabhan *et al*., 2010). This is confirmed by prenatal androgenisation of pregnant mice, which resulted in enlarged visceral adipocyte differentiation in young female offspring (Roland *et al*., 2010) and prenatal androgen (PA) exposure in normal fed (regular chow fed) Sprague-Dawley rats resulted in increased triglyceride and cholesterol concentrations in their female adult offspring (Demissie *et al*., 2008), and a high fat diet in a different cohort of mice following the same treatment regime developed further increased hepatic triglyceride content in female adult offspring, (Demissie *et al*., 2008). Interestingly both normal and high fat fed offspring rats offspring from androgenized pregnancies showed increased fasting serum insulin concentrations with no change in their glucose levels, suggesting the link between prenatal androgens and postnatal weight gain underlying metabolic alterations such as obesity and metabolic syndrome (Demissie *et al*., 2008). Similarly, reduced visceral adiposity with improved insulin resistivity in post-pubertal female adult sheep (20 months old) from early prenatally androgenized (d30-d90) pregnancies suggests that there is a period of developmental adaptation in these animals (Veiga-Lopez *et al*., 2013) because the same group had earlier demonstrated reduced insulin sensitivity in the PA young female adult sheep offspring (androgenised from d60-90 gestation) (Padmanabhan *et al*., 2010).

Clearly then, there is an identified need to further understand pancreatic development in PCOS models, and how prenatal environments can alter such development with consequences related to hyperinsulinaemia, if we are to understand the relationship between pancreatic function and insulin sensitivity in both these models and also PCOS. The following sections will discuss pancreatic development and the postnatal role of steroids during this process, relevant to the experiments designed and performed in this thesis.

1.1 The pancreas - structure, function, regulation and development

1.1.1 Basic Anatomy

The name pancreas is derived from the Greek word, ‘pan’ meaning ‘all’ and ‘creas’ meaning ‘flesh’ (Slack 1995). In humans, the pancreas weighs around 70-150 grams and measures 15-25 cm in length (Slack 1995) draining to the duodenum by the ampulla of Vater, which is where the main pancreatic duct is connected with common bile duct (Slack 1995) (Figure 1.2). The pancreas is comprised of two different types of glandular tissue, the exocrine pancreas; and the focus of this thesis, the endocrine pancreas. Exocrine function comprises secretion of digestive enzymes (proteases, lipases, amylases and nucleases) from acinar/duct cells. The Islets of Langerhans are the endocrine component of the pancreas containing alpha (α)- cells secreting glucagon (Nadal *et al*., 1999) beta (β) cells secreting insulin (Quesada *et al*., 2006) somatostatin releasing delta cells, PP cells secreting polypeptides and more recently identified epsilon (ε) - cells producing ghrelin (Prado *et al*., 2004). All of these endocrine cells collectively account for only 1-2% of total pancreatic tissue (Bouwens and Rooman, 2005).

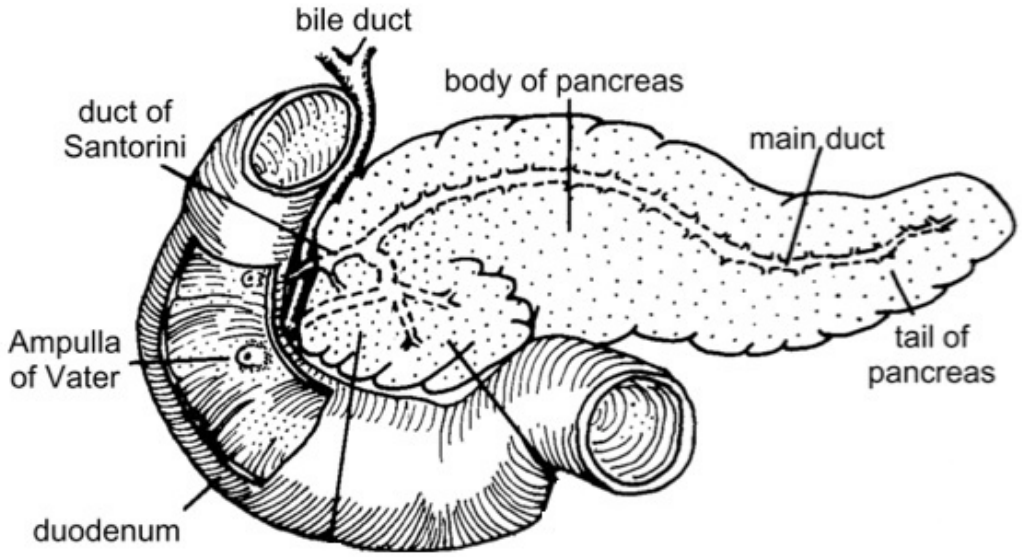


Figure 1.2 Anatomy of the human adult pancreas. (Slack 1995).

1.1.2 Pancreas- function

The regulation of blood glucose concentrations (endocrine) and digestion (exocrine) are the key functions performed by the pancreas (Gesina *et al*., 2004). Circulating glucose concentrations must be maintained in a precise range independent of dietary ingestion. This process of blood glucose homeostasis involves the liver, adipose tissue, brain, skeletal muscle and endocrine pancreas (Fritsche *et al*., 2008). Insulin secretion remains at low levels during fasting because of low plasma glucose concentrations. During this stage the compensatory hormone glucagon, along with corticosteroids and adrenalin aid in promoting the production of glucose from hepatic storage, and subsequent release into the circulation. Glucagon, a counter regulatory hormone to insulin, acts in response to insulin-induced hypoglycemia by raising glucose concentrations (Freychet *et al*., 1988) and thereby maintains blood glucose homeostasis. Glucagon is secreted in a pulsatile fashion by the α-cells of the islets (Opara *et al*.,1988) inducing hepatic glucose output via glycogenolysis and gluconeogenesis processes (Weigle and Goodner, 1986). Glucagon activates PKA phosphorylation, which then activates glycogen phosphorylase kinase, which phosphorylates (serine-14) glycogen phosphorylase. The phosphorylation of glycogen phosphorylase leads to increased glycogen breakdown (glycogenolysis) and produces glucose 6-phosphate (G-6-P), which is then converted into glucose by glucose-6-phosphatase (G-6-Pase), increasing the glucose pool for hepatic output (Johnson *et al*.,1997). Glucagon also regulates glucose concentration by stimulating hepatic gluconeogenesis mechanism acting via phosphoenolpyruvate carboxykinase (PEPCK) and CREB cycle finally leading to glucose secretion (Larsson and Ahrén, 2000; Okar and Lange, 1999). Whilst insulin is required for much of the peripheral uptake of glucose, it is imperative that circulating glucose concentrations are maintained within strict limits, as neuronal tissue glucose uptake is not insulin mediated and thus neuronal glucose regulation is a reflection of circulating concentrations. Insulin acts in a paracrine fashion and inhibits glucagon release via activation of the insulin receptor- phosphatidylinositol 3-kinase (PI3K) pathway (Kaneko

et al., 1999) and also increases K_{ATP} channel activity in rat α-cells via membrane hyperpolarization, thus inducing an inhibitory effect on glucagon production (Franklin *et al.*, 2005).

During the fed stage, where glucose concentrations rise due to absorption from the gut, insulin secretion from pancreatic beta cells is increased which then suppresses the glucose level in the body by promoting glucose uptake via muscles and adipocytes (insulin mediated glucose disposal (IMGD). Insulin also prevents liver hepatocytes from producing glucose by inhibiting the processes of gluconeogenesis and glucogenolysis (Fritsche *et al*., 2008). Increased translation of pre-pro-insulin transcript regulates the synthesis of insulin in response to acute glucose stimulation, whilst prolonged exposure to glucose results in the synthesis of insulin through insulin gene transcription (Poitout *et al*., 2006. β-cells are electrically excitable, playing a key role in regulation of secretion (Drews *et al*., 2010). The final insulin secretory response in β-cells is in turn driven by oscillations of membrane potential via Ca^{2+} influx (Rorsman *et al*., 2000).

1.1.3 Glucose stimulated Insulin Synthesis (GSIS) in pancreatic beta- cells

Insulin is secreted in response to glucose in a biphasic manner (Barbosa *et al*., 1998). Glucose transporter 2 (GLUT2) mediates the entry of glucose into beta cells through facilitated diffusion (Jiang *et al*., 2008), conferring regulation of insulin secretion at a cellular level. In response to increased glucose concentrations, glucokinase (GK) from the nucleus is released into the cytosol pool (Agius *et al*., 1995; van Schaftingen *et al*., 1997). The result being that glucose in the β-cell is now phosphorylated to glucose-6-phosphate (Matschinsky *et al* 1998) (Figure 1.3). In this way, GK is considered as a pancreatic beta cell glucosensor and plays a critical role in glucose stimulated insulin secretion (Iynedjian 2009). Pancreatic β-cells express low levels of lactate dehydrogenase (Schuit *et al*., 1997; Sekine *et al*., 1994) and high levels of pyruvate carboxylase activity (Schuit *et al*., 1997)

meaning pyruvate is synthesized by glycolysis in the β-cells and later enters the Kreb's cycle (Schuit *et al*., 1997). Following glucose phosphorylation by GK, adenosine triphosphate (ATP) is generated both by glycolysis and Kreb's cycle in the mitochondria (mitochondrial metabolism) leading to closure of ATP-sensitive K+ channels (K_{ATP} channels) due to an increase in both intracellular diadenosine polyphosphates (DPs) (Ashcroft, 2006) and ATP/ADP ratio (Ashcroft *et al*.,1984). As a consequence, depolarization of the plasma membrane occurs due to the closure of ATP sensitive K+ channels, leading to extracellular calcium influx (Ashcroft, 2006).

This electrical activity regulates the secretory response in β-cells that consists of oscillations of the membrane potential ranging from electrical silent periods to Ca^{2+} action potential originating depolarizing plateaus (Rorsman *et al*., 2000). The increased carbon dioxide (CO_2) influx produced from glucose into the Krebs cycle leads to production of intermediates such as malate (Brun *et al*., 1996; Schuit *et al*., 1997) glutamate (Maechler and Wollheim, 1999) and citrate (Brun *et al*., 1996; Schuit *et al*., 1997), which leave the mitochondria and accumulate into the cytosol stimulating insulin release (Prentki *et al*., 1997). Malate efflux promotes electron transfer from cytosolic NADH to NADPH (MacDonald, 1995), whilst citrate produces acetyl esters using malonyl coenzyme A (CoA) as a precursor and finally the combination of ATP and proton dependent step, the glutamate efflux from the mitochondria leads to the uptake of glutamate by the secretory vesicles, also referred to as large dense-core vesicles, which leads to exocytosis of insulin secretory granules (Maechler and Wollheim, 1999) (Figure 1.3).

Cytosolic Ca^{2+} induced insulin-containing secretory granules act synergistically with the cyclic adenine monophosphate (cAMP) pathway (Wang and Iynedjian, 1997) on the exocytosis process, transporting the insulin secretory granules to the plasma membrane of β-cells and finally releasing insulin into the circulation (Flatt 1996).

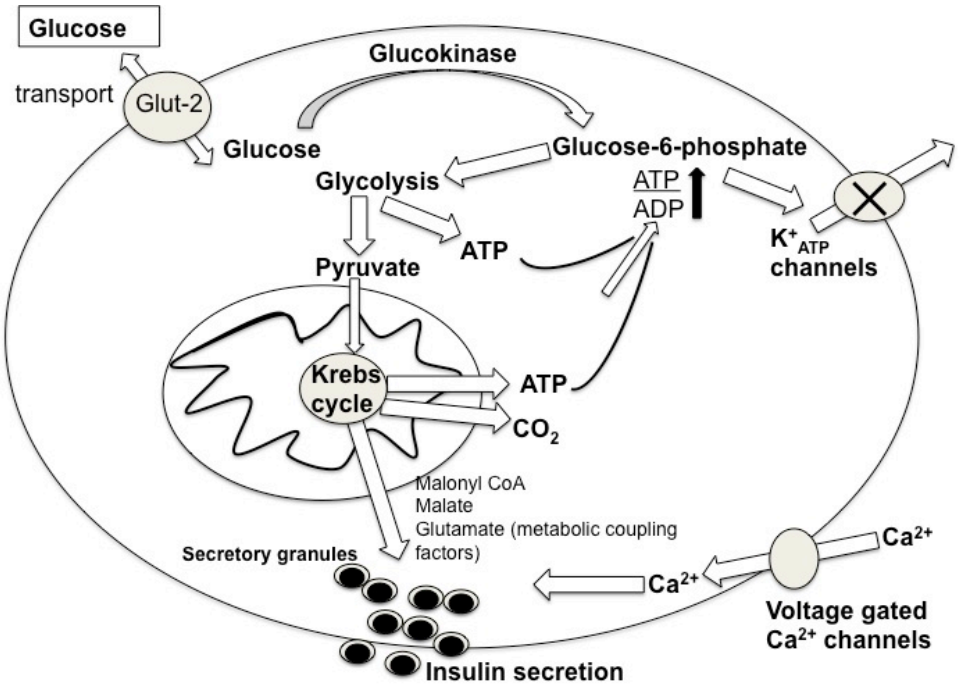


Figure 1.3 Schematic representation of glucose stimulated insulin secretion (GSIS) in pancreatic β-cells.

In brief, after the glucose uptake by Glut-2, glucokinase acts as a rate limiter by converting glucose to glucose-6-phosphate. This increase in glucose metabolism leads to production of ATP via glycolysis, pyruvate oxidation and reducing equivalent shuttles, resulting in increased ATP/ADP ratio, leading to inhibition of KATP channels and membrane depolarization. This in turn leads to activation of voltage gated Ca2+ channels and in combination with coupling factors exocytosis of insulin granules is activated resulting in insulin secretion.

1.2 Pancreatic Development in PCOS animal models

Understanding of pancreatic development owes much to research driven by alarming increases in the rates of development of type-2 diabetes mellitus and insulin resistance (Hill and Duvillié, 2000). Consumption of a high-calorie diet, changes in lifestyle and lack of exercise have lead to an increase in global prevalence of diabetes and obesity (Kasuga, 2006). About 60-90% of human clinical study cases of diabetes are known to suffer from obesity

related type-2 diabetes mellitus (Anderson *et al*., 2003), which is further interlinked to insulin resistance (Ludvik *et al*.,1995).

The prenatal period is considered to be one stage where beta cells obtain their capacity for insulin secretion. With direct relevance to this thesis, prenatal androgen exposure in female mice has been shown to result in a dampened response to glucose by islets *in vitro*, suggesting *in utero* exposure of androgens resulted in altered β-cell function (Roland *et al*., 2010). Similarly, maternal androgen exposure in sheep at d62-d102 increases the expression of genes (*Pdx-1, IGFR-1, INSR and INS*) involved in β-cell development and function during fetal life, which translates *in vitro* to increased insulin secretion from the female fetal pancreas at d90 gestation (Rae *et al*., 2013). As later in the thesis the effects of prenatal steroid exposure on fetal pancreatic development and function will become a focus of the work, it is therefore important to understand the development of pancreas under normal physiological conditions.

Much of the work that informs literature on embryonic development of the pancreas is derived from rodent and human studies, however, there are important differences with regards to the timing of the β-cell differentiation between human and mouse (Piper *et al.*, 2004; Sarkar *et al*., 2008), meaning caution must be exercised in extrapolations from one species to another. In this regard, for a better understanding of the pancreatic morphogenesis in humans, the sheep is perhaps a better comparator animal than a rodent (Green *et al.*,2010) due to sheep being a large animal model to study fetal physiology (Green *et al*.,2010; Padmanabhan and Veiga-Lopez, 2013) and also there being striking similarities between human and ovine pancreatic development (Green *et al*., 2010). Numerous processes that occur during intrauterine life in humans and sheep only occur postnatally in rodents, underscoring species differences in development, and emphasizing that the correct animal model is critical; whilst rodents may be convenient in terms of study, they are not a universally relevant or applicable animal surrogate of human development.

Mammalian pancreatic development follows 3 key steps, namely primary transition, secondary transition and isletogenesis (Green *et al*., 2010). The pancreas is derived from the endoderm region of the developing embryo and after the primary transition, the cells protodifferentiate to form the pancreatic anlagen (Pictet *et al*.,1972). Around embryonic day 9.5 in the mouse (33% of total gestational age of 28 days), and in humans (Piper *et al*., 2004) around dGA (days gestational age) 25-26 (9.6% of total gestational age 265 days), two epithelium buds form, one arising dorsally from the upper duodenal part of foregut, while the second arising from the ventral to the hepatic endoderm, which then fuse (Slack, 1995). During the primary transition, a dense outgrowth is formed when the pancreatic precursor (epithelial progenitor) cells expand, become lobulated and finally elongate into branches (Piper *et al*., 2004). This elongation is associated with a condensed mesodermal covering in rodents (Piper *et al*., 2004). In contrast, the pancreatic buds elongate into a loose mesenchymal bed in humans around 26-41 dGA (12.6% of total gestational age) (Piper *et al*., 2004) and between 24-29 dGA (18% of total gestational age) in the sheep (Cole *et al*., 2009). Later, progenitor cell expansion culminates with 'differentiated state' or the secondary transition in mouse between embryonic day 13.5-15.5 (50% of total gestational age 28 days) (Pictet *et al*., 1972) however, in conjunction with progenitor cell expansion, the secondary transition in humans begins at 52dGA (19.6% of total gestational age of 265 days) and in sheep before 24dGA (16.3% of total gestational age 265 days) (Cole *et al*., 2009; Piper *et al*., 2004).

Pancreagenesis involves two other processes; islet cell replication and isletogenesis. The mitotic activity of the newly formed endocrine cells remains quiescent until term embryonic day 19 (E19) in mouse (Jensen *et al*., 2000). In humans, the β-cell proliferation commences at 8 weeks gestational age (21.1% of total gestation of 265 days) and replication continues throughout gestation (Kassem *et al.*, 2000). A similar time frame is seen in sheep where β-cell differentiation and proliferation occurs simultaneously during pancreatic

development (Cole *et al*., 2009). Isletogenesis or formation of Islet-like structures occurs in humans around 11 weeks gestational age (Piper *et al*., 2004), and around 33dGA in sheep (22.7% of total gestational age of 147 days) (Cole *et al*., 2009). Cole *et al* (2009) describe isletogenesis in sheep pancreatic development, whereby islets develop by undergoing an epithelial-mesenchymal transition (EMT) from individual epithelial progenitor cells of the pancreatic tubule. In brief, the EMT process, which is driven by the SNAIL family of pancreatic developmental molecules, helps in making the epithelium behave like a β-cell and produce insulin while still remaining part of the epithelium and then the β-cell is extruded from the epithelium by forming a 'bottle shape' (Cole *et al*., 2009). Finally, once the individual β-cell is separated, it then replicates to form a β-cell mass (Cole *et al*., 2009). The following section describes in detail some of the known key genes involved in β-cell development and function during fetal life.

1.2.1 Pancreatic Duodenal homeobox 1 (PDX-1)

PDX-1 plays a key role in β-cell survival, pancreatic precursor cell maturation and differentiation in the developing gut (Butler *et al*.,2007; Kim and Hebrok, 2001). PDX-1 gene belongs to the mammalian *Parahox* gene cluster, representing mammalian genes that are involved in development and found outside the classical *Hox* (Homeobox) gene cluster (Brooke, Garcia-Fernàndez, and Holland, 1998). The latter *Hox* gene cluster comprises three genes: *Pdx1* (β-cells), *Gsh1* (α-cells) and Cdx2/3 (α-cells) (Rosanas-Urgell *et al*.,2005). PDX-1 is involved in initiating differentiation and morphogenesis of the mouse pancreatic epithelial progenitor cells, which later restricts to mature β-cells in adult life (Offield *et al*., 1996) and is also involved in transactivation of β-cell specific genes (Sander and German, 1997) such as somatostatin, insulin, glucose transporter-2 (Glut-2) and glucokinase in rats (Gremlich *et al*., 1997). Similar cellular pattern of PDX-1 expression are seen in humans (Piper *et al*., 2004) and PDX-1 expression and localization was observed at 33 dGA in sheep fetal pancreatic β-cells (Cole *et al*., 2009). *Pdx-1* gene expression and immune-detection was also evident in adult mouse

pancreatic islets (Rosanas-Urgell *et al*., 2005). This is an indication of the dual action of PDX-1, because it is an essential factor in early pancreatic development and also a key transcription factor for proper functioning of pancreatic islets and glucose homeostasis during adulthood.

PDX-1 is involved in regulation of insulin gene transcription via glucose metabolism (Wu *et al*., 1999). In brief, PDX-1 is activated by glucose via insulin-dependent cell signaling, which involves phosphatidylinositol 3-kinase (PI3K). This leads to activation of the cytoplasmic form of PDX-1 and translocation to the nucleus where it binds to specific gene promoter sequences initiating transcription (Wu *et al*., 1999).

Thus pancreatic agenesis is observed in PDX-1 null (knock out) mice, due to inhibition of branching and morphogenesis of the initial buds (Jonsson *et al*., 1994) and a similar condition is noticed in a homozygous mutation of the *Pdx-1* gene in humans (Stoffers *et al*., 1997), underscoring the importance of PDX-1 in the pancreatic developmental process. *Pdx-1* point mutation and frame shift mutations are observed in heterozygous human patients, who subsequently suffer from a subtype of maturity onset of diabetes in young (MODY), namely familial early-onset type 2-diabetes (Ashizawa *et al*., 2004).
In the pancreatectomy rat model (Px) and Zucker diabetic fatty (ZDF) rat model, PDX-1 protein expression levels were significantly reduced (Zangen *et al*., 1997), and in the Px rat model, both Glut-2 and insulin gene expression were reduced (Zangen *et al*., 1997). As PDX-1 is involved in regulation of insulin and Glut-2 gene expression (Gremlich *et al.*, 1997), it is arguable that downregulation of PDX-1 in these animal models results in the pathogenesis of pancreatic β-cell failure and type-2 diabetes (Weir *et al*., 1997). Certainly, pancreatic islets exhibit reduced protein expression of PDX-1 and reduced islet area in both new born pups and neonatal life (28 postnatal) rats of rat dams who were exposed to low protein diet during gestation and lactation (Arantes *et al*., 2002). In a maternal protein restriction sheep model, β-cell function was determined at the molecular level, which revealed that *Pdx-1* gene expression was unaltered during fetal life, however it was upregulated in

young adult lambs from low protein diet pregnancy, further leading to impaired insulin secretion (Gatford *et al*., 2008). Thus the above studies suggest that intrauterine environment (low protein diet) can have an effect on *Pdx-1* expression in terms of β-cell mass and function. Furthermore, from the above evidence, it is arguable that maternal environmental insults can act upon the developing pancreas, and the effects are evident even during postnatal life as seen in terms of impaired pancreatic endocrine function in sheep (Gatford *et al*., 2008). Recently, our group investigated the effects of maternal *in utero* androgen exposure (TP) at d62-d102 in sheep, which showed us that *Pdx-1* gene expression was significantly increased in female fetal pancreas (Rae *et al*., 2013), and was associated with altered *in vitro* fetal insulin secretion and a hyperinsulinemic condition during adult life (Rae *et al*., 2013), which would back up these studies.

1.2.2 Insulin like growth factors (IGFs) and Insulin like growth factor receptors (IGFRs)

Insulin-like growth factors (IGFs) during pancreatic development comprise of type 1 (IGF-1) and type 2 (IGF-2), whose receptors belong to protein tyrosine kinase receptor family. Both insulin and IGF ligands can bind to either insulin receptor or IGF-1 receptor. IGF-1 is a key hormone in pancreatic islet cell development, cell proliferation, growth and metabolism (Kenyon, 2010). IGF signaling is thought to play an important role in the linkage of low birth weight to development of insulin resistance, obesity and type-2 diabetes mellitus (Eriksson *et al.*, 2002; Godfrey and Barker, 2001). IGF-1 acts via IGFR-1 and/or insulin receptor, where the latter two have similar homology (Byrne *et al*., 2002). IGF-1 binds to the extracellular domains of the insulin receptor or IGFR-1, where the signal is mediated through auto-phosphorylated tyrosine residues of the intracellular β-subunits of the insulin receptor, which leads to phosphorylation of insulin receptor substrate-2 (IRS-2) (Burks and White, 2001). The phosphor-inositol 3-kinase (PI3K) pathway is activated upon IRS-2 protein phosphorylation, which drives expression of IGFs effects on glucose homeostasis. The downstream signal transduction of PI3K results in several

IGF mediated effects such as gene expression, mitogenesis, cell growth and protein synthesis. PI3K pathway activates transcription factors such as Pdx-1, via activating phosphokinase B (PKB), also known as Akt, and target of rapamycin (mTOR) in the downstream signaling (Withers and White, 2000). PKB in turn regulates gene expression via inactivation of transcription factor forkhead in rhabdomuosarcoma (FoxO1), which enhances the activity of signal translator and activator STAT-3 dependent promoters (Kortylewski *et al*., 2003). mTOR acts as an inhibitor on the translational process by inhibiting 4E-binding protein (4E-BP1), an cell cycle by activating $p70^{s6k}$. The latter will finally induce an increase in β-cell size due to the translation of ribosomal proteins and elongation factors such as cyclin D and E (Kortylewski *et al*., 2003).

Apoptosis remains the key cause of β-cell death in insulin secreting β-cells or pancreatic islets when exposed to different pathological conditions like diabetes (Butler *et al*., 2007). In case of β-cell failure, disturbances in the insulin/insulin growth factor (IGF) signaling pathway results in decreased expression of *Pdx1* (Chan-Chen *et al*., 2008). IGF-1 acts on β-cell apoptosis by activating phosphokinase-B and also inhibiting Bcl-2 associated death protein (BAD) activity, thereby giving such signalling its displaying its antiapoptotic activity (Henshall *et al*., 2002) and further maintaining/increasing cell numbers by inhibiting apoptosis (Bryne *et al*., 2002). All the pathways mentioned activated via the IGF-1 signalling pathway (Burks and White, 2001; Rhodes and White, 2002; Withers and White, 2000) have clear implications for fetal development since pancreatic β-cells clearly depend on the interactions of these growth factors (Holt, 2002) for the proper β-cell growth and function. Foe example, pancreatic *IGF-I, IGF-II* and insulin receptor (*INSR*) gene expression are all significantly upregulated in young lambs who are prenatally exposed to protein restriction or fetal growth restriction with downstream consequences of unchanged β-cell mass but impaired β-cell function (Gatford *et al*., 2008). In addition, prenatal androgenization significantly increased the mRNA expression of *IGF-1* in the liver of adult offspring but not during fetal life in an ovine model of PCOS

(Hogg *et al*., 2011). In the same sheep model of PCOS, mRNA expression of pancreatic *IGFR-I*, *INSR* (insulin receptor) and *INS* (insulin gene) are significantly upregulated during fetal life in response to prenatal androgenisation and postnatal changes in insulin secretion are one such legacy of the prenatal androgenic environment (Rae *et al*., 2013). Collectively, the above findings suggest that metabolic alteration in terms of IGF/insulin signaling can be permanently affected during *in utero* life, and consequences felt in adult life.

1.3 Is the Pancreas a sex steroid dependent tissue?

Sex steroid hormones (SSH) are so-called because of their role in reproductive function. Comprising of androgens (androstenedione, dihydrotestosterone (DHT), testosterone, dehydroepiandrosterone) estrogens (estrone, estriol and estradiol) and progestagens (progesterone), the major sources of these are adrenal glands, testis, ovary and placenta. Such steroids play fundamental roles throughout life, including sexual differentiation, reproductive axis function, spermatogenesis and the menstrual cycle (Wilson and Foster, 1992). In terms of the process and regulation of steroidogenesis, this is dealt with in detail in later sections on the adrenal gland and ovary.

In terms of gonadal steroid production, in brief, under hypothalamic regulation via gonadotropin releasing hormone secretion (GnRH), the anterior pituitary secretes gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH), which control steroidogenesis in the gonads of both males and females (Santoro *et al*., 1986) as illustrated in Figure 1.4. The hypothalamic pituitary adrenal axis operates to produce steroids in an analagous fashion, utilizing hypothalamic CRH (Corticotrophic releasing hormone) to drive anterior pituitary ACTH culminating in production of sex steroids and glucocorticoids from the adrenal glands (Figure 1.4). In both cases, negative regulations is affected by the steroids produced (Figure 1.4).

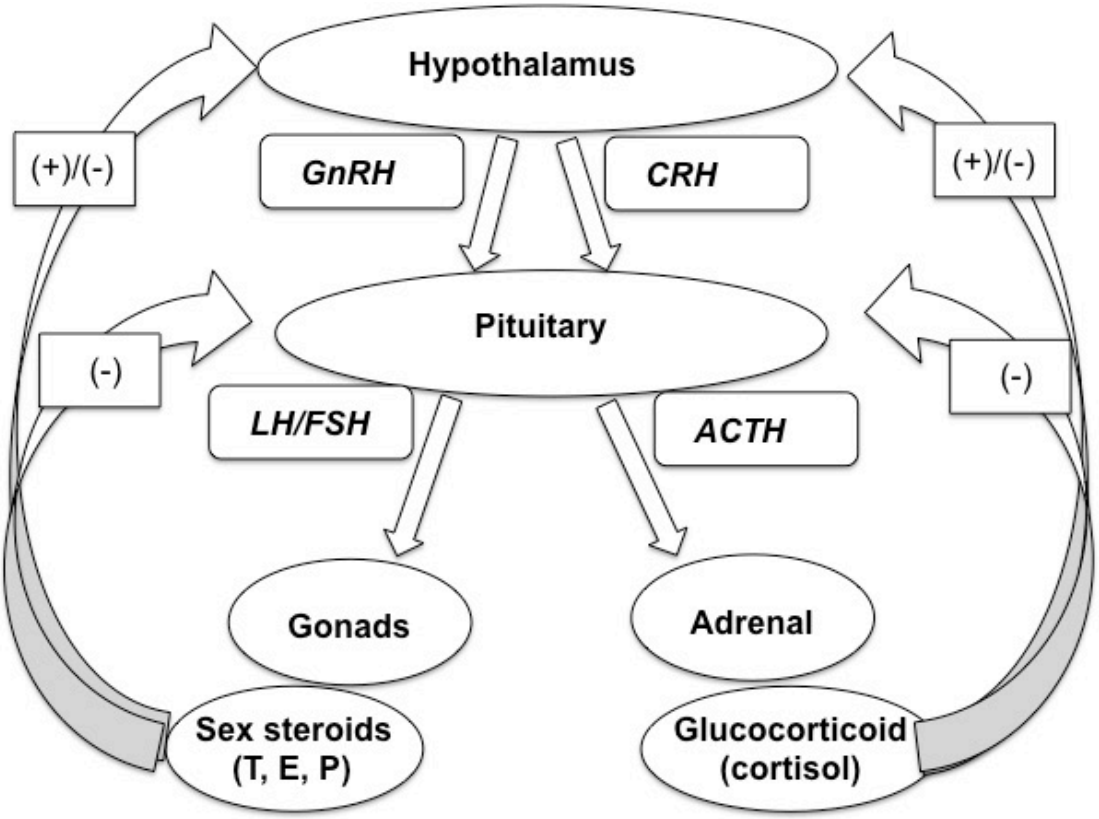


Figure 1.4 The hypothalamic pituitary gonadal (HPG) and hypothalamic adrenal axes (HPA) acts via negative feedback mechanism.
(GnRH- gonadotrophic releasing hormone; LH-luteinizing hormone; FSH-follicle stilumating hormone; T-testosterone, E-estrogen, P-progesterone; CRH- Corticotrophic releasing hormone; ACTH-adrenocorticotrophic hormone).

The presence of sex steroid hormone (SSH) receptors in pancreatic tissue suggests a relationship between SSH and pancreatic tissue (Greenway *et al*., 1981). Progesterone receptors (PR) are expressed in 75% of glucagon secreting cells and 20% of insulin secreting cells in pancreas (Doglioni *et al*., 1990). Importantly, androgen receptor (AR) mRNA expression in rat pancreas (Diaz- Sanchez *et al*., 1995), ovine fetal pancreas (Rae *et al*., 2013) and human fetal and adult pancreas (Corbishley *et al*., 1986) has been demonstrated and estrogen receptor (ER)-α and ER-β are also expressed by insulin secreting β-cells (Alonso-Magdalena *et al*., 2008; Nadal *et al*., 1999).

Hence it is clear that the pancreas has the capability of responding to all classes of SSH.

Steroid metabolism potential of pancreatic tissue is indicated by expression of steroidogenic enzymes such as 3-β hydroxysteroid dehydrogenase (3β-HSD) and 17-β hydroxysteroid dehydrogenase (17β-HSD), both have been localized in the canine endocrine pancreas (Mendoza-Hernández *et al*., 1996; Mendoza-Hernández *et al*., 1988). Enzymes involved in androgen biosynthesis have been detected in rat pancreatic tissue; examples include P450scc and P450 17-α both of which are critical in *de novo* synthesis of androgen (Ogishima *et al*., 2008). Additionally, testosterone is involved in regulation of insulin gene expression, and insulin secretion *in vitro* in cultured adult rat islets (Morimoto *et al*., 2001), suggesting the possibility of a role in insulin secretion *in vivo*.

1.3.1 Androgens and Pancreatic function in PCOS

Androgens are the sex steroids involved in the development of a male phenotype during fetal life; sexual characteristics at the time of puberty and also maintenance of the male sexual behavior and function. In females, androgens are a precursor for ovarian estradiol production, and are regulators of ovarian follicular recruitment (Burger, 2002). 5-α reductase enzyme peripherally converts testosterone into a more potent androgen dihydrotestosterone (DHT) (Azzouni *et al*., 2012). Both androgen and DHT bind to and activate the androgen receptor (AR), a 110kDa protein (Radmayr *et al*., 2008), which is a key transcription factor for androgen mediated signaling.

AR belongs to the steroid nuclear receptor superfamily (Heinlein and Chang, 2002) and its phosphorylation is mediated via serine residues with ligand bound to AR (Gioeli *et al*., 2002). Kinase recruitment and serine phosphorylation, which protects AR from proteolytic degradation, is promoted by androgen binding (Blok *et al*., 1998). The phosphorylated conformational

change promotes AR movement from the cytoplasm into the nucleus, which is followed by binding of AR with the androgen response element (ARE) (van Royen *et al*., 2007). This binding of tissue specific AREs to AR results in recruitment of histone acetyl transferase enzymes, further leading to androgen dependent gene transcription (Heinlein and Chang, 2002).

Androgenic effects on endocrine pancreatic function can occur via both genomic and non-genomic mechanisms. For instance, androgen receptor mRNA was expressed and downregulated due to testosterone administration in rat pancreas (Díaz-Sánchez *et al*., 1995). *In vivo* insulin mRNA and *in vitro* insulin concentration was significantly upregulated in prepubertal male rat pancreas exposed to testosterone, however, both were decreased due to gonadectomy (testosterone deprivation), (Morimoto *et al*., 2001). Testosterone administration in adult male rats resulted in increased insulin mRNA, insulin protein abundance and insulin release (S Morimoto, Fernandez-Mejia *et al*., 2001), suggesting a direct role of androgens in pancreas islet function and also a new potential target for treating hyperandrogenic conditions such as PCOS associated metabolic disorder (Bruns *et al*., 2004b). Other studies also demonstrate the protective effect of testosterone. Compared to gonadectomised male rats, apoptotic beta-cell mass index (apoptotic nuclei/ total cells nuclei) was significantly reduced in male gonadectomised rats who were administered with testosterone enanthate, (Morimoto *et al*., 2005). This effect was completely reversed due to anti-androgen (flutamide) treatment indicating a classical androgen receptor mediated mechanisms in the pancreas (Morimoto *et al*., 2005). These rats, later supplemented with testosterone and administered with STZ showed androgen-mediated cytoprotective activity by inhibiting β-cell apoptosis via induction of antioxidant enzyme catalase and super oxidase dismutase (Palomar-Morales *et al*., 2010). Endocrine gland-derived vascular epithelial growth factor (EG- VEGF) mRNA expression is increased in rat pancreatic islets treated with testosterone whereas the reverse is observed in treatment with flutamide (Morales *et al*., 2008). Dehydroepiandrosterone decreased glucose driven insulin release in BRIN-BD11 cell line, which is

derived from RINmF rat insulinoma cells. The serum insulin concentration was less in case of streptozotocin (STZ) treated Wistar females rats compared in males, in which the former developed hyperglycemia and β-cell destruction and were more susceptible to diabetes at a high rate when compared to males (Vital *et al*., 2006). However, there remains the possibility of sex-specific β-cell responses to androgens since the majority of these studies have utilized male castrate animals thus avoiding complexing features of the ovarian cyclical variations in circulating steroids. Hence male animals have been pre-exposed to relatively high concentrations of androgens during fetal life, whereas female animals have not, thus one focus of this thesis will be to examine potential differences attributable to sex in terms of androgenic pancreatic responses.

There is also some evidence of nongenomic effects of testosterone in rat pancreatic β-cells. Physiological concentrations of testosterone administered *in vitro* in isolated rat pancreas, resulted in rapid stimulation of Ca^{2+} uptake and subsequent insulin secretion (Grillo *et al*., 2005). Dehydroepiandrosterone (DHEA), on the other hand, had an inhibitory effect on carbachol induced insulin secretion in human pancreatic islets and carbachol induced Ca^{2+} release and insulin secretion in rat insulinoma (INS-1) cell. Activity of carbcachol induced Ca^{2+} release by DHEA was blocked by pertussis toxin activity, suggesting DHEA acting via non-genomic mechanism (G-protein receptors) of androgens in mediating the signal transduction in pancreatic β-cells (Liu *et al*., 2006).

Not only does testosterone have protective effects on pancreatic β-cells, but it is also involved in discordant effects on diabetes. Female non-obese mice (NOD) mice, a model for spontaneous type-1 diabetes, became diabetic at a higher frequency than males, however further diabetic development was prevented by treating the mice with androgens (Fitzpatrick *et al*, 1991 and Fox, 1992), and castration in the NOD males result in a similar condition observed in females prior to androgen replacement (Fitzpatrick *et al*, 1991 and Fox, 1992). In context of metabolic disturbances associated with PCOS

and relevant to this thesis, prenatal androgen exposure in animal models such as rats have altered metabolic function in terms of altered glucose tolerance, and β-cell dysfunction in adult female mice (Roland *et al*.,2010) and in the ovine model of PCOS (Rae *et al*., 2013), in terms of upregulated *Pdx-1* mRNA expression *in vivo* and increased *in vitro* insulin secretion during fetal life in females, and the legacy of hyperinsulinemia and increase insulin secreting β-cells later in adulthood, suggesting a role for prenatal androgens in programming long-term alterations in metabolic function similar to conditions such as PCOS in women (Dumesic *et al*., 2007). All these studies suggest that androgens influence responsible for glucose mediated insulin secretion, pancreatic beta cell destruction and development of diabetes. Collectively, these observations suggest that androgens can alter glucose mediated insulin secretion and pancreatic β-cell function, both in a contemporary way, and also via developmental perturbation with a potential lifelong, health-relevant legacy.

1.3.2 Estrogens and pancreatic function in PCOS

Estrogens are known to play important roles in blood glucose homeostasis, such as maintaining normal insulin sensitivity during pregnancy and menstrual cycle (Livingstone and Collison, 2002; Louet *et al*., 2004) and glucose stimulated insulin secretion (GSIS) both *in vivo* and *in vitro* (Alonso-Magdalena *et al*., 2006; Nadal *et al*., 1998). Furthermore, increased or decreased estrogen levels outside physiological ranges can promote insulin resistance and type-2 diabetes (Ding *et al*., 2007; Godsland, 2005; Livingstone and Collison, 2002). Ovariectomy or menopause leading to low estrogen concentrations is therefore associated with impaired glucose tolerance and insulin resistance (Godsland, 1996). In addition, adult male mice treated with E2 (estradiol) for 4 days (100μg/kg/day), demonstrated alterations in glucose tolerance, became insulin resistant and finally hyperinsulinemic, illustrating the potential for estradiol concentrations to alter glucose homeostasis in the short term (Ropero *et al*., 2008).

Estrogen receptor- α (ERα) and estrogen receptor-β (ERβ) are the two nuclear receptors, through which estrogens mediate their effects (Losel *et al*., 2003; Sutter-Dub, 2002). Both the receptors are expressed by insulin secreting β-cells (Alonso-Magdalena *et al*., 2008; Liu and Mauvais-Jarvis, 2010). Only ERα agonist treatment showed significant increase in insulin secretion *in vitro* indicating that estrogenic action on insulin biosynthesis is mediated by ERα (Alonso-Magdalena *et al*., 2008). Genetic polymorphisms of ERα gene in humans is associated with type-II diabetes and metabolic syndrome (Gallagher *et al*., 2007), and ERα knockout mice display increased rates of obesity and insulin resistance (Barros and Gustafsson, 2011; Ropero *et al*., 2008). That reduction in β-cell mass and pancreatic insulin content observed in both male and female wild type mice exposed to a single dose of streptozotocin (STZ) (Vital *et al*., 2006) was further exacerbated in aromatase knockout (ArKO) male and female mice and could be rescued to some extent by supplementation with estradiol treatment (Vital *et al*., 2006), demonstrating the protective effect of estradiol via ERα. Impaired glucose metabolism and insulin resistance is also found in human patients suffering from aromatase deficiency due to a point mutation of the aromatase gene (Zirilli *et al*.,2008). Estradiol is known to regulate the expression of several genes involved in islet physiology. In female Wistar rats, insulin gene expression and circulating insulin levels vary across the estrous cycle in accordance with circulating estradiol and progesterone levels (Morimoto *et al*., 2001). Estrogen deprived (ovariectomised, OVX) female Sprague-Dawley rats show decreased pancreatic glucokinase gene expression, resulting in decreased insulin secretion capacity, however, insulin levels return to normal after estrogen replacement, clearly suggesting the direct effect of estrogens on β-cell gene expression (Choi *et al*., 2005). Estradiol also regulates the expression of *Pdx-1* gene, a key transcription factor involved in development of the pancreas and also transcription of insulin gene (Choi *et al*., 2005). In addition to classical, genomic signaling effects of estrogens, estradiol may also exerts its effects via non-genomic actions. Fort example, rapid effects of 17-β estradiol in islet cells leads to activation of cGMP/PKG signaling in male mice (Nadal *et al*., 1998). Nadal *et al* (1998), also found increased expression of intracellular

Ca^{2+} and decreased K^{ATP} channel activity due to 17β-stradiol effect in male pancreatc β-cell, suggesting augmenting of insulin secretion. On the contrary, inappropriate or pathophysiological estrogenic signaling may lead to adverse effects, for example, the environmental disrupting compound Bisphenol A (BPA), which has estrogenic properties, can promote postprandial hyperinsulinemia and insulin resistance in healthy male mice (Alonso-Magdalena *et al*., 2006). This study also suggested that BPA and naturally occuring estrogens have similar potency in upregulation of insulin secretion (Alonso-Magdalena *et al*., 2006).

Estradiol valerate (EV) treatment in rats during prepubertal life staring at day 14 through to neonatal life (Rosa-E-Silva *et al*., 2003) and letrozole (nonsterdoidal aromatase inhibitor to block conversion of androgen to estrogen) starting at postnatal day 21- 3 months (early) (Mannerås *et al*., 2007) or at postnatal day 42- 3 weeks (late) administration (Baravalle *et al*, 2006; Kafali *et al*, 2004) are the two main models used to study the role of prenatal estradiol programing. Letrozole administration to rats at two different time points causes ovarian hyperandrogenism (Mannerås *et al*., 2007; Baravalle *et al*, 2006 and Kafali *et al*, 2004). In early letrozole treated rats insulin sensitivity, visceral fat and lipid profiles were unaffected (Mannerås *et al*., 2007). Given that androgens such as testosterone can be metabolized to estradiol via P450 aromatase, which is found in many tissues including the placenta, a sub-aim of this thesis is to delineate which effects in androgen exposure models are truly androgenic in nature, and which may be indirect via metabolism to estrogenic steroids.

1.3.3 Glucocorticoids and pancreatic function

Glucocorticoids such as corticosterone and cortisol are the class of steroid hormones secreted by the zona fasiculata, a component of the outer cortical region of the adrenal gland (Endoh *et al*., 1996 and Miller, 2008). After entering the cytoplasm of the target cells via diffusion, glucocorticoids exert effects via binding to glucocorticoid receptors (GR) (Funder, 1992). GR

belongs to the superfamily of the steroid nuclear receptors. GR-α and GR-β are the two variants of GR produced by alternative splicing (Duma *et al*., 2006) with a molecular weight of 94kDa and 90kDa respectively (Giguère *et al*., 1986). The triggering of phosphorylation, dimerization and translocation of GR into the nucleus occurs due to the binding of GC to its receptor isoform GRα, which in turn binds to particular elements on DNA resulting in repression or enhancement of hormonal effective gene transcription (Kumar and Thompson, 1999). Outcomes include stimulation of hepatic gluconeogenesis via GR and induction of key enzymes such as glucose- 6-phosphate and phosphoenolpyruvate carboxykinase.(PEP-CK) (Hanson and Reshef, 1997). By activating glycogen synthase and inactivating, glycogen phosphorylase, a glycogen-mobilizing enzyme, glucocorticoids can also stimulate glycogen synthesis and furthermore decrease translocation of glucose transporters (GLUT 4) to the cell surface thereby inhibiting peripheral glucose uptake (Dimitriadis *et al*., 1997).

GCs are involved in regulation of many pathways such as those involved in stress responses, blood pressure maintenance, metabolism, fluid and electrolyte homeostasis and response to infection (Reynolds, 2010). Endogenous levels of glucocorticoids are maintained not only by HPA axis activity (discussed below), but also by intracellular 11β-hydroxysteroid dehydrogenases (11β-HSD), which catalyse extra-adrenal, peripheral interconversion of inactive 11-keto metabolites and active glucocorticoids (Nixon *et al*., 2012). Numerous fetal tissues and placenta express GRs from early embryonic stages (Cole *et al*., 1995; Speirs *et al*., 2004). Placental 11β-HSD 2 expression protects the developing fetus tissues from the high levels of maternal glucocorticoids by converting the active glucocorticoid cortisol into its inactive form cortisone (J R Seckl, 1997). In this role, and in its role in numerous cells expressing the mineralocorticoid receptor (MR), 11βHSD2 is thought of as a gatekeeper enzyme, since it prevents occupation of MR by GCs and thereby protects against apparent mineralocorticoid excess syndrome (AME) (Michael *et al*., 2003). During development, glucocorticoids are known to promote organ maturation and precocious organ maturation is

induced by exogenous glucocorticoid (Bian *et al*., 1992; Fowden, 1995). However, exposure to excess glucocorticoids during prenatal life leads to low birth weight in both humans (Bloom *et al*., 2001; French *et al*., 1999) and animals (Nyirenda, *et al*., 1998). Hepatic GR and PEP-CK mRNA expression levels were increased in adult male wistar rat offspring who were exposed to excess prenatal glucocorticoids during late gestation, which further led to glucose intolerance (increased hepatic gluconeogenesis) suggesting the programming effect of glucocorticoids (Nyirenda *et al*., 1998). The other isotype of 11βHSD, the type 1 enzyme, plays a role in activation of cortisone to cortisol, thereby regulating, in concert with 11βHSD2, glucocorticoid concentrations locally in many tissues (Tomlinson *et al*., 2004). Glucocorticoids (GC) also play an important role in fetal pancreatic development. That pancreatic transcription factor *Pdx-1* gene expression was significantly downregulated *in vitro* in the embryonic pancreatic buds of wistar rats in response to prenatal GC exposure, suggests a role for glucocorticoids in modifying the balance of β-cell specific transcription factors during development (Gesina *et al*., 2004). Prenatal glucocorticoid exposure during late gestation resulted in downregulation of hepatic IGF-II mRNA expression *in vivo*, by suppressing the promoter 4 activity in the fetal sheep (Li *et al*., 1998). Repeated maternal glucocorticoid (betamethasone- d125 and d146) administration in sheep decreased cord plasma insulin concentration at 125 days of gestation and increased cord plasma glucose levels in the ovine fetus at day 146 gestation (Sloboda *et al*., 2002). Early maternal glucocorticoid (cortisol) exposure to pregnant sheep resulted in hyperinsulinemia in adult male offspring suggesting the long term effects of cortisol during early pregnancy might lead to altered pancreatic function and diabetes (De Blasio *et al*., 2007). Prenatal glucocorticoid treatment in this thesis is used to some extent as a surrogate of stress to investigate its effects on metabolic function during fetal and postnatal life.

In summary, it is clear that the pancreas, and perhaps also its development, can be altered in terms of function by steroids. Since the origin of such steroids is mainly the gonads and the adrenal gland, a central tennat of this

work is the premise that there is some form of steroidal interplay between the pancreas and the adrenal gland during development. Given adrenal hyerpandrogenism is thought to contribute to overall androgen load in PCOS, in addition to the pancreas, the adrenal is also a focus of this thesis, in terms of asking the question of whether not its function can be programmed by *in utero* exposure, and hence the following sections deal with adrenal development in detail with respect to PCOS.

1.4 Adrenal Gland and PCOS

1.4.1 Hypothalamic-Pituitary-Adrenal (HPA) axis

In mammals, basal and stress related homeostasis are modulated by the hypothalamic-pituitary-adrenal (HPA) axis, whose effects are primarily mediated via the production of cortisol. Corticotrophic releasing hormone (CRH) (Vale *et al*., 1981) and arginine vasopressin (AVP) are released from the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) (Vale *et al*.,1981). Both CRH and AVP neuropeptides are released into the hypophysial portal circulation (Antoni, 1986) and reach the anterior pituitary where they bind to their respective receptors (corticotrophic releasing hormone receptor (CRHR) (Perrin and Vale, 1999) and vasopressin receptor (V_3) (Hernando *et al*., 2001) and act synergistically to stimulate adrenocorticotrophic hormone (ACTH) secretion from corticotrophin cells into the general systemic circulation. ACTH binds to the melanocortin type-2 receptor (MC2-R) present in the parenchymal cells of the adrenocortical zona fasiculata region (principal target) (Figure 1.5).

The activated MC2-R initiates the stimulation of the cyclic adenosine-monophosphate pathway (cAMP), which induces steroidogenesis and secretion of adrenal steroids such as glucocorticoids, mineralocorticoids and adrenal androgens (Cone *et al*., 1996; Mountjoy *et al*., 1992). During the initial step of glucocorticoid biosynthesis, ACTH specifically initiates the conversion of cholesterol to pregnenolone (Raffin-Sanson *et al*., 2003; Simpson and Waterman, 1988). Glucocorticoids, being the downstream

effectors of the HPA axis regulate physiological changes or effects via intracellular receptors (Bamberger *et al*., 1996), where the effects are usually adaptive. However, changes such as excess or inadequate activation of HPA axis can lead to development of pathological conditions (McEwen and Stellar, 1993).

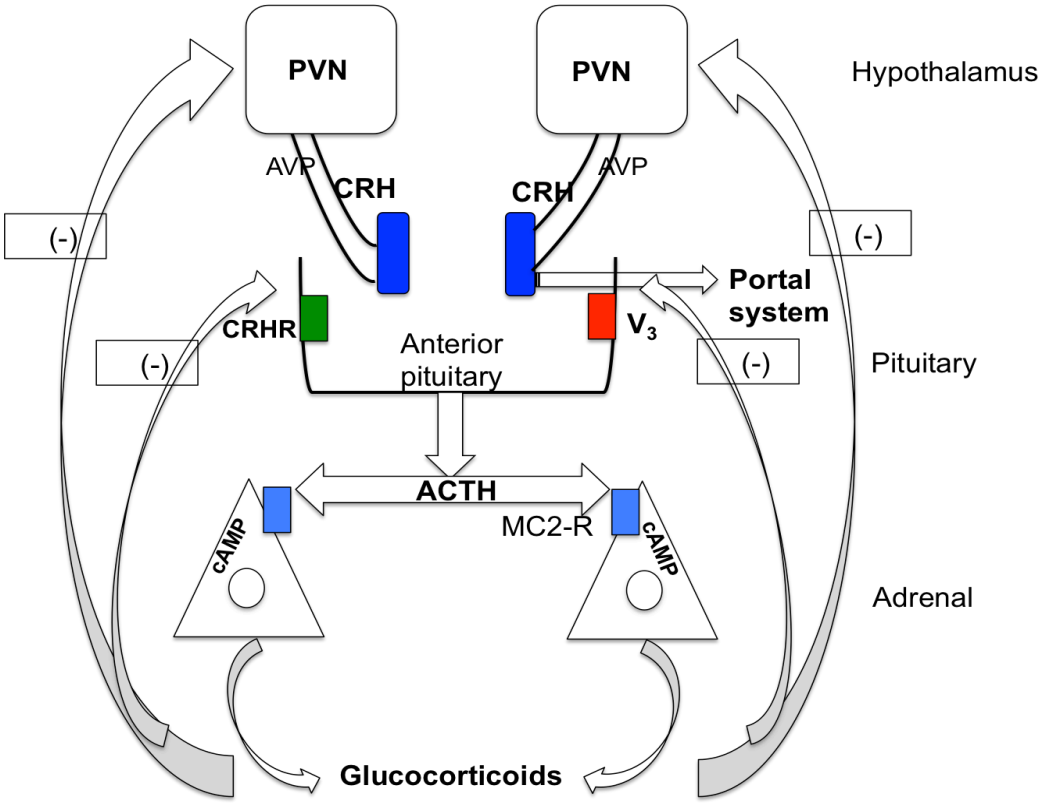


Figure 1.5 Schematic representation of the HPA axis.
In response to stress, CRF and AVP are released from the PVN of the hypothalamus and into the portal vessels and reach the anterior pituitary gland, then binding to CRHR and V3 receptors respectively. CRH and AVP act synergistically to induce ACTH release into systemic circulation. The circulating ACTH binds to melanocorticotrophic receptor (MC2-R) in the zona fasiculata of the adrenocortical region, driving release of glucocorticoids into the circulation. Finally glucocorticoids regulate physiological events and regulate further HPA axis activation by negative feedback.

Stress can be defined as a state of real or perceived threat to homeostasis (Dobson and Smith, 2000). A complex range of stress responses such as

immune, endocrine and nervous systems are required in order to maintain homeostasis due to a stimulus (stressor) (Carrasco and Van de Kar, 2003; Chrousos and Gold, 1992). This activation of stress response initiates a number of physiological and behavioral changes, which further aid survival of the individual when encountered with a homeostatic challenge. The physiological changes in response to stress includes increased cardiovascular, respiratory and metabolic rate with inhibitory effects on general functions such as feeding, digestion, growth, reproduction and immunity (Habib *et al*., 2001; Sapolsky *et al*., 2000).

1.4.2 Adrenal cortex, zonation and function

The adrenal glands occupy a position superior to each kidney having a flattened pyramidal shape (Tortora and Grabowski, 2003), consisting of an inner medulla and outer cortex. The adult adrenal cortex consists of three histological and functionally distinct zones, the zona glomerulosa (ZG) located just below the outermost capsule, which secretes mineralocorticoids; the zona fasiculata (ZF) the mid zone, which secrets glucocorticoids and the innermost zona reticularis (ZR), which is responsible for secretion of adrenal androgens such as dehydroepiandrosterone (DHEA) and its sulphated form (DHEA-S). All three steroid hormones are synthesized via a cascade of steroidogenic enzymes (Miller and Auchus, 2011). Aldosterone is the mineralocorticoid secreted in response to rennin-angiotensin II system (AT-II) in the zona glomerulosa. This hormone regulates several functions such as renal sodium retention, blood pressure and intravascular water homeostasis (Williams, 2005). Glucocorticoids such as cortisol are secreted by the zona fasiculata in response to the HPA activity described above (Habib *et al*., 2001). The adrenal medulla is located to the centre of the adrenal gland, which, via sympathetic nervous stimulation, secretes the cathecolamines, epinephrine and nonepinephrine.

Specific binding of adrenocorticotrophic hormone (ACTH) to its G-protein coupled receptor; MC2R (ACTH-receptor) induces steroidogenesis in the adrenal cortex (Di blasio *et al*., 1990), culminating in the classical

glucocorticoid secretory response. However, *in vivo* and *in vitro* studies have demonstrated not only glucocorticoid release, but also adrenal androgens (DHEA and DHEAS) are secreted in response to ACTH stimulation (Xing *et al*., 2010). Within the context of this thesis, the question arises as to whether or not the adrenal gland could perhaps be a source, additional to the ovary, of excess androgens commonly observed in PCOS-animal models.

1.4.3 Fetal Adrenal Development and function

The cells of the human adrenal cortex are a part of the adrenogonadal primordium within the urogenital ridge, originating from the intermediate mesoderm (Sucheston and Cannon, 1968; Wrobel and Süss, 1999). Adrenocortical cells develop early at 4 weeks of human embryonic development (Parker *et al*., 2002) and by week 8, the cortex is separated from the gonadal primordium into a distinct adrenal primordium (Mesiano and Jaffe, 1997). In comparison, the adrenal gland in the ovine fetus can be observed as early as dGA 28 (19% of total gestational age of 147 days) (Wintour *et al*., 1975), however clear zonation becomes apparent only around dGA 60 (41% of total gestational age of 147 days) (Webb, 1980). The human fetal adrenal cortex is comprised of three zones namely, the inner fetal zone (FZ) (analogous to zona reticularis) and outer definitive zone (DZ) (analogous to zona glomerulosa) both consisting of eosinophilic cells larger in size in the former and small densely packed cells in the latter (Goto *et al*., 2006; Hanley and Arlt, 2006). In humans, a third zone, the transition zone (TZ) is present in between the FZ and DZ functioning analogous to adult zona fasiculata (Figure 1.6) (Kempná and Flück, 2008). In sheep fetal adrenal gland, the outer zone is equivalent of adult zona glomerulosa while the inner zone corresponds to the zona fasiculata (Robinson *et al*., 1979; Webb, 1980). Although both human and ovine fetal adrenal glands share development similarities, the ovine adrenal gland lacks a specific fetal zone during its development (Robinson, 1979) and the zona reticularis becomes evident only during postnatal life (1 month old lamb) (Naaman-Répérant and Durand, 1997).

The fetal adrenal medulla is formed later in gestation by the pheochromoblasts that are derived from the neural crest which migrate through the fetal adrenal cortex at early as 6 weeks of gestation (Cooper *et al*., 1990 and Ehrhart-Bornstein *et al*., 1997). There are two hypotheses put forth to explain the adrenal zonal specification. Firstly, the cell migration theory explains that the cells, which migrate, centripetally gaining zone-specific characteristics, are stem cells that exist subcapsularly and are the cells that are the precursors differentiating in the ZG. The second theory proposes that all the zones in the fetal adrenal cortex are derived from the undifferentiated stem cells (Mesiano and Jaffe, 1997).

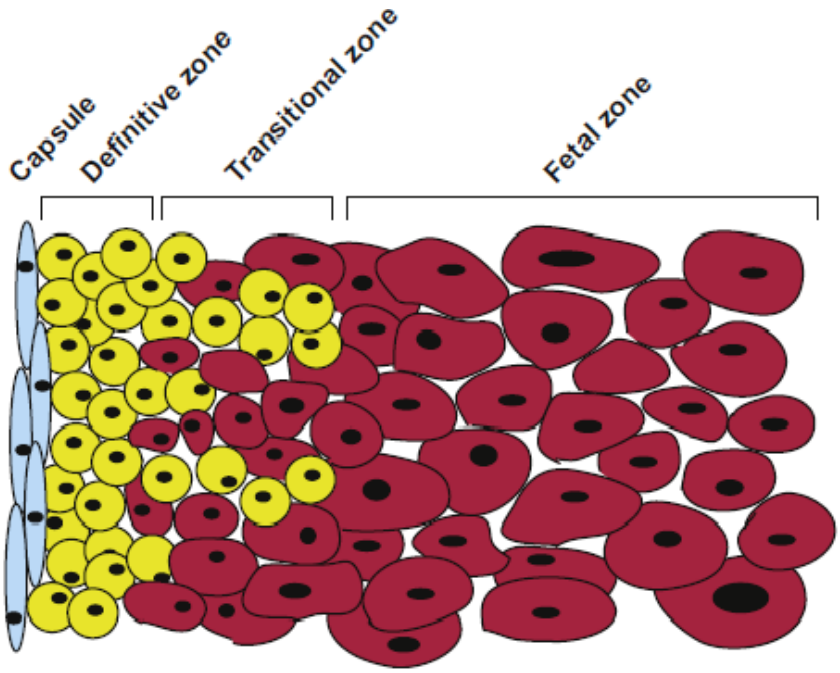


Figure 1.6 Structure of the different zones during fetal adrenal cortical development. Adapted from (Lalli and Paolo Sassone, 2003).

By 7 weeks of gestation in humans, fetal adrenal gland express steroidogenic enzymes (Hanley and Arlt, 2006) and in humans ACTH starts its control on cortisol synthesis by 8 weeks of gestation (38% of total gestational age of 147 days) (Goto *et al*., 2006). In sheep, fetal adrenal gland grows in a biphasic fashion, where the first growth period is rapid occurring between dGA 60-dGA120. At dGA60 zona glomerulosa cells produce aldosterone, and the second growth period occurring after dGA120 (81% of total gestational age of

147 days) sees development and maturation of zona fasiculata (Boshier and Holloway, 1989; Webb, 1980). The adrenal cortex in the ovine fetus secrets cortisol only during the first half of gestation (Wintour *et al*., 1995) and also last fifth of gestation, but remains quiescent between dGA90-dGA120 (term ~147days) (Wintour *et al*., 1975), possibly due to the inadequate production of ACTH from the fetal pituitary during prior to this time. However, it is apparent that cortisol secretion during the last fifth of gestation helps in development of organs and is critical in the onset of parturition (Liggins, 1994a, Liggins, 1994b).

1.4.1 Adrenal Steriodogenesis: initial steps

Although most of the cholesterol required for the synthesis of steroid hormones is provided through low-density lipoproteins (LDL), the adrenal cortex can also synthesize cholesterol *de novo* from acetate (Mason and Rainey, 1987). Sterol response element binding protein (SREBPs), a group of transcription factors, regulate the genes involved in fatty acid and cholesterol biosynthesis (Horton *et al*., 2002). 3-hydroxy-3-methylglutaryl co-enzyme-A reductase is the rate-limiting enzyme in this synthesis pathway (Miller and Auchus, 2011). LDL cholesterol esters, which enter the adrenal cells through receptor-mediated endocytosis, are converted to free cholesterol prior to steroid hormone synthesis (Brown *et al*., 1979). Mitochondria are an important site in steriodogenesis; cholesterol is transported from outer mitochondrial membrane to the inner mitochondrial membrane via steroid acute regulatory protein (StAR) (Figure 1.7) (Ponting and Aravind, 1999). P450 side chain cleavage (P450scc) catalyzes the first true step in adrenal steriodogenesis by converting cholesterol to pregnenolone in the ZG, ZF and ZR (Parker and Schimmer, 1995). The pattern of steroid synthesis in adrenal gland is complex as each of the adrenal cortex regions secretes different steroids, the mineralocorticoids, glucocorticoids (cortisol) (which involves additional enzymes such as CYP21 and CYP11B1) and weak adrenal androgens (DHEA and DHEAS) whose

synthesis shares some enzymes common to all zones (Auchus & Rainey, 2004).

1.4.1.1 3β-hydroxysteroid dehydrogenase (3β-HSD)

3β-hydroxysteroid dehydrogenase (3β-HSD) is expressed in human fetal adrenal glands from 7-12 weeks of gestation (Goto *et al*., 2006). 3β-HSD converts pregnenolone to progesterone in the zona glomerulosa, 17-hydroxypregnenolone to 17-hydroxy progesterone in zona fasiculata and dehydroepiandrosterone (DHEA) to androstenedione in zona reticularis respectively (Figure 1.7) (Lee *et al*., 1999; Payne and Hales, 2004).

1.4.1.2 P450c17

P450c17 or CYP17 enzyme, which is expressed only in the ZF and ZR region of the adrenal cortex, performs a dual function. 17α-hydroxylase activity converts progesterone to 17-hydroxy progesterone in the ZF and pregnenolone to 17-hydroxy- pregnenolone in the ZR respectively (Payne & Hales, 2004). 17, 20-lyase activity of P450c17 enzyme, which is expressed only in the ZR converts 17-hydroxy- pregnenolone to dehydroepiandrosterone (DHEA) and 17-hydroxy progesterone to androstenedione (Auchus, 1998 and Lee-Robichaud *et al*., 1995).

1.4.1.3 CYP21A (21-hydroxylase)

21-hydroxylase (P450c21) converts progesterone to deoxycortisone in the zona glomerulosa and 17-hydroxyprogesterone to 11-deoxycortisol in the zona fasiculata (Payne & Hales, 2004). CYP21 is expressed during late gestation in ovine fetus due to its role in regulation of parturition (Myers *et al*., 2005; Warnes *et al*., 2004) and in humans around 14 weeks of gestation (Coulter and Jaffe, 1998; Narasaka *et al*, 2001).

1.4.1.4 CYP11B (11β-hydroxylase)

Progesterone is metabolized to aldosterone by P45011βB2 (CYP11B2) in the zona glomerulosa (Payne and Hales, 2004). CYP11B1 activity helps in hydroxylation of 11-deoxycortisol to cortisol in the zona fasiculata (Payne & Hales, 2004). CYP11B1 is also expressed in developing sheep (Coulter *et al*., 2000), rodents (Mellon *et al*., 1995), and human fetal adrenal (Coulter and Jaffe, 1998).

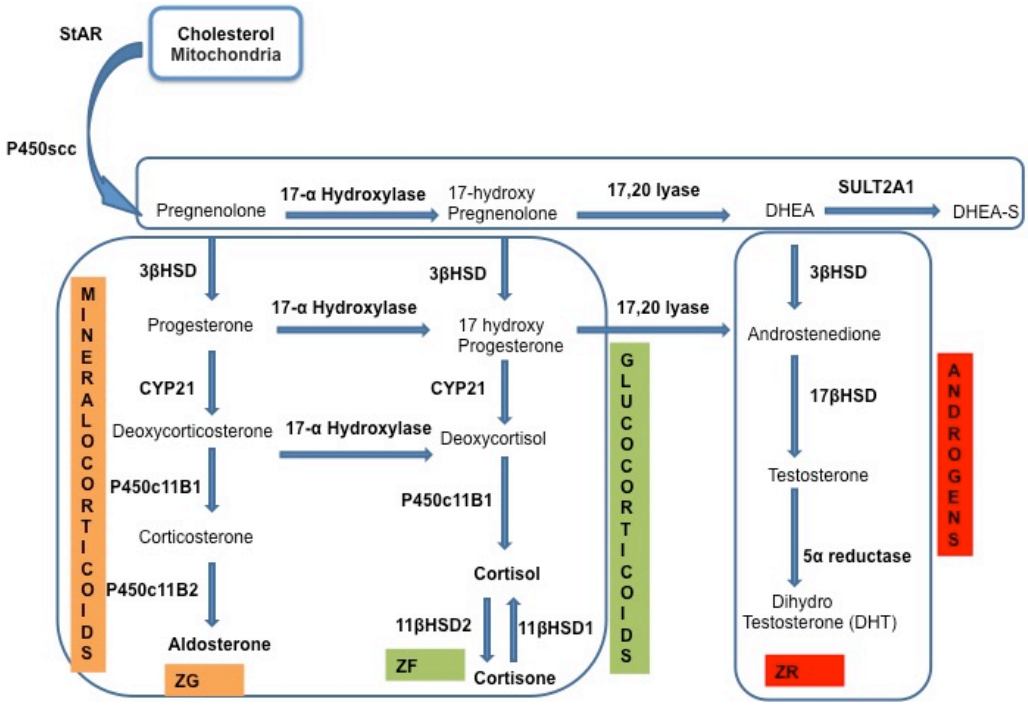


Figure 1.7 Schematic representation of the adrenal steroidogenic pathway. (ZG- zona glomerulosa; ZF- zona fasiculata; ZR- zona reticularis)

1.4.1.5 SULT2A1

SULT2A1 catalyzes dehydroepiandrosterone (DHEA) to its sulphated form, DHEAS, which is the abundantly produced adrenal androgen within the zona reticularis (Rainey and Nakamura, 2008).

1.4.1.6 11β-HSDs

11βHSDs are involved in interconversion of glucocorticoids within the zona fasiculata region of the adrenal gland depending on the available co-factor.

11β-HSD type 1 converts inactive cortisone to active cortisol utilising NADPH co-factor and 11βHSD type 2 converts active cortisol (11βHSD-2) to inactive cortisone in presence of NAD+ as a co-factor cortisol gets inactivated (Figure 1.7) (Michael *et al.*, 2003).

1.4.1.7 5-α reductase

Testosterone is converted to a more potent androgen dihydrotestosterone (DHT) by the activity of 5α reductase (Figure 1.7) (Siiteri and Wilson, 1974).

1.5 Adrenal Hyperandrogenism and PCOS

In addition to the ovarian androgen excess in PCOS, excess adrenal androgen secretion is also observed (Hague *et al.*, 1990; Rosenfield, 1999). PCOS related adrenal androgen excess in women accounts for 20-30% of excess androgens, which is manifested by elevated levels of circulating androstenedione, DHEA and DHEAS (Carmina et al., 1992; Yildiz and Azziz, 2007) along with hyper-responsive ACTH stimulated DHEA and androstenedione (Azziz *et al*, 1998). Abnormal regulation of P450c17α in the adrenal cortex is observed in women with PCOS (Rosenfield *et al*., 1990). PCOS is also found in patients suffering from non-congenital adrenal hyperplasia (NCAH) and congenital adrenal hyperplasia (CAH) leading to adrenal androgen excess due to abnormal functioning of 21-hydroxylase in addition to luteinizing hormone hypersecretion and ovarian hyperandrogenism (Carmina and Lobo, 1994; Levin *et al*., 1991 and Moran *et al*., 2000). PCOS phenotype is also seen in patients with CYP21 mis-sense mutations causing excess androgen secretion (Witchel and Aston, 2000; Witchel *et al*., 1997). During fetal development, the adrenal gland produces large amounts of dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) (Yuen and Mincey, 1987), which declines after birth and remains low until adrenarche. Adrenarche is the increased production of adrenal DHEA and DHEAS occurring during 6-8 years of age in humans (Parker, 1991). Adrenarche is considered to be a relatively recent evolutionary phenomenon as it has been identified in the Old World Monkeys (*Hominoidea*

superfamily), that includes humans and chimpanzees (Richard *et al.*, 2004; Genazzani *et al*., 1983).

In utero environment alterations during fetal adrenal development can have a bearing upon long-term adrenal function. Pregnant Sprague-Dawley rats exposed to ethyl alcohol (EtOH) during the second week of gestation demonstrated significantly increased mRNA expression of corticotrophic releasing hormone (CRH) at postnatal day 21 (in response to electroshocks), (Lee *et al*.,1990). In a different study, pregnant rats exposed to the endocrine disrupting compound di- (2-ethylhexyl) Phthalate from day 14 until birth showed chronic activation of the adrenal gland and decreased aldosterone secretion during postnatal life (day 60) in adult male rats (Martinez-Arguelles *et al*., 2014), suggesting the susceptibility of altered fetal environment on the adrenal development and the long term consequences in terms of altered adrenal function. However, since the latter study examined only male rats, it is important to consider that there are sex-specific differences in response to a range of hormones (Giussani *et al*.,2011; Wierman, 2007), hence in the current thesis adrenal from both male and female sheep fetuses and offspring were assessed where possible. Adrenal steroidogenesis was altered in adult rats in response to maternal undernutrition and elevated ACTH-R levels were observed indicating altered hypothalamic pituitary adrenal (HPA) axis in both male and female adult offspring that were subjected to maternal under nutrition *in utero* (Khorram *et al.*, 2011). *In utero* maternal androgen over exposure in rhesus monkeys recapitulates PCOS features such as elevated basal circulating adrenal DHEA and DHEAS in the female adult offspring (Zhou *et al*., 2005). Hence it is clear that prenatal androgenization carries not only a consequence of altered ovarian steroidogenesis, but also adrenal steroidogenesis alterations, however, mechanisms underpinning such altered function remain unknown.

1.5.1 Glucocorticoids and PCOS

Fetal circulating glucocorticoid levels are increased during late gestation in several pre-cocial species (Fowden *et al*, 1998). This increase in fetal plasma glucocorticoid in essential for initiation of labor and delivery in sheep (Liggins, 1968; Magyar *et al.*, 1980). Independent of the effects on parturition, glucocorticoids are also crucial in maturation of fetal lung (Surbek *et al*., 2012). Placental 11β-hydroxysteroid dehydrogenasetype-2 acts as a barrier, protecting the fetus from excess maternal circulating glucocorticoids by converting the active glucocorticoids such as cortisol to its inactive form (cortisone) (Benediktsson *et al*., 1993). However, *in utero* glucocorticoid exposure during fetal adrenal development can have a bearing upon adrenal development, resulting in long term functional alteration, for example, prenatal glucocorticoid exposure and further exposure to carbenoxolone, a placental 11βHSD2 inhibitor, resulted in reduced growth in male rat offspring along with hyperglycemia (Lindsay *et al*. 1996). The synthetic glucocorticoid, dexamethasone is a poor substrate for 11βHSD-2 (Brown *et al*., 1996) and in sheep exposure to prenatal synthetic glucocorticoids early in gestation leads to decreased fetal plasma cortisol and increased expression of key adrenal steroidogenic enzymes in both sexes, and decreased placenta 11βHSD-2 gene expression only in male fetuses during near term (d125), therefore altering the fetal pituitary-adrenal axis development (Braun et al., 2009a). Prenatal glucocorticoid exposure to pregnant rats from day 13 to term resulted in elevated *ACTH-R* mRNA expression in both male and female offspring and also elevated urinary aldosterone and corticosterone secretion (Waddell *et al*., 2010). Glucocorticoids have also been proposed to alter signal transduction in the adrenal cortex, which in turn regulates ACTH sensitivity (Picard-Hagen *et al*.,1995).

Collectively, the above descriptions serve to highlight the critical importance of each of the steroidogenic enzymes and moreover, the correct balance of these enzymes, in maintaining adrenal function and overall homeostasis, and thereby also serves to illustrate how developmental disruption of adrenal

steroidogenic function can have serious health consequences throughout developmental life, and beyond.

1.5.2 Adrenal associated metabolic abnormalities in PCOS: a co-conspiracy between the pancreas and the adrenal gland?

Approximately 50-70% of PCOS patients suffer from insulin resistance, glucose intolerance and hyperinsulinemia (Legro *et al*., 1999). Insulin augments ovarian androgen production from follicular theca cells (Nestler *et al*., 1998) and thus may also be a reason behind excess adrenal androgen. There is a possibility that changes in adrenal androgen secretion are the end result of abnormal insulin secretion. Insulin like growth factor-II (IGF-II) acting via insulin like growth factor receptor type-1 regulates P45017 activity, which may play a role in increased androgen secretion in cases such as PCOS (Mesiano *et al*., 1997). Reports to date have not been successful in demonstrating any effect of insulin on adrenal androgen production in PCOS patients. There is disagreement with the increased DHEAS levels in hyperandrogenic women who were experimentally given short-term hyperinsulinemia (Buyalos *et al*.,1997; Falcone *et al*.,1990; Moghetti *et al*., 1996). Secretion of adipocytokines and other inflammatory products (Ehrhart-Bornstein *et al*., 1998) and estrogen in the adipose tissue suggests a role for obesity in altering adrenal function (Forney *et al*., 1981). Obese patients with both abdominal and visceral adiposity show a higher degree of cortisol secretion and metabolism (Stewart *et al*.,1999; Vicennati and Pasquali, 2000; Vicennati *et al*., 1998). Understanding the cause and effect remains a key priority in terms of adrenal and pancreatic dialogue leading to altered function in PCOS.

1.6 Is there a Male phenotype of PCOS?

In recent years, fetal programming of PCOS related metabolic syndrome has been examined in terms of trans-generational inheritance of the syndrome. This may potentially be mediated through epigenetic mechanisms, such as

DNA methylation (Ozanne and Constância, 2007; Pinney and Simmons, 2010). Heritability of adrenal androgen secretion was observed in 68- weight and ethnicity compared unrelated control men and 119 brothers of 87 unrelated women with PCOS (Legro *et al*., 2002). Several studies have indicated that impaired glucose tolerance (IGT) occurs through maternal lineage rather than paternal or grand paternal inheritance of diabetes (Benyshek *et al*., 2006; Blondeau *et al*., 2002; Zambrano *et al*., 2006). Most of the male related PCOS research is based on epidemiological evidence (Recabarren *et al*., 2008; Sam *et al*., 2008; Yilmaz *et al*., 2005) and very little is understood about this inheritance of PCOS, in part due to lack of a male phenotype. However, premature baldness is seen in men in PCOS families (Carey *et al*., 1993; Ferriman and Purdie, 1979; Mao *et al*., 2001). Insulin resistance, a metabolic phenotype of PCOS is seen in brothers, sisters and mothers of women with PCOS (Kaushal *et al*., 2004; Norman *et al*., 1996; Sir-Petermann *et al*., 2002; Yildiz *et al*., 2003). Recabarren *et al*., (2008) have shown that sons of women with PCOS exhibit high body weight from infancy in addition to insulin resistance as the subjects get older, indicating risk for development of metabolic syndrome. From animal studies, experimentally, in the absence of hyperandrogenism, a decrease in β-cell compensation and insulin sensitivity is demonstrated in prenatally androgenized male rhesus monkeys (Bruns *et al*., 2004). In ovine PCOS/excess androgen exposure models, midgestational androgen exposure alters testis development in terms of reduced testicular gene expression, Leydig cell function (Connolly *et al*., 2013) and decreased sensitivity of the testes to luteinizing hormone in young male sheep (Recabarren *et al*., 2013) suggesting altered testicular function, in male relatives of women with PCOS. There is a lack of sufficient research in male animal models with regard to possible male phenotypes of PCOS. Therefore, this study sets out to investigate the metabolic disturbances in prenatally androgen over-exposed male sheep fetuses.

1.7 Therapeutic Management of PCOS

Hyperandrogenism, hyperinsulinemia and insulin resistance are identified as significant contributors in the pathophysiology of PCOS as they play a key role in PCOS related metabolic and reproductive disturbances (Diamanti-Kandarakis, 2008). An initial treatment option for PCOS, depending on the obesity of the patient, is weight loss, since obesity is seen in about 35%-60% of women with PCOS. Weight loss of around 5% of the initial weight can lead to re-establishment of the menstrual cycle alongside decreased circulating glucose and androgens concentrations in PCOS women (Patel and Nestler, 2006). Weight loss accompanied with good diet, can ameliorate metabolic effects due to decreased hyperinsulinemia in insulin resistance women (Reaven, 2005) and exercise is known to play a key role in this process (Bruner *et al*., 2006), however, knowledge on optimal type, duration and frequency of exercise is lacking. Clomiphene citrate (CC) is a selective estrogen receptor antagonist, which interferes with negative feedback of estrogen at the hypothalamus, thereby increasing HPG axis activity, resulting in effective ovulation in case of PCOS women suffering from infertility issues such as oligo- or anovulation (Homburg, 2005).

Metformin, a biguanide, is used as a pharmaceutical option to treat both metabolic and reproductive abnormalities in PCOS women. Metformin has pleotropic actions on several tissues such as ovary, liver, endothelium, skeletal muscles and adipose tissue (Palomba *et al*., 2009). It activates glycolytic enzymes such as pyruvate kinase and hexokinase in the liver, thus stimulating glycolysis and glucose entry into the liver. Metformin is also an insulin sensitizer used to treat type 2 diabetes -mellitus (T2DM) resulting in increased insulin mediated hepatic glucose uptake (Diamanti-Kandarakis *et al*., 2010). Phosphorylation of extracellular signal related kinase 1-2 (ERK1-2) involved in lipolysis is stimulated by high glucose concentrations and tumour necrosis factor-α (TNF-α). This signaling is inhibited by metformin in the primary rat adipocytes, which indicates the anti-lipolytic activity of metformin in decreasing the systemic levels of free fatty acids (FFA) (Ren *et al*., 2006).

Metformin treatment can also have some effects in terms of normalization of androgen concentrations, extending its efficacy across the broad clinical spectrum of PCOS (Kolodziejczyk *et al*.,2000). There is reduction in androgen production in the ovaries (Mansfield *et al*.,2003; Rice *et al*., 2009), reduced activity of steroidogenic enzymes such as CYP17, P450 side chain cleavage (P450scc), 3-β hydroxysteroid dehydrogenase (3β-HSD), steroidogenic acute regulatory protein (StAR) and 17α- hydroxylase/17,20 lyase (CYP17) in women with PCOS treated with metformin, which may be a downstream consequence of a reduction in circulating insulin concentrations (Diamanti-Kandarakis *et al*., 2010).

Glucocorticoids are used to treat PCOS patients with high adrenal androgen secretion (Parsanezhad *et al*. 2002). Anti-androgen treatment in PCOS patients with hirsutism results in reduced new hair growth and also slows down the growth of already present terminal hair (Calaf *et al*., 2007). A key issue that remains in the therapy described is that it treats only the symptoms of PCOS and not the origins, and it is urgently required that we understand the origins and underlying causes of this syndrome for better treatments than metformin which shows varying degrees of success across the spectrum of PCOS.

1.8 Animal Models

Several studies have demonstrated fetal programming of PCOS traits in numerous species by inducing excess testosterone exposure prenatally. Similar to PCOS patients, prenatally testosterone treated monkeys and sheep manifest hyperandrogenism (Eisner *et al*., 2002; Padmanabhan *et al*., 2006), polycystic ovaries (West *et al*., 2001), hypergonadotropism (Dumesic *et al*., 1997), hyperinsulinemia (Recabarren *et al*., 2005) and neuroendocrine feedback defects (Sharma *et al*., 2002). Subsequently, a salient feature of such offspring is hyperandrogenic anovulation (Abbott *et al*., 2005).

Exposure to prenatal excess testosterone in sheep (Birch *et al*., 2003), rats (Foecking *et al*.,2005) and monkeys (Abbott *et al*., 1998) leads to ovulatory dysfunction later in adulthood. Sheep exposed from gestational age 30 to 90 days to prenatal testosterone induces a poly-follicular phenotype by increasing the proportion of growing follicles (Steckler *et al*., 2005). Increase in the pituitary responsiveness to gonadotropin releasing hormone (GnRH) and reduced sensitivity of hypothalamus to progesterone and estradiol negative feedback induces LH hypersecretion in prenatally testosterone treated sheep (Sarma *et al*., 2005).

PCOS animal models, in addition to recreating the reproductive phenotypes observed in humans, can also recapitulate the metabolic phenotype of PCOS, since downstream consequences of prenatal androgen excess such as increased adiposity, insulin resistance and hyperinsulinemia have also been observed (Bruns *et al*., 2007; Padmanabhan *et al*., 2010; Rae *et al*., 2013; Roland *et al*., 2010). Prenatal androgen exposure in sheep altered fetal pancreatic gene expression and function and also had effects during postnatal life in terms of excess insulin secretion in absence of insulin resistance (Rae *et al*., 2013). Collectively, it is evident that animal models can provide increased insight into understanding the pathogenesis involved in clinical conditions such as PCOS.

1.8.1 Benefits of Sheep as model for PCOS

Using sheep as a model for PCOS study over other animals has benefits due to similarities between ovine and human pre and postnatal development/growth (Padmanabhan and Veiga-Lopez, 2013) and in terms of litter sizes, sheep, like humans, have capability of producing one or two offspring weighing about 3-6kg (Remacle *et al*., 2007). Sheep have been successfully used for many years to study reproductive biology, and also fetal development with relevance to humans (Harding and Bloomfield, 2004). They are amenable to a wide variety of procedural manipulations, which includes ultrasound monitoring of ovarian follicular dynamics, behavioral interaction

studies in the natural setting, non-invasive sequential monitoring of ovarian follicular dynamics, repetitive hormonal sampling, neurotransmitter measuring due to the large brain size, detailed hormonal profiling and cost effectiveness when compared to non-human primates (Padmanabhan and Veiga-Lopez, 2013).

1.9 Objectives of this thesis

Several studies in the past have made use of animal models such as rodents, monkeys and sheep where the pregnant mother was administered with excess androgens and the effects were monitored in the fetus and postnatal life, which has aided understanding of the developmental origins of PCOS. The main limitation of the maternally androgen injected model is that testosterone administered via a maternal route can be metabolized to estrogens via placental aromatase enzyme activity, which begs the question of whether the effects observed in the fetus are direct consequences of androgenic excess or via metabolism, estrogenic excess. Additionally, maternal androgen administration can alter maternal glucose dynamics, hence complexing effects of androgens and glucose. Therefore, this thesis has used a unique and novel sheep model throughout, where the synthetic androgen, testosterone-propionate (TP) was administered directly into the fetus using ultrasound guided injection technique, thus bypassing maternal and placental metabolic activity. This permits direct examination of effects of fetal steroidal excesses, and in addition permits estrogenic effects to be studied without the occurrence of abortion due to estrogenic activity in the pregnant mother. A further refinement utilized in these studies is that steroid treatments were commenced mid-gestation – previous studies have utilized d30 of gestation as the start of treatment, however, this coincides with the process of sexual differentiation in sheep, and has the unwanted effect of making female lambs display a male external genetalia phenotype, which of course does not occur in human PCOS patients. Mid-gestational exposure prevents this occurrence, with female lambs displaying no overt genital phenotype attributable to prenatal steroid exposure. A third strength of these

studies is that wherever possible, effects noted in female offspring are contrasted with effects noted in male offspring, and thus a deliverable is information regarding the effects of pre-exposure to androgens (due to endogenous androgen secretion from fetal testes) on responses to administered steroids. The tissues chosen as a focus of this study are the pancreas and adrenal gland, highlighted above as potential foci of prenatal steroid action with relevance to metabolic alterations, which may have life long health consequences in offspring. To address the question of permanency of effects, and to identify the legacies, if any, of altered prenatal steroid exposure, studies were conducted both on fetal tissues and postnatal tissues derived from offspring delivered from such experimentally manipulated pregnancies.

1.9.1 Aims

This thesis will focus on prenatal steroid (androgenic, estrogenic and glucocorticoid) excess exposure on both female and male (wherever possible) fetuses and offspring.

The overarching aims are:

1. To delineate the effects of androgens from estrogens in the development and subsequent postnatal function of the pancreas and adrenal glands.
2. To study the effects of prenatal glucocorticoids on metabolic development and function, again focusing on the pancreas and the adrenal gland.
3. To determine, with respect to questions 1 and 2, the differences of gender in terms of response to fetal steroidal over exposure.

Chapter 2 General Materials and Methods

2.0 Materials and methods

This section details the general material and methods employed in this thesis. All chemicals and reagents were purchased from Sigma-Aldrich, UK, unless otherwise stated. Any work that has been carried out in conjunction with others is duly acknowledged in the chapters in which the specific work is presented.

2.1 Animal Husbandry

Scottish Greyface ewes were the animals used throughout this thesis, which were purchased by, housed and cared for at the Marshall Building, Roslin, Edinburgh, authorised by UK Home Office Project License Number 60/3744 and the animal work was carried out after authorisation by local ethical committee approval, under legislation of the Animals (Scientific Procedures) Act 1986.

2.1.1 Mating and pregnant ewe husbandry

Ewes were fed to gain comparative body condition (scores 2.5-3) before they were estrous cycle synchronized. Synchronization was by inserting a vaginal Chronogest CR sponge, which was impregnated with 20mg of flugestone acetate (progesterone analogue) (Intervet UK Ltd, Buckinghamshire, UK). Later the sponges were withdrawn the same day initiating the estrous cycle. The Scottish Greyface ewes were then mated with Texel rams under natural breeding conditions and pregnancy was later confirmed by ultrasound scan. Singleton, twin and triplet pregnancies were evenly divided into treatment and control experimental groups. Pregnant ewes were fed with Excel EweNuts (0.5 kg daily, Carrs Billington, Lancashire, UK) and Crystalyx Extra High Energy Lick (Caltech Soway Mills, Cumbria, UK), plus hay (*ad libitum*) and housed in groups in spacious enclosures. To prevent dominant ewes from being overfed at the expense of smaller ewes, the former were housed separately. All ewes were vaccinated against Clostridial diseases and

pasteurellosis 4-6 weeks prior to lambing by treating these animals with Heptavac P Plus (Intervet UK Ltd.).

2.1.2 Fetal Treatments

This thesis deals only with direct fetal treatments, which occurred at d62 and 82 of gestation (ovine pregnancy is ~147 days). The pregnant ewes were anaesthetized by an intramuscular injection of 10mg Xylazine (Rompun, Bayor Plc Animal health Division, Berkshire, UK) and left to settle for 10 min, followed by a dose of 2.0mg/kg I.V ketamine (Ketaset, Fort Dodge Animal health, Southampton, UK) intravenously. Under sterile surgical conditions, injections were carried out using an ultrasound vaginal probe fitted with a needle guide (20G Quincke spinal needle), which was inserted through the uterine wall and into the fetal flank. The delivery of the injection (200μl) into the fetal flank was confirmed on ultrasound image, and any obvious leakages or uptake into the fetal circulation were recorded. The fetuses were injected at gestational d62 and d82 of gestation directly with testosterone propionate (TP- 20mg; AMS Biotechnology (Europe) Ltd., Abingdon, UK) or diethylstilbestrol (DES-50μg) or dexamethanose (DEX-100μg) dissolved in vegetable oil (Sainsbury's SO organic range) (containing 5% ethanol) at a stock concentration of 100mg/ml, using a 20G Quincke spinal needle (BD Biosciences, Oxford, UK) under ultrasound guidance. Dr. Colin Duncan performed ultrasound and Professor Alan McNeilly, Dr Mick Rae, Dr Kirsten Hogg and Dr Fiona Connolly carried out the fetal injections in accordance with personal licenses. Afterwards, experimental treatment ewes were administered a post-operative dose of antibiotics (1mg/25kg:i.m., Stepcare: Animalcare Ltd., York, UK).

2.1.3 Husbandry of lambs and young adult offspring

Lambs were naturally suckled and if necessary supplemented with powdered lamb milk (Shepherdess SCA Mill, North Yorkshire, UK), then gradually weaned onto hay, before being removed from dams at 12 weeks. Lambs were

administered with Heptavac P Plus vaccine (Intervet UK Ltd.) at 3 and 7 weeks of age. Various parameters were assessed in the offspring (both lamb and young adult) from steroid manipulated pregnancies such as gene expression, glucose tolerance (GTT) and Synacthen testing, which are detailed in the specific chapters in which these methods are included.

2.2 Animal Sacrifice

2.2.1 Fetal, lamb and adult animal sacrifice

Euthanasia was performed in accordance with the regulatory outline of Schedule 1: Appropriate Methods of Humane Killing of the Animals (Scientific Procedures) Act 1986. At day 90 of gestation, pregnant ewes and their fetuses were euthanized by intravenous overdose of sodium pentobarbitone (150mg/kg; i.v.; Euthetal; Merial Animal Health Ltd., Essex, UK) and lambs at 3 months age and young adults at 11 months age as detailed in table 2.1 were killed similarly.

2.2.2 Tissue collection

A range of tissue samples destined for numerous different studies (many out with the scope and remit of this thesis) were collected from fetuses (d90 gestation), lambs (3 months age) and young adults (11 months of age). Tissues such as adrenal and pancreas were divided into two pieces, one each for fixing and freezing. Immediately after dissection, a part from each tissue type was either snap frozen in dry ice followed by storage at -80^{0}C, to prevent RNA degradation or placed in Bouins fixative for 24 hours (to prevent tissue degradation and maintain tissue integrity) prior to storage in 70% ethanol for subsequent histological processing. In adult cohorts, an additional muscle and liver sample was collected and snap frozen on dry ice and stored at -80^{0}C.

Gestational Day (d)	Treatment	Concentration	Collection Stage
d62 and d82	Fetal Treatment Regime		
	Vehicle control	_	Fetal d90
			Lamb (12weeks)
			Adult (11 months)
	TP	20mg	Fetal d90
			Lamb (12weeks)
			Adult (11 months)
	DES	50μg	Fetal d90
			Lamb (12weeks)
			Adult (11 months)
	DEX	100μg	Fetal d90
			Lamb (12weeks)
			Adult (11 months)

Table 2.1 The pregnant ewe treatment regime and resulting offspring.

Timings of treatment regimes for pregnant ewes and a breakdown of treatment cohorts, listing concentration of TP delivered, age of sacrifice and collection.

2.3 Gene expression analysis

2.3.1 RNA extraction

RNA extraction methods employed were based upon tissue type and structural characteristics e.g composition of fibrous, lipid or blood material, explained in specific chapters.

RNA was isolated from pancreas (fetal only) and adrenal (fetal, lamb and adult) tissues by making use of the Qiagen RNeasy Mini Kits (QIAGEN Ltd., Crawley, UK). Working under sterile conditions, approximately, ~30mg of either pancreatic or adrenal tissue piece was weighed (without thawing, all procedures carried out on dry ice) from the snap frozen tissue and transferred to a separate RNase- free magnetic bead (Qiagen Ltd) containing 2ml eppendorf tube placed over dry ice. This was followed by addition of 600µl of RLT lysis buffer (Qiagen RNeasy Mini Kit, Crawley, UK) to each tube supplemented with 10ul per ml of β-mercaptoethanol and these tubes were loaded for homogenization onto a tissue lyser (Qiagen Tissue Lyser LT, Qiagen) and processed for ~3 minutes at 50Hz. The guanidine thiocyanate present in the RLT buffer inactivates RNases and denatures proteins present in the sample. Following homogenisation, the tissue samples were centrifuged at 13000 rpm for 3 minutes and the supernatant was collected into a 1.5ml eppendorf. The supernatant (Lysate) was mixed with 600µl of 70% ethanol (prepared from 100% ethanol diluted in distilled water) and washed thoroughly by using RNeasy minispin columns (Qiagen Ltd) by centrifugation for 5 seconds at 13000 rpm. Then, the columns were washed with RW1 buffer for 15 seconds at 13000 rpm followed by washes using RPE buffer for 15 seconds at 13000 rpm and a final wash with RPE buffer for 2 minutes at 13000 rpm and the flow through was discarded. Finally, spin columns were placed in new 1.5ml eppendorfs and centrifuged twice for 1 minute at 13000 rpm using RNase free water (33µl final volume) and the eluted samples were collected in sterile eppendorfs and stored at -80^{o}C for further use.

2.3.2 RNA concentration measurement

Prior to cDNA synthesis, RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Purity of RNA concentration was determined by 260/280 ratio and a value of ~2.0 was considered indicative of suitable purity for downstream applications.

2.3.3 DNaseI digestion

RNA extracted from the above tissue samples was prepared for DNaseI treatment to rid the samples genomic DNA. The concentration of RNA measured via Nano Drop was normalized to 2µg, by adding RNase- free water to make a final volume of 15µl for each sample. The DNaseI mastermix comprised 10X DNase1 buffer (2µl/ sample)(Sigma Aldrich), and DNaseI enzyme (3µl/sample) making up a final volume of 5µl, to be added to each of the RNA sample making a final volume of 20µl (Table 2.3). These samples were then incubated at room temperature for 15 minutes followed by the addition of 1µl of EDTA (Sigma Aldrich) stop solution, which chelates the calcium and magnesium ions to stop the DNase activity. The samples were then loaded onto a bench time PCR thermocycler (Thermal cycler, 2720, Applied Biosystems, Life Technologies, Glasgow, UK) and heated at 70^{o}C for 10 minutes to completely denature the DNase enzyme.

2.3.4 Complementary DNA (cDNA) synthesis

To carry out the reverse transcription step, the RNA samples from the above DNase treatment were divided into two (run in duplicates to ensure sufficient production of cDNA to cover all analyses initially envisaged but also to ensure that future analyses that may be desirable are also encompassed by a single synthesis) aliquots of 10µl each. 1µl of random nonamer primers (PrimerDesign, Southampton, UK) was added to each sample tube and incubated at 65^{0}C for 5 minutes on the thermocycler. Both RT+ve and RT-ve sample mastermixes were prepared. The RT+ve mastermix, which was made up to a final volume of 10µl mastermix per reaction contained 2µl of 10X RT buffer, 2µl of 100nM DTT, 1 µl of dNTP, 1µl of RT enzyme and 4µl of PCR water (Entire Kit- Precision Reverse Transcription kit, PrimerDesign) (Table 2.2). The RT-ve mastermix excluded the RTase enzyme and instead 1µl of PCR water was added to make up the final volume of 10µl. The cDNA synthesis was paused after the annealing reaction and 10µl of the extension mastermix was added to each sample appropriately, before resuming the

reaction for the extension process, which was programmed at 4°C for 5 minutes, 25°C for 5 minutes, 55°C for 20 minutes, 70°C for 15 minutes and finally 4°C for 5 minutes. Once the reaction was completed, the samples in duplicate were pooled together and mixed to make a final volume of 40µl and stored at -20°C until further use.

DNase Treatment	
Reagent	Volume (µl)
10X DNase buffer	2µl
DNase 1 enzyme	3µl
3µg RNA diluted in Nuclease free H_2O (final volume)	15µl
EDTA	1µl
Total	21µl (2 aliquots of 10.5 µl each (duplicates) for cDNA synthesis
cDNA synthesis	
Random Nonamers	1µl
10X RT buffer	2µl
10mM dNTPs	1µl
100mM DTT	2µl
RTase	1µl
Nuclease free H_2O	4µl
DNase treated RNA (100ng/µl)	10.5µl
Total	21.5µl X 2 (Final volume 43µl)

Table 2.2 The cDNA reagents, volume and final concentrations required for cDNA synthesis.
(dNTP- dinucleotide tri phosphate; DTT- *Dithiothreitol*).

2.3.5 Quantitative Real-Time PCR (q-RT-PCR)

Quantitative real time PCR allows the mRNA quantification in a specific and reproducible manner, where the amplification is proportional to an increase in

fluorescence as measured by a detector thereby quantifying gene expression. SYBR Green detector was utilised in this thesis.

2.3.5.1 SYBR Green qRT-PCR chemistry

SYBR Green is a dye, which intercalates to dsDNA and then emits green fluorescence, such that the amount of fluorescence emitted (λ_{max} = 520 nm) is directly proportional to the quantity of product present in each cycle. However, during the denaturation step of the PCR cycle, dsDNA is denatured by high temperature to single stranded DNA and hence the fluorescence capacity of SYBR Green is lost, as it is no longer intercalated. But when primers anneal (annealing step) to their target and extension occurs, then dsDNA is produced and subsequently fluorescence occurs due to SYBR green intercalation, which is measured at the end of the every single amplification cycle. The mRNA abundance is determined by the cycle threshold (Ct) value, which is the fluorescence threshold reached above that of background fluorescence, and is recorded during the exponential phase of the reaction. The above cycles are repeated 40 times such that even low abundance transcripts can be detected. Inclusion of RT-ve samples gave an indication of where fluorescence was due to factors other than amplicon accumulation e.g primer dimerization- (as a quality control method all data had to be a minimum of 6 cycles earlier than respective RT-ve detection-personal communication, Primer Design Ltd). In practice >10 cycles earlier than negative controls was the normal situation. To ensure reaction specificity a disassociation (melting) curve was included in all runs, which was performed by the PCR instrument at the end of PCR cycling. A single high peak indicates a single fluorescent product (gene of interest) therefore discriminating between non-specific and specific binding of the fluorescent reporter and more than one peak would invalidate results (Figure 2.1). To validate the cDNA synthesis, each and every sample was checked using *GAPDH* primers, as *GAPDH* is a more stable and constitutively expressed gene at high levels in most tissues.

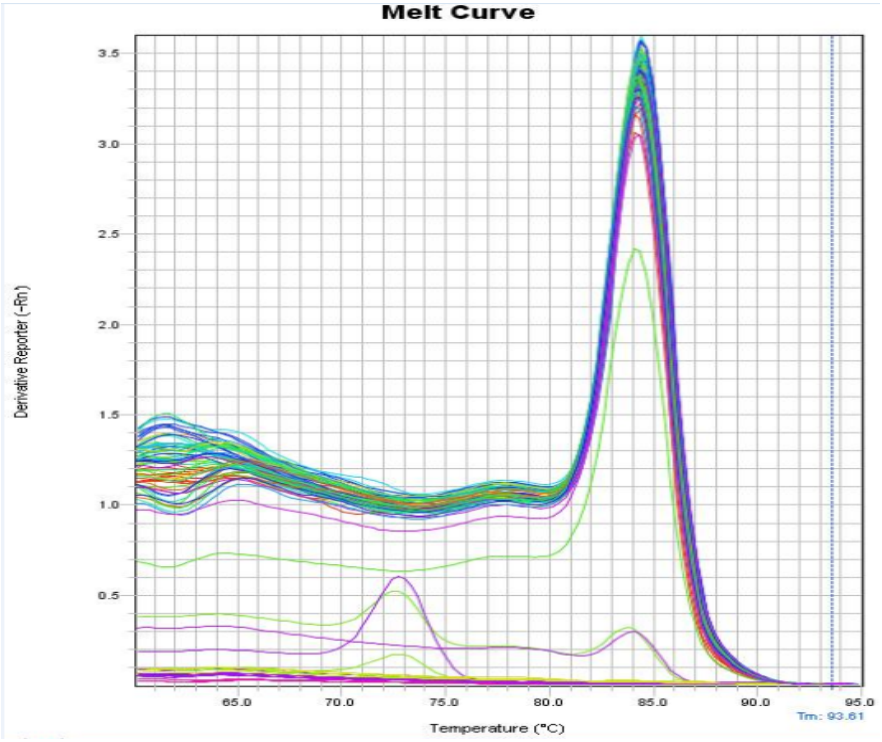


Figure 2.1 Dissociation or melting curve using SYBR Green detection method.

PDX-1 primers used in female fetal pancreas generated this curve.

2.3.6 GeNorm reference gene analysis

Even though the highly expressed *GAPDH* was used to validate the cDNA quality, GAPDH regulation can differ under specific conditions. Therefore, this thesis analysed a set of 12 ovine reference genes to determine the most stable housekeeping gene(s) under the experiment conditions (steroid manipulations) employed, using the geNormPLUS reference gene selection kit (PrimerDesign, UK) for each tissue. The 12 ovine housekeeping genes chosen were as follows: *18s, YWHAZ, aCOA, RPS26, B2M, MDH1, RPS2, RPL19, ATP synthase, GAPDH, Cyp1A1* and *β-actin*. Four samples were chosen from each treatment group (Control, TP, DES and DEX) and two PCR plates (MicroAmp®, Applied Biosystems) were setup for analysis (1 for control and TP; 1 for DES and DEX). Firstly, the four chosen cDNA samples were diluted to 1:10 dilution, (7µl of cDNA was combined with 63µl of PCR water) to make sure that 5µl of cDNA was available for each sample well. A volume of 15µl of Mastermix was prepared for all 12-reference genes, which comprised

of 10µl 2X SYBR Green Mix, 1µl primers (0.5µl of forward and reverse each), and 4µl of PCR water. 5µl of the chosen cDNA sample was added to the 96-well plate across the entire row followed by the addition of 15µl of mastermix down the column making up a final volume of 20µl per well. The PCR plates were then loaded into the qRT-PCR instrument and once the reaction was completed the data was analysed using BioGazalle qBase Plus software (Biogazalle, Ghent, Belgium) which determined the stable reference genes for both pancreatic and adrenal samples separately. Lists of stable reference genes used are mentioned in the materials and methods within specific chapters. Figure 2.2 represents an example of most stable reference genes analysed for female fetal pancreas at d90 gestation- the 'housekeeper' reference value used was the geometric mean of the Ct values of *G3PD*, *ATPsynth* and *β-actin*.

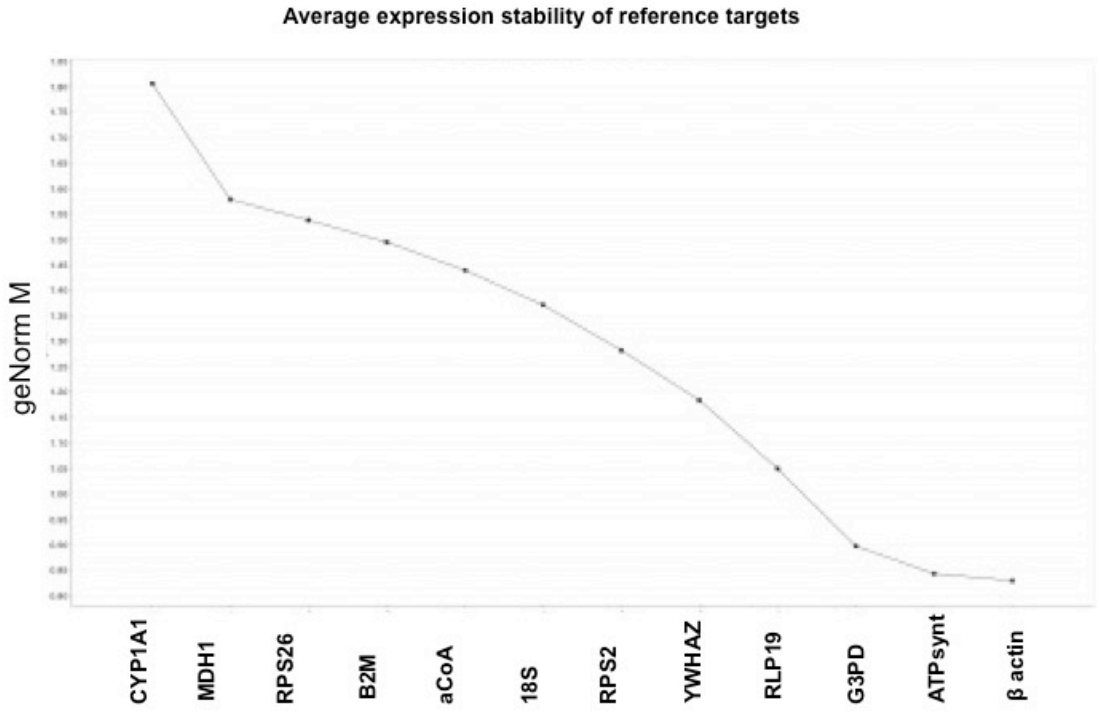


Figure 2.2 Representational image of stable reference genes analysed using geNorM analysis in the female fetal pancreas at d90 gestation.

2.3.7 SYBR Green qRT-PCR protocol

A mastermix solution of 19µl per reaction, sufficient for 70 reactions was prepared consisting of 10µl of 2X SYBR green with ROX PCR mix, 0.25µl of forward primer (25 µM), 0.25µl of reverse primer (25 µM) and 8.5 µl of PCR water and added into a 96-well plate inside a PCR preparation hood (UV sterilised and HEPA filtered, using only dedicated pipettes for this stage of the analyses). 1µl of cDNA sample, negative samples or reference sample (endogenous control/calibrator sample-comprising of a pool cDNA from a number of samples of the tissue being analysed) and PCR water in duplicate according to the well plan were added. The 96-well plate was then sealed with optical adhesive film MicroAmp®, Applied Biosystems) to prevent further evaporation of samples and centrifuged for uniform mixing of samples. The PCR plate was then loaded on to a real-Time PCR machine (StepOnePlus™ Real Time PCR System, Applied Biosystems).

Step		Temperature (^{0}C)	Time (min/sec)
Enzyme activation		95	10 minutes
Denaturation	x40 cycles	95	15 seconds
Primer annealing/extension		60	1 minute
Melting curve stage		95	15 seconds
		60	15 seconds
		95	15 seconds

Table 2.3 QRT-PCR cycling stages performed by ABI 7900HT fast Real Time PCR instrument.

2.3.7.1 SYBR Green qRT-PCR data analysis

Real time PCR data was compiled using ABI 7900HT v2.2 software (Applied Biosystems) and exported as an excel file. Sample duplicates were checked for precision (<0.5 Ct drift was deemed acceptable) and the dissociation curve was inspected to confirm specificity of PCR product. Finally, delta (Δ) Ct method was employed to analyse raw C_t values and obtain an expression

values normalised against housekeeping gene(s) (depending on the tissue type) as follows:

ΔC_t = Mean C_t of Gene of Interest- Mean C_t of housekeeping gene (geometric mean of stable housekeeping gene panel)

Once ΔC_t was calculated for particular gene of interest, later the comparative $\Delta\Delta C_t$ was calculated for the same as follows:

$\Delta\Delta C_t$ = ΔC_t of Gene of Interest- ΔC_t of reference(endogenous control/calibrator) sample

Finally, the fold difference of expression levels of gene of interest relative to reference sample was calculated using the formula $2^{(-\Delta\Delta Ct)}$.

2.4 Histology

2.4.1 Tissue fixation

Tissue blocks fixed in Bouins solution for 24 hours after collection were transferred to 70% ethanol. Samples were processed in an automated tissue processor (Leica Microsystems, Milton Keynes, UK) as per manufacturer's instructions, using the processing parameters detailed in the Table 2.4. Later, the tissues were embedded into paraffin wax blocks using the paraffin-embedding centre (Leica–EG 1160, Leica Microsystems) and left to cool/solidify and stored at room temperature prior to further use.

2.4.2 Tissue processing and sectioning

Paraffin embedded fetal and adult pancreatic blocks were cut into 5µm thin sections using a rotary microtome (RM2125 RT, Leica Microsystems, Heidelberger, Germany) and sections placed on the surface of a water bath (Grant Instruments, Cambridge, UK) to float at 41^{o}C prior to transference onto

charged slides (Superfrost® Plus, Thermo Scientific, Epson, UK), then dried in an oven at 65^0C for 30 minutes to bond the tissue to the glass.

2.4.3 Dewaxing and rehydration

The sections were dewaxed by submerging in xylene for 10 minutes (2 separate baths, 5 minutes each) and rehydrated using a standard ethanol series (100% ethanol, 95% ethanol, 70% ethanol) and finally distilled water (5 minute each bath).

Solution	Time	Temp
Ethanol 70%	1hr 30 min	Ambient
Ethanol 80%	1hr 30 min	Ambient
Ethanol 90%	1hr 30 min	Ambient
Ethanol 95%	1hr 30 min	Ambient
Ethanol 95%	1hr 30 min	Ambient
Abs Ethanol	2hr	Ambient
Abs Ethanol	2hr	Ambient
Xylene	1hr	Ambient
Xylene	1hr	Ambient
Xylene	1hr	Ambient
Paraffin wax	1hr	60^oC
Paraffin wax	1hr	60^oC
Paraffin wax	1hr 30 min	60^oC

Table 2.4 Steps involved in automated tissue processor.
(Leica Microsystems)

2.4.4 Immunohistochemsitry

Immunohistochemistry (IHC) bridges between three different scientific disciplines namely, immunology, histology and chemistry. IHC involves the localisation of antigens (Ag) (in this case specific proteins) within tissue sections by making use of specific antibodies (Abs). Post antigen-antibody binding, normally a secondary antibody (raised against the host species of the primary antibody) is used, labelled with either a fluorophore or a reactive

moiety which after a histochemical reaction can be visualised using under light microscopy or in the case of fluorescent reporters with suitable excitation/emission microscopy. In this thesis an indirect IHC technique was employed, where primary antibody applied will bind to the epitope on the surface of antigen within the tissue. A labelled secondary biotinylated (conjugated to biotin) antibody binds to the unlabelled primary antibody. The streptavidin-peroxidase detection method was employed to detect the biotinylated portion of the secondary antibody, as the Avidin (conjugated to horseradish peroxidase-HRP) in the ABC complex has high affinity for the biotin label on the secondary antibody. The HRP conjugated to the Avidin now bound to the biotin label of the secondary Ab reacts with 3,3'-diaminobenzidine (DAB) dye, a commonly used organic compound, oxidising it to form an insoluble brown precipitate.

2.4.5 Antigen Retrieval

Fixation with reagents such as the Bouins solution used here can cause unwarranted masking of antigens. This thesis employed the heat induced method of antigen retrieval as it works through breaking the cross linkage between the Ags, however the actual mechanism is still unknown, but appears to be pH and heat dependent; in this regard both acidic and alkaline conditions can be employed. Following dewaxing and rehydration processes, slides were incubated in a container of 0.1M-citrate buffer, pH 6.0 (final volume of 500ml) for low-pH antigen retrieval by heating them in microwave on full power for 5 minutes X 2 with 1-minute rest each, and microwaved on full power (750W) again for 5 minutes before resting for 20 minutes. Before proceeding to next step, the slides were cooled by washing under running tap water.

2.4.6 Blocking

IHC involves several steps to prevent/minimise background and non-specific staining. Endogenous peroxidase activity must be quenched in order to avoid false-positive signal generation where the detection system relies upon

exogenous peroxidase activity. In this thesis, following antigen retrieval, slides were treated with 3% hydrogen peroxide (H_2O_2) (diluted using distilled H_2O) for 5 minutes on a shaker, which quenches the peroxidase activity. This was followed by two separate changes of washes in TBS for 5 minutes each.

An additional blocking step was included where tissues were incubated in a humidified chamber for 30 min using 2.5% normal horse serum (Vectastain Universal Quick Kit R.T.U, Vector Laboratories, Peterborough, UK, PK-7800) – the serum chosen is the species of the secondary Ab. Proteins present in the serum can therefore bind to non-specific sites present in the tissue so that they will be inaccessible to the secondary antibody.

2.4.7 Primary Antibody

Even though both monoclonal and polyclonal antibodies are used in IHC, this thesis employed only monoclonal antibodies. An advantage of using monoclonal antibodies (mAbs) over polyclonal is that the former binds to a single specific antigen-binding site (epitope) and therefore reduces the possibility of cross-reactivity with other Ags. Further details of primary antibodies are mentioned in specific chapters. Following incubation with blocking serum, slides were cleared of excess serum and primary antibody (diluted appropriately in blocking serum solution) was applied. Negative control slides were treated with IgG at an equivalent concentration (from same species as primary Ab) to primary Ab and all slides were incubated overnight at 4^{o}C.

2.4.8 Colourimetric detection, counterstaining and mounting

Following overnight incubation with the primary antibody, sections were washed with TBS containing 0.1% tween (TBST) twice for 5 minutes each and incubated with biotinylated secondary antibody (Anti-Rabbit/Anti-Mouse IgG (H+L), for 30 minutes (Vectastain Universal Quick Kit R.T.U, Vector Laboratories, Peterborough, UK PK-7800) at room temperature. The negative

controls were washed separately. Then sections were washed twice in TBST (0.1% Tween) for 5 minutes each and incubated with Streptavidin-HRP Conjugate (ABC) (Vectastain Universal Quick Kit R.T.U, Vector Laboratories, Peterborough, UK, PK-7800) complex for 1 hour, and then washed twice in TBST for 5 minutes before colorimetric detection. This was followed by addition of DAB (3'3- diaminobenzidine [SK-100, Vector Laboratories, Burlingame, U.S.A]) substrate as per manufacturer instructions, and incubated for 30 seconds-1 minute until sufficient staining was evident by monitoring under the microscope for suitable colour development. Slides were then washed in distilled water for 5 minutes followed by counterstaining with haematoxylin for 15 seconds, then 'blued' under tap water until all haematoxylin had changed colour from purple to blue. The slides were then dehydrated in an ascending alcohol series (70% ethanol, 90% ethanol, 100% ethanol) for 5 minutes each and placed in xylene for 5 minutes prior for mounting. Finally, the sections were mounted with Pertex (mounting medium) prior to applying cover slips and air dried for 24 hours before analysis or storage.

2.4.9 Light microscopy and imaging

Cells in the sections that were positive for antigen of interest were localised and counted (detailed in specific chapters) under the 10X objective of a light microscope (Leitz Wetzlar, Germany). Protein immunolocalisation images were captured under 10x objective using a Olympus light microscope (Olympus BH-2, UK) fitted with a Olympus DP25 camera (Olympus Corporation, UK). Images were processed using cellSense® Image processing software v.1.4 (Olympus Corporation, GmbH) and were exported as Jpeg files.

2.5 Western Blotting

Western blotting (immunoblotting) methods are based on antigen-antibody interactions. A known quantity of total protein is separated based on

molecular weight (Mw) using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Seperated proteins are then transferred from the gel to a membrane prior to probing of interest-specific antibody and a labelled secondary antibody. Fluorescent detection is employed in this thesis to visualise protein bands: the fluorophore attached to the conjugated secondary antibody is excited by specific wavelength light and a photo sensor such as charged coupled device (CCD) camera equipped with appropriate emission filters detects emission. The captured digital image of the western blot can then be used for quantitative analysis such as determination of protein band size.

2.5.1 Protein Extraction and Precleaning

A small piece of tissue (~30mg) was cut on dry ice and added to 2ml tubes containing magnetic beads and 200μl of Tris Lysis buffer [50mM Tris/HCl (pH 8.0), 1% (v/v) Triton X-100, 150mM NaCl, 5mM EDTA, complete protease inhibitor tablets (1ml of 0.1% of lysis buffer comprising of protenase phosphatase), which was followed by homogenizing the tissue on Tissue Lyser LT (Qiagen, Germany) for 2 minutes. Each sample was then transferred to a different 2ml tube and centrifuged at 4°C for 5 minutes at 10000g after which the protein supernatant was removed and stored.

The protein samples were precleaned using Sepharose conjugate (Rec-Protein Sepharose 4B Conjugate, Invitrogen Ltd, USA), washed in lysis buffer twice and then added to each sample at a 0.1v/v dilution. The samples were then rotated for 2 hours at 4°C followed by microcentrifugation at 4°C for 10 minutes at 10000g. Later, the supernatants were transferred to clean 2ml tube and stored at -80°C until further analysis.

2.5.2 Protein concentration measurement

Protein concentration was measured using Bradford dye-binding (Protein dye-BioRad) assay (Bradford 1976). The Coomassie Blue G-250 dye within the

Bradford reagent binds to the protein sample, which shifts the absorbance from 465nm to 595nm and this change in absorbance at 595nm is directly proportional to the amount of protein present in any given sample. Bradford reagent was prepared by adding 100mg of Comassie Brilliant Blue G-250 (Bio-Rad) to 50ml of 95% ethanol. To this, 100ml of 85% (w/v) phosphoric acid was added and the solution was placed on a stirrer for 30 min, then made up to a final volume of 1L by adding dH_2O and filtered through Whatman grade 1 filter paper. 5μl of protein samples were added to the wells of 96-well plates (Costar 96-well Polystyrene plate, NY, USA) followed by addition of 250μl of Bradford reagent and incubated on a plate shaker at 37^0C for 3 minutes. The microplate was then read at 595nm using microplate reader (Dynex Technologies, MRXII) and software Revelation V.4.25. The software extrapolated the concentrations of samples by providing a standard curve constructed from the different dilutions of bovine serum albumin (BSA) used as standards, ranging from 0-3mg/ml. Samples were diluted using dH_2O in order to fall within the linear range of the standard. Quality control was included in every assay to ensure assay precision. Inter- and intra assay CVs were <5% and <2%, respectively.

2.5.3 Preparation of Gels

1.5mm thick polyacrylamide gels were made in advance and stored at 4^0C for up to 2 days before use. Glass plates (Mini PROTEAN® System, BioRad Laboratories Ltd, USA) were used to make up resolving and stacking gel as outlined in Table 2.5, however, TEMED and APS were added just before casting the gels. The glass plates were assembled and the resolving gels poured carefully (making sure of no leakages and also keeping free from any air bubbles) up to the level 1cm below the comb teeth. Finally, 4% stacking gel was poured gently, avoiding air bubbles before the 10-well comb (1.5mm spacer) (Mini PROTEAN® Comb 10-well, BioRad Laboratories Ltd, USA) was inserted and incubated to polymerize for 30 min.

Reagent	Resolving (12%)	Stacking (4%)
Acrylamide	4ml	670μl
Tris-HCl	2.5ml (1.5M; pH 8.0)	2.4ml (0.5M; pH 6.8)
20% (w/v) SDS	50μl	50μl
dH_2O	3.4ml	3.075ml
APS (10%w/v)	50μl	50μl
TEMED	15μl	15μl

Table 2.5 Components of western blot resolving and stacking gels used in SDS-PAGE.

2.5.4 Western Blot Protocol

All reagents used in this protocol were purchased from Sigma-Aldrich, USA, unless otherwise stated. All samples were diluted to 1μg/μl in lysis buffer, (0.1M Tris-HCl pH 6.8, 20% glycerol, 4% (w/v) SDS, 1% bromophenol blue and 3% β-mercaptoethanol) (final volume of 100μl). Protein samples were denatured by heating at 70^0C for 5 minutes and were loaded (10/20μg per well depending on the protein of interest) with the addition of a full-range pre-stained protein molecular weight marker (PageRuler™Plus, Thermo Scientific, USA; Product No. 26619; Lot No. 00112289) into one well per gel and electrophoresed at 200V for 60 minutes. Sponges, blotting paper (Gel Blotting Paper Gb002; Scientific laboratories Ltd, Nottingham, UK), and nitrocellulose membrane (Amersham™ Hybond ECL, 0.45μm thick, GE healthcare Life Sciences, UK) were cut to size and soaked in transfer buffer before use. The gel was carefully separated from the glass plates by making use of transfer buffer (pH 8.0) and was 'sandwiched' between sponges (x2), filter paper (x2) and nitrocellulose membrane (x1) as shown in Figure 2.3. The cassettes were placed between electrodes in the gel tank and incubated with an ice block to prevent the apparatus from over heating and finally the blot transfer was run at 25V for 2 hours. Transfer was confirmed visually by placing the blot in 0.2% Ponceau S staining solution. Later the blots were blocked in 5% BSA in TBST

for 1 hour at room temperature. Blots were then incubated overnight at 4^0C with the primary antibody of interest (diluted in 5% TBST).

Blots were washed extensively 4 times for 5 minutes each using wash buffer (TBST) and then incubated in darkness for 2 hours with fluorescent secondary antibody specific to the species of the primary antibody. Blots were again washed using TBST every 5 min (x4) before visualisation. The blots were scanned under the Odyssey® Infrared Imaging System (LI-COR Biosciences, Cambridge, UK) using appropriate filters (700/800nm channel) and finally proteins bands were visualised and quantified using the software Image Studio Lite™ v.2.0. The size of the visualised protein band was confirmed with the position of the molecular weight marker.

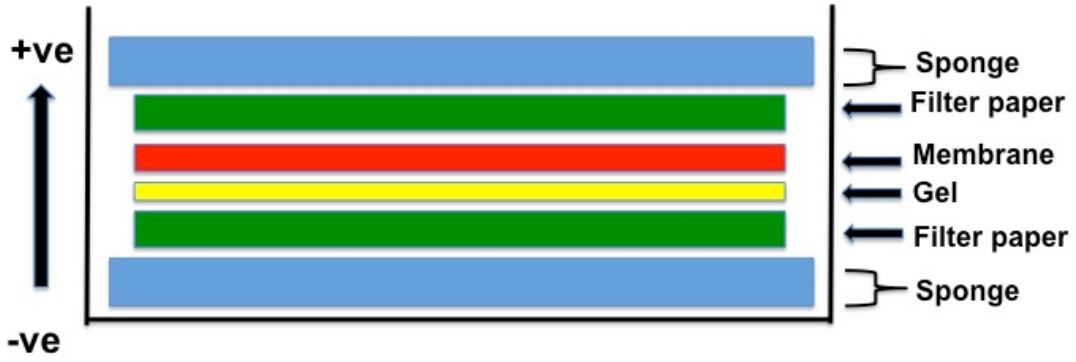


Figure 2.3 Gel assembly set up utilised during transfer process for western blotting.

Contents	Running Buffer	Transfer Buffer	Wash Buffer (TBS)
Tris	0.025M	0.025M	20mM
Glycine	0.192M	0.192M	_
SDS	0.1%	_	_
NaCl	_	_	0.15mM

Table 2.6 Components of buffers utilised in western blotting protocol.

2.6 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is the 'gold standard' to measure plasma steroid concentrations, as it is highly sensitive allowing the detection and quantification of small amounts of antigen in the sample. RIA works on the 'competitive binding' principle, where a competition exists between unbound antigen (analyte) within the given sample and radioactive labelled antigen (tracer) (usually gamma emitting radioiodine (^{125}I) to a given concentration of primary antibody specific to antigen of interest), therefore inversely measuring the quantity of antigen (analyte). The analyte (amount of hormone of interest in a sample) competes with the tracer for antibody binding, displacing it, so that as the concentration of the antigen in the serum sample increases, the quantity of bound radioactive tracer decreases. The bound antigens are separated from unbound antigens by adding secondary antibody specific to the primary antibody, and a further wash using appropriate wash buffer. The bound fraction of antigen-antibody-secondary antibody complex is centrifuged to form a pellet, and the supernatant containing unbound antigen is discarded. Finally, the radioactivity of the bound fraction is measured using a gamma counter. By making use of known antigen concentrations (calibrated standard concentration of the hormone being measured), a standard curve is generated, allowing sample concentrations to be derived from the linear portion of the standard curve.

2.6.1 Serum Extraction

To separate steroids from binding proteins present in plasma samples and thereby to maintain accuracy of the assay, sheep plasma samples, along with standards and quality controls (high, medium and low) were extracted using a diethyl ether/ethanol ice bath. The protocol is as follows: 200μl of standards controls and sheep plasma samples were added into separate glass tubes, (16x150mm), followed by addition of 2ml diethyl ether (Fischer Scientific) and vortexed for 10 minutes using a multi-vortex. Tubes were then snap frozen in a tray containing ethyl alcohol (VWR International, France) and dry ice. After a

few seconds, the ether/organic layer containing extracted steroids was decanted into fresh glass tubes, 12x75mm (Borosilicate Glass, Pyrex®) and dried down under a stream of nitrogen on hot block at 40^0C. The dried extracts were reconstituted in 250μl of reconstitution buffer (Table 2.7) stored at 4^0C prior to assay.

2.6.2 Radioimmunoassay protocol

RIA was carried out over three days, in which the tracer and the testosterone antibody were added on day 1, secondary antibody incubation on day 2 and separation of the antigen-antibody complex on day 3. The assay was set up in duplicate in 10x75mm polypropylene tubes (Sarstedt ltd, Leicester, UK), and in each case total counts (T0), non-specific binding (NSB; assay buffer and tracer), (B0; assay buffer, primary antibody and tracer) standards, low, medium and high quality controls (QC) were included along with unknown samples. Firstly, the plasma extracts were resuspended in 250µl of testosterone assay buffer (10% Triton X in PBS in 0.1% BSA) at room temperature for 1-hour prior to start of the experiment. Later, both primary antibody (Rabbit anti-testosterone-19, AMS Biotechnology) (1:250000 working dilution made up in assay buffer) and tracer (Testosterone ^{125}I, MP Biomedicals) diluted in assay buffer to give a count rate of 15,000cpm/100μl) were added to the tubes. Total count (T0) tubes received only 100µl, and NSB tubes, tracer and assay buffer (thus any counts bound in these tubes are a reflection of non-specific binding, not Ab binding, and are used for correction of assay data); and both tracer (100µl) and antibody (100µl) were added to the tubes containing standards and sample. Tubes were vortexed, covered with paraffin laboratory film and incubated overnight at 4^0C to attain equilibrium.

Following overnight incubation, 100µl of secondary antibody, a combination of normal rabbit serum (NRS; 1: 600 dilution; SAPU, Carluke, Lanarkshire, Scotland) and donkey anti-rabbit serum (DARS; 1:60 dilution; SAPU, Carluke, Lanarkshire, Scotland), both diluted in assay buffer was added to all the tubes

except the total counts (T0). Tubes were vortexed, covered using laboratory film and incubated overnight at 4^0C.

On day 3, 2ml of wash buffer (polyethylene glycol 4%, saline 0.9%, triton 0.2%) was added into each tube (apart from T0 tubes) and centrifuged (Sigma laboratories, 6K15) at 3000G for 30 minutes. Supernatant was discarded and tubes inverted on tissue paper to remove residual liquid before counting radioactivity on the gamma counter (LKB Wallac, 1261, MultiGamma), which gave the raw γ counts.

2.6.3 RIA analysis

The assay was analysed using computational software AssayZap (Biosoft, Cambridge, UK), which analysed the raw data (γ counts per min) generated from each bound fraction (pellet) plotted a standard curve automatically from a pre-determined method file and calculated the results based on the interpolation of unknown values with those of standard binding curve. Data were exported to an Excel file for further analysis. Intra and inter-assay co-efficient of variation (CVs) was determined by the quality control samples included in each assay and were 8.7% and 7.9% respectively.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) works on the antigen-antibody binding principle similar to immunohistochemsitry and is an alternate to radioimmunoassay to quantify protein (antigen) in a sample. The 96-well plate is coated with a primary capture antibody and the added sample antigen binds to specific binding sites. A second detection primary antibody added binds to sample antigen creating a ‘sandwich’ between two layers of antibody, hence the name sandwich ELISA, which is employed in this thesis. A secondary detecting antibody conjugated to HRP or AP is directed against primary detection antibody, in case the latter is not conjugated to an enzyme for detection purpose. The amount of bound antigen is determined using

colorimetric substrates such as 3,3',5,5'- tetramethylbenzidine (TMB), which initiates an enzymatic colorimetric reaction post binding to HRP producing blue colour. The enzymatic reaction is terminated by adding an acidic stop solution (e.g HCl), which results in a colour change from blue to yellow, and the colour intensity is directly proportional to the amount of antigen present in the sample. The plate is spectrophotometrically read at 450nm. This thesis employed two ELISA, one for cortisol, which was optimised and validated before measuring actual samples (detailed in section 2.7.1) and the other for insulin (using commercially available kit; section 4.2.2). As the two assays were performed at two different places, two different locations, two different instruments and software were used to determine the standard curve and calculate the concentrations of hormones in the unknown samples.

2.7.1 Cortisol Enzyme Immunoassay - optimization and validation

During certain stress responses (such as trauma, travel stress, castration, intense heat, infection and diseases) plasma cortisol levels are elevated. Therefore, measurement of cortisol concentration in blood is an indicator of presence of stress (Sapolsky *et al*., 2000). There are EIAs developed for measurement of plasma and salivary cortisol in cattle (Chacon Perez et al., 2004), dogs (Ginel *et al*., 1998) and pigs (Kaneko *et al*., 2003). Even though there are commercially available validated ELISA kits for plasma cortisol in sheep, these were prohibitively expensive and could not be made available for this study. Thus, it was essential to develop, optimize and validate a specific ELISA protocol to measure cortisol in sheep plasma samples with a dynamic measurement range wide enough to encompass both basal and increased plasma concentrations in order to assess adrenal function in terms of glucocorticoid output from Synacthen (ACTH analogue) challenges.

2.7.2 Standards for cortisol calibration curve

1mg/ml standard stock solution of cortisol was prepared in ethanol. 500ng/ml final concentration (standard spiking solutions) of cortisol standard was prepared by diluting the stock in steroid-striped sheep serum to achieve the following calibration standard concentration range: 0ng/ml, 10ng/ml, 25ng/ml, 50ng/ml, 100ng/ml, 250ng/ml and 500ng/ml.

2.7.3 Optimization

Developing an ELISA protocol to measure cortisol in sheep plasma consisted of optimizing several parameters such as anti-cortisol CMO concentration used for coating the micro titre plate, blocking buffer constituency, primary antibody concentration, horseradish peroxidase (HRP) conjugate concentration, concentration of cortisol standards and finally incubation times of TMB. The following summarises the parameters optimised and outcomes of these trials:

a. CMO concentration range tested: 1:5000-1:10000; *1:10000 was found to be optimal for further experiments*

b. Blocking buffer: 1% gelatin dissolved PBS: BSA concentration range tested: 0.5%, 2% and 4% BSA in PBS; *2% BSA in PBS was found optimal which provided a sensitive standard curve.*

c. Primary antibody concentration range tested: 1:5000-1:20000 dilutions were tested for each concentration where a different variable was being optimised-under the condition that were chosen to be optimal. *1:10000 dilution gave most sensitive standard curve*.

d. HRP secondary Ab concentration range tested: 1:2500-1:10000 tested for each of the two different preparations, against the full range of primary antibody concentrations tested- *1:10000 Goat anti-rabbit, (Immunopure, Thermo Scientific, Rockford, USA) was found optimal in terms of sensitive standard curve obtained when tested along with 1:10000 of primary antibody.*

e. TMB incubation time range tested: 5- 10 minutes- *10 minutes incubation time was optimal, which was based on, the sensitive standard curve obtained.*

Overall optimization from the above trials indicated that using 1:10000 of CMO to coat the plate, 2% BSA in PBS blocking buffer, 1:10000 of primary antibody and 1:10000 of secondary antibody (HRP conjugate) (Goat Antirabbit, ImmunoPure, Thermo Scientific, Rockford, USA) gives a sensitive sigmoidal standard curve (Figure 2.4).

2.7.4 Validation of the technique

Cortisol assay specificity was determined by calculating the percentage cross-reactivity with a range of different steroids supplied by Sigma, USA and intra and inter-assay CVs were also determined.

2.7.4.1 Specificity- Cross reactivity test

The cross reactivity test showed acceptable levels of cross reactivity with all steroids tested apart from 11-deoxycortisol (22.8% cross reactivity) and corticosterone (18% cross reactivity). Since 11-deoxycortisol is found at a concentration of 100 times lower than cortisol and does not have any glucocorticoid or mineralocorticoid effect (Lopez-Calderon, 1999), it is very unlikely that this steroid could be a source of false elevation of cortisol in data sets obtained. Corticosterone, on the other hand is not the dominant hormone in sheep unlike in rats and birds, it showed 18% cross reactivity in our experiments, however, since this is not the primary end product of glucocorticoid synthesis in sheep, then similar to 11-deoxycortisol, corticosterone is present at much lower concentrations in cortisol dominant species, and thus is unlikely to pose real issues in terms of inaccuracy in sheep. It should also be remembered that the primary purpose of development of this assay was to measure cortisol pre and post adrenal stimulation in sheep, and that the other steroid under examination would be testosterone; neither testosterone, nor the other steroids tested showed any appreciable cross reactivity and hence the cortisol ELISA was deemed

precise enough for the use intended. Therefore the hormone does not interfere with the assay sensitivity.

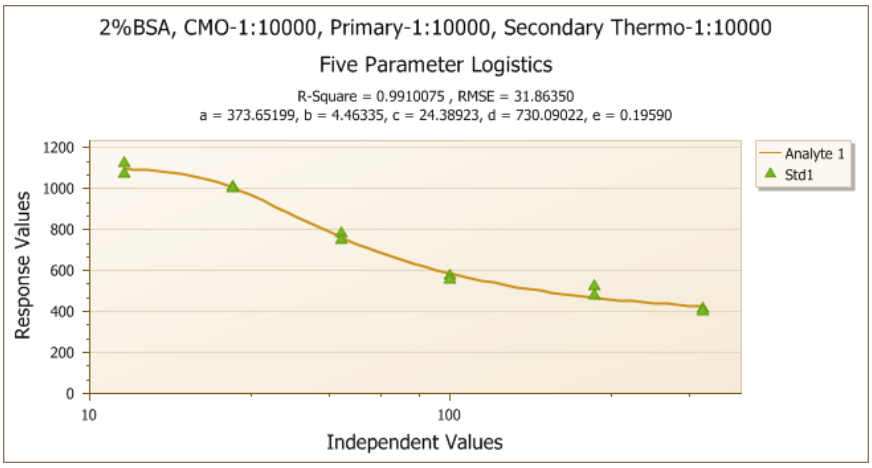


Figure 2.4. Cortisol standard curve representing selection of optimal concentrations of CMO, blocking buffer, primary and secondary antibody used in optimizing the EIA.

A. CMO- 1:10000, blocking buffer-2%BSA in PBS, Primary Antibody- 1:10000, secondary antibody- 1:10000.

2.7.5 Intra-assay and interassay coefficient of variances (CVs)

Two different intra-assay CVs were calculated based on the cortisol concentration obtained from the samples which were <100ng/ml and >100ng/ml. The intra-assay CV for samples <100ng/ml concentration was 6.6% and for those samples >100ng/ml the CV was 11.8%. This compares well with commonly used commercial ovine cortisol ELISA eg. Abnova (<100ng/ml=9.4%; >100ng/ml=6.2%) and was considered acceptable for use. Since all samples from experimental animals showed less than 100ng/ml cortisol, 6.6% intra-assay CV was considered to be the most accurate CV in terms of the data generated. Inter-assay CV was calculated over 10 different cortisol assay plates based on a QC of intermediate range (318ng/ml) of cortisol. This gave an inter-assay CV of 10.1%, again comparing reasonably well with commercially available assays (eg.Abnova interaasay CV of 8.6-15%).

Hormones	% Cross-reactivity
11-deoxycortisol	22.8%
Corticosterone	18%
Androstedione	0.47%
Cortisone	10.4%
DHEA	<0.1%
Progesterone	2.7%
Testosterone	<0.1%
DHT	<0.1%
Estradiol	<0.1%

Table 2.7. Percentage cross reactivity of different steroids tested against cortisol using the enzyme immunoassay (EIA) using the optimized parameters.

Intra-assay CV	CV
<100ng/ml	6.6%
>100ng/ml	11.6%

Table 2.8. Intra-assay coefficient of variation expressed as percentages of low and high concentrations.

2.7.6 Serum extraction

See details of extraction procedure for testosterone assay (Section 2.6.1).

2.7.7 Enzyme linked immunosorbent assay (ELISA) protocol – Cortisol

The optimized protocol was as follows. A 96-well plate (Microlon®, Germany) was coated with 200μl of Cortisol- carboxymethyloxime (CMO) (Biogenesis, Poole, England) conjugate anti-antigen IgG (1:10000 dilution) in coating buffer

(Table 2.9). Plates were then covered with parafilm (Greiner Bio-One, Stonhouse, Glasgow) and incubated overnight at 4^0C. Plate contents were discarded and the plate was washed five times in wash buffer (Table 2.9) using an automatic plate washer (CAPP, Wash™) and then tapped on a paper towel to remove any residual liquid in the plate. The plate was blocked by adding 250μl of 2% blocking buffer in BSA for 1 hour at room temperature to avoid non-specific binding of the antigen. The plate was washed X5 in wash buffer prior to adding the sample and primary antibody. 200μl volume containing 100μl of the sample or standards or controls and 100μl of cortisol primary antibody (1:10000 dilution) was added to the 96- well plate in duplicate according to the plate well plan using a Hamilton diluter instrument (Microlab®, Switzerland) for accuracy and the plate covered with parafilm and incubated for 2 hours at room temperature. Plates were then washed five times in wash buffer and dried on paper towels followed by addition of 100μl of 1:10000 dilution of donkey anti-sheep horse radish peroxidase (HRP) (ImmunoPure® ThermoScientific, USA) (detection antibody) using a multichannel pipette and incubated for 1 hour at room temperature. The plate was given a final wash five times in wash buffer and then 100μl of tetra methyl benzidine (TMB) (Millipore, Termacula, California) was added and incubated in darkness until a blue colour developed. Once the colour was developed, 100μl of stop solution (1M H_2SO_4 ; Table 2.9) was added to stop HRP activity.

Buffer	Chemicals/Stock	pH	Final volume	Manufacturer
Coating buffer	Na_2CO_3 :2.12g $NaHCO_3$:2.02g	9.6	1L	Sigma, USA
Blocking Buffer	PBS (NaCl :8g KCl :0.2g Na_2HPO_4 :1.44g KH_2PO_4 : 0.24g) + 2% BSA	7.4	1L	Sigma, USA
Assay Buffer	DPBS + 0.1% BSA +preservative	7.4	1L	BioWhittaker®, Lonza, Germany
Wash Buffer	Tris 250mM: 30.285g NaCl 250mM :43.83g 19mL of Conc.HCl	7.4	1L	Sigma, USA
Stop Solution	1M Sulphuric Acid	–	–	–

Table 2.9. Constituents of reagents used for Cortisol ELISA optimization and validation.

Finally, the plate was read on a plate reader (Multiskan Ex, LabSystems) at 450nm using Multiskan Transmit software to obtain absorbance values of standards, controls and samples. The absorbance data obtained from the plate reader was opened using Microsoft excel software and copied onto MasterPlex™ (MiraiBio Group, Hitachi Software solutions, USA) which plotted

a standard curve (five parameter logistics cubic spline) and measured the concentration of the samples and quality control (QCs) included in each which were used to calculate assay coefficient of variance.

2.8 Statistical Analysis

Statistical analysis was performed using the computational software GraphPad Prism v6.0 (GraphPad Software Inc., San Diego, CA). The majority of data dealt with in this thesis was the comparison of raw data between control and 3 other treatment groups (TP, DES and DEX). One-way ANOVA was employed for fetal control, TP and DES treatment and Student's unpaired t-test was performed Control and DEX treatment and control groups between sexes. In all cases, data was considered significant where $P<0.05$. Where any data manipulation/transformation was required prior to statistical testing this is mentioned in each specific case.

Chapter 3 The effects of direct prenatal steroidal excess exposure on developing fetal pancreas

3.0 Introduction

Variables in the maternal or placental environment, such as hormonal or nutritional indices, can have effects on the developing fetus, which can later lead to metabolic associated health consequences during adulthood (Barker, 2004; Godfrey and Barker, 2001). The fetal pancreas is one such tissue, which is vulnerable to *in utero* exposure to altered nutritional or hormonal environments (Petrik *et al*., 1999; Rae *et al*., 2013) with consequences observed in adult life in both humans and animal models (Kanaka-Gantenbein, 2010; Rae *et al*., 2013; Roland *et al*., 2010).

Fetal pancreatic development in sheep beings at approximately dGA 25-26, where two parts of the epithelium forms distinctive buds (Slack, 1995), however after primary (24dGA) and secondary (24-29dGA) transition, the islet like structures are formed around d33GA (Cole *et al*., 2009) (Figure 3.1) and finally distinctive insulin (β) and glucagon (α) secreting cells are present around d40 (A L Fowden and Hill, 2001). This study focuses on the effects of midgestation (d62 & d82) direct *in utero* steroid exposure (Figure 3.1) on the developing pancreas in terms of gene expression, islet morphology and function, as we know from our previous work that midgestational (d62-102) maternal androgen exposure in pregnant sheep leads to altered fetal pancreatic gene expression and function in female fetuses. This was carried into adtulhood further leading to altered pancreatic β-cell mass (Rae *et al*., 2013) and function in adulthood (Hogg *et al*., 2011) and is relevant to clinical conditions such as polycystic ovary syndrome (Dumesic *et al*., 2007). Women with PCOS suffer from not only reproductive abnormalities (Teede *et al*, 2010) but also metabolic associated abnormalities such as hyperinsulinemia, insulin resistance, hyperglycemia, obesity and type 2 diabetes (Dantas *et al*., 2013).

The rationale behind the direct fetal injection of steroid employed here is that there are certain drawbacks in the previous maternally androgen exposed models, where placental aromatase can metabolise androgens to estrogens including estrone and estradiol, meaning prenatal androgen exposure leads to

elevated fetal circulating estrogen levels in addition to male androgen concentrations in female fetuses (Rae *et al*., 2013).

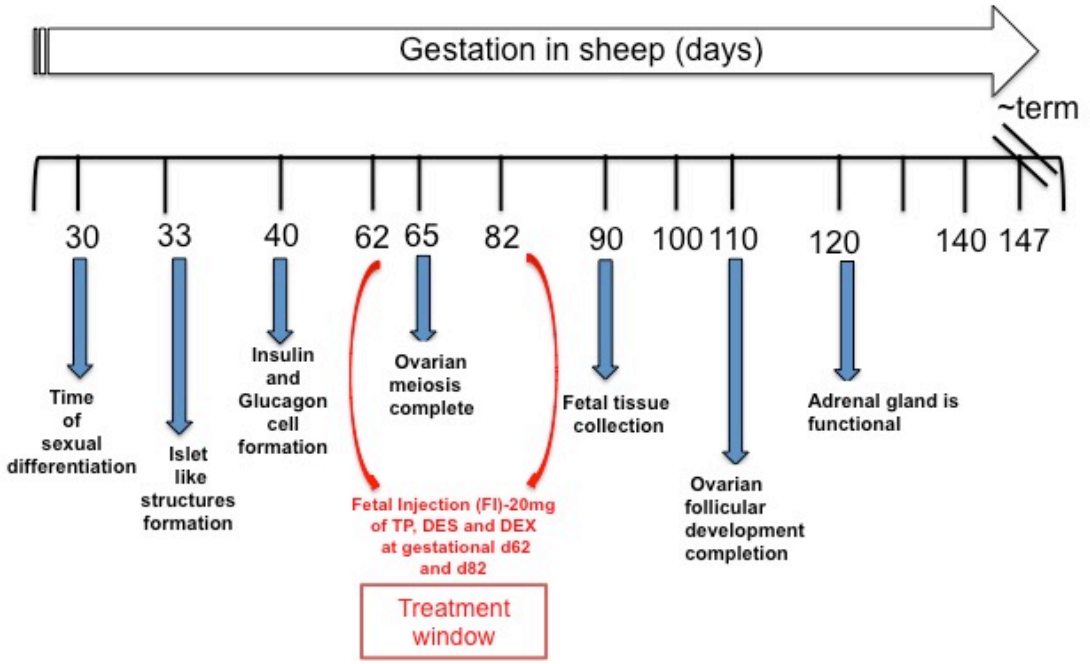


Figure 3.1. Sheep fetal development and mid-gestational direct fetal steroid treatment regime in our sheep model.
At d33 gestation, islet-like structures are formed in sheep fetus and later by d40 gestation the islets are differentiated into insulin and glucagon secreting cells (Cole *et al*., 2009). Sheep fetuses were directly exposed to TP, DES and DEX (20mg) at d62 and d82 gestation and the fetuses were sacrificed at d90. Term~147 days

This then begs the question as to whether the effects observed are androgenic or estrogenic. Recently, it has been shown that testosterone exposure to pregnant ewes from d30-d90 led to increased fetal serum estradiol and estrone levels (Almudena Veiga-Lopez et al., 2011), which may have programming effects in adulthood (Steckler *et al*., 2007; West *et al*., 2001). Another reason behind developing the direct fetal injection model was to allow manipulation of the dose of steroids to which the fetus was exposed. Dihydrotestosterone (DHT), which is a non-aromatizable androgen, has been used in sheep studies to delineate androgenic effects from estrogenic during fetal programming (Steckler *et al*., 2007; West *et al*., 2001), where the

pregnant mother was injected with TP or DHT. However, this does not give a clear picture for dosage control and thus the amount of DHT or TP reaching the fetus via placental route could remain variable. In addition, there could be androgenic placental effects, which could modulate potential estrogenic effects, masking the true situation.

In the current chapter, we have investigated the effects of direct administration of different classes of steroids such as TP (aromatizable androgen), DES (synthetic estrogen) and DEX (synthetic glucocorticoid) on the developing female fetal pancreas. Readouts from these experimental manipulations are expression profiles of key genes involved in regulating β-cell development and function at d90 gestation. This study also utilized male fetal pancreas samples, wherever possible, in order to examine differences between the male and female fetal pancreas, and differences in their responses to prenatal steroid excess.

The aims of this chapter were to measure the effect of mid-gestation direct steroid exposure on the developing pancreas, addressing the following research questions:

a. Do excesses of androgens, estrogens or glucocorticoids have effects on key genes associated with pancreatic development?
b. If effects of genes involved in pancreatic development are noted, is there evidence of downstream structural alterations in the developing pancreas?
c. Do consequences of steroidal excesses during development have functional effects upon insulin secretion from developing fetal pancreas?
d. Are responses to steroidal excesses fetal sex-specific?

3.1 Materials and methods

3.1.1 Experimental animals

Ethical approval, animal husbandry, treatment regime, sacrifice, fetal tissue collection are detailed in section 2.1-2.2. The female and male fetuses assessed in this chapter are listed in the table 3.1.

Analysis	**Female fetuses**	**Sample Number (n)**
RNA (qRT-PCR) study	Injection at d62 and d82, collection and assessed at d90	C=7;TP=7(20mg) DES=5 (50μg); DEX=4 (100μg)
Histology	Injection at d62 and d82, collection and assessed at d90	C=5;TP=5 (20mg) DES=5 (50μg); DEX=4 (100μg)
Tissue Culture	Injection at d62 and d82, collection and assessed at d90	C=5;TP=7 (20mg) DES=4(50μg); DEX=4 (100μg)
Fetal injected fetuses	**Male fetuses**	**Sample Number (n)**
RNA (qRT-PCR) study	Injection at d62 and d82, collection and assessed at d90	C=7;TP=7(20mg) DES=5 (50μg);DEX=4 (100μg)
Histology	Injection at d62 and d82, collection and assessed at d90	C=5;TP=5 (20mg) DES=6(50μg); DEX=6 (100μg)

Table 3.1 Animal treatment cohorts discussed in this chapter, treatment regime and relevant sample numbers.

3.1.2 Tissue collection

Fetal pancreas from both male and female fetuses were collected after dissection at d90, where one part of the tissue was snap frozen at -80^{0}C and the other half was stored in Bouins fixative for 24h prior to transfer to 70% ethanol and embedded in paraffin wax for histological studies. In all cases tissue was sampled from the mid portion of the fetal pancreas to make samples as comparable as possible.

3.1.3 Analysis of Gene expression in the fetal pancreas

Fetal Pancreatic tissues were collected and the RNA extracted was reverse transcribed to cDNA as detailed in Chapter 2, Section 2.3.1-2.3.3), then qRT-PCR was performed to measure the relative gene expression (details in Chapter 2, Section 2.3.5). Table 3.2 is the summary of forward and reverse primers used to quantitate gene expression of genes of interest and housekeeping/reference genes identified as stable by GENORM algorithm analysis.

Genes of Interest	Forward primer(5'-3')	Reverse primer(5'-3')
INS	CCAGCGGGAAATCAAGAGAGA	CCCTAGGGAGCTGGTCACTT
INSR	GCTTCGAGGCTGCACCAT	AGCTCAGCTGCCAGGTTGTT
PDX-1	CAGAGCCGGAGGAGAACAAG	CTGGAGATGTATTTGTTGAAAAGG
GLUT-2	CGAAATTGGGACCATCTCACAT	CACCGATAGCACCCCTGAGT
IGF-1	GCTTCCGGAGCTGTGATCTG	GACTTGGCGGCCTTGAGA
IGF-II	GGCGGGGAGCTGGTGGACA	TCGGTTTATGCGGCTGGATGGT
IGFR-I	GAATCTGCCAAGGAGACTCG	CCTGACAGCTGGACTACAGGCATCA
IGFR-II	ATGAAGCTGGACTACAGGCATCA	GCTCGCCGTCCTCAGTTTC
AR	GCCCATCTTTCTGAATGTCG	CAAACACCATAAGCCCCATC
ER-α	GAATCTGCCAAGGAGACCG	CCTGACAGCTCTTCCTTCTG
ER-β	GAGGCCTCCATGATGATGTC	GGTCTGGAGCAAAGATGAGC
GR	AAGTCATTGAACCCGAGGTG	ATGCCATGAGGAACATCCAT
Reference genes	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
GAPDH	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG
RPL19	SEQUENCE NOT AVAILABLE	SEQUENCE NOT AVAILABLE
ATP Synth	SEQUENCE NOT AVAILABLE	SEQUENCE NOT AVAILABLE
β-actin	SEQUENCE NOT AVAILABLE	SEQUENCE NOT AVAILABLE

Table 3.2 List of forward and reverse primer sequences (genes of interest and reference genes) for ovine fetal pancreas.

Where sequences are unavailable, this is due to the primers used being part of the GENORM kit as supplied, where sequence information was manufacturers proprietary information.

3.2 Fetal Pancreatic Histology

Fetal pancreatic tissue was immersion fixed in Bouins solution at the time of collection for 24h, and then embedded in paraffin wax as detailed in Chapter 2, Section 2.4.1-2.4.3.

3.2.1 Tissue sectioning

Paraffin embedded fetal pancreatic blocks were cut into 5μm thin sections using a microtome (RM2125 RT, Leica Microsystems, Heidelberger, Germany) and wax ribbons were placed on surface of the water bath (Grant Instruments, Cambridge, UK) to float at 41^{o}C. Each slide contained three sections/animal (selected sections 1, 11 and 21) separated by 50μm apart each to avoid the same cells being represented in more than one section. Sections collected onto charged slides (Supraflost Plus, Thermo Scientific, Epson, UK) were dried in an oven at 65^{0}C to bond the tissue to the glass and finally slides were stored at room temperature for immunohistochemical studies.

3.2.2 Immunohistochemistry

Immunohistochemistry on fetal pancreatic tissue was carried out as detailed in Chapter 2, section 2.4.4. Table 3.3 lists the antibodies used for fetal pancreas.

3.2.3 Cell counting

Cells in the sections (3 section/animal) that were positive for insulin and glucagon (separate slides) were visually scored under the 10X objective of a light microscope (Leitz Wetzlar, Germany). Five random fields were chosen

from each section and the stained cells were counted in a 10 by 10 grid. A mean of the counts from the 5 fields in each section was calculated and divided by 1.16 (correction factor- to correct to mm^2) to obtain the number of stained cells per mm^2 per section. In the same way, a mean value from all three sections per slide was taken into account to get a value for each animal subjected to analysis.

Antigen to be detected	Antibody	Dilution	Manufacturer	Secondary antibody
Insulin	Mouse monoclonal anti-insulin antibody	1:1000	abCam®	GAMB (Vector laboratory)
Glucagon	Mouse monoclonal glucagon antibody	1:4000	abCam®	GAMB (Vector laboratory)

Table 3.3. List of antibodies analysed by Immunohistochemsitry in fetal pancreas.

Listed are the concentrations of primary antibody and secondary antibodies applied were universal goat-anti mouse biotinylated (GAMB) antibody.

3.3 Tissue culture

Fetal pancreatic tissue biopsies collected from the fetal injected (FI) fetuses were minced finely under aseptic conditions and later re-suspended in collagenase (3mg/ml) (Sigma Aldrich, Poole, UK) containing HEPES buffered saline solution (HBSS) and incubated at 37^{o}C for 20 min. Suspensions were further disaggregated by passing through a 10G needle 3 times, then centrifuged (600g, 10 min). Collagenase containing medium was aspirated, then 2ml HBSS (wash solution) was added and the centrifugation step repeated, prior to re-suspension in 1ml culture medium (Dulbecco's Modified Eagle medium (DMEM), 2mM *L*-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin, 0.25% bovine serum albumin (BSA), pH 7.4 (final glucose concentration 5.5mM) (Sigma Aldrich). 100µl aliquots of cell suspension were

added to 300µl of culture medium containing glucose (5.5mM, euglycaemic), and incubated for 3 hours at 37^{o}C/5% CO_2 (balance was air) with gentle agitation in sterile tubes with vented lids. At the end of the incubation time, samples were centrifuged (600g, 15 min), then supernatants removed and frozen at -20^{o}C until insulin measurement. Cell pellets were washed twice with 1ml of PBS, pH7.4 after re-suspension and were centrifuged to repellet and stored at -20^{o}C until protein determination. Prior to performing protein estimation assays, pancreatic cell pellets were resuspended in lysis solution (50nM Tris-HCl, pH8.0, 150mM NaCl, 0.1% Triton-X-100) and sonicated for 2 minutes, followed by a one-minute incubation on ice, then a further 2 minutes sonication on ice.

3.3.1 Hormone determination

ELISA kits for ovine insulin (ALPCO, Salem, NH) were used to measure insulin in tissue culture supernatants. The data were collected by making use of a Dynex Technologies Revelation 4.25 plate reader (dual wavelength detection) using a detection wavelength of 450nm and a reference wavelength of 630nm as per manufacturer instructions. Curve fitting was cubic spine fit. Samples were assayed in duplicate on a single plate per experiment to avoid any plate-to-plate variation. In our hands this ELISA kit had an intra assay coefficient of variation of 4.5% and sensitivity of 0.14ng/ml.

3.3.2 PCR data derivation and Statistical Analysis

A comparative C_t analysis was used in order to establish the mRNA expression of the genes of interest (GOI) relative to untreated samples. The geometric mean of the most stable housekeeping genes analysed (using GeNorm analysis) for both female (*ATPsynth* and *β-actin*) and male tissues (*ATPsynth, RPL19, GAPDH*) was utilised as a housekeeping score in order to determine the ΔC_t value (C_t of target gene- C_t of housekeeping gene). The $\Delta\Delta C_t$ value for each gene of interest was determined by calculating the difference between the ΔC_t of a sample from that of ΔC_t of a reference

sample, constructed from a pool of ovine pancreatic samples (sufficient was prepared such that all analyses included this reference sample in all PCR runs). $2^{(-\Delta\Delta Ct)}$ was calculated for each gene of interest, in which the data was converted to a fold change further to give relative expression of the gene in treated samples as compared to control (untreated) animals.

All the above, along with the cell counts data from immunohistochemistry, and *in vitro* insulin secretion experiments, was transferred to GraphPad Prism (v.6.0, Graphpad software, San Diego, California, USA). Log transformation, ($base_{10}$), was carried out where data sets displayed unequal skew. Statistical analysis was carried out relevant to research questions, hence, due to the interest in comparing and contrasting effects of TP and DES, data from Control, TP and DES were analysed using one-way ANOVA (analysis of variance) followed by Tukey's *post-hoc* test to determine specific differences between groups. Differences between Control and DEX group were analysed using unpaired two-tailed Student's t-test. Results are presented as $\pm$SEM and p-value, where $P<0.05$ was considered significantly different.

3.4 Results

3.4.1 Effect of direct fetal steroid exposure on ovine male and female fetal pancreas

qRT-PCR analysis on fetal female and male pancreas was performed in order to determine changes in the relative expression levels of genes due to the prenatal over-exposure of TP, DES and DEX in comparison to control groups (listed in the figure 3.2-3.5 below). Since DES is a synthetic estrogen, and TP, an aromatizable androgen, analysis of these treatment groups together permitted resolution of androgenic and estrogenic effects. Dexamethasone (DEX), a synthetic glucocorticoid, was compared against control groups independent of the above analysis to determine the relative gene expression levels in the fetal pancreas in response to elevated glucocorticoids, as a surrogate of maternal/fetal stress and aberrant adrenal function. Insulin and glucagon immunohistochemistry was quantified in fetal pancreata from both sexes (Figure 3.7.2 and 3.8.2). Furthermore, *in vitro* insulin secretion in response to static, euglycaemic glucose load was also measured. This latter analysis was only conducted in female tissue due to practical constraints of time during tissue collection sessions (Figure 3.9).

3.4.1.1 Male and female comparative fetal pancreatic gene expression

Pdx-1 mRNA expression was significantly higher ($P<0.01$; Figure 3.2.A) in male fetal control animals compared to female controls and there was a trend towards increase in *AR* ($P=0.0542$; Figure 3.2.G) gene expression in male controls compared to female control animals. None of the other genes analysed showed any significant difference between sexes in terms of gene expression analysed in this study.

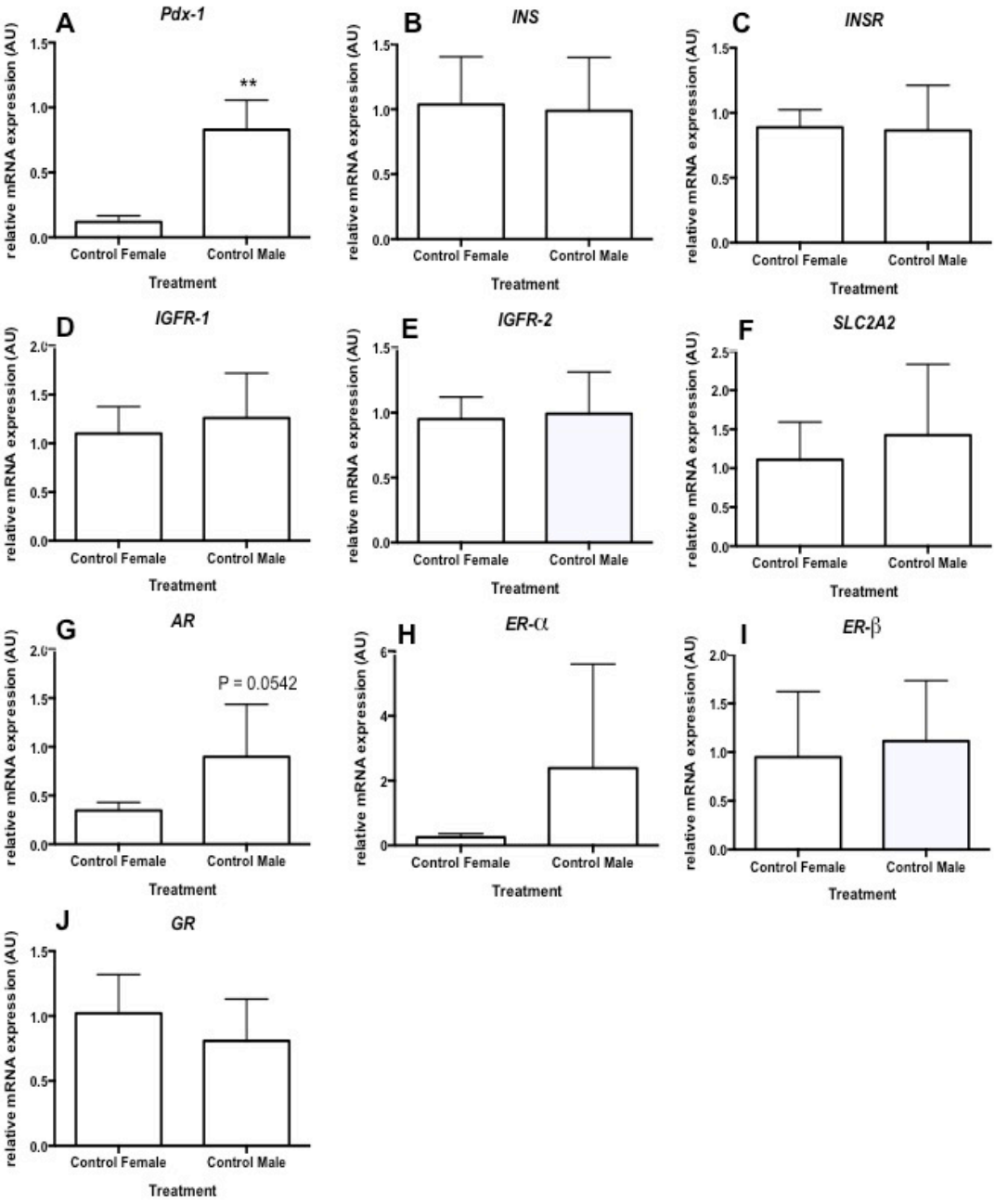


Figure 3.2 Comparative gene expression analysis in male and female control fetal pancreas at d90 gestation the d90 fetal control. Female Control (n=7), Male Control (n=7) ***P*<0.01.

3.4.1.2 Fetal pancreatic gene expression responses to TP and DES treatments

Figures 3.3 and 3.4 summarize the effects of TP and DES on the developing pancreas due to steroid application in both female and male fetuses

respectively. *PDX-1* (key transcription factor driving insulin gene transcription) mRNA abundance was significantly increased (*P<0.001*; Figure 3.3.A) by TP treatment in females, but no effect was seen with DES treatment in females and males showed no effect with either treatment (Figure 3.4.A). Insulin gene (*INS*) mRNA abundance was significantly increased (*P<0.05;* Figure 3.3.B) by TP treatment, but was unaffected by DES in females, whereas there was no significant effect in male pancreas from either treatment (Figure 3.4.B). Insulin receptor *(INSR)* was significantly increased by TP treatment (*P*<0.05; Figure 3.3.C), but not with DES treatment, in females and there was no effect of either treatment in males (Figure 3.4.C). IGF- receptor type-1 (*IGFR-1*) mRNA abundance displayed a strong trend (P=0.06;Figure 3.3.D) towards increased expression driven by TP treatment in females, and once again there was no change with either treatments in male fetuses (Figure 3.4.D). *IGFR-II* mRNA abundance was not significantly altered by TP or DES treatment in either sexes (Figure 3.3.E and Figure 3.4.F). *Slc2a2*, the gene coding for membrane bound transport protein GLUT-2, which imports glucose in β-cells and is also a mediator of insulin secretion was unaffected in females (Figure 3.3.F) by either TP nor DES treatment, however, it was significantly decreased by TP treatment in males (Figure 3.4.F). Androgen receptor (*AR*) mRNA abundance was significantly increased (*P<0.05;* Figure 3.3.G) by TP treatment when compared to control group in female fetuses only.

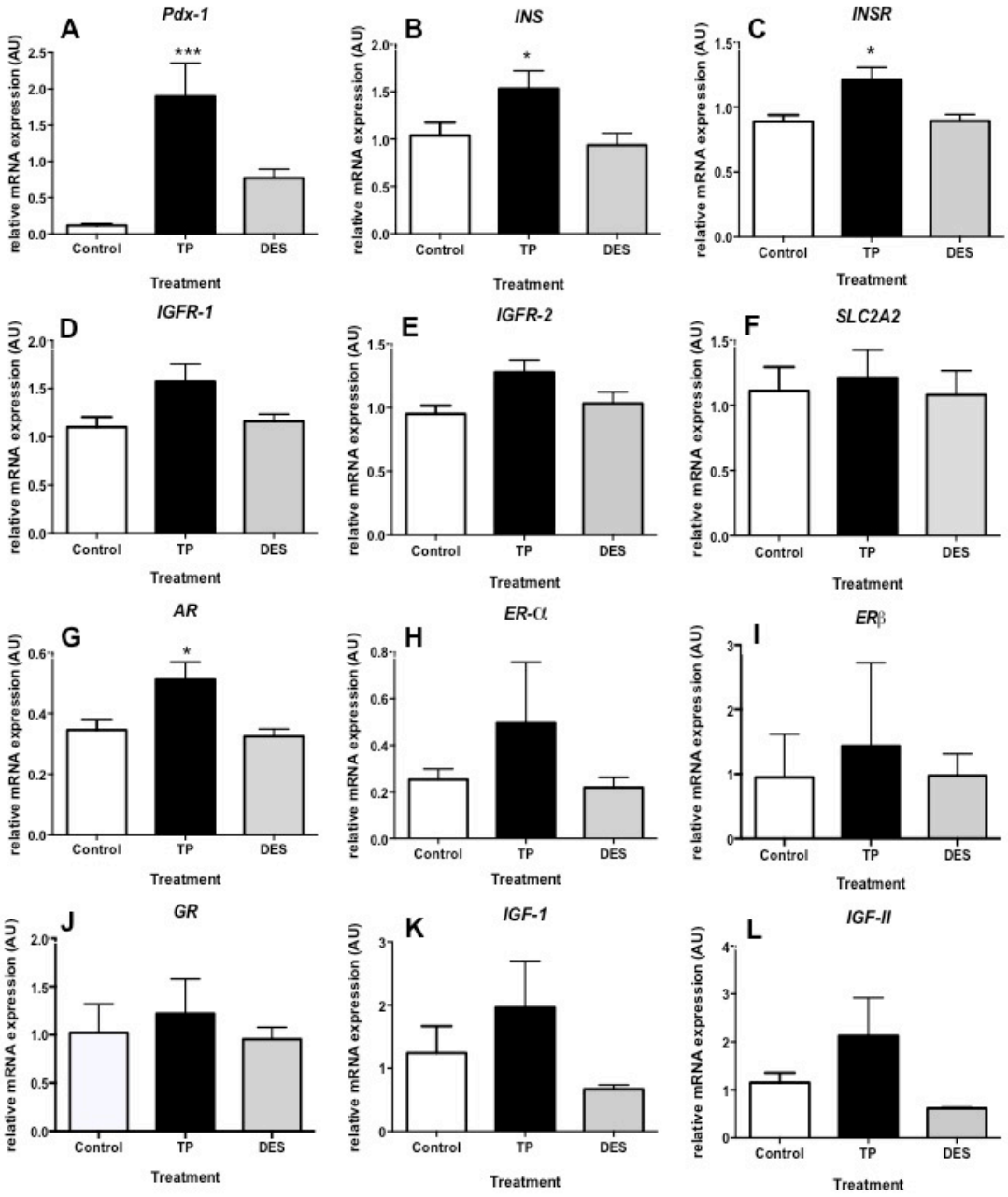


Figure 3.3 Effect of fetal TP and DES exposure on expression of *PDX-1*, *INS, INSR, IGFR-1, IGFR-II, Slc2a2, AR, ERα, ERβ, GR, IGF-I and IGF-II* (A-L) in female fetal pancreas at d90 gestation in comparison to controls. (Control n=7;TP n=7; DES n=5) **P*<0.05*;* ****P*<0.001

Estrogen receptors, namely *ERα* (Figure 3.3.H; Figure 3.4.H)*, ERβ* (Figure 3.3.I; Figure 3.4.I) and glucocorticoid receptor (*GR)* (Figure 3.3.J and Figure 3.4.J), were unaffected by any treatment in either fetal gender. Insulin like growth factor type-1 (*IGF-1*) and type-2 *(IGF-II*) mRNA abundance (studied

only in females) were not significantly altered by any treatment in females (Figure 3.3.K-L).

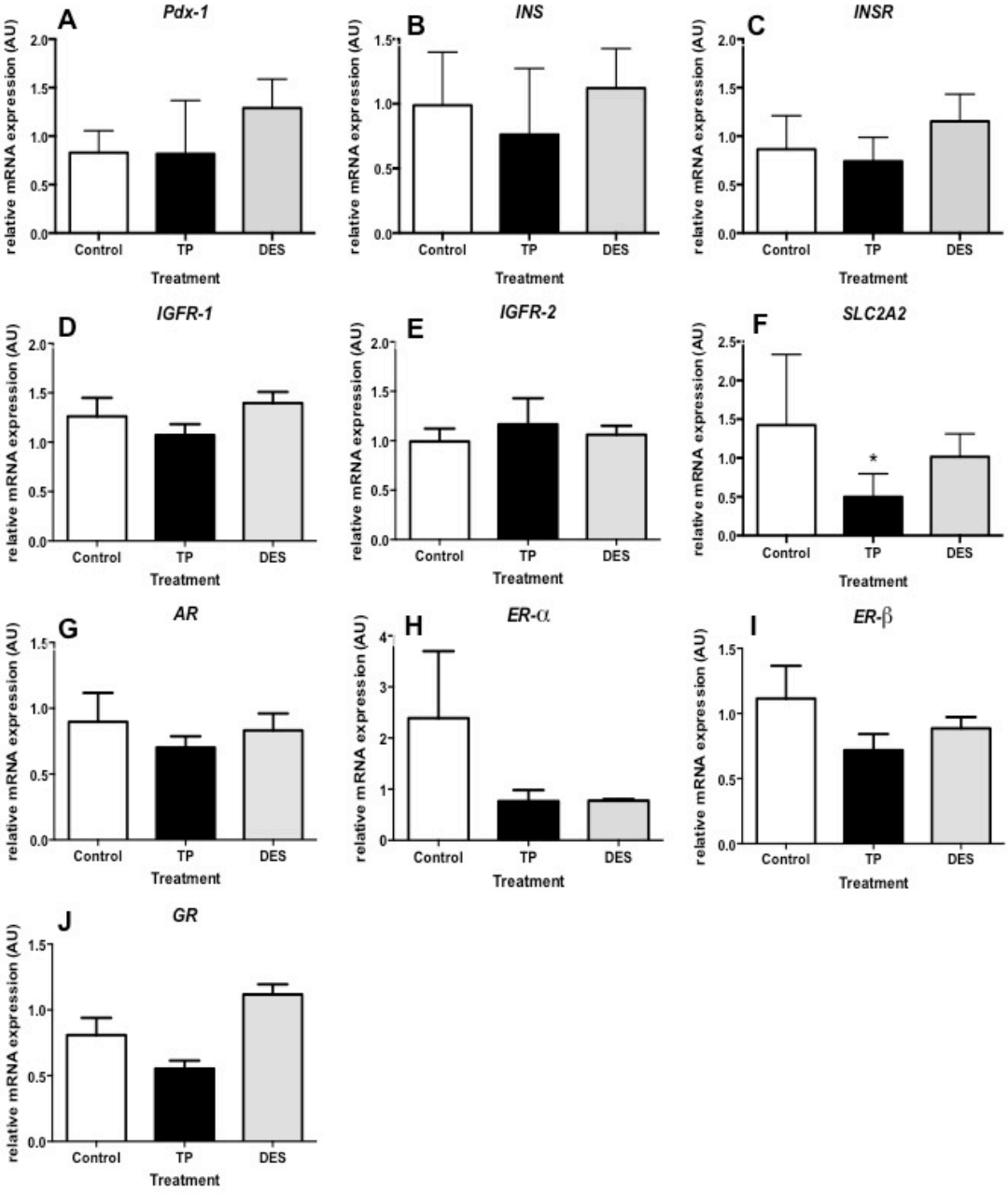


Figure 3.4 Effect of fetal TP and DES exposure on expression of *PDX-1*, *INS, INSR, IGFR-1, IGFR-II, SLC2A2, AR, ERα, ERβ* and *GR* (A-J) in male fetal pancreas at d90 gestation in comparison to controls. (Control n=7;TP n=7; DES n=5) **P*<0.05.

3.4.1.3 Fetal pancreatic gene expression responses to DEX exposure

PDX-1 (*P*<0.001; Figure 3.5.A) and *ER-*α (*P*<0.05; Figure 3.5.H) mRNA abundances were significantly increased by DEX treatment in female fetal pancreas, but no effects were noted in males (Figure 3.6.A; Figure 3.6.H-F). Insulin receptor *(INSR)* mRNA expression (*P*<0.01; Figure 3.5.C) was significantly decreased in the fetal female pancreas, but was unaffected in male pancreas (Figure 3.6.C). *GR* mRNA abundance was unaffected by DEX treatment in females (Figure 3.5.J), however was significantly increased (~2 fold) in DEX treated males (*P*<0.01; Figure 3.6.J) compared to controls. *INS*, *IGFR-I, IGFR-II, Slc2a2, ER*β, *IGF-1 and IGF- II* (studied only in females) mRNA abundances were not altered in either fetal sex by DEX treatment.

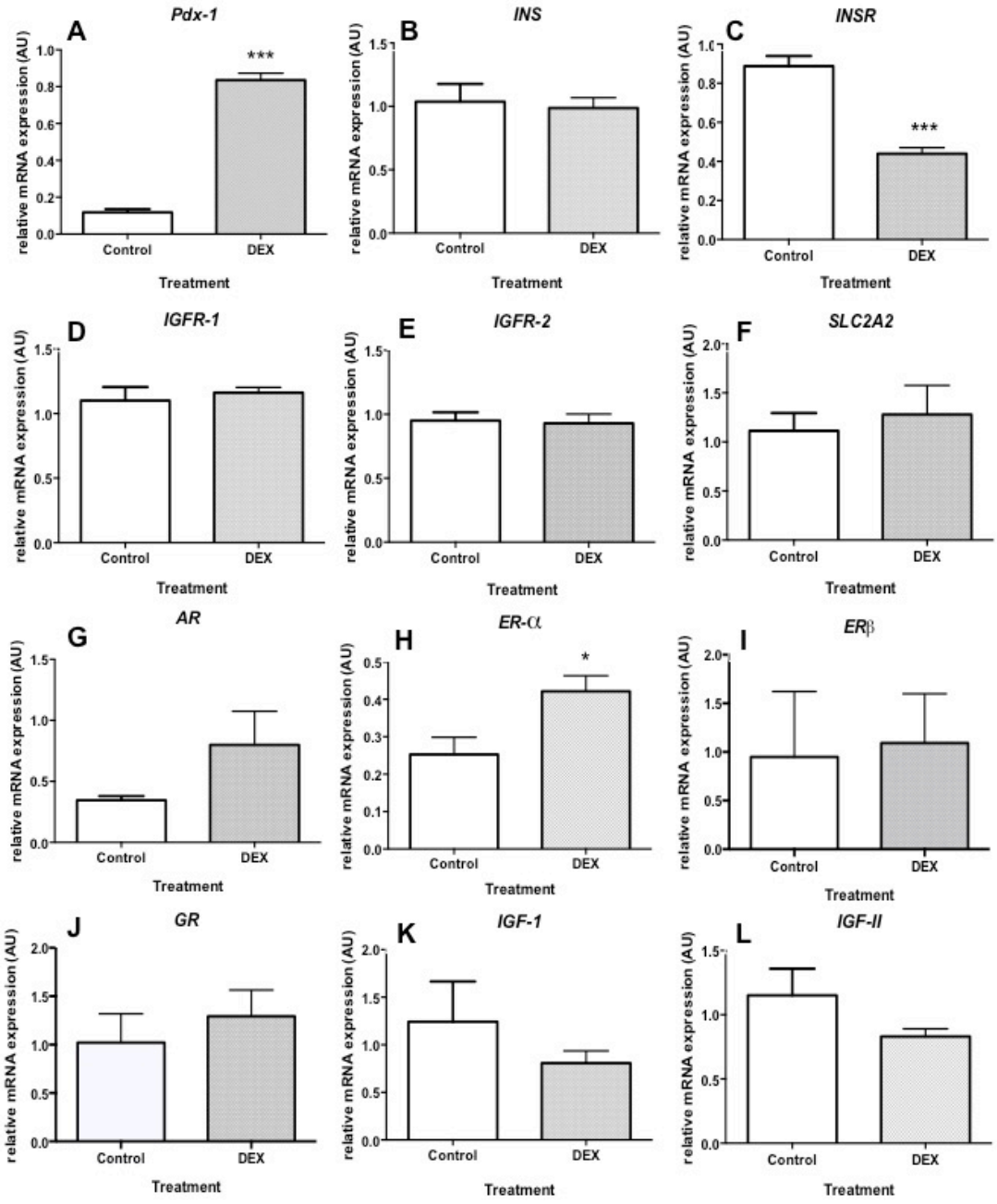


Figure 3.5 Effect of fetal DEX exposure on expression of *PDX-1*, *INS, INSR, IGFR-1, IGFR-II, SLC2A2, AR, ERα, ERβ, GR, IGF-I and IGF-II* (A-L) in female fetal pancreas at d90 gestation in comparison to control (Control n=7; DEX n=4)**P*<0.05*;* ****P*<0.001.

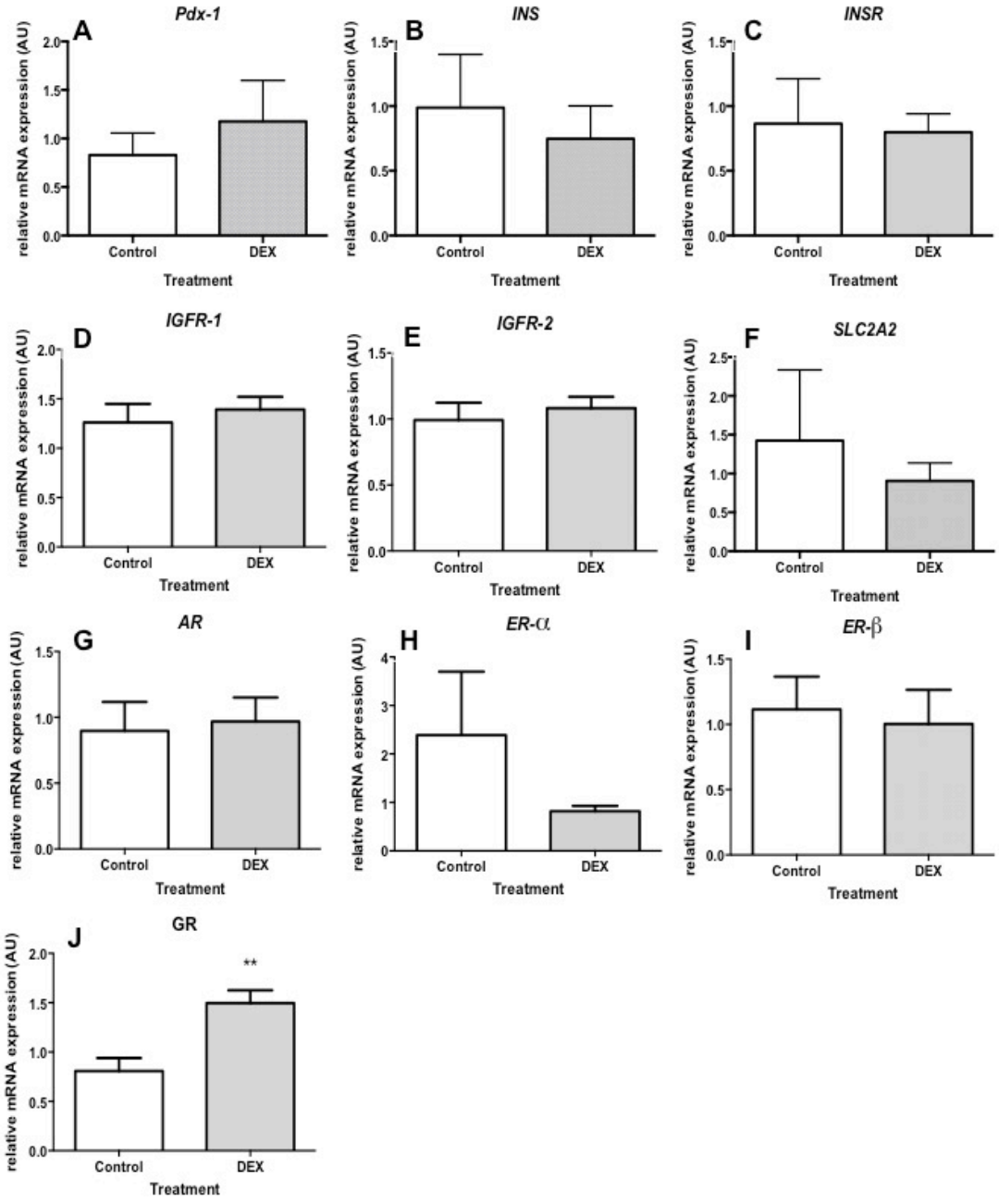


Figure 3.6 Effect of fetal DEX exposure on expression of *PDX-1*, *INS, INSR, IGFR-1, IGFR-II, SLC2A2, AR, ERα, ERβ* and *GR* (A-J) in male fetal pancreas at d90 gestation. (Control n=7; DEX n=4)***P*<0.01.

3.5 Effects of excesses of TP, DES and DEX upon pancreatic β-cell morphology in females and male fetuses.

In order to investigate the effect of mid-gestation steroid exposure on female and male ovine fetal pancreatic morphology, alpha and beta cell numbers were quantified for each treatment group.

3.5.1. α- Cell Morphology

Number of glucagon secreting α-cells were significantly increased in male control animals ($P<0.01$; Figure 3.7.1.A) compared to female controls. However, none of the prenatal steroids (TP, DES and DEX) had any effect on α-cell numbers in either fetal sex (Figure 3.7.1 B-E).

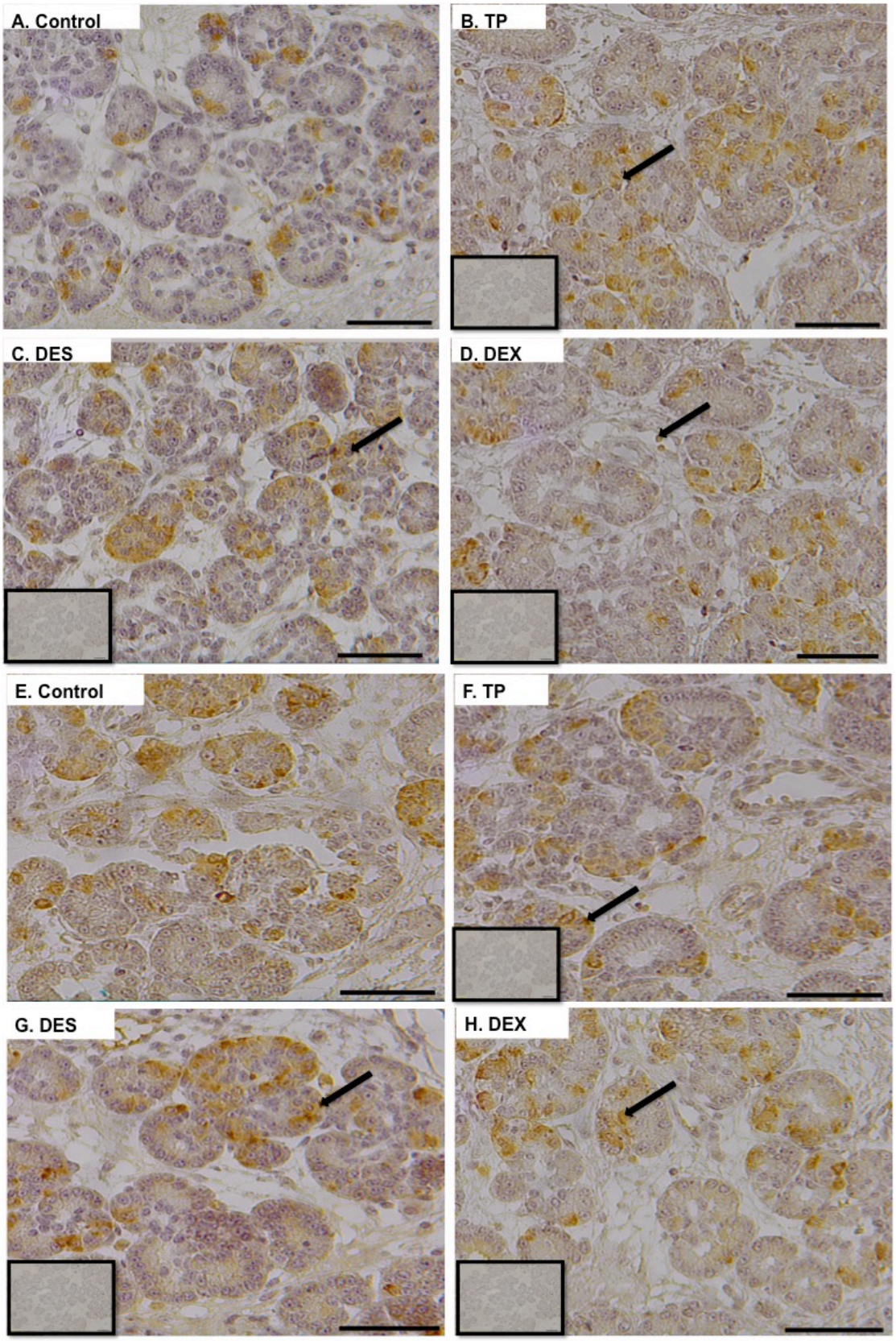

A. Control
B. TP
C. DES
D. DEX
E. Control
F. TP
G. DES
H. DEX

Figure 3.7 Effect of direct prenatal steroid treatment on glucagon secreting α-cell numbers in the female and male ovine fetal pancreas at gestational d90
A) Glucagon secreting α-cells in female control animals. (B-D) Glucagon secreting α-cells in female TP, DES and DEX animals respectively (Control n=5, TP n=5, DES n=5, DEX, n=4) (E-H). E) Glucagon secreting α-cells in male Control animals. (F-G) Glucagon secreting α-cells in male TP, DES and DEX animals (Control n=5, TP n=5, DES n=6, DEX, n=6). Arrows indicate glucagon-secreting α-cells. Inset boxes are negative control sections for comparison. Scale- 50μm

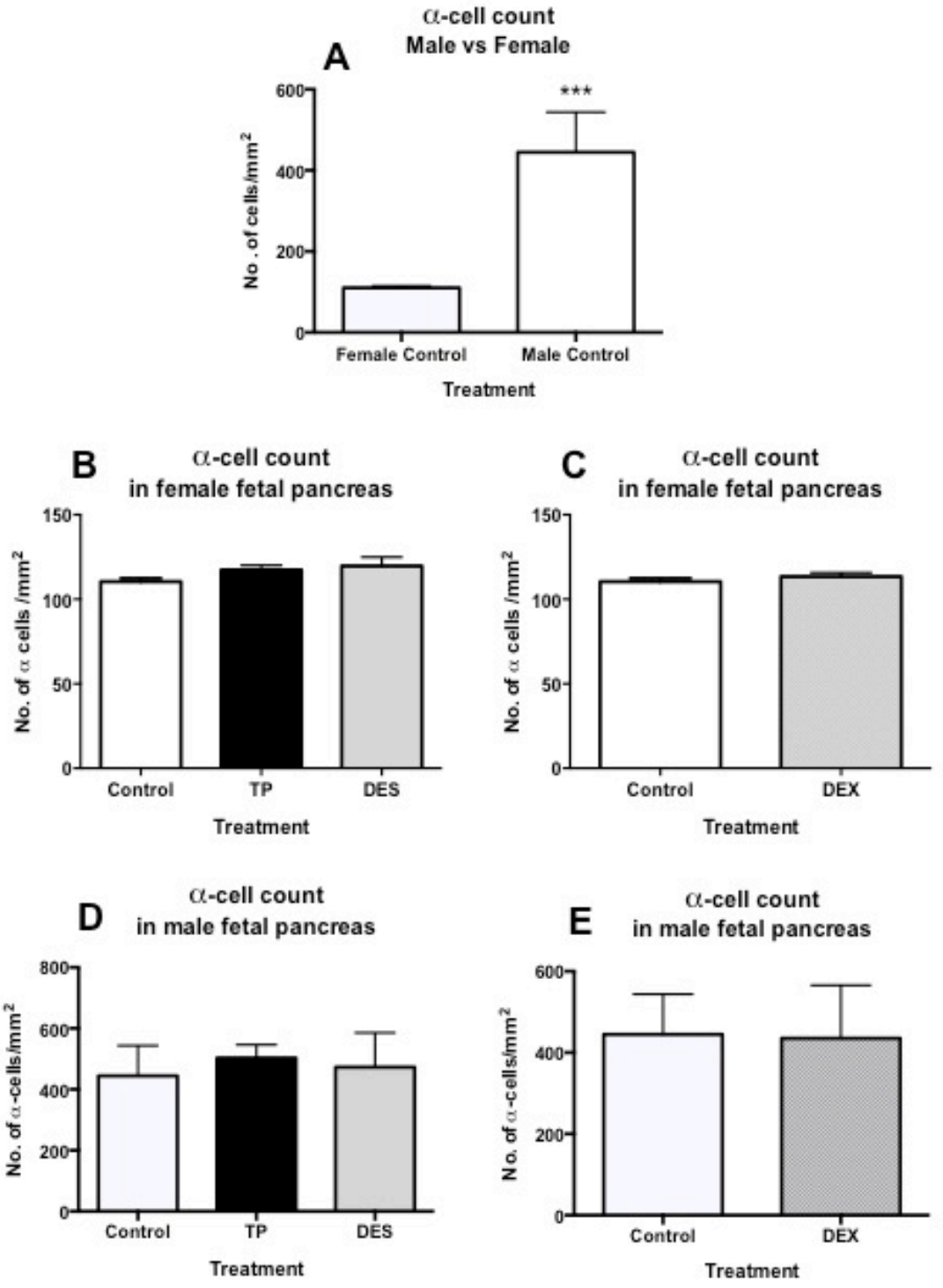


Figure 3.7.1 Effect of direct prenatal steroid injection on α-cell numbers in the male and female ovine fetal pancreas at gestational d90.

A) α-cell counts between male and female control B) Female- α-cell number after direct fetal injection of TP or DES compared to Control (Control n=5, TP n=5, DES n=5). C) female- α-cell number after direct fetal injection of DEX compared to Control (DEX n=4). D) Male- α-cell number after direct fetal

injection of TP and DES compared to Control in males. E) α-cell number after direct fetal injection of DEX compared to Control (DEX n=6) in males. Values represent mean ± SEM.

3.5.2 β-cell counts/islet morphology

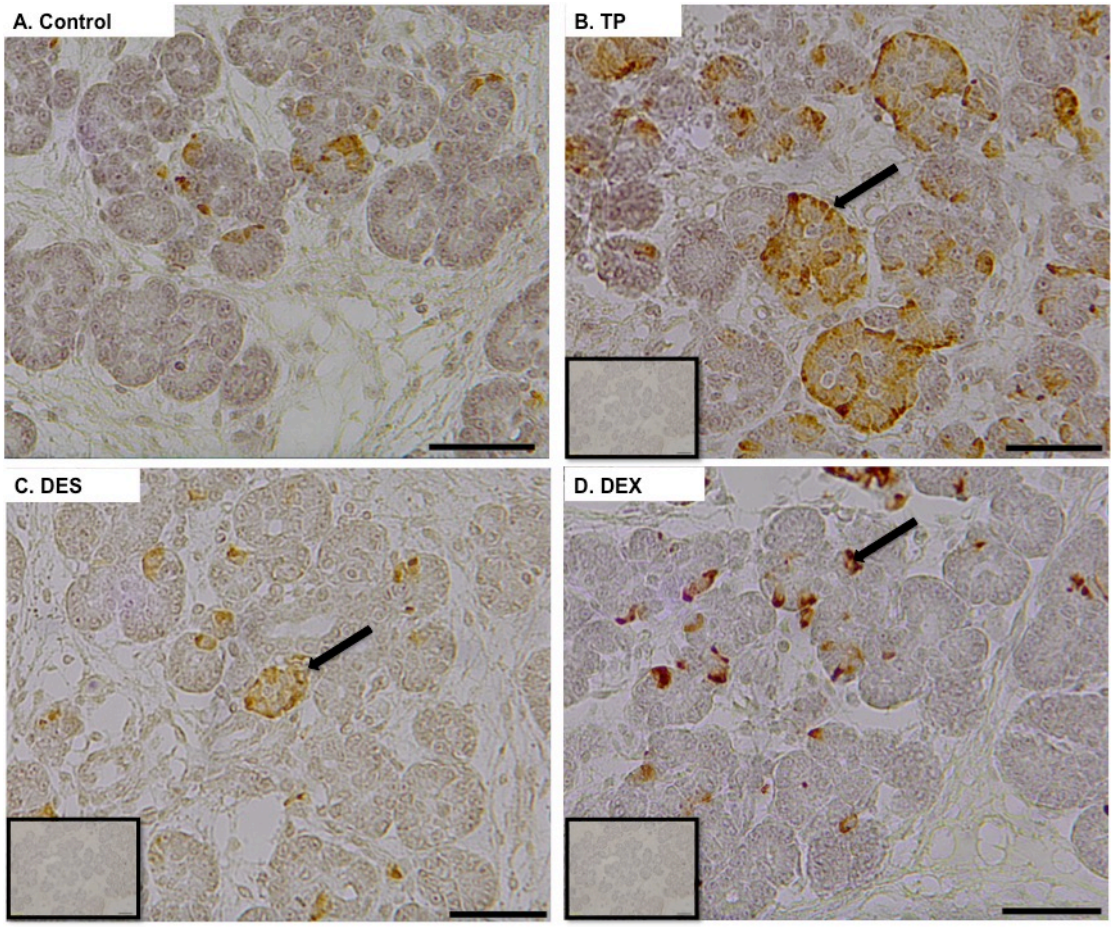


Figure 3.8. Effect of direct prenatal steroid treatment on β-cell numbers in the female ovine fetal pancreas at gestational d90

(A-D). A) Insulin secreting β-cells in Control animals B) Insulin secreting β-cells were significantly increased (*P*<0.01) by TP treatment but not with DES (C) or DEX (D) in female fetal pancreas (DES; Control n=5, TP n=5, DES n=5, DEX, n=4). Arrows indicate insulin-secreting β-cells. Inset boxes are negative control sections for comparison. Scale- 50μm

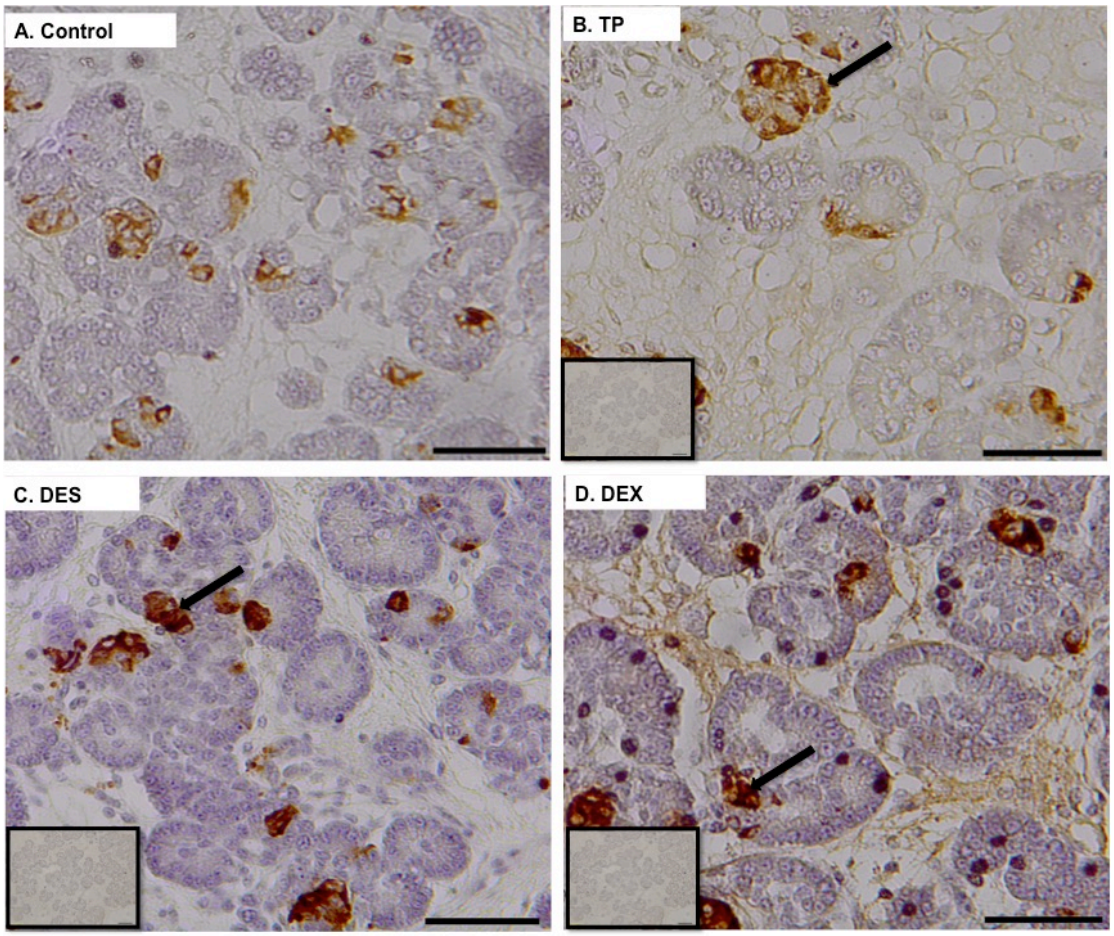


Figure 3.8.1 Effect of direct prenatal steroid treatment on insulin secreting β-cell numbers in the male ovine fetal pancreas at gestational d90.

A) Insulin secreting β-cells in Control animals. B-D) Insulin secreting β-cells in Control, TP, DES and DEX animals (Control n=5, TP n=5, DES n=6, DEX, n=6). Arrows indicate insulin-secreting β-cells. Inset boxes are negative control sections for comparison. . Scale- 50µm

Number of insulin secreting β-cells was significantly increased in male control animals compared to female controls (P<0.05; Figure 3.8.2.A). Interestingly, number of insulin-secreting β-cells were significantly increased in female fetuses (P<0.001) in response to TP treatment (Figure 3.8.B and Figure.3.8.2.B) but not with DES, which had no effects in either sex. DEX treatment also had no effect on β-cell numbers in either sex.

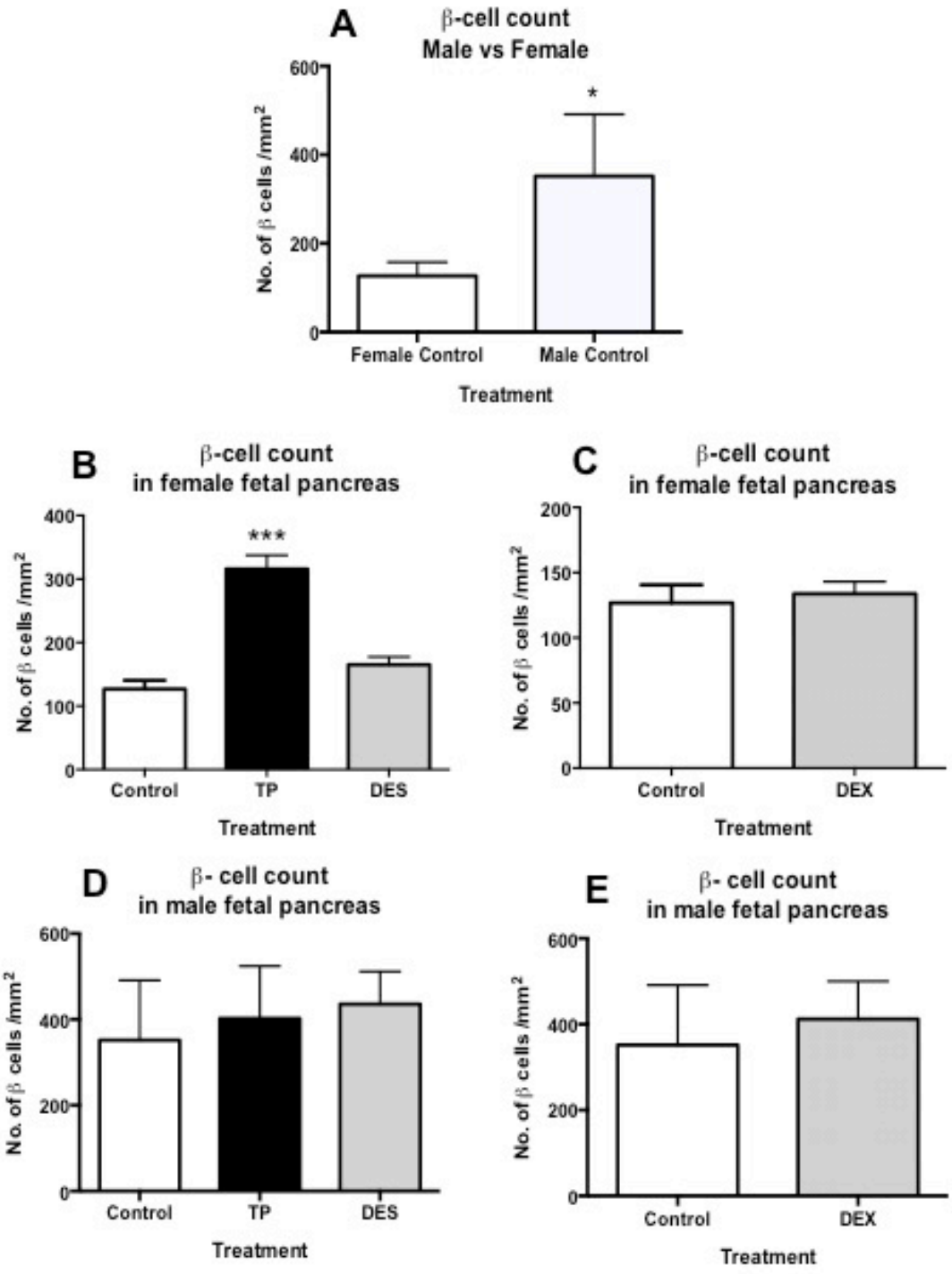


Figure 3.8.2 Effect of direct prenatal steroid injection on β-cell numbers in the female and male ovine fetal pancreas at gestational d90.

A) Insulin secreting β-cells in female and male fetal control pancreas (Control n=5 B) β-cell number exposed to direct fetal injection of TP and DES compared to Control in females (Control n=5, TP n=5, DES n=5). C) β-cell number exposed to direct fetal injection of DEX compared to (Control n=5;

DEX n=4) in females. D) β-cell number exposed to direct fetal injection of TP and DES compared to Control in males (Control n=5, TP n=5, DES n=6).E) β-cell number after direct fetal injection of DEX compared to Control (Control n= 5; DEX n=6) in males. One way ANOVA used for C, TP and DES and student unpaired t-test for C and DEX (non parametric). Values represent mean ± SEM. *P<0.05; *P<0.01

3.5.3 Functional analysis- direct prenatal androgen (TP) exposure altered *in vitro* insulin secretion in female fetal pancreas

In vitro insulin secretion was significantly increased (P<0.05; Figure 3.9.A) by prenatal TP treatment in female fetal pancreatic tissue culture in response to euglycaemic glucose concentrations (5.5mM) but not with DES or DEX (Figure 3.9.B) treatment.

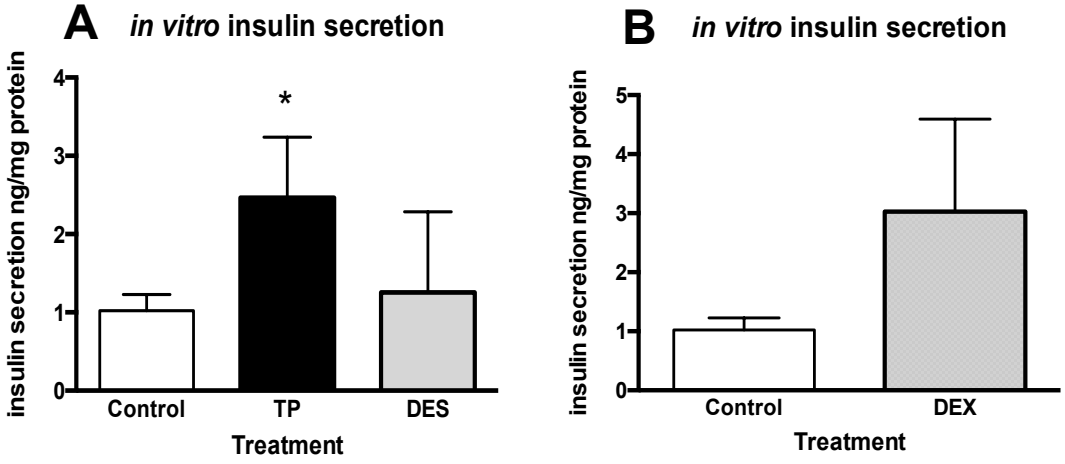


Figure.3.9 In vitro insulin secretion in response to euglycaemic culture.
A) Insulin secretion post glucose stimulation (5.5mM) *in vitro* by TP treatment and DES in female fetal pancreatic culture (Control n=5, TP n=7, DES n=7) B) insulin secretion after direct fetal injection of DEX in females (Control n=5, DEX n=4). Values represent ±SEM compared to control. *P<0.05.

3.6 Discussion

During fetal development, nutritional or hormonal challenge occurring during critical periods of organogenesis can lead to alteration of developmental processes (Godfrey and Barker, 2001). As the pancreas is a sex steroid responsive tissue (Morimoto *et al*., 2010), *in utero* steroid exposure can give rise to altered fetal pancreatic gene expression, structural changes (Rae *et al*., 2013) and functional changes during adulthood (Roland *et al*., 2010), which may underpin health issues later in adult life as seen in clinical conditions such as PCOS (Dumesic *et al*., 2007). It is evident from several animals models of polycystic ovary syndrome, such as rodents, sheep and monkeys that maternal steroid (androgen) exposure during pregnancy can lead to metabolic abnormalities such as altered glucose tolerance, β-cell morphology, insulin resistance and hyperinsulinemia during adulthood (Bruns *et al*., 2004; Hogg *et al*., 2011a; Rae *et al*., 2013; Roland *et al*., 2010).

The effects of testosterone on fetal development could be androgenic or via metabolism to estrogens in the maternally androgen exposed sheep model (Veiga-Lopez *et al*., 2011), where placental aromatase can metabolise androgens to estrogens including estrone and estradiol, begging the question as to whether the effects observed in the metabolic tissues are androgenic or estrogenic (Veiga-Lopez *et al*., 2011). Earlier, we observed that maternal androgen exposure in sheep from d62-102 gestation increased plasma estradiol concentrations in female fetuses at d90 gestation (Rae *et al*., 2013) and androgen exposure to pregnant ewe from d30-d90 lead to increased fetal serum estradiol and estrone levels (Almudena Veiga-Lopez et al., 2011), which may have programming effects in adulthood (Steckler *et al*., 2007; West *et al*., 2001). Therefore, the current study also looked at DES exposed groups from d90 gestation as a direct comparator group against the TP treated fetuses. Finally, DEX exposed fetuses were included as a surrogate of stress and altered HPA axis activity.

3.6.1 Effects of excesses of steroid treatment on female and male ovine fetal pancreas

Previous work from our laboratory indicated AR colocalisation with insulin, indicative of β-cell androgen receptivity (Rae *et al,* 2013). AR has also been observed in the rat pancreas, and has testosterone mediated insulin promoter activity (Morimoto *et al*., 2001). Key regulators involved in both β-cell development and function, such as *IGF1* and *IGF2* (Fujimoto and Polonski, 2009; Gatford *et al*., 2008) mRNA expression were unaffected by either FI-TP or FI-DES exposure in female sheep fetuses in this study, however we observed a strong trend (*P*=0.06) towards increased *IGFR1* expression in females due to FI-TP but not FI-DES exposure. Maternal androgen administration *in utero* in sheep resulted in elevated expression of *IGFR-1* in female fetal pancreas (Rae *et al*., 2013), and the trend towards increased expression seen here, given its female specificity, would appear likely to ascribe this modulation of expression to an androgenic effect (Rae *et al*., 2013). Although not β-cell specific, IGF's are of key importance in pancreatic islet cell development and function (van Haeften and Twickler, 2004). IGFs initiate key signalling pathways relevant to cell differentiation and maturation (Rhodes and White, 2002; Withers and White, 2002 and Burks and White, 2001), which have clear implications for fetal life (Holt, 2002).

Here we demonstrate that insulin receptor (*INSR*) mRNA abundance was significantly increased by TP treatment and not by DES in the female fetal pancreas. This is consistent with our previous work, where *in utero* maternal androgen (TP) exposure (d62-102) to pregnant sheep led to a significant increase in *INSR* mRNA abundance in d90 female fetal pancreas (Rae *et al,* 2013). Thus our previously reported effect of TP on *INSR* mRNA was an androgenic effect, since DES could not replicate this. Whilst there was no difference in terms of basal *INSR* expression between male and female control fetuses, the lack of response to TP in males could be due to pre-exposure to endogenous androgens secreted by the fetal testes from d30 onwards. Insulin receptor facilitates insulin gene transcription downstream

from the insulin-signalling pathway (Saltiel and Kahn, 2001). Illustrating the importance of insulin signalling in β-cells, specific knockout of β-cell *INSR* (βIRKO) during postnatal life (4 months age) led to selective loss of insulin secretion in response to intraperitoneal glucose load in mice (Kulkarni *et al*., 1999). Another study from the same group demonstrated decreased glucokinase and *Slc2a2* gene expression along with beta cell mass in βIRKO mice female offspring (Otani *et al*., 2004), which clearly suggests that any changes in *INSR* mRNA may have a significant bearing upon insulin secretion.

In addition to the latter elevated *INSR* expression, *INS,* a β-cell specific gene was also significantly increased due to TP and not DES treatment in females in terms of mRNA expression. Our previous work also demonstrated elevated *INS* gene mRNA abundance in female fetal pancreas whose mothers were exposed to androgens during pregnancy (Rae *et al*., 2013), which clearly suggests that the effect observed in previous study was androgenic with no estrogenic contribution. Okada *et al*., (2007), have also shown that insulin acting via insulin receptor is important for β-cell proliferation, suggesting that any changes in the level of insulin receptor may lead to alteration in β-cell function. This is supported by the studies of Morimoto *et al*., (2001), where testosterone increased *INS* mRNA abundance in male adult Wistar rats, however in the current study we saw no changes in the *INS* mRNA in male fetuses exposed to TP nor DES, which is again consistent with our previous work, where *INS* gene expression was unaltered in male fetal pancreas due to TP exposure (Rae *et al*., 2013), suggesting sex-specific differences in such androgen responses (Palomar-Morales *et al*., 2010). Maternal androgenic exposure route to some extent at least is compensated for by reduced endogenous testicular androgen synthesis (Connolly *et al*., 2013). However, direct fetal injections employed here are less likely to be explained by this due to the larger doses delivered to the fetus suggesting that there may be sex-specificity in terms of effects that are additional to pre-exposure. Interestingly, *INSR* expression was significantly decreased by DEX treatment in females and the lack of this effect in males also points towards sex specificity of the

developing pancreas in terms of responses to steroid excess. To our knowledge this is first study to show such decreased expression of *INSR* mRNA in response to glucocorticoid excess in the female developing pancreas. Previously, in a prenatal protein restriction sheep model, increased pancreatic *INSR* expression in female lamb offspring was noted, indicative perhaps of a permanent programmed change in pancreatic function, but there was no change in β-cell mass observed (Gatford *et al*., 2008).

In this study*, PDX-1,* also a β-cell specific gene, was significantly increased in terms of mRNA expression due to prenatal TP but not DES, once again in females only. IGF-1 signalling regulates β-cell mass upstream of PDX-1 (Babu *et al*., 2007) In our previous study, maternal TP exposure led to increased *PDX-1* mRNA expression in female fetal pancreas (Rae *et al*., 2013). Thus these data indicate that the mode of action of TP on *PDX-1* expression in our previous study was androgenic and not via metabolism to estrogens. Interestingly, basal *PDX-1* mRNA abundance was also significantly greater in males compared to females; consistent with the idea that androgens regulate the expression of this gene. Increased *Pdx-1* expression in male fetuses could be due to the fact that *PDX-1* expression and localization is seen at 33dGA in sheep fetal pancreatic β-cells (Cole *et al*., 2009) coincidental with sexual differentiation at this gestational time point and thus endogenous androgen production. This then raises the hypothesis that prenatal androgenisation may to some extent give rise to a phenotypically male β-cell located in a female pancreas. As an aside, if as these data suggest, pancreatic sensitivity to androgenic steroids may be differentially set in male and females during the time of sexual differentiation, with functional consequences, then this may have implications in terms of origins of cells for transplantation. PDX-1 is involved in initiating differentiation and morphogenesis of the mouse pancreatic epithelial progenitor cells, later restricted to mature β-cells in adult life (Offield et al., 1996), hence increased *PDX-1* expression noted here has the potential to drive increased β-cell mass and in tandem with increased *INS* expression, also insulin secretion. PDX-1 is also involved in transactivation of β-cell specific genes (Sander et al., 1997)

such as *INS* in rats (Gremlich *et al*., 1997), hence there is likely a functional relationship between elevated expression of *PDX-1*, *INS* (acting via *INSR* or *IGFR-1*) and *in vitro* insulin secretion in our FI-TP injected female fetal pancreas at d90 gestation, underscoring functional protein translation changes with potential consequences for long-term consequences due to steroidal alteration during fetal life (Rae *et al*., 2013). Summarising the changes in gene expression discussed above, if anything these would predict the possiblilty of increased insulin secretion and increased β-cell numbers in TP- exposed females.

Examination of pancreatic tissues from d90 gestation revealed insulin secreting β-cell numbers were indeed increased in female fetal pancreas due to FI-TP exposure and not FI-DES nor FI-DEX suggesting that androgen overexposure stimulated β-cell mass regulation as predicted by the gene expression alterations discussed above. Interestingly, both basal α and β-cell numbers were significantly higher in males compared to females, once again suggesting that the FI-TP females may to some extent have a masculinised β-cell phenotype in an otherwise 'female pancreas'. However, there was no alteration in terms of α-cell numbers by any of the treatment groups applied in this study. This is in agreement with maternal nutrition manipulation: pancreatic developmental studies, where the sheep fetuses have demonstrated plasticity with regards to β-cell but not α-cell development (Ford *et al*., 2009).

In terms of pancreatic function, as predicted by mRNA analyses and β-cell counts, TP treatment was associated with increased insulin secretion from isolated pancreatic tissue in response to a fixed, euglycaemic dose (5.5mM) of glucose *in vitro* in female fetuses. This is in accordance with our previous work in sheep, where *in vitro* insulin secretion was significantly increased in the prenatally androgenised female fetal pancreatic cultures. In the current study we did not measure fetal plasma concentrations because fetal insulin secretion is responsive to maternal glucose fluctuations. Instead we opted to examine potential secretory responses *in vitro*, and this demonstrated

increased insulin secretion in female fetuses exposed to TP during development, but not those exposed to either DES or DEX, indicative of an androgenic mode of action congruent with histological and molecular analyses. Similarly, rat female offspring, derived from pregnancies where maternal exposure to dihydrotestosterone (DHT) occurred, exhibited excess insulin secretion in cultured islets and impaired glucose sensing compared to control animals (Roland *et al*., 2010) indicating that the effects reported here may have consequences postnatally. In summary, excess fetal androgenic signalling in female fetuses was associated with increased β-cell numbers and gene expression alterations, which likely underpin increased β-cell numbers and functionally, increased insulin secretion.

Given the presence of AR in insulin secreting β-cells (Rae *et al*., 2013) and upregulated *AR* expression in this study by TP treatment and not DES or DEX treatment in female pancreas, it is possible that these TP-treated pancreata may be more responsive to androgens. Interestingly, *AR* gene expression in control males as compared to females showed a trend towards being higher (*P*=0.054) and, in combination with basal *Pdx-1* mRNA expression being higher in male controls than females, suggests androgens may enhance the receptive capacity of the developing pancreas to endogenous androgens. Again this points to the possibility of there being distinct male or female pancreatic phenotypes derived from fetal life steroid exposures.

Both TP and DEX treatments caused increased *PDX-1* expression in female fetuses. However, unlike the scenario discussed above with FI-TP, there was no change in expression of *INSR*, *INS*, *IGFR-1,* β-cell numbers or *in vitro* insulin secretion, associated with DEX stimulation. This is difficult to explain from current data sets, however it may suggest that since IGF signalling is so important for islet and β-cell development (Fujimoto and Polonski, 2009 and Gatford *et al*., 2008) and as there is a trend towards increase in *IGFR-1* gene in the current study by TP (which was also evident in maternally exposed androgenized pregnancies- Rae *et al*., 2013) and not by DES or DEX, then it is possible that androgens mediate their effects via both IGF and insulin

signalling pathways, driving up downstream insulin gene transcription and insulin secretion.

In addition to changes in pancreatic *AR* expression, estrogen receptor α (*ER-*α) mRNA expression was also significantly increased following DEX treatment in females and increased *GR* in males, effects not replicated by TP or DES. Again, this demonstrates intrinsic differences between male and female pancreatic tissue, which we again suggest may at least in part be due to endogenous exposure to androgens from d30 onwards in males via testicular development. ERα is expressed in insulin secreting β-cells (Alonso-Magdalena *et al.*, 2008) and treatment with ERα agonist in Swiss albino male mice caused a significant increase in insulin secretion *in vitro,* indicating that estrogenic action on insulin biosynthesis is mediated by ERα (Alonso-Magdalena *et al*., 2008). Steroids bind to their specific receptors and this steroid-receptor complex binds to a hormone response element (HRE) of DNA further activating gene transcription (Hut *et al*., 1997). ER and GR interactions are highly specific, because of their differences in DNA- binding regions and also as the cognate response elements remain sequence specific (Mader *et al*., 1989 and Klock *et al*., 1987). ER and GR can alter transcription by binding to activator protein-1 (AP-1) (Gaub *et al*., 1990; Webb *et al*., 1995) and, as pancreas is also known to express high levels of AP-1 (at least in the case of pancreatic cancer cells) (Shin *et al*., 2009), in the current study, there is a possibility that exogenous DEX treatment augments interaction of ERα mRNA at the AP-1 response element site further leading to an increase in ERα. Our data is supported by Hut *et al* (1997) studies, which demonstrated that transcriptional properties of ER are integrated with GR via the AP-1 response element. However, we did not see any morphological or functional change in response to DEX treatment in female or male fetal pancreas. This study has for the first time, indicated elevated *GR* expression in male fetal pancreas due to prenatal DEX exposure, suggesting potential pancreatic sensitivity to glucocorticoids such as cortisol, and it is important to note that enhanced cortisol secretion can lead to glucose intolerance and insulin resistance in males (Walker *et al*., 1998). Maternal glucocorticoid

(dexamethasone) exposure in pregnant sheep resulted in excess insulin secretion in adult male offspring suggesting the long term effects of glucocorticoid excess during early pregnancy might lead to altered pancreatic function (De Blasio *et al*., 2007).

Collectively, the data gathered in the current study suggests that the increased mRNA expression of *INSR, INS*, *PDX-1, IGF-1* and *AR,* β-cell numbers and *in vitro* insulin secretion in female pancreas during fetal life in response to androgenic excess is a direct androgenic effect with no estrogenic contribution. Given previous evidence of permanent alterations in PDX-1 originating in fetal life and remaining evident postnatally (Gatford *et al*., 2008) the potential consequences for adult health were investigated and are reported in Chapter 4. At this stage it is also intriguing to consider that previously implied altered β-cell function in animal models of PCOS, and indeed human clinical PCOS (Abbott *et al* 2005; Goodarzi *et al*., 2005) may have an early life origin and thus such models/ conditions may have a primary pancreatic phenotype masked by altered pancreatic function in response to downstream development of insulin resistance.

Chapter 4 The effects of prenatal steroidal excess on ovine adult pancreatic function

4.0 Introduction

In Chapter 3 the effects of direct fetal steroid exposure to Testosterone propionate (TP), Diethylstilbestrol (DES) and Dexamethasone (DEX) on pancreatic development and the potential legacy of this in terms of adult health and disease was examined. As a result of the fetal androgen (TP) exposure, genes involved in fetal pancreatic development (*IGRF1* and *AR*) and function (*INS*, *INSR* and *PDX-1*) were altered in terms of expression, which was further associated with increased numbers of insulin secreting β-cell numbers. Functionally, this was in turn associated with increased insulin secretion *in vitro* due to TP treatment in the female fetuses at d90 of gestation. Thus the conclusions drawn were that there were sex-specific and potentially direct effects of androgens (TP) on the developing fetal pancreatic beta cells. Whilst such studies invariably lead to speculation as to what the postnatal legacy of such altered *in utero* environments could be, in order to definitively address such speculation a follow up study on adult pancreas was necessary to determine any long-term legacy of fetal steroid exposure.

We therefore hypothesize, based on fetal data presented in Chapter 3, that fetal steroid exposure permanently programs the female adult sheep pancreas in terms of insulin secretion leading to hyperinsulinemia.

4 .1 Materials and methods

4.1.1 Animal Husbandry

The tissues and blood samples used in this study were collected from lamb (2 months) and adult (11 months old) Scottish Greyface ewes derived from directly steroid manipulated fetuses as explained in materials methods chapter section.

Study	Treatment	Time of analysis/ sacrifice	Sample Number (n)
Lamb (females only)			
Fetal Treatment	Injection at d62 and 82 gestation (20mg TP)	2 months old postnatal	Control=4, TP=13,
Adult (females only)			
Fetal Treatment	Injection at d62 and 82 gestation (20mg TP; 50μg DES; 100μg DEX)	11 months old postnatal	Control=6, TP 20mg=13; Control=4;DES= 7;DEX=11

Table 4.1 The experimental cohorts for the study of female adult pancreatic structure and function, treatment regime and corresponding sample numbers.

Differing numbers of animals in each study group, and limited steroids studied at lamb stage are reflective of focused effort where main effects were noted balanced with available resources.

4.1.2 *In vivo* function tests and tissue collection

In this study only female animals were studied. This was due to a combination of two factors. Firstly in none of the work reported here did we have male offspring beyond lamb stages of life due to the practical constraints of housing large groups of post-pubertal rams. Secondly, and specific to this aim of the part of the project, since pancreatic effects of steroidal excesses during fetal life were only noted in terms of female fetuses, it was decided that resources would be employed in this direction only, thus permitting all steroid classes to be examined in a single sex offspring cohort.

Glucose tolerance tests (GTT) were carried out in both female lamb (2 months) female adult (11 months) animals, which had been overnight fasted. Bolus glucose administration (I.V) (500mg/ml, 20ml volume, therefore 10g glucose) occurred immediately after collecting the basal/zero time blood sample via jugular venipuncture. In lambs, blood was collected 15 and 30 min post glucose administration.

In adult animals (same animals as lamb cohort), blood was again collected at 0 min and also at 15 and 30 minutes post glucose administration and simultaneously, muscle biopsies were collected and snap frozen at -80°C from 15 time points post-glucose administration for both RNA and protein study. Blood samples were collected into sodium fluoride combined anticoagulant (Sarstedt Ltd, Numbrecht, Germany) S-monocuvettes for glucose measurement and heparinized glass tubes for insulin determination. Blood samples were placed upon ice and then centrifuged at 3000rpm for 15min at 4°C, then the plasma fraction was carefully aspirated and stored at -20°C until further analysis.

Two weeks later, the adult sheep were sacrificed and liver and pancreas tissue collected was snap frozen and stored at -80^{0}C. 15 minutes prior to sacrifice, in order to make hepatic samples comparable with previously collected muscle biopsies in terms of contemporary glucose exposure, a 10g glucose bolus was given intravenously as described above. Thus both muscle and hepatic biopsies were controlled in terms of glucose exposure at the time of collection. A portion of pancreatic tissue was stored in Bouins fixative solution for 24 hours, transferred to different grades of alcohol (see section 2.4.1-2.4.2) for histological processing and embedded in paraffin wax.

4.1.3 Glucose measurement

A colorimetric glucose assay kit (Alpha laboratories Ltd, Eastleigh, UK) was used to measure glucose concentrations using a Cobas fara centrifugal analyzer (Roche Diagnostics LtD), which was operated by Dr Forbes Howie, The University of Edinburgh. Glucose gets oxidized into gluconic acid and hydrogen peroxide via glucose oxidase, which, in the presence of peroxidase enzyme reacts with 4-animoantupyrine and hydroxynenzioic acid, forming a red compound at the end of the reaction. Colour intensity was monitored at 500nm, which is proportional to the concentration of glucose in the sample. Assay sensitivity was 0.2mmol/L and intra and inter assay CVs were <2% and <3%, respectively.

4.1.4 Enzyme linked immunosorbent assay (ELISA)- Insulin measurement

Insulin ELISA (‘sandwich’ enzyme immunoassay) was carried out using a commercially available ELISA kit (ALPCO Diagnostics, 80-INSOV-E01, Salem, NH, USA) for the determination of sheep plasma insulin, according to manufacturers instructions without any modifications. Optical density (OD) was measured on a spectrophotometer (Molecular Devices, CA, USA) at 450nm wavelength. A standard curve was plotted using Softmax Pro software (Molecular Devices) using a cubic spine curve fit as recommended by ELISA

manufacturers protocols, and insulin concentrations of the samples were determined. The assay sensitivity was 0.14ng/ml and inter and intra assay CVs for insulin ELISA were (<6%) and (<5%) respectively.

4.1.5 Pancreatic Immunohistochemistry

Adult pancreas was fixed in Bouins solution for 24 hours prior to processing and blocking in paraffin wax. Sections (5μm) were cut and mounted onto charged microscope slides as explained in section 3.2.1. Pancreatic sections underwent immunohistochemical staining as described in section 2.4.4. Antibodies and optimized working concentrations are detailed in Table 4.

Receptor Type	Antibody	Dilution	Manufacturer	Secondary antibody
Insulin	Mouse monoclonal anti-insulin antibody	1:1000	AbCam®	Goat anti-mouse universal biotinylated secondary antibody (Vector laboratory)

Table 4.2. Insulin antibody analyzed by Immunohistochemsitry in fetal pancreas and the concentrations of primary antibody and secondary antibodies applied were universal goat-anti mouse biotinylated (GAMB).

4.1.6 Cell counting

Cells in the sections that were positive for insulin were counted under the 10X objective of a light microscope (Leitz Wetzlar, Germany). 5 random fields were chosen from each section and the insulin stained cells were counted using a 10 by 10 grid. A mean of the counts from the 5 fields was calculated and divided by 1.16 (correction factor) to obtain the number of cells per mm^2 per section. In a similar fashion the mean value for all three sections per slide was taken to get a value for every animal subjected to analysis.

4.1.7 RNA extraction

As muscle and liver are fibrous and lipid-rich tissues, extracting RNA by column based methods results in low RNA yield due to column blockage issues. Therefore in this study, a TRI-reagent, phenol-based method to extract RNA from muscle and liver tissues was utilised. The TRI-reagent method is a combination of lysis and purification of tissue samples with guanidine-thiocyanate and that of phenol and chloroform to achieve RNA isolation. Approximately ~50mg of muscle/liver tissue was added into a 2ml RNase free tubes (autoclaved) containing magnetic beads and 1ml of Trisure (Trisure™, BioLine, USA, Cat No. BIO-38033) reagent and homogenized on the tissue lyser (Qiagen Tissue Lyser) at 50Hz for 4 minutes. Homogenates were then transferred into clean, autoclaved 1.5ml tubes and incubated at room temperature for 5 minutes. Following the incubation, 200µl of chloroform was added and mixed vigorously for 15 seconds by shaking and incubated for 5 minutes at room temperature, which was followed by centrifugation at 12000g (114000rpm, 4^0C) for 20 minutes. After centrifugation, the upper aqueous phase was carefully collected without disturbing the lower organic phase into a clean 1.5ml tube, then 500µl of isopropanol was added and incubated at room temperature for 10 minutes. Samples were then centrifuged at 12000g (4^0C) for 15 minutes, and supernatant aspirated and discarded leaving behind the pellet at the bottom of the tube. 1ml of 75% ethanol was added, vortexed and then centrifuged at 7500g and 4^0C for 10 minutes to wash the nucleic acid pellet. After pipetting and air-drying the ethanol for ~5-10 minutes, the pellet was dissolved in 20µl of RNase- free water (Sigma Aldrich). Finally, RNA concentration was measured using a spectrophotometer (NanoDrop 1000, Fisher Scientific UK, Ltd, Leicestershire, UK) before being stored at -80^0C. Selected samples were also scanned using an Agilent Bioanalyser 2100 to ensure that isolates were to sufficient quality (RIN>7.5) DNase treatment, cDNA synthesis and qRT-PCR were performed in adult muscle and liver (see section 2.3.3 and 2.3.5) The primer sequences used for PCR are listed in Table 4.2.

Gene	Primers 5'-3'	Primer sequence	Species of genome used to design primer	Manufacturer
GLUT 1	Forward	TGCTGAGCGTCATCTTCATC	Bovine	Eurofins, UK
GLUT 1	Reverse	GGCTCTCCTCCTTCATCTCC	Bovine	Eurofins, UK
GLUT 2	Forward	CGAAATTGGGACCATCTCACAT	Ovine	Primer Design, UK
GLUT 2	Reverse	CACCGATAGCACCCCTGAGT	Ovine	Primer Design, UK
GLUT 4	Forward	ACCTTATGGCCACTCCTCCT	Bovine	Eurofins, UK
GLUT 4	Reverse	CTCAGCCAACACCTCAGACA	Bovine	Eurofins, UK
IRS 1	Forward	ATCATCAACCCCATCAGACG	Bovine	Eurofins, UK
IRS 1	Reverse	GAGTTTGCCACTACCGCTCT	Bovine	Eurofins, UK
INSR	Forward	GCTTCGAGGCTGCACCAT	Ovine	Primer Design, UK
INSR	Reverse	AGCTCAGCTGCCAGGTTGTT	Ovine	Primer Design, UK
GAPDH	Forward	GGCGTGAACCACGAGAAGTATAA	Ovine	Primer Design, UK
GAPDH	Reverse	AAGCAGGGATGATGTTCTGG	Ovine	Primer Design, UK

Table 4.3 List of primer sequences used in qRT-PCR for muscle and liver.

4.2 Western blotting

Protein extraction, purification, gel preparation and western blotting were performed as explained in section 2.5.1-2.5.4. The antibodies used for western blotting are listed in Table 4.4.

Antibody	Antibody information	Dilution	Amount of protein loaded	Manufacturer
AKT	Rabbit polyclonal antibody	1:2000 in 5%BSA/ TBST	10µg	No.9272, Cell Signaling Technology.Inc, Danvers, MA 01923 USA
Phospho- AKT	Rabbit polyclonal antibody	1:500 in 5%BSA/ TBST	40µg	No.9271, Cell Signaling Technology.Inc, Danvers, MA 01923 USA
ERK/ P44/42 MAPK	Rabbit polyclonal antibody	1:2000 in 5%BSA/ TBST	10µg	No.9102, Cell Signaling Technology.Inc, Danvers, MA 01923 USA
Phospho- ERK/ P44/42 MAPK	Rabbit monoclonal antibody	1:500 in 5%BSA/ TBST	40µg	No.4377, Cell Signaling Technology.Inc, Danvers, MA 01923 USA
Anti- β-actin	Mouse monoclonal antibody	1:4000 in 5%BSA/ TBST	-	Ab6276,AbCam , UK
Secondary antibody for AKT/ERK/ pAKT/pERK	Goat Anti-rabbit fluorescence antibody	1:20000 in TBST/ 0.02%SDS	-	No.926-68021 IRDye® 680LT Goat anti-Rabbit IgG (H + L), Licor Biosciences Ltd,UK
Secondary antibody for β-actin	Goat Anti-mouse fluorescent antibody	1:20000 in TBST/ 0.02%SDS	-	No.926-32210 IRDye® 800CW Goat anti-Mouse IgG (H + L), , Licor Biosciences Ltd,UK

Table 4.4 List of primary and secondary antibodies used for western blotting.

4.3 Statistical analysis

Graph Pad prism v.6.0 (San Diego, USA) was used to perform all statistical analysis. Difference between Control vs TP cohort (2010 cohort), Control vs DES (2012 cohort) and Control vs DEX (2012 cohort) was measured using Student's unpaired two-way t-test. One-way ANOVA was used to analyze the difference between different time points (0,15,30) with the analyzed blood samples. The same software was used to calculate area under the curve (AUC) (a built-in algorithm utilising trapezoidal rule) for insulin and glucose secretion post glucose administration.

4.4 Results

4.4.1 Direct prenatal steroidal exposure altered β-cell numbers in female adult pancreas at 11 months age postnatal

In order to investigate the effect of mid-gestation steroid exposure on female adult pancreatic morphology, insulin secreting beta cell numbers (per mm^2) were quantified for each treatment group after performing the immunohistochemistry.

Insulin secreting β-cells/mm^2 were significantly increased (P<0.01) in female adult pancreas by TP treatment (Figure.4.1.A and Figure 4.1.1 A-B) but not with DES (Figure.4.1.B an Figure 4.1.1.C-D) compared to control animals. However, prenatal DEX treatment (Figure.4.1.C and Figure 4.1.1.E) significantly decreased (P<0.05) β-cell numbers in female adult pancreas compared to control animals.

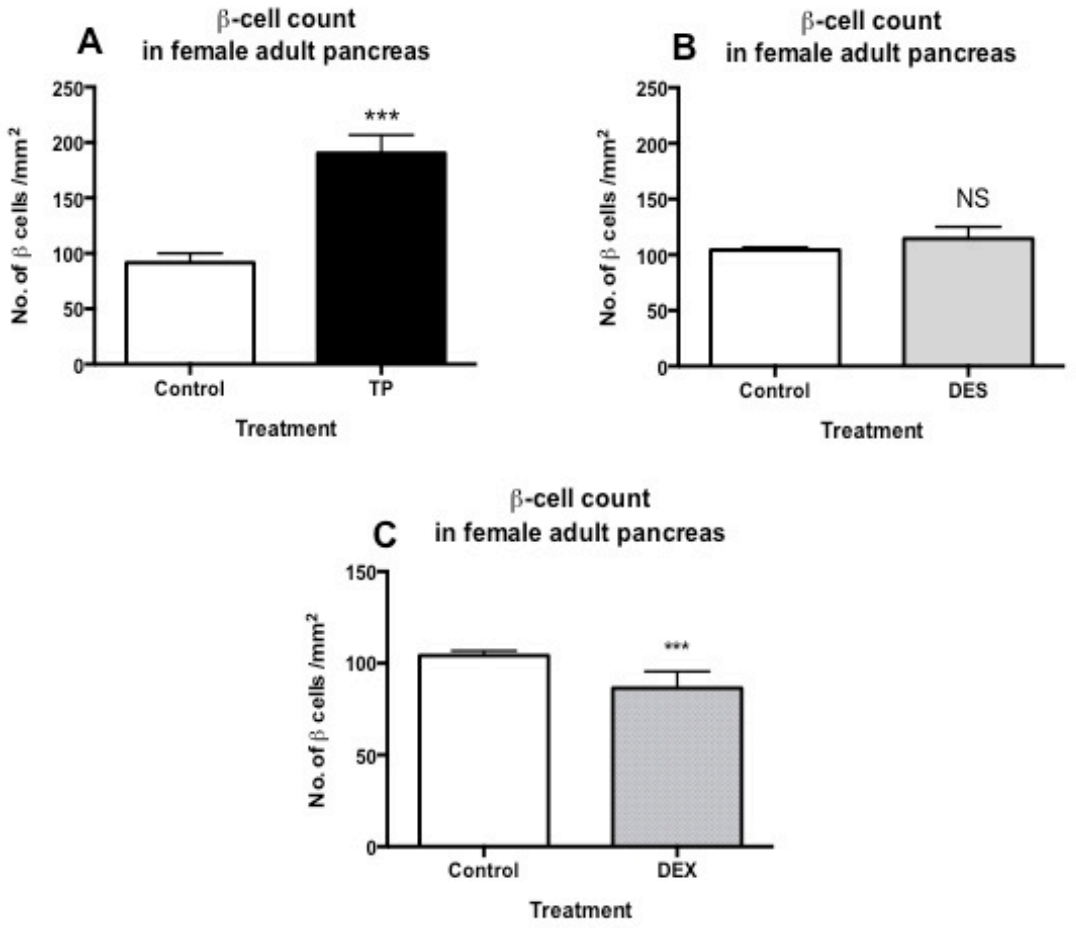


Figure 4.1 β-cell content in the female adult offspring from FI-TP and control treatment pregnancies were assessed by immunohistochemistry.

A) Insulin secreting β-cells exposed to TP treatment B) Insulin secreting β-cells exposed to DES treatment C) Insulin secreting β-cells exposed to DEX treatment (Control n=6, TP n=13; Control n=4, DES n=6, DEX, n=9). *** *P<0.001*

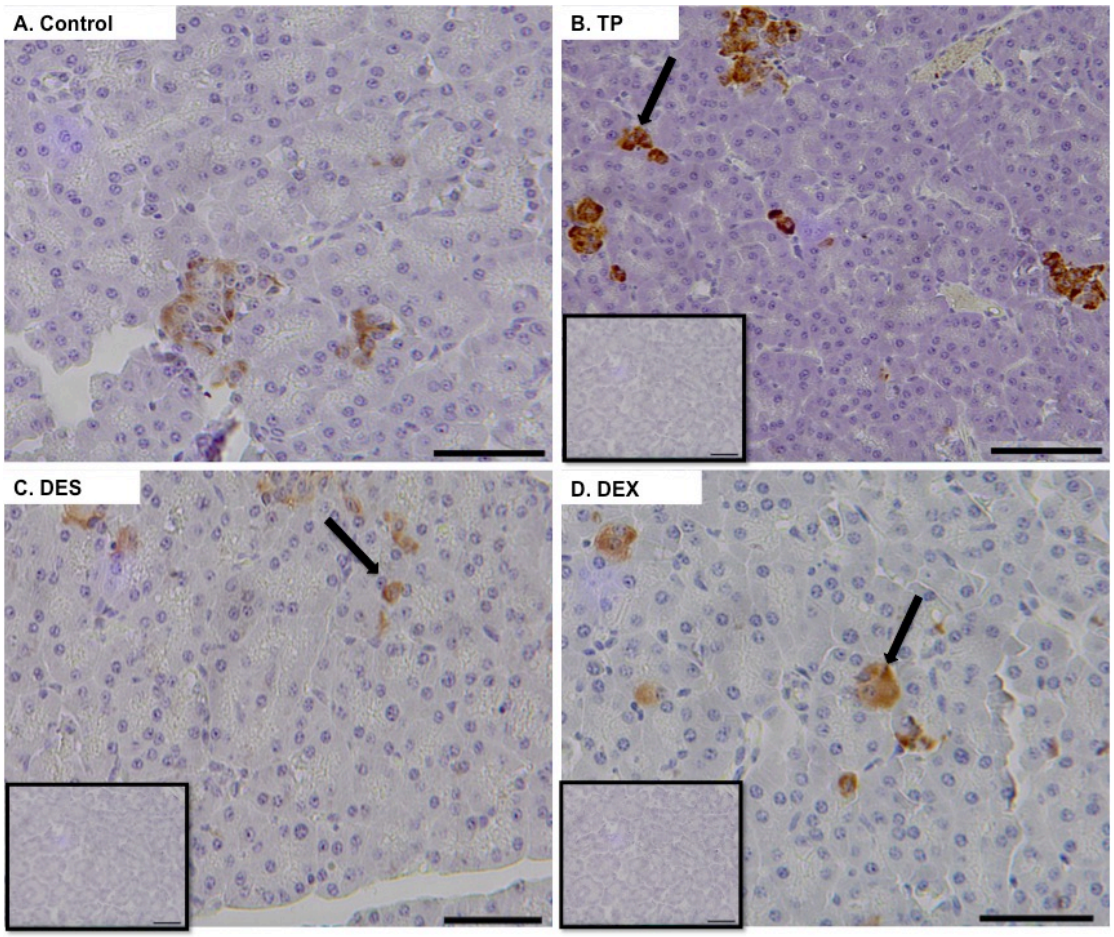


Figure 4.1.1 Immunohistochemical representation of insulin secreting β-cells in the pancreas of female adult offspring.

(A-B) Insulin secreting β-cells were significantly increased (*P*<0.01) by TP treatment ((Control n=6, TP n=13) (C-D) prenatal DES exposure has no effects on adult beta cell numbers E) prenatal DEX treatment decreased beta cell numbers in female adult pancreas; Control n=4, DES n=6, DEX, n=9). Scale- 50µm.

4.4.2 Direct prenatal androgen exposure altered female adult pancreatic function

In order to determine potential effects of direct prenatal steroid exposure on insulin/glucose homeostasis, blood samples were collected at 0 time point in adult female (11 months old) animals who were then administered with an

intravenous bolus glucose (10g) glucose and blood collected post stimulation (15 and 30 min).

None of the steroids that the ewes were exposed to prenatally had any effect upon plasma glucose concentrations (Figure 4.2.A-F) in female adult animals, suggesting that these animals had unaltered glucose dynamics.

However, glucose stimulated insulin concentrations showed a significantly augmented increase at 15 (P<0.05) and 30 min intervals (P<0.01) (Figure 4.2.1 A-B) in female adult animals by FI-TP treatment compared to controls. There was also a trend towards increased basal insulin concentrations in the TP treated animals (Figure 4.2.1.A), but this was not significant. FI-DES treatment had no effect on insulin secretion (Figure 4.2.1.C-D) pre and post glucose stimulation in female adult offspring compared to controls.

Similarly, FI-DEX was not associated with altered insulin secretion in response to glucose challenge (Figure 4.2.1 E-F), as compared to cohort control animals.

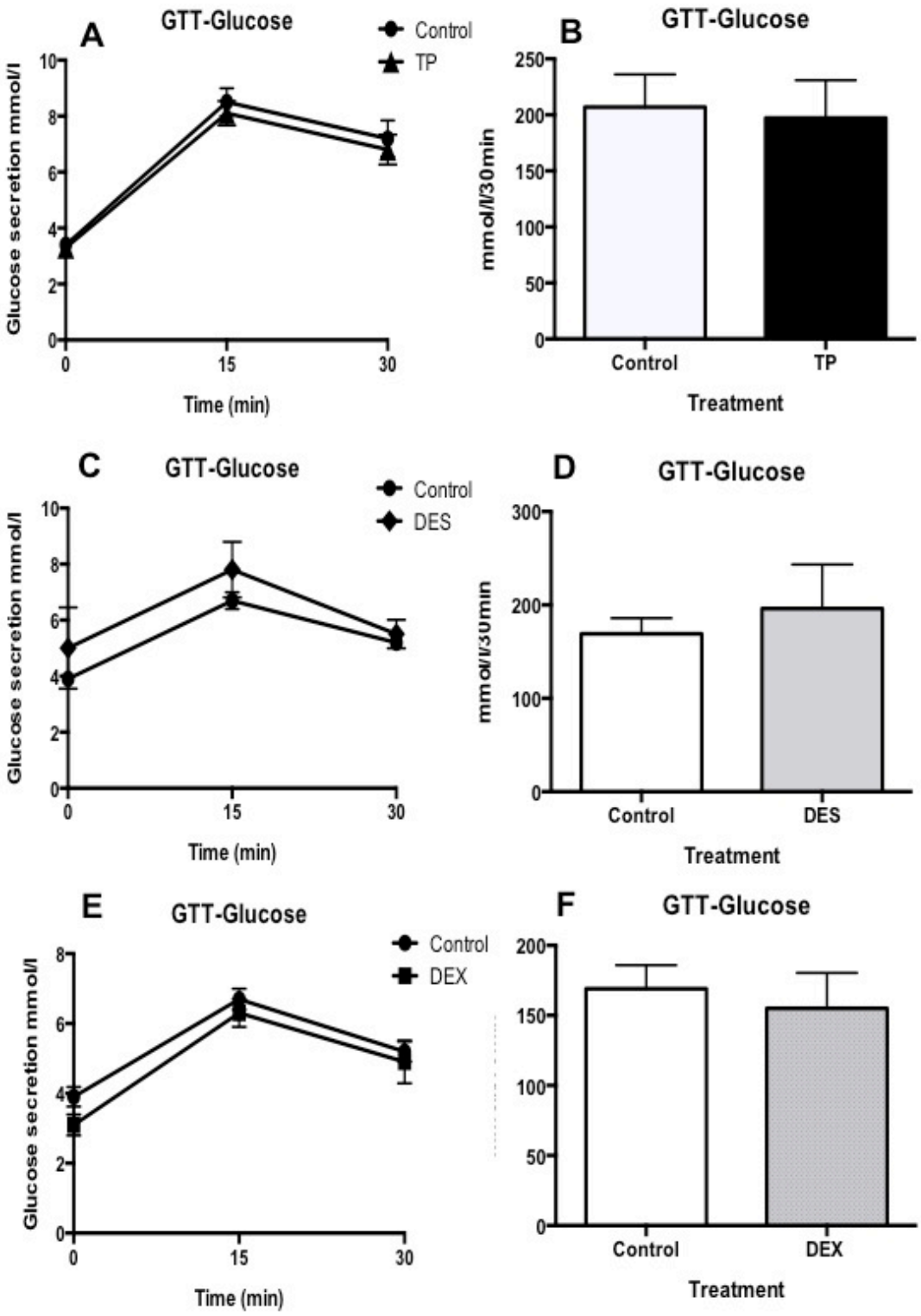


Figure 4.2. in vivo glucose secretion in response to glucose administration in female adult animals at 11 months old postnatal.

(A-B) TP treatment had no effect on glucose secretion in FI adult female sheep (11 months) post glucose stimulation compared to controls in response to glucose administration

(Control, n= 6, TP, n=13). (C-D) DES and (E-F) DEX treatment also had no effect glucose secretion in FI adult female sheep (11 months) compared to controls in response to glucose administration. (2012 cohort, Control, n=4; DES, n=7 and DEX, n=11. Values are represented as ± SEM

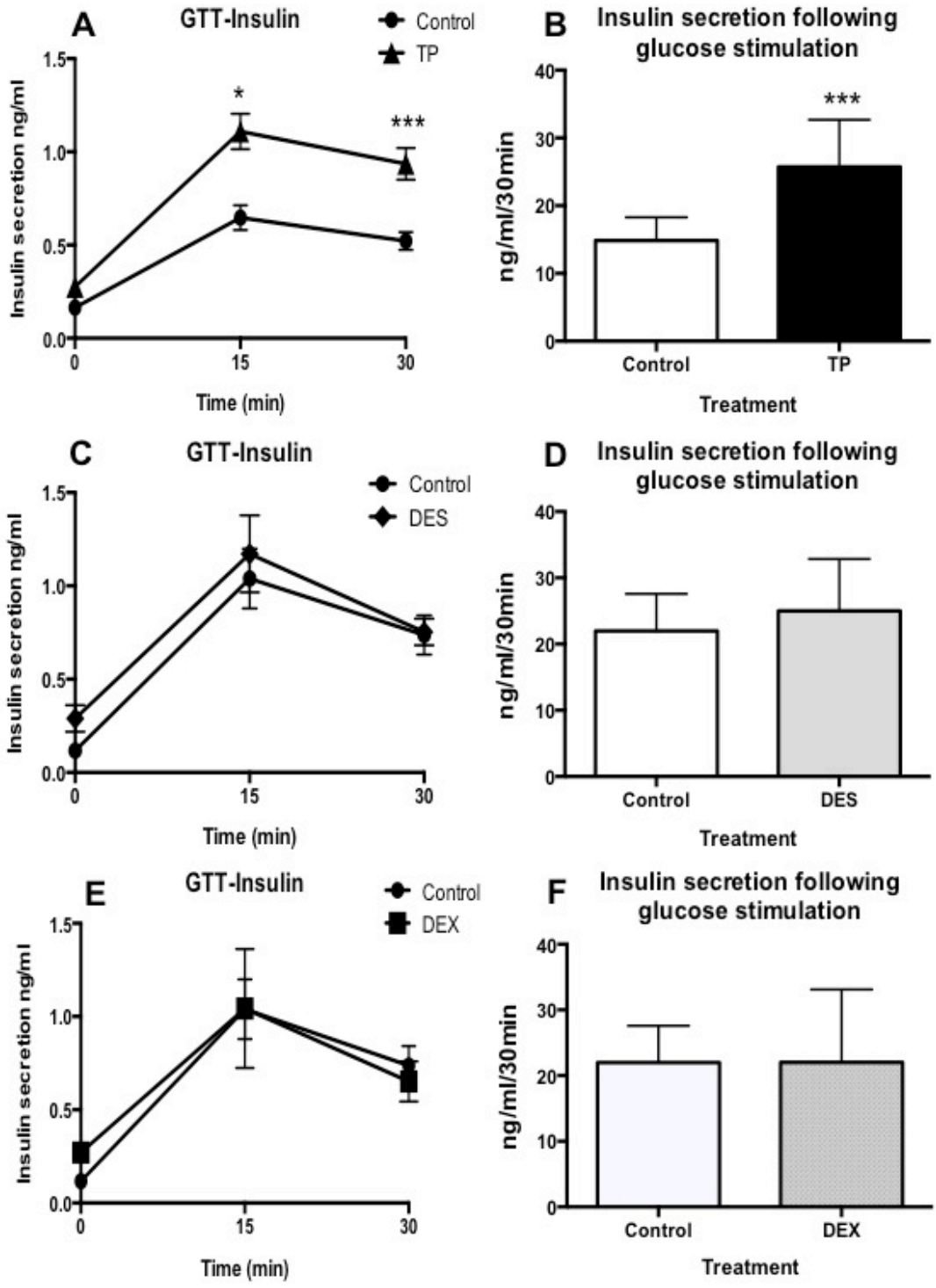


Figure 4.2.1 Insulin secretion in response to external glucose administration in female adult animals at 11 months old postnatal.

(A-B) TP treatment increased insulin secretion in FI adult female sheep (11 months) at 15 (* $p<0.05$) and 30 min (*** $p<0.01$) post glucose stimulation compared to controls in response to glucose administration (2010 cohort, Control, n= 6, TP, n=13. C-D) DES and E-F) DEX treatment had no effect on

insulin secretion in FI adult female sheep (11 months) compared to controls. (Control, n=4; DES, n=7 and DEX, n=11. Values are represented as ± SEM. *P<0.05; *** P<0.01

4.5 Prenatal androgen exposure has no effect on peripheral insulin signaling associated gene expression in muscle and liver in female adult offspring

Since we observed increased β-cell numbers and also an exaggerated insulin secretory response to glucose, but no alterations in glucose concentrations under test conditions in TP exposed offspring, it was important to focus on any potential of altered insulin signaling in these ewes. To this end, we assessed insulin signaling in terms of mRNA encoding relevant genes, and downstream pathway activation (phosphorylation of MAPK and AKT pathways) in muscle and liver biopsies, which had been bolus glucose exposed for 15 minutes prior to collection. Due to the labour and expense intensive nature of such analyses, only control and TP treated animals were analysed. There was so significant difference in the mRNA abundance levels of GLUT-1 (*SLC2A1*), GLUT-2 (*SLC2A2*) (expressed only in liver), GLUT-4 (*SLC2A4*), *INSR and IRS-1* in liver tissues post glucose stimulation (Figure 4.3 A-E) or skeletal muscle biopsies (Figure 4.4 A-D) due to prenatal androgen exposure.

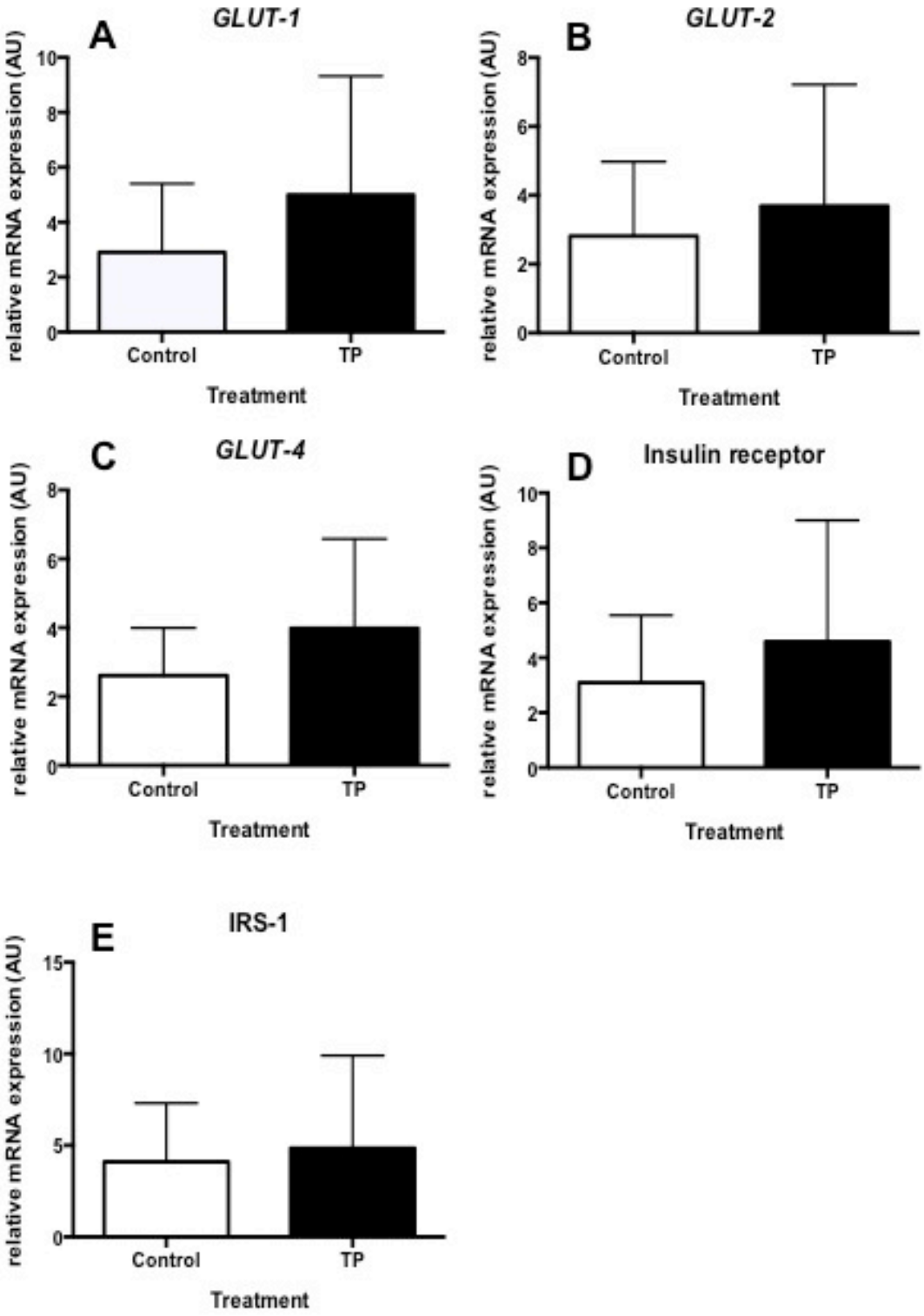


FIGURE 4.3 EFFECT OF FETAL TP EXPOSURE ON EXPRESSION SLC2A1, SLC2A2, SLC2A4 INSR, IRS-1 (A-E) IN FEMALE ADULT LIVER AT 11 MONTHS OLD. (BOLUS GLUCOSE WAS ADMINISTERED 15 MIN PRIOR TO SACRIFICE).

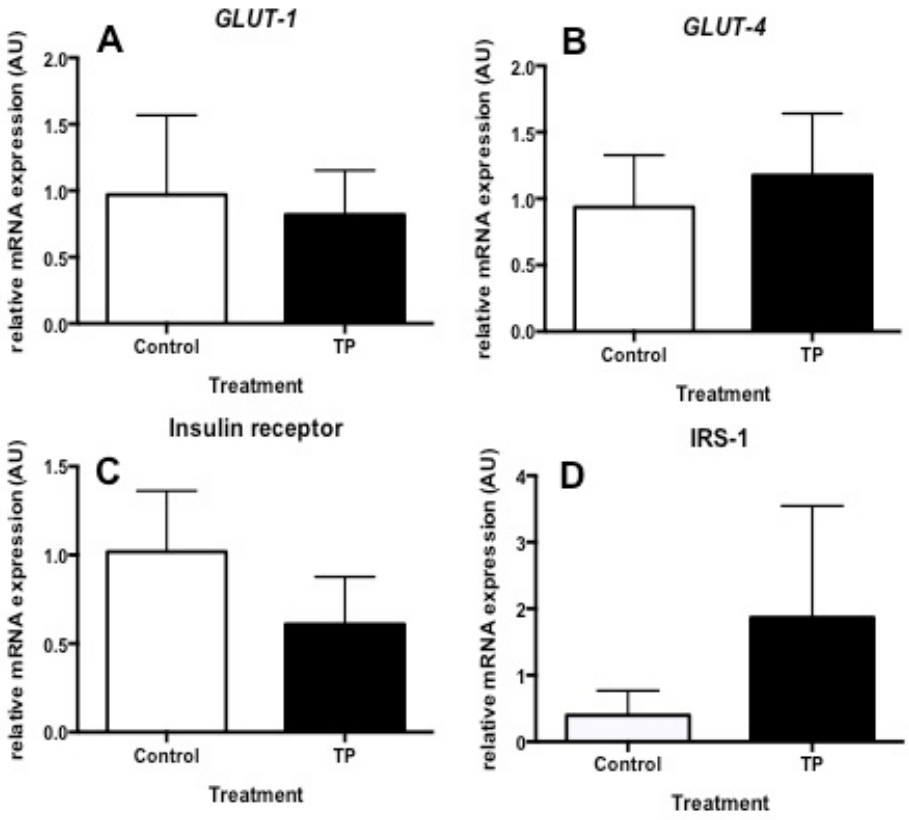
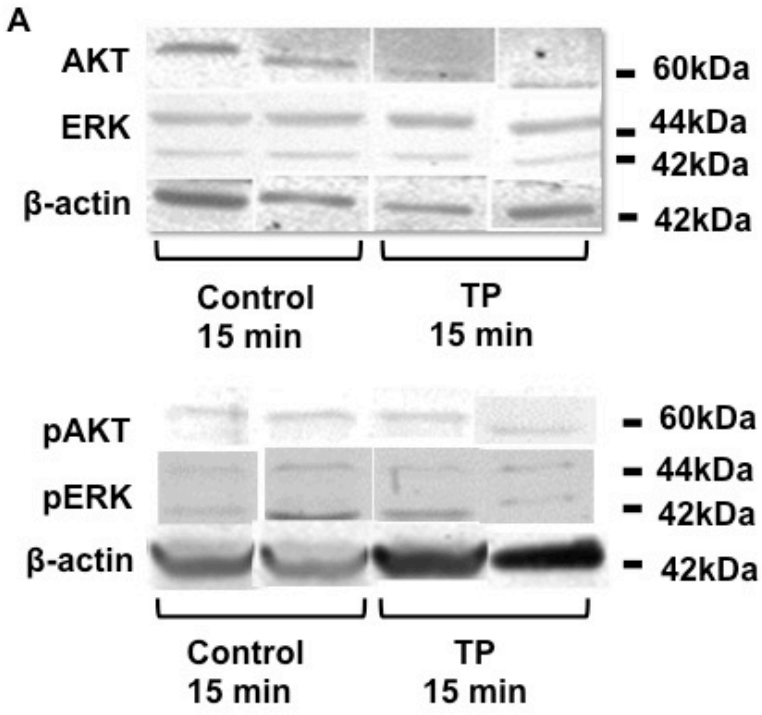


Figure 4.4 Gene expression analysis in the fetal TP injected female adult skeletal muscle biopsies at 11 months old.
Fetal TP exposure had no effect on *GLUT1, GLUT4, INSR, IRS-1* (A-D) gene expression in the 11 months old female adult skeletal muscle.

4.5.1 Prenatal androgen exposure has no effect on peripheral insulin signaling in muscle or hepatic protein expression in female adult offspring

The effect of prenatal androgen exposure on insulin signaling molecules AKT, ERK1/2 and their respective phosphorylated proteins (pAKT and pERK) was determined using western blotting. The blots compared control and TP treated samples from both muscle (Figure 4.5 A) and liver (Figure 4.6.A) for total AKT, total ERK, phospho AKT and phospho ERK. β-actin was used as a positive control to ensure correct amounts of protein was loaded to each well and to correct for such discrepancies.

There was no significant difference between the ratio of total AKT:phospho AKT (Figure 4.5.B), total ERK:phospho ERK for whole ERK band (Figure 4.5.C) or the individual bands (44kDa and 42kDa) (Figure 4.5.C-D) in the muscle biopsies following glucose administration (15 min) in prenatal TP treated female adult animals compared to controls.


A
AKT
ERK
β-actin
60kDa
44kDa
42kDa
42kDa
Control
15 min
TP
15 min
pAKT
pERK
β-actin
60kDa
44kDa
42kDa
42kDa
Control
15 min
TP
15 min

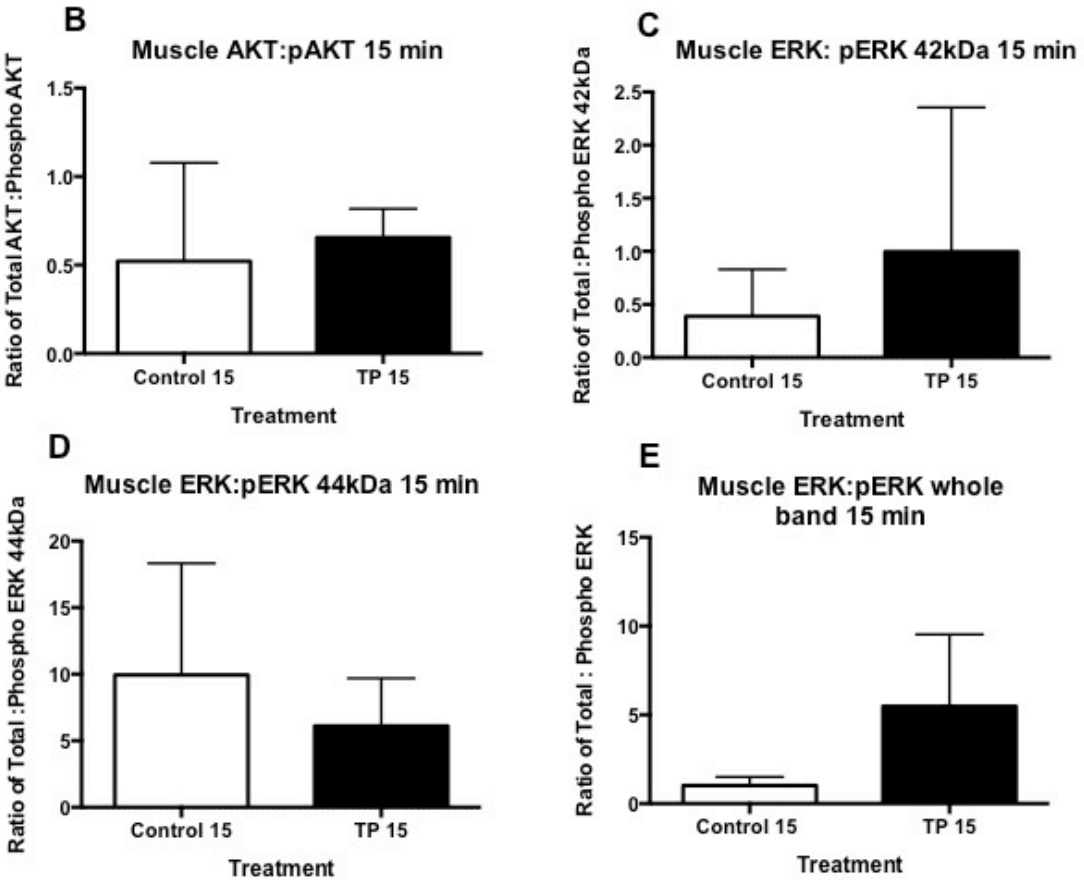

B
Muscle AKT:pAKT 15 min
Ratio of Total AKT :Phospho AKT
1.5
1.0
0.5
0.0
Control 15
TP 15
Treatment
C
Muscle ERK: pERK 42kDa 15 min
Ratio of Total :Phospho ERK 42kDa
2.5
2.0
1.5
1.0
0.5
0.0
Control 15
TP 15
Treatment
D
Muscle ERK:pERK 44kDa 15 min
Ratio of Total :Phospho ERK 44kDa
20
15
10
5
0
Control 15
TP 15
Treatment
E
Muscle ERK:pERK whole band 15 min
Ratio of Total : Phospho ERK
15
10
5
0
Control 15
TP 15
Treatment

Figure 4.5. A) Immunoblots for total AKT, total ERK, phosphorylated AKT, phosphorylated ERK and β-actin protein expression in skeletal muscle biopsies after glucose bolus in in fetal TP exposed adult female offspring.
B) Ratio of total AKT: phospho AKT between control and TP treated animals. C) Ratio of total : phospho ERK (42kDa) between control and TP treated animals. D) Ratio of total: phospho ERK (44kDa) between control and TP treated animals. E) Ratio of total: phospho ERK (whole band) between control and TP treated animals. Values are represented as mean ± SEM

4.5.2 Prenatal androgen exposure has no effect on peripheral insulin signaling protein expression in female adult liver

In the liver samples collected 15 minutes after glucose stimulation, there was no significant difference in the ratios of total AKT: phospho AKT (Figure 4.6.B), total ERK:phospho ERK for whole ERK band (Figure 4.6.C) or the individual bands (44kDa and 42kDa) (Figure 4.6.C-D) in prenatal TP treated female adult animals compared to controls.

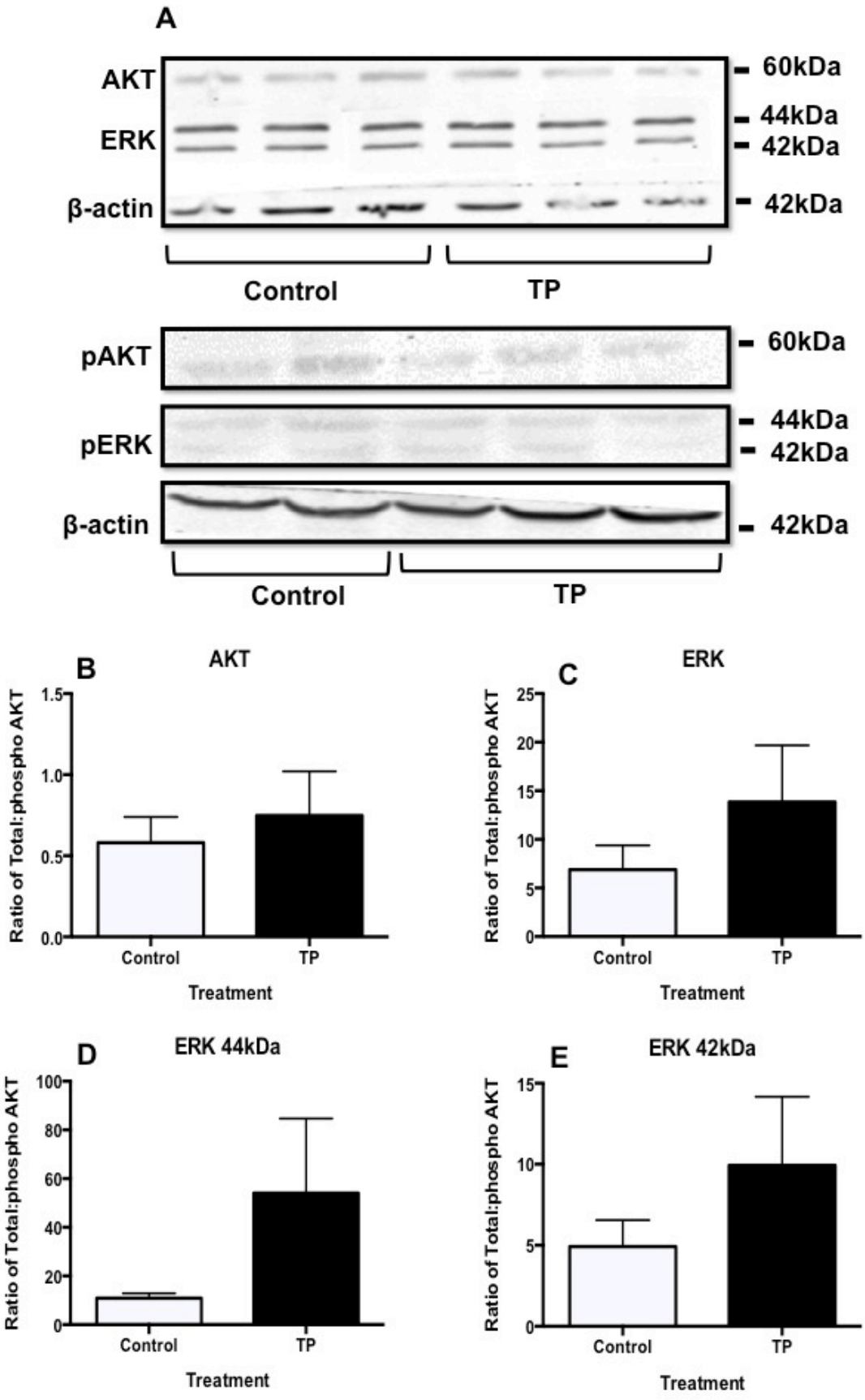

A
AKT
ERK
β-actin
60kDa
44kDa
42kDa
42kDa
Control
TP
pAKT
pERK
β-actin
60kDa
44kDa
42kDa
42kDa
Control
TP
B
AKT
Ratio of Total:phospho AKT
1.5
1.0
0.5
0.0
Control
TP
Treatment
C
ERK
Ratio of Total:phospho AKT
25
20
15
10
5
0
Control
TP
Treatment
D
ERK 44kDa
Ratio of Total:phospho AKT
100
80
60
40
20
0
Control
TP
Treatment
E
ERK 42kDa
Ratio of Total:phospho AKT
15
10
5
0
Control
TP
Treatment

Figure 4.6 A) Western immunoblots for total AKT, total ERK, phosphorylated AKT, phosphorylated ERK and β-actin protein expression in liver at sacrifice two weeks later after glucose bolus in adult female offspring from prenatal androgen exposure compared to control animals.
Quantification of total:phosphorylated AKT and ERK protein in liver at sacrifice after two weeks post glucose bolus in adult female offspring from A) Ratio of total AKT: phospho AKT in female adult liver between control and TP treated animals. B) Ratio of total: phospho ERK (whole band) between control and TP treated animals. C) ratio of total: phospho ERK (44kDa) between control and TP treated animals D) Ratio of total : phosphor ERK (42kDa) between control and TP treated animals. Values are represented as mean ± SEM of total: phospho in control and TP treated animals.

4.6 Direct fetal androgen exposure caused increased insulin secretion in prepubertal females

Data from adult animals demonstrated exaggerated insulin secretion in response to glucose associated with direct fetal exposure to androgens (TP) at d62/82 in the female adult offspring. To investigate the developmental period during which this pancreatic phenotype might arise, pancreatic function was also assessed in prenatally androgenized female lambs at 10-12 weeks of age (Figure 4.7). There were no significant changes observed in female lambs that were exposed to any of the prenatal steroid exposures we have utilised (Figure 4.7). However, there was a strong trend towards increased insulin secretion in animals exposed to TP prenatally, and this was absent in animals exposed to DES or DEX. Hence, although not significant in a statistical sense, this mirrored the data obtained in adult animals; indicative that the altered pancreatic function noted in fetal and adult life was likely present in pre-pubertal animals also.

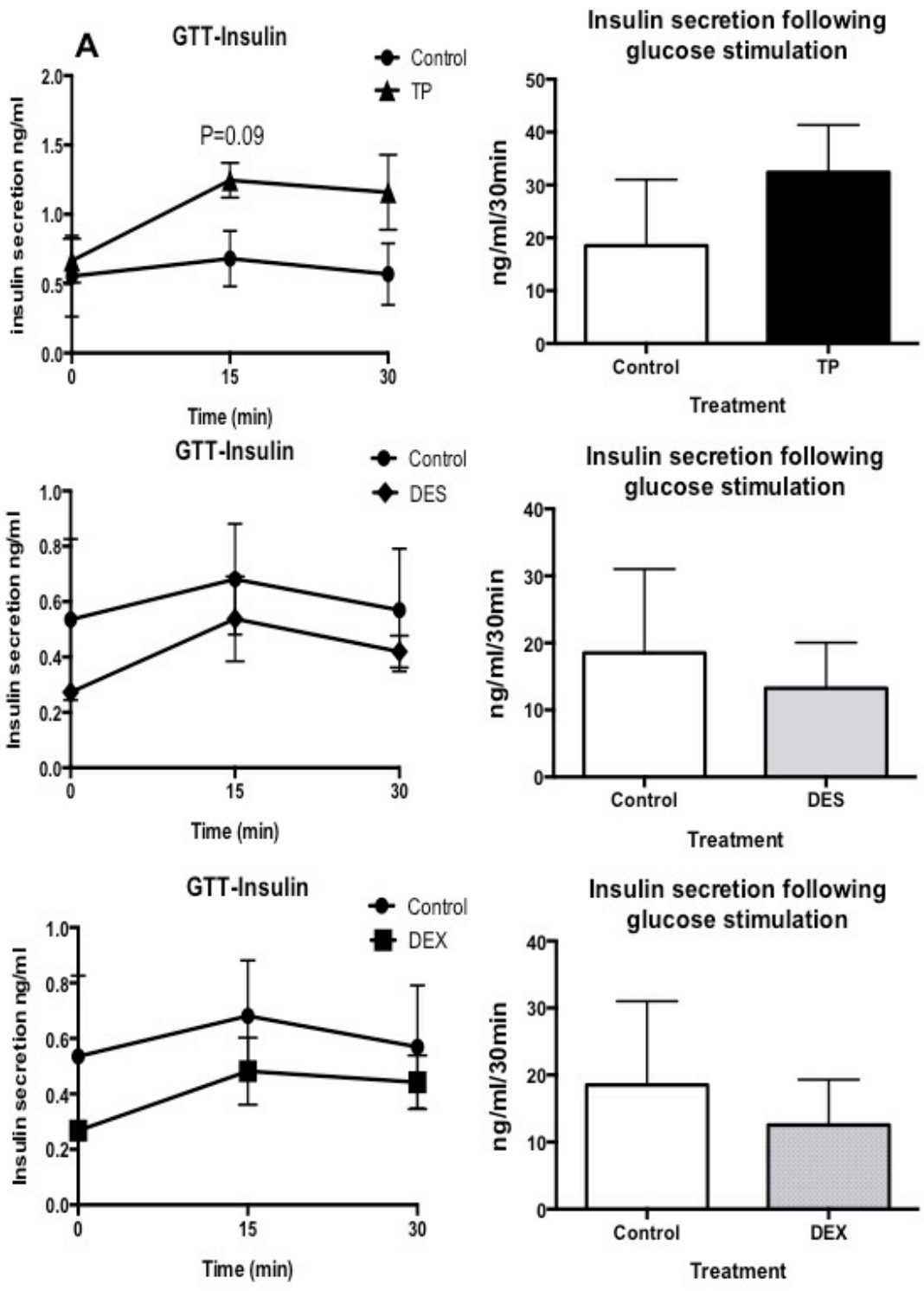


Figure 4.7 The hormonal secretion following pancreas stimulation (GTT) in fetally steroid exposed female lambs.

Blood was sampled at a basal time point (0 min) and 15 and 30 min post GTT stimulation by ELISA. (A-B) Insulin secretion in fetal TP exposed lambs, (C-D) Insulin secretion in fetal DES exposed lambs, (E-F) Insulin secretion in fetal DEX exposed lambs.

4.7 Discussion

In Chapter 3, the data derived from fetal life samples demonstrated that there were no changes in glucagon secreting α-cells in response to altered steroidal environments during pregnancy. However, during fetal life, and exclusively in response to excess androgen administration, there were increased β-cell numbers, increased expression of key pancreatic development genes and increased pancreatic β-cell function in the female fetal pancreas. The work reported in this chapter was a follow up of these fetal findings, and hence was focused on assessing the effects of direct fetal administration of different classes of steroids (TP, DES and DEX) (DES and DEX assessed wherever possible) on β-cell morphology and function in the female prepubertal lamb (function only) and adult offspring to ascertain whether there was a programming effect of the steroids that could have relevance to postnatal health.

The current study showed that direct prenatally androgenized pregnancies were associated with increased pancreatic β-cell numbers and elevated *in vivo* insulin secretion in response to glucose stimulation in absence of notable peripheral or hepatic insulin resistance in the female adult offspring, suggestive of permanent androgenic programming of pancreatic function.

4.7.1 Legacy of direct prenatal androgen and not estrogen exposure on pancreatic morphology and function in FI d62/82 model

Direct FI-TP exposure leads to an increase in the numbers of insulin secreting β-cells in female adult offspring (11 months old). As there was no such effect due to prenatal DES exposure, this strongly suggests an androgenic mode of action on β-cell/islet morphology that has persisted into adulthood. This is in accordance with our previous study, where female fetuses exposed to MI-TP from gestational d62-d102 similarly had increased β-cell numbers in female

adult offspring (Rae *et al*., 2013). The current data however provide additional information as regards mechanism of action, since by bypassing maternal and placental metabolism the applied androgens were unlikely to be sufficiently metabolized to estrogens, suggestive therefore that MI-TP and FI-TP androgenisation effects on pancreatic function are directly attributable to hyperandrogenaemia with no estrogenic contribution. This suggestion is 'double locked' by the lack of effects of DES, a potent estrogen receptor agonist. We therefore conclude that the effects observed here and in previous studies are indeed androgenic in nature, and furthermore, that these would appear to be potentially at least a direct extension of the effects observed in fetal life, which were to some extent predictive of the altered pancreatic structure and function observed here.

As a consequence of increased β-cell numbers, FI-TP (but not FI-DES) prenatally exposed female adult offspring had increased plasma insulin concentrations post GTT stimulation and also increased total insulin secretion $(AUC)_{insulin}$ with no changes in glucose concentrations nor $(AUC)_{glucose}$. Hence glucose-handling issues were not evident at this young adult stage of life, suggesting a direct action of fetal androgens exposure in altering β-cell function. This is again in accordance with previous studies in sheep, where MI-TP administered female adult offspring showed increased plasma insulin levels with no changes in glucose dynamics (Hogg *et al*., 2011), clearly suggesting that effects observed during adulthood are androgen specific. Importantly, the observed exaggerated response to glucose in terms of insulin secretion raises the question as to whether or not hyperinsulinaemia is necessarily always a compensatory response to insulin resistance, or possibly a primary pancreatic phenotype preceding, and possibly predisposing to IR.

To further investigate whether or not altered female fetal pancreatic development could directly underpin the adult female pancreatic phenotype observed in response to FI-TP, or if such an adult phenotype develops progressively as the animal matures, pancreas function was assessed in the 10-12 week lamb. Although not statistically significant, there was a trend

towards increased insulin secretion, in an identical fashion to that observed in adulthood, and this trend was also androgen specific. This implies that there are indeed androgenic effects inherent in pancreas tissue, and that the altered structure and function observed herein is the legacy of the fetal changes observed in chapter 3.

Similarly, prenatal androgenisation from d40-80 gestation in monkeys disrupts glucoregulation leading to hyperglycemia and excess insulin secretion in adult monkey dams and their female offspring (Abbott *et al*., 2010) and also in adult female Sprague-dawley rats (E16-19) (Demissie *et al*., 2008), suggesting early androgenization can disrupt future glucose homeostasis. Therefore, careful considerations are necessary with regards to the timing of steroid exposure and cross-species comparisons. This was one of the design drivers of our midgestational model (d62/82 Fi-TP), which may be more physiologically relevant than those that have been previously used to study effects of steroid hormones during pregnancy on adult health and disease.

Similar to the increased β-cell numbers in adult offspring from FI-TP pregnancy in the current study, a recent study in a PCOS monkey model demonstrated infants and adult offspring from prenatally androgenized (maternal route) pregnancies had decreased total islet size but increased islet numbers in infants at 45 postnatal days but no significant morphological differences were observed in terms of islet size in adult animals compared to controls (Nicol *et al*., 2014). Many factors such as overexposure to androgens (Rae *et al*., 2013) and fetal undernutrition (Gatford *et al*., 2008) in sheep and maternal hyperglycemia in monkeys (Abbott *et al*., 2010b) within the intra uterine environment can affect islet morphology during postnatal life. As the mothers of the above infants from prenatal androgenisation (d40-80 GA) had hyperglycemia (Nicol *et al*., 2014), it remains unclear as to whether or not the effects observed in infants were direct consequences of excess prenatal androgen signaling in the pancreas, or indirect via altered maternal glucose. However, the data presented in our study here in sheep implies the possibility that direct androgenic effects may occur on pancreatic β-cell morphology, as

due to its unique fetal route of administration, effects noted here were independent of maternal glucose alterations due to elevated maternal androgens. In addition to the pancreatic morphology changes discussed above, overexposure to androgens during fetal life was also associated with a hyperinsulinemic phenotype (increased basal and stimulated AUC ($_{insulin}$) in absence of glucose intolerance (normoglycemic), which clearly suggests that prenatal androgens excess had a direct action on the pancreas in terms of development and subsequent postnatal function.

Since we recorded no change in glucose handling in FI-TP female adult offspring in the presence of increased insulin response, this then begs the question as to whether or not insulin resistance (IR) was present to cause excess insulin secretion (hyperinsulinemia). The alternative would be a primary pancreatic alteration- this is critically important in both PCOS research and indeed Type-2 Diabetes Mellitus (non-insulin dependent diabetes mellitus-NIDDM) research given current debate over whether or not hyperinsulinemia may predispose to IR development (Cao *et al*., 2010). It is not an exaggeration to state that understanding of the possibility that hyperinsulinaemia could underpin IR would represent a frame shift in how diseases such as NIDDM are considered. By 40 years of age, nearly 20% of obese PCOS women suffer from impaired glucose tolerance or NIDDM (Dunaif *et al*.,1987) and this incidence increases with age compared to controls (Dahlgren *et al*., 1992). The reason behind this is ascribed to the insulin resistant characteristic of PCOS (Holte *et al*., 1994). However, only a subset of these insulin resistant women develop further NIDDM, which begs the question as to whether there is pancreatic dysfunction that contribute towards predisposition to NIDDM in PCOS. In support of this is a study conducted in PCOS women, in which multiple regression analysis revealed that there is a strong correlation between β-cell function (but not insulin resistance) and bioavailable testosterone concentrations in PCOS women compared to controls, suggesting that there could be a under-recognized importance for β-cell function in PCOS (Goodarzi *et al*., 2005).

Pancreatic β-cell dysfunctions are also observed in PCOS animal models exposed to excess androgens *in utero*. Blunted intracellular calcium levels were observed in response to glucose in isolated islets from mice exposed to excess androgens *in utero* (Roland *et al*., 2010). Early prenatal androgen exposure resulted in decreased insulin disposition index (DI- relative index of the relationship between acute pancreatic insulin responsiveness and the ability of insulin to induce glucose uptake) and late gestation androgen exposure increased insulin DI in response to induced glucose tolerance test compared to control animals in adult female rhesus monkeys (Eisner *et al*.,2000), suggesting impaired β-cell function regardless of the timing of prenatal steroid exposure.

Ras-ERK (extracellular-signal-regulated kinase) pathway and insulin receptor substrate (IRS)- protein kinase B (PKB) pathway are the two best characterized insulin signaling pathways (McKay and Morrison, 2007; White, 1998), that are activated by IRS-1 proteins and involved in cell proliferation, resulting in growth and mitogenesis (McKay and Morrison, 2007) and insulin receptor tyrosine kinase mediated initiation of IRS phosphorylation (White, 1998) respectively, leading to phosphoinositide 3-kinase activation (Alessi *et al*., 1996; Lawlor *et al*., 2001; Hanada *et al*., 2004) activation. As a result of insulin mediated AKT activation, many metabolic processes are regulated such as glucose transport (Eguez *et al*., 2005), gluconeogensis (Logie *et al*., 2007) and glycogen synthesis (Cross *et al*., 1995). Altered insulin signalling in terms of ERK and AKT phosphorylation at the molecular level and at different stages of insulin signalling is evident in human PCOS cases in different tissue types such as skin fibroblasts (decreased insulin receptor serine phosphorylation), skeletal muscle biopsies, adipocytes, myotubes and ovarian theca cells (decreased IRS-1 tyrosine phosphorylation) (Chu *et al*., 2004; Dunaif *et al*., 1995; Li *et al*., 2002; Qui *et al*., 2005) in association with insulin resistance and hyperinsulinemia (Cao *et al*., 2010; Rajkhowa *et al*., 2009).

In order to investigate whether the exaggerated insulin secretion observed in FI-TP female adult was associated with any molecular peripheral insulin

resistance, insulin signalling was assessed in terms of gene expression of genes key to insulin signalling, and downstream phosphorylation of signaling molecules in insulin target tissues (muscle, liver). There were no alterations in terms of expression of genes involved in insulin signalling and neither were there any effects upon phosphorylation of signaling proteins downstream of insulin receptor activation (ERK, AKT, pERK, pAKT), which clearly suggests the absence of insulin resistance in these 11 months old offspring. Combined with the fetal excess insulin secretion noted in chapter 3, this suggests the possibility of primary pancreatic alterations as a consequence of FI- TP exposure. Rae *et al*., (2013) also observed no alterations in terms of peripheral insulin signaling in the MI-TP sheep female adult offspring, suggesting that hyperinsulinemia (Hogg, Wood, et al., 2011) precedes insulin resistance in these models. Female adult offspring in the current study were of normal weight, which could also be one of the reasons behind the absence of insulin resistance at 11 months age, suggesting that the relationship between insulin resistance (IR) and hyperinsulinemia is weaker than perhaps would be assumed, similar to descriptions of lean PCOS women (Ciampelli *et al*., 1997). Due to the fact that insulin resistance reflects the presence of obesity related PCOS, and hyperinsulinemia being a primary metabolic feature at least in lean PCOS women, both entities may represent two distinct features of PCOS related insulin disorder (Ciampelli *et al*., 1997).

Among PCOS associated metabolic aberrations such as impaired glucose tolerance (IGT) and type 2 diabetes, insulin resistance is believed to be playing a critical role in pathogenesis (Legro *et al.*, 1999; Ehrmann *et al*., 1999). Peripheral insulin resistance in PCOS women is of a magnitude similar to that observed in patients suffering from type 2 diabetes mellitus (T2DM) alone, independent of obesity (Ovalle and Azziz, 2002). Impaired glucose tolerance is evident in about 40% of obese PCOS women, with 10% of them developing T2DM (Legro *et al.*, 1999; Ehrmann *et al*., 1999). Progression of the risk from PCOS associated insulin resistance into T2DM indicates that both the diseases might be sharing a common molecular defect, however the reasons for impaired insulin sensitivity/action in the peripheral tissues at the molecular level is still unclear.

4.7.2 Direct prenatal FI-DEX had no effect on pancreatic function in adulthood

Direct prenatal glucocorticoid exposure led to decreased β-cell mass in female adult offspring but no significant changes in *in vivo* insulin or glucose secretion were noted. Given that the suppresive effects of DEX in terms of β-cell numbers were subtle as compared to the more pointed increase in β-cell numbers seen in the case of TP treatments, it remains a possibility that the DEX effects do not translate into a measureable functional difference. However, decreased β-cell numbers could have health implications if the pancreas in these animals was placed under stress by eg. insulin resistance, as it may have a decreased dynamic range of plasticity (in terms of insulin secretory capability) to cope with increased demand. Late gestational dexamethasone exposure during fetal life, mimicking maternal stress, resulted in decreased β-cell pool and impaired glucose homeostasis during adulthood in female Wistar rats (Nyirenda *et al*.,1998) supporting the concept of long term metabolic programming effects due to prenatal DEX treatment. Maternal exposure to dexamethasone during late gestation also resulted in reduced β-cell mass in the fetal rat pancreas, suggesting direct effects of *in utero* exposure to dexamethasone (Dumortier *et al*., 2011). Even though there were significantly decreased β-cell numbers and a slight trend towards decreased insulin secretion in female adult offspring in the current study, these were not of statistitical significance.

4.8 Conclusion

In conclusion, direct fetal androgenization (TP) in sheep (FI) has demonstrated a permanent legacy of a primary pancreatic alteration resulting in increased insulin secretion in the absence of peripheral insulin resistance.

Chapter 5 The effects of direct prenatal steroidal excess exposure on developing fetal adrenal

5.0 Introduction

The human fetal adrenal cortex comprises of three zones namely; the inner fetal zone (FZ) (analogous to zona reticularis) and outer definitive zone (DZ) (analogous to zona glomerulosa) both consisting of eosinophilic cells larger in size in the former and small densely packed cells in the latter (Hanley *et al*., 2001 and Goto *et al*., 2006) and a third layer, the transitional zone, which develops between DZ and FZ at the end of gestation analogous to adult zona fasiculata (Mesiano *et al*., 1993). The adrenal gland in the ovine fetus can be observed as early as gestational day (dGA) 28 (Wintour *et al*., 1975). Although both human and ovine fetal adrenal glands share development similarities, the ovine adrenal gland lacks a specific fetal zone during its development (Robinson, 1979) and the zona reticularis becomes evident only during postnatal life (1 month old lamb) (Naaman-Répérant and Durand, 1997). Ovine fetal adrenal glands develop in a biphasic fashion, with rapid development occurring between gestational d60-120G. At d60G zona glomerulosa cells begin to produce aldosterone, and then the second growth maturation period occurring after dGA120 sees development and maturation of the zona fasiculata (Boshier and Holloway, 1989; Webb, 1980). The adrenal cortex in the ovine fetus secrets cortisol only during the first half of gestation (Wintour *et al*., 1995) and the last month of gestation, remaining relatively quiescent between d90-120G (term ~147days) (Wintour *et al*., 1975), likely due to the inadequate production of ACTH from the fetal pituitary during midgestation. However, it is apparent that cortisol secretion during the last few weeks of gestation helps in development of organs and is critical in the onset of ovine parturition (Liggins, 1994a, 1994b). In addition to the ovarian androgen excess, excess adrenal androgen secretion is also observed in PCOS women (Hague *et al.*, 1990; Rosenfield, 1999), which accounts for an estimated 20-30% of androgen excess, and is manifested by elevated levels of circulating androstenedione, DHEA and DHEAS (Carmina *et al*., 1992; Yildiz and Azziz, 2007) along with hyper responsive ACTH stimulated DHEA and androstenedione (Azziz *et al*, 1998). Abnormal regulation of P450c17α in the adrenal cortex is observed in women with PCOS (Rosenfield *et al*., 1990). *In utero* environment alterations during fetal

adrenal development can have a bearing upon long-term adrenal function, evident from maternal androgen over exposed rhesus monkeys, which recapitulate features seen in PCOS women such as elevated basal circulating adrenal DHEA and DHEAS in the female adult offspring (Zhou *et al*., 2005). Hence it is clear that prenatal androgenization carries not only a consequence of altered ovarian steroidogenesis (Hogg *et al*., 2012), but also altered adrenal steroidogenesis, however, mechanisms underpinning such altered function remain unknown.

5.1 Objectives/Hypothesis

The aims of this chapter were to measure the effect of mid-gestation direct steroid exposure on the postnatal adrenal from steroid manipulated pregnancies, addressing the following research questions?

a. Do excesses of androgens, estrogens or glucocorticoids have effects on key genes associated with adrenal development/function?
b. Are responses to steroidal excesses fetal sex-specific?

5.2 Materials and methods

5.2.1 Animal Husbandry and Treatment regime- midgestation (d62/82) FI-TP, FI-DES, FI-DEX

Animal husbandry was as detailed in section 2.1-2.1.1 and fetal treatment regime of pregnant ewes was followed as explained in section 2.1.2. Lamb and adult offspring husbandry was as detailed in section 2.1.3.

5.2.2 Early gestation maternal androgenisation in combination with mid-gestation FI-DEX

The rationale behind this study was due to the increased expression of genes coding for adrenal steroidogenic enzymes in male fetal adrenals from FI-DEX exposed pregnancies. Only female fetal samples were studied in this study, the rationale being that our initial data derived from male and female fetuses (described in this chapter) indicated that this was the most direct way of answering the question that was raised by these initial findings. Initial findings appeared to indicate the possibility that differences observed in response to midgestational DEX between males and females may be due to endogenous, early gestation 'self-androgenisation' occurring in males. The fetal injection treatment regime was identical to that described in section 2.1.2 except that the pregnant ewes were injected (maternal injection-MI) with 100mg/ml of testosterone propionate (TP) (AMS Biotechnology (Europe) Ltd., Abingdon, UK) at d30 and d45 gestation, followed by fetal injection of dexamethasone (20mg) (FI-DEX) at d62/82 gestation. Vehicle controls (C) received vegetable oil (Sainsbury's So organic range) and 5% ethanol alone. Maternal injections were delivered (i.m.; 1ml) into the flank. This protocol was designed to mimic d30 testicular androgenisation that occurs in males in treated female fetuses to determine if this could alter their response to DEX in a similar way to that which we had observed in males in initial gene expression examinations. Animal sacrifice, tissue and plasma sample collection were detailed as in section 2.2.1-2.2.2. Table 5.1 illustrates the animals assessed in this chapter.

Analysis	Fetal sex	Sample Number (n)
Female fetuses		
RNA (qRT-PCR) study	Injection at d62 and d82, collection and assessed at d90	C=6;TP=6(20mg) DES=6 (50μg); DEX=5(100μg)
RNA (qRT-PCR) study	MI Injection at d30 and d45 followed by FI-DEX-d62 and d82	C=2; DEX=3 (100μg)
Male fetuses		
RNA (qRT-PCR) study	Injection at d62 and d82, collection and assessed at d90	C=6;TP=6 (20mg) DES=6 (50μg); DEX=6 (100μg)

Table 5.1 The experimental animals included in the assessment of fetal adrenal gland, treatment regime and sample numbers.

5.2.3 Gene expression analysis- Quantitative real time-PCR

RNA extraction, cDNA synthesis and qRT-PCR protocols were performed as explained in section 2.3.1-2.3.5. The expression of range of genes in fetal adrenal was assessed. The primer details for each gene are listed in the table 5.2.

Genes of Interest	Forward primer(5'-3')	Reverse primer(5'-3')
STAR	GCATCCTCAAAGACCAGGAG	CTTGACACTGGGGTTCCACT
CYP11A1	CAACGTCCCTCCAGAACTGT	CAGGAGGCAGTAGAGGATGC
HSD3B	GGAGACATTCTGGATGAGCAG	TCTATGGTGCTGGTGTGGA
CYP17A1	AGACATATTCCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT
CYP21A	Not available	Not available
CYP11B1	AGAAGTACACGCCCTTGGTG	AGCGCGTGGATAAAGTTCAG
HSD11B1	AGCATTGTGGTCGTCGTCTCCT	CCTTGGTCGCCTCATATTCC
HSD11B2	TAAGGCGAGATTAGGTAGGTTG	ACCCTTCAAATCACAGCACTG
HSD17B	Not available	Not available
SRD5A1	ATGTTCCTCGTCCACTATGC	GTAGCCATTATAGGTGCAGAAGA
SRD5A2	GCCGTTTCCAGTTGTATTCCT	AGCAGGGTATTCAGCACAGTA
AR	GCCCATCTTTCTGAATGTCG	CAAACACCATAAGCCCCATC
ER-α	GAATCTGCCAAGGAGACCCG	CCTGACAGCTCTTCCTTCTG
ER-β	GAGGCCTCCATGATGATGTC	GGTCTGGAGCAAAGATGAGC
GR	AAGTCATTGAACCCGAGGTG	ATGCCATGAGGAACATCCAT
MR	CTTCGCCTTCTATGATCCTTG	AGGGTGGAGAGCAGGTTATC
PTCH	GGACAAACTTTGACCCTTTGG	CATGACCAACTTCAGCCTTATTC
SHH	CTGCTCTACAGCGACTTCCTC	GCGGACCAACTTCAGCCTTATTC
ACTH-R	ATGAAACACATTCTCAATCTG	AACGTTTTCCAAAATCTTGTAC
Reference gene		
GAPDH	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG
ATPSynth	SEQUENCE NOT AVAILABLE	SEQUENCE NOT AVAILABLE
RPS2	SEQUENCE NOT AVAILABLE	SEQUENCE NOT AVAILABLE
YWHAZ	SEQUENCE NOT AVAILABLE	SEQUENCE NOT AVAILABLE

Table 5.2. List of forward and reverse primer sequences (genes of interest and references genes) for ovine fetal adrenal.

Where no sequences are available, this is due to the primers used being part of the GENORM kit as supplied, where sequence information was manufacturer's proprietary information.

5.2.4 Statistical analysis

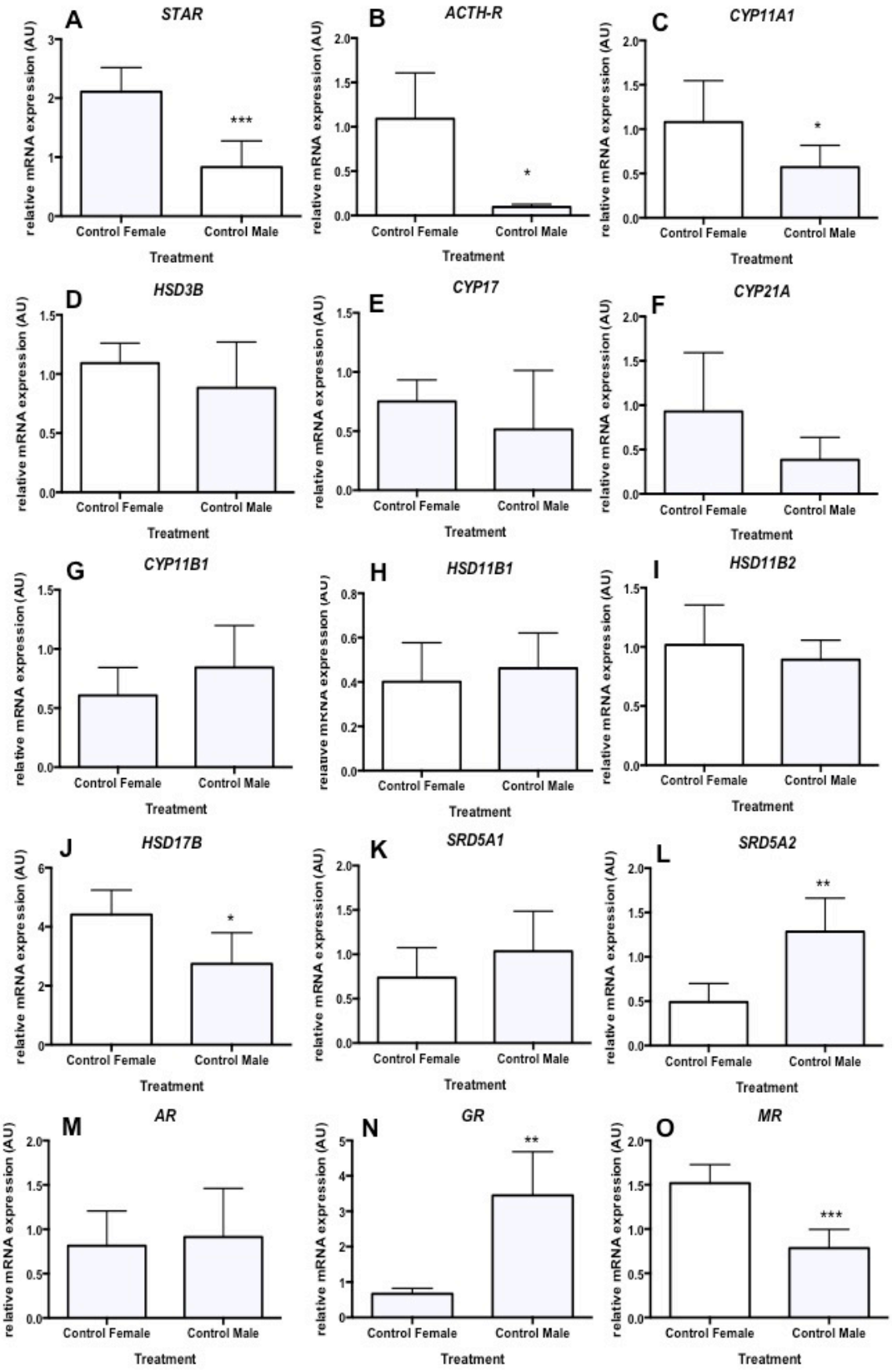
Real time PCR analysis was carried out to establish relative mRNA expression of genes as detailed in section 3.3.2. Housekeeping genes used to calculate relative gene expression were *RPS2, YWHAZ, ATPsynth* for male fetal adrenal and *GAPDH* and *RPS2* for female fetal adrenal determined via GeNorm algorithm as described in section 2.3.8. Due to the interest in comparing and contrasting effects of TP and DES, data from Control, TP and DES were analysed using one-way ANOVA (analysis of variance) followed by Tukey's *post-hoc* test to determine specific differences between groups. Differences between Control and DEX group were analysed using unpaired two-tailed Student's t-test. Results are presented as $\pm$SEM and p-value, where $P<0.05$ were considered significant.

5.3 Results

In order to investigate the effects of FI exposure to TP, DES and DEX on fetal adrenal development in both males and females, the genes coding for adrenal steroidogenic enzymes and steroid receptors were assessed. Since DES is a synthetic estrogen, and TP, an aromatizable androgen, analysis of these treatment groups together permitted resolution of androgenic and estrogenic effects previously noted in models where maternal treatments, and hence opportunity for generation of estrogens from androgens has occurred. Dexamethasone (DEX), a synthetic glucocorticoid, was compared against control groups independent of the above analysis.

5.3.1 Gender associated fetal adrenal steroidogenic gene expression

Basal levels of *STAR* ($P<0.001$; Figure 5.1.A), *ACTH-R* ($P<0.05$; Figure 5.1.B), *CYP11A1* ($P<0.05$; Figure 5.1.C), *HSD17B* ($P<0.05$; Figure 5.1.J) and *MR* ($P<0.01$; Figure 5.1.O) were significantly lower in male fetal adrenal than in females. However, basal *SRD5A2* ($P<0.01$; Figure 5.1.L) and *GR* ($P<0.01$; Figure 5.1.N) expression was significantly higher in male fetal controls compared to females. None of the other genes analysed showed any significant difference between sexes in terms of gene expression analysed in this study.


A
STAR
relative mRNA expression (AU)
***
Control Female
Control Male
Treatment
B
ACTH-R
relative mRNA expression (AU)
*
Control Female
Control Male
Treatment
C
CYP11A1
relative mRNA expression (AU)
*
Control Female
Control Male
Treatment
D
HSD3B
relative mRNA expression (AU)
Control Female
Control Male
Treatment
E
CYP17
relative mRNA expression (AU)
Control Female
Control Male
Treatment
F
CYP21A
relative mRNA expression (AU)
Control Female
Control Male
Treatment
G
CYP11B1
relative mRNA expression (AU)
Control Female
Control Male
Treatment
H
HSD11B1
relative mRNA expression (AU)
Control Female
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HSD11B2
relative mRNA expression (AU)
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J
HSD17B
relative mRNA expression (AU)
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Control Female
Control Male
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K
SRD5A1
relative mRNA expression (AU)
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SRD5A2
relative mRNA expression (AU)
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Control Female
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MR
relative mRNA expression (AU)
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Control Female
Control Male
Treatment

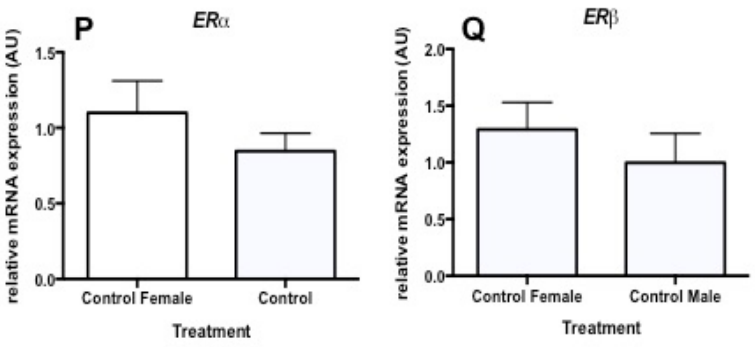
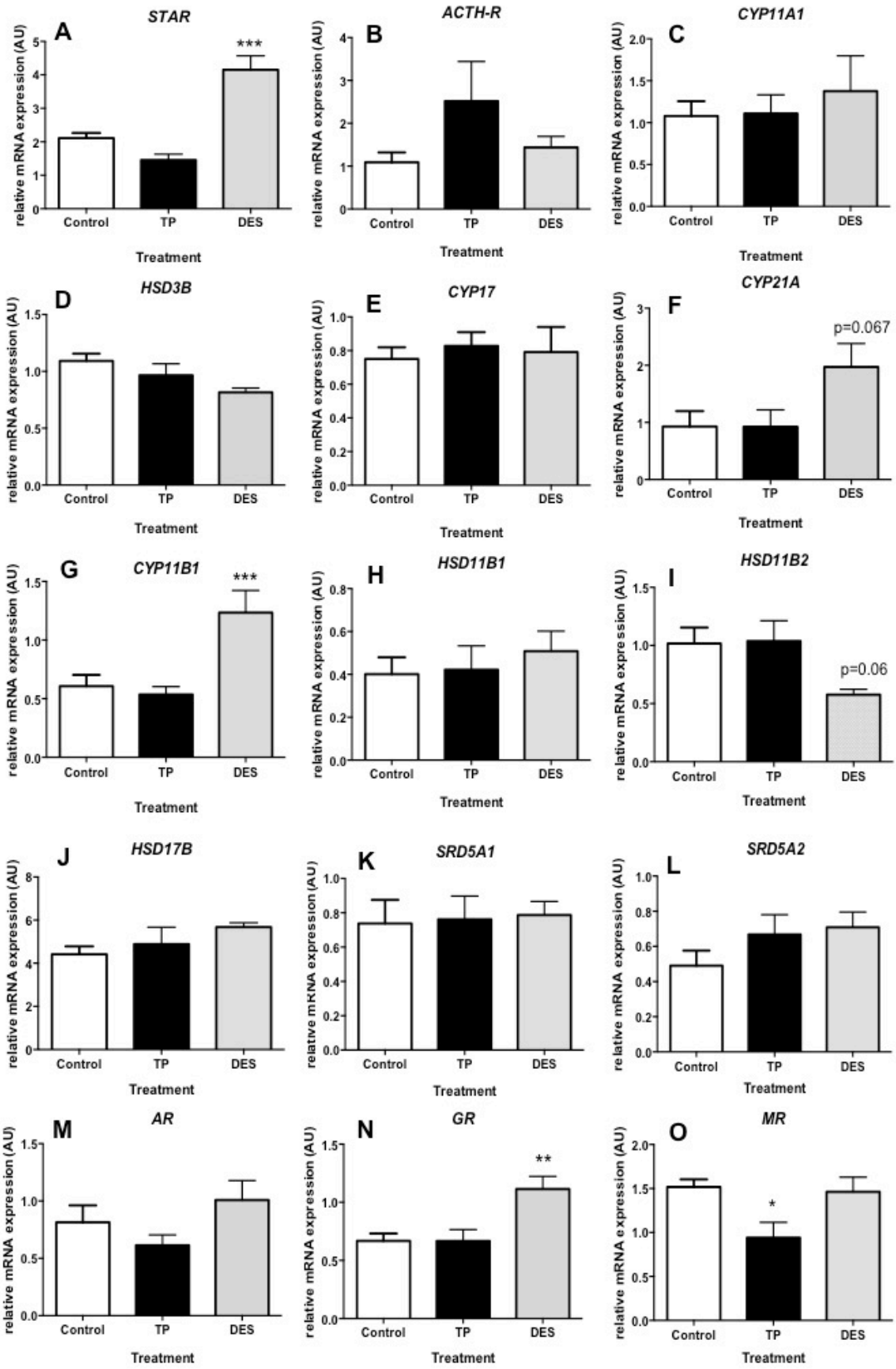


Figure 5.1 Effect of gender on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, CYP11B1, HSD11B1, HSD11B2, HSD17B, SRD5A1, SRD5A2, AR, MR, GR, ERα and ERβ in female and male fetal adrenal at d90 gestation.
(Control, Female, n=6; Male, n=6). Values represent mean ± SEM. **P*<0.05, ** *P*<0.01, *** *P*<0.001.

5.3.2 Effect of excess of prenatal TP and DES treatment on female and male fetal adrenals

Figures 5.2 and 5.3 summarize the effects of TP and DES on the developing adrenal in female and male fetuses, respectively. Steroid acute regulatory protein mRNA (*STAR*) was significantly increased (~ 2 fold) only by DES treatment in both females (*P<0.001*; Figure 5.2.A) and males (*P<0.05*; Figure 5.3.A) compared to control animals. Adrenocorticotrophic hormone (*ACTH-R)* mRNA abundance was significantly increased by DES treatment and not by TP only in males (*P<0.001*; Figure 5.3.B) compared to controls. *CYP21A* showed a strong trend towards increased expression by DES treatment only in females (*P*=0.067; Figure 5.2.F). *CYP11B1* mRNA abundance was significantly increased (*P<0.001*) by DES treatment only in females (Figure 5.2.G) and not by TP compared to controls. *HSD11B2* mRNA abundance showed a strong trend towards a decrease in expression by DES treatment and not by TP only in females (*P*=0.06; Figure 5.3.H-I). *HSD17B* mRNA abundance was significantly decreased by DES and not by TP treatment only in males (*P<0.01;* Figure 5.3.J) compared to control animals.

GR (*P<0.01*); Figure 5.1.N) and *ERβ* (*P<0.05*; Figure 5.2.Q) mRNA abundance was significantly increased by DES only in females compared to control animals. Interestingly, mineralocorticoid receptor (*MR*) mRNA abundance was significantly decreased by TP treatment only in females (*P<0.05*; Figure 5.2.O) but had no significant change by DES treatment in either sex. The remainder of the genes analysed were not altered by TP or DES treatment in either sex.


A
STAR
***
B
ACTH-R
C
CYP11A1
D
HSD3B
E
CYP17
F
CYP21A
p=0.067
G
CYP11B1
***
H
HSD11B1
I
HSD11B2
p=0.06
J
HSD17B
K
SRD5A1
L
SRD5A2
M
AR
N
GR
**
O
MR
*
relative mRNA expression (AU)
Control
TP
DES
Treatment

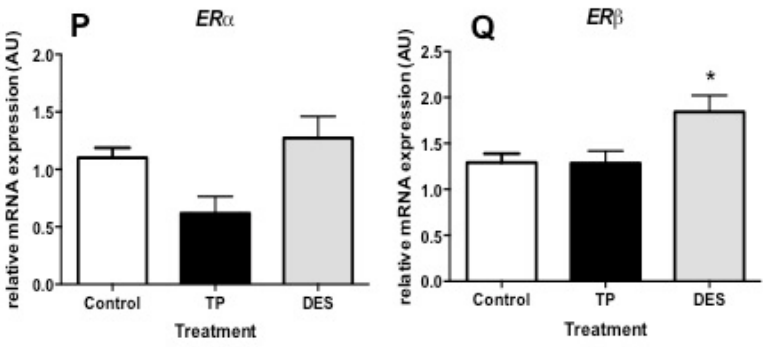
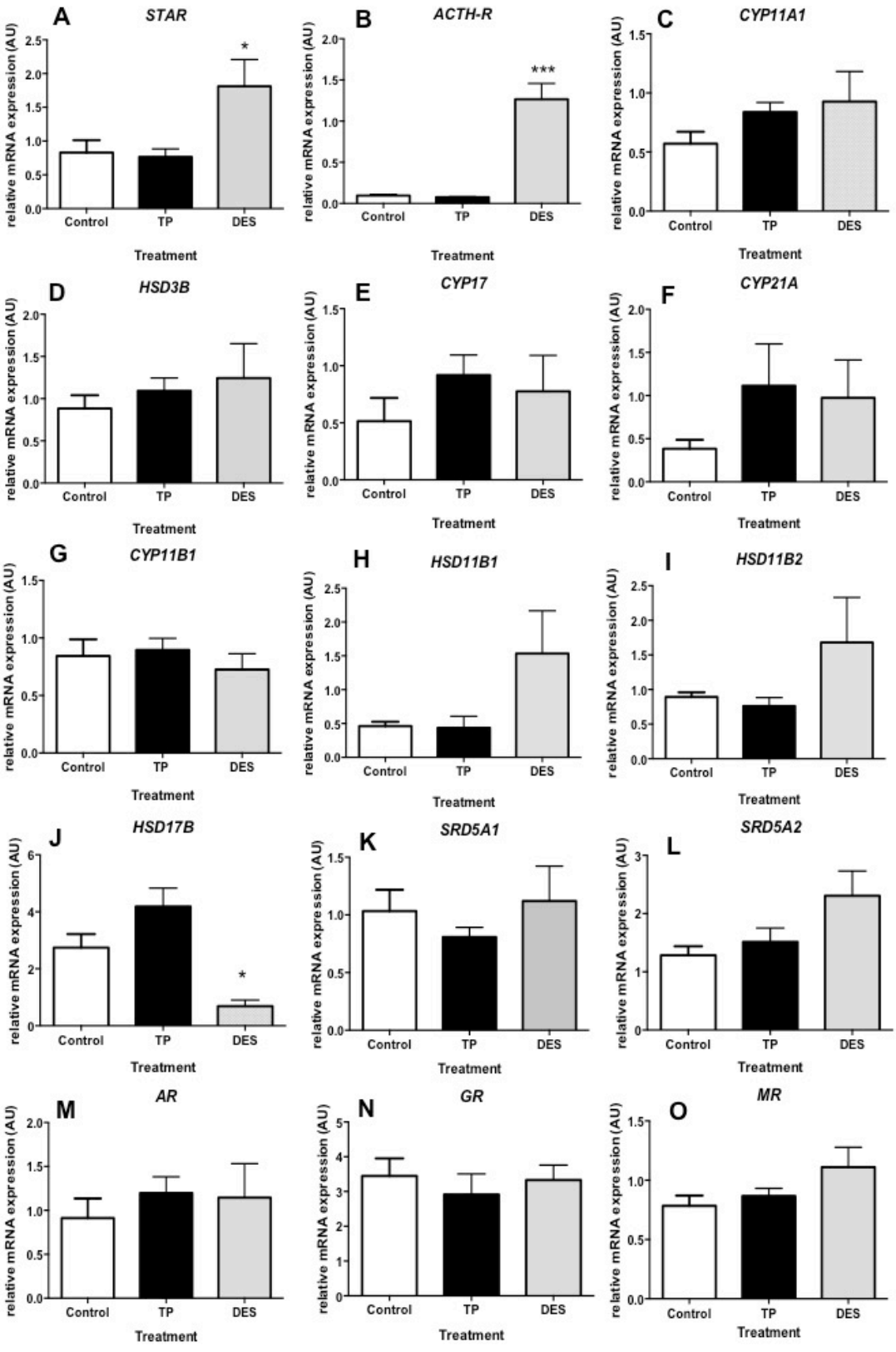


Figure 5.2 Effect of fetal exposure to TP and DES on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, CYP11B1, HSD11B1, HSD11B2, HSD17B, SRD5A1, SRD5A2, AR, MR, GR, ERα and ERβ in female fetal adrenal at d90 gestation (Control n=6, TP n=6, DES n=5). Values represent mean ± SEM. * *P<0.05,* *** *P<0.001*.


A
STAR
*
B
ACTH-R
***
C
CYP11A1
D
HSD3B
E
CYP17
F
CYP21A
G
CYP11B1
H
HSD11B1
I
HSD11B2
J
HSD17B
*
K
SRD5A1
L
SRD5A2
M
AR
N
GR
O
MR
relative mRNA expression (AU)
Control
TP
DES
Treatment

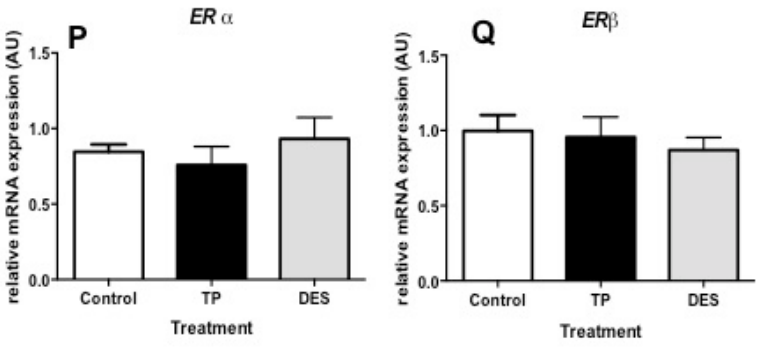


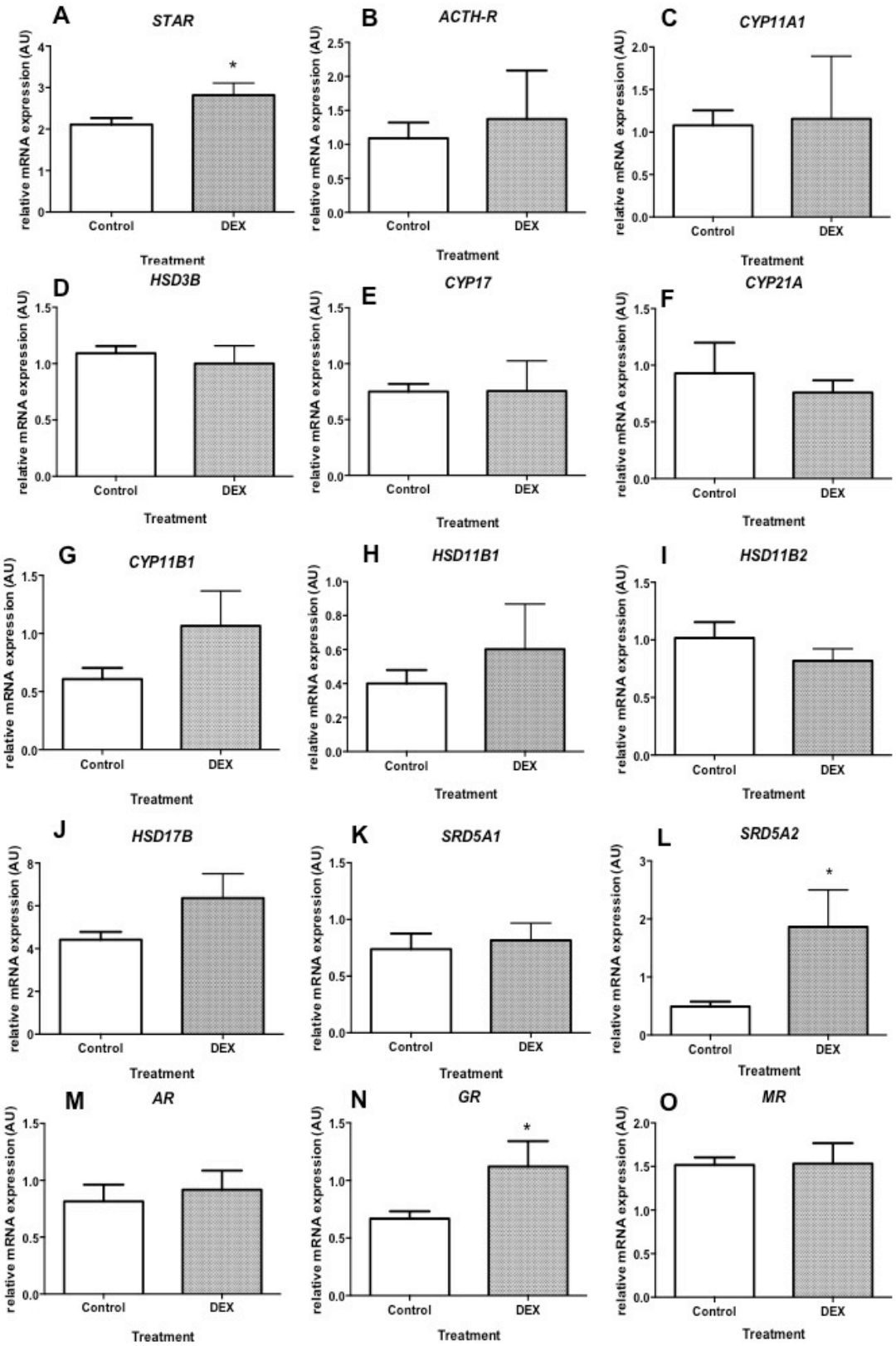
Figure 5.3 Effect of fetal exposure to TP and DES on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, CYP11B1, HSD11B1, HSD11B2, HSD17B, SRD5A1, SRD5A2, AR, MR, GR, ERα and ERβ in male fetal adrenal at d90 gestation
(Control n=6, TP n=6,DES n=6,DEX n=6). Values represent mean ± SEM. * *P*<0.05, *** *P*<0.001.

5.3.3 Effect of FI-DEX treatment on female and male fetal adrenal

STAR mRNA abundance was significantly increased by DEX treatment in both females (*P<0.05*; Figure 5.4.A) and males (*P<0.001*; Figure 5.5.A). *ACTH-R* (*P<0.001*; Figure 5.5.B), *CY11A1* (*P<0.001*; Figure 5.5.C), *HSD3B* (*P<0.001*; Figure 5.5.D) and *CYP17* (*P<0.05*; Figure 5.5.E) mRNA expression were significantly increased by DEX treatment only in males compared to control samples. *HSD11B1* mRNA abundance was unaltered in females, but significantly increased by DEX treatment only in males (*P<0.01;* Figure 5.5.I) compared to controls. Other genes analysed were unaffected by DEX exposure in either sex.

SRD5A1 mRNA abundance was significantly increased (*P<0.05;* Figure 5.5.K) by DEX treatment only in males, whereas, *SRD5A2* mRNA abundance was significantly increased in both females (*P<0.05*; Figure 5.4.L) and males (*P<0.001*; Figure 5.5.L).

Genes encoding for steroid receptors such as androgen receptor (*AR*), estrogen receptor (*ER*) mineralocorticoid receptor (*MR*) and glucocorticoid receptor (*GR*) were also altered during fetal life due to prenatal DEX exposure. *AR* mRNA abundance was significantly increased (*P<0.001*; Figure 5.5.M) only in males, while *GR* mRNA expression was significantly increased in both females (*P<0.05*; Figure 5.4.N) and males (*P<0.001*; Figure 5.5.N) by DEX treatment. Finally, *ER*α (*P<0.05*; Figure 5.5.P) mRNA expression was significantly increased only in males and *ER*β (*P*=0.06; Figure 5.5.Q) showed a strong trend towards increase in its expression by prenatal DEX exposure. The remainder of the genes analysed were unaffected.


A
STAR
relative mRNA expression (AU)
Control
DEX
Treatment
B
ACTH-R
C
CYP11A1
D
HSD3B
E
CYP17
F
CYP21A
G
CYP11B1
H
HSD11B1
I
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L
SRD5A2
M
AR
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MR

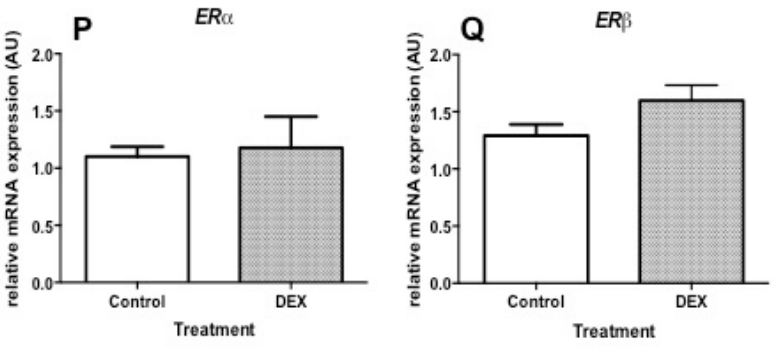
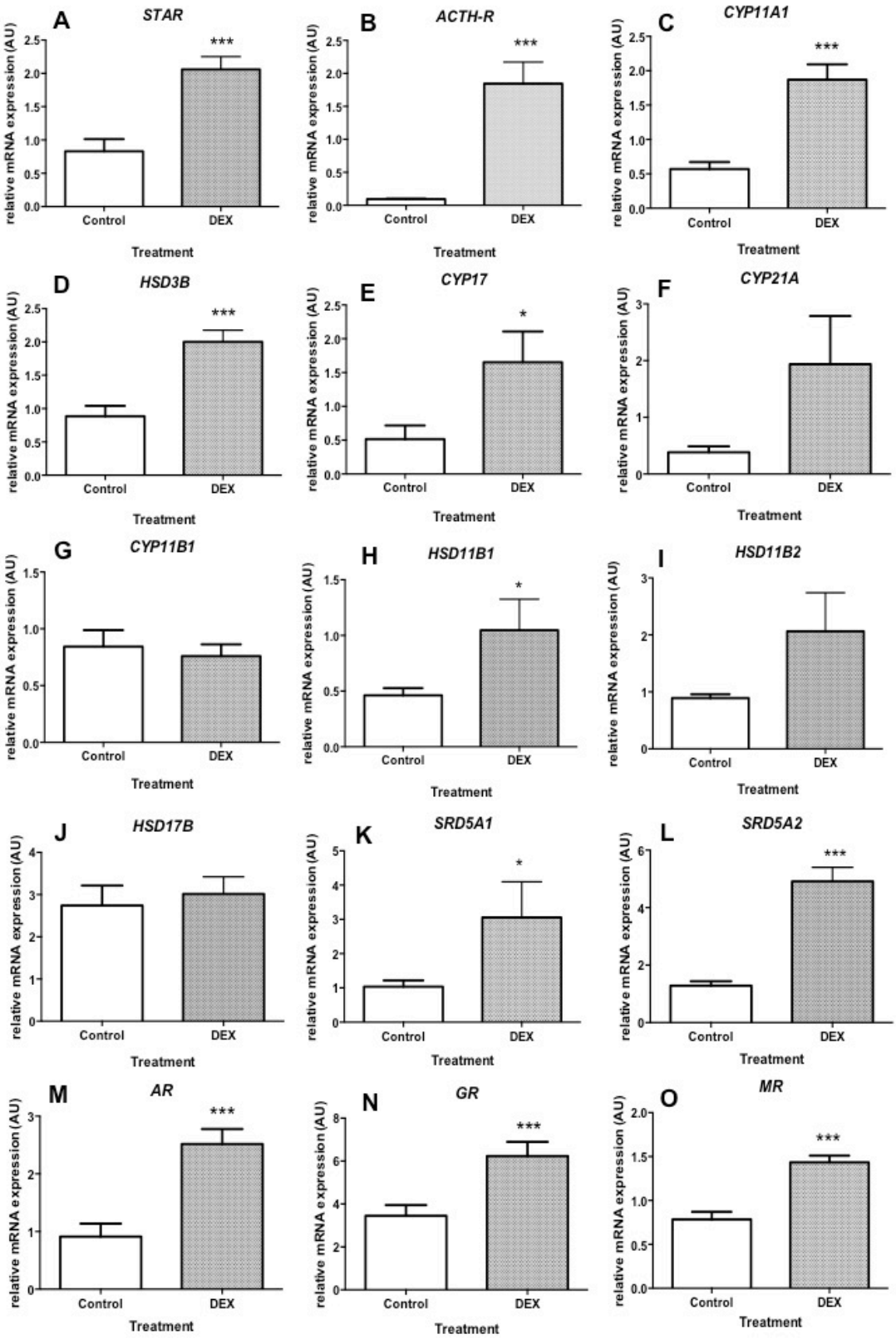


Figure 5.4 Effect of fetal exposure to DEX on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, CYP11B1, HSD11B1, HSD11B2, HSD17B, SRD5A1, SRD5A2, AR, MR, GR, ERα and ERβ in female fetal adrenal at d90 gestation.
(Control n=6, DEX n=5). Values represent mean ± SEM. **P*<0.05.


A
STAR
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HSD17B
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SRD5A1
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AR
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N
GR
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O
MR
***
relative mRNA expression (AU)
Control
DEX
Treatment

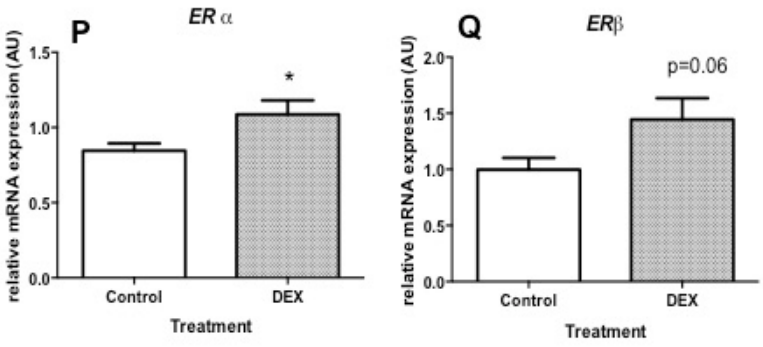

Figure 5.5 Effect of fetal exposure to DEX on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, CYP11B1, HSD11B1, HSD11B2, HSD17B, SRD5A1, SRD5A2, AR, MR, GR, ERα and ERβ in male fetal adrenal at d90 gestation.
(Control n=6, DEX n=6). Values represent mean ± SEM. * *P*<0.05, *** *P*<0.01.

5.4 Effect of MI-TP (d30 gestation) and FI- DEX (d62 and d82 gestation) on female fetal adrenal steroidogenic gene expression.

As FI-DEX treatment at d62&82 had such a profound effect on male steroidogenic associated gene expression, but not female, we hypothesized that sexual differentiation (testis formation at d30 in sheep) and subsequent 'natural androgenisation' could be the reason behind this sexually dimorphic response. Therefore, a subset of animals exposed to testosterone (MI-TP) at d30 gestation (maternal route) was created, thus phenocopying male testicular androgenisation in females followed by FI-DEX at d62&82. Then a subset of genes, which were robustly elevated by DEX in male fetuses were studied in these 'masculinised female' fetuses. DEX treatment upon a background of androgen excess from earlier in gestation had no significant effect on the mRNA abundance of genes encoding for *STAR, CYP11A1, HSD3B, SRD5A1, AR, MR* and *GR* (Figure 5.6). Hence early gestation masculinisation did not appear to induce a male-type response to later gestation DEX in female fetuses.

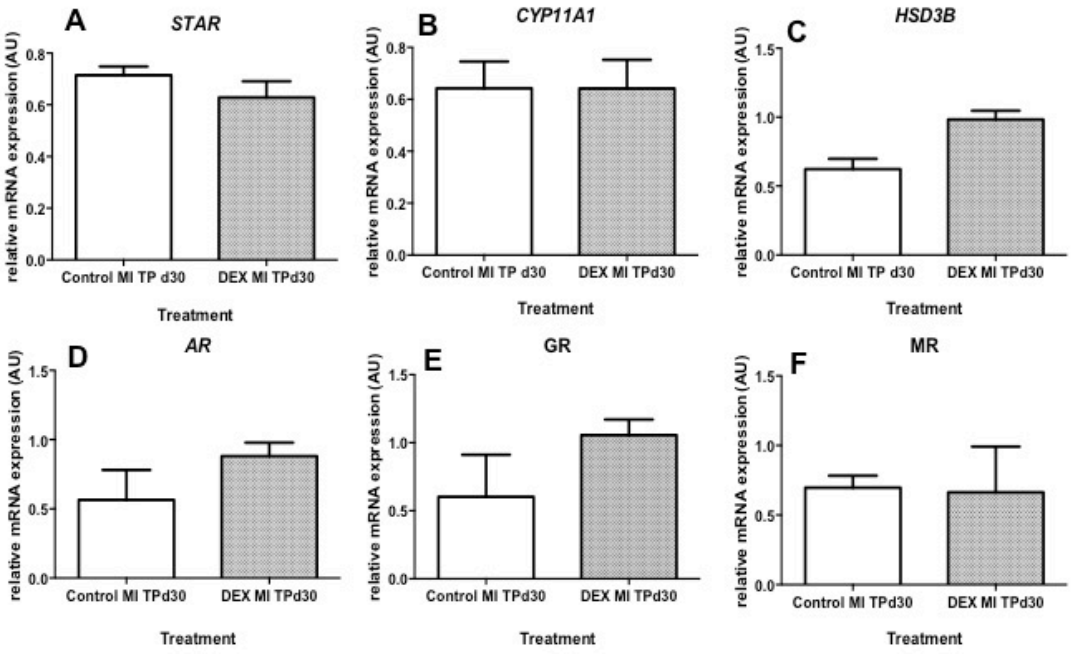

Figure 5.6. Effect of MI-TP d30 followed by direct fetal exposure to DEX at d62 & 82 on expression of genes coding for STAR, CYP11A1, HSD3B, AR, MR and GR in female fetal adrenal at d90 gestation.
(Control n=2, DEX n=3). Values represent mean ± SEM.

5.5 Effect of d30 MI-TP on basal female fetal adrenal gene expression

STAR mRNA abundance was significantly decreased (*P*<0.001; Figure 5.7.A) in MI-TP (d30) treated females as compared to untreated controls, reducing expression down to a similar level as observed in control male fetuses. *CYP11A1* mRNA abundance followed a similar trend (*P*=0.07; Figure 5.7.B) as did *MR* mRNA abundance (*P*<0.01; Figure 5.7.F). None of the other genes analysed had any effect due to MI-TP at d30 in female fetuses.

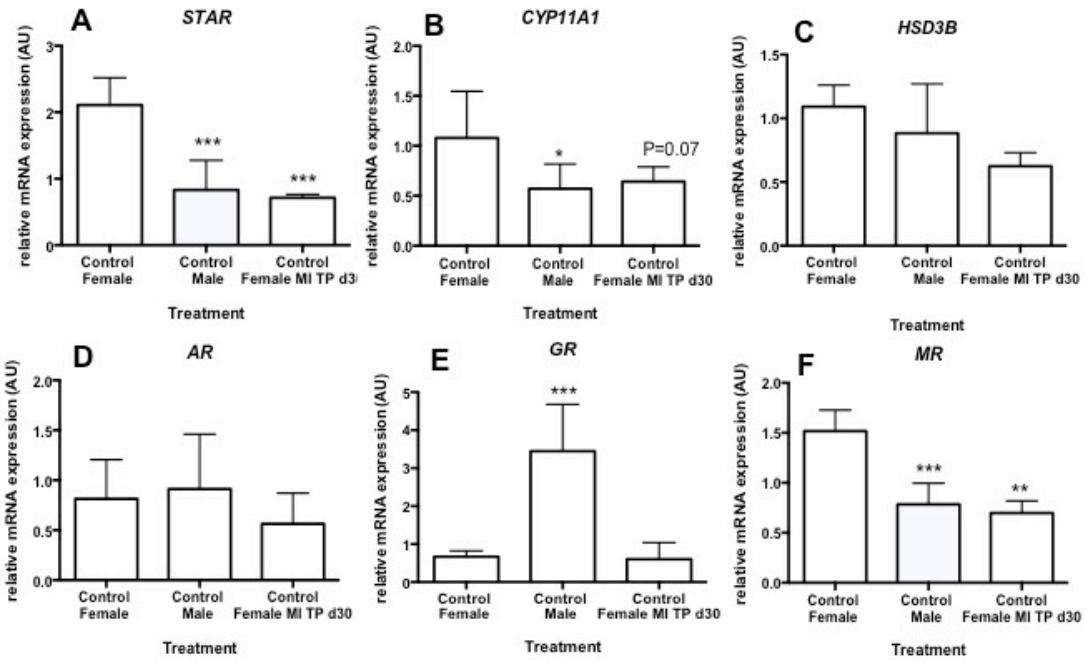

Figure 5.7 Effect of vehicle treatment (FI d62&82 in comparison with MI-TPd30) on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, AR, GR and MR, in fetal adrenal at d90 gestation.
(Control, Female, n=6; Male, n=6; MI-TP d30, n=2). Values represent mean ± SEM. **P*<0.05, ** *P*<0.01, *** *P*<0.001.

5.5 Discussion

In this study, the effects of exposure to excess prenatal steroids (TP, DES and DEX) were assessed in both male and female fetal adrenal glands in terms of steroidogenic gene expression. Whilst the fetal adrenal gland is active during the first half and last month of gestation, it remains quiescent during midgestation (Wintour *et al*., 1975), likely due to the inadequate production of ACTH from the fetal pituitary prior to this time. Therefore, adrenal samples collected in this study from midgestational steroid manipulated pregnancies may not be optimal in terms of timing of examination of steroidogenic enzyme expression, however, gross alterations in gene expression should be evident. Timing of collection of tissues for fetal adrenal study resulted from the collection times (d90) which in fact designed as part of a larger, multi-organ study. Additionally, another aim of this study was to examine potential differences between male and female fetuses in terms of adrenal responses to exogenous steroids, and such potential gender-specific responses should be visible even during a quiescent period since respective receptor expression has already been confirmed (Rae and Duncan, unpublished). Hence, this work focused upon gender-differential responses by utilising steroidogenic GOIs as potential markers of sex-specific adrenal development.

5.5.1 Gender associated differences in fetal adrenal gene expression

Basal *ACTH-R*, *STAR* and *CYP11A1* mRNA expression levels were lower in males than in females at d90 gestation, suggesting that steroidogenic potential is lesser in the male than the female adrenal gland. Lower *STAR* and *CYP11A1* is unlikely to be a consequence of lowered ACTH responsiveness (lower *ACTH-R*) as there is no ACTH drive in fetal sheep at this time of gestation (Wintour *et al*., 1975). There was also lower *HSD17B* expression in males than in females at d90, which might indicate perhaps a lesser androgenic capacity in males than in females. Speculatively, endogenous

androgen exposure around d30/35 from fetal testis (Quirke *et al*., 2001) could be the possible reason behind low adrenal steroidogenic capacity in males as compared to females. Connolly *et al*., (2013) have demonstrated decreased fetal testicular steroidogenic enzyme gene expression and LH secretion along with abnormal leydig cell development, in response to direct *in utero* androgen exposure. Therefore, decreased adrenal androgenic potential in males may be indicative of a compensatory mechanism of adrenal gland to protect testicular development. i.e if there was significant androgen secretion from male fetal adrenal during development, the adrenal androgens could possibly act upon the hypothalamic-pituitary-testicular axis, thereby causing lowered LH and thus affecting Leydig cell distribution and testicular androgen secretion, in a similar fashion to that of exogenous androgens (Connolly *et al*., 2013). ACTH secretion is negatively regulated by androgens during adulthood (Giussani *et al*., 2000), however, as noted above there is no ACTH secretion in the sheep during midgestation. Increased *SRD5A2* in males as compared to females suggests the potential of the adrenal gland to regulate testicular androgenic signaling by potentiating androgenic signalling through metabolism of weaker androgens into more potent forms (Söderström *et al*., 2001). In contrast, *MR* expression was lower in males than in females, however the functional significance of this is unknown. In summary, as the above gene expression changes are occurring during a quiescent period in adrenal gland development and function, the effects may not be of a functional relevance during postnatal life. Nonetheless it was intriguing that *GR* expression was significantly higher in males than in females, suggesting possible increased sensitivity to glucocorticoids in males.

5.5.2 Are gender differences in adrenal gene expression due to endogenous androgen exposure?

A limitation to this study was the relatively small sample size. Nonetheless, *STAR*, *CYP11A1* and *MR* were masculinized, in that they were reduced to male levels, by excess androgen exposure at d30 in female fetuses. This suggested that the sex differences observed in these genes are likely due to

earlier exposure (d30/35) to endogenous testicular androgens in males. In contrast, excess androgen exposure at d30 had no effect on *GR* expression in females, indicating that higher *GR* in males is independent of testicular androgens during sexual differentiation. However it could be a more fundamental gene factor that triggers dimorphic differences between male and female adrenal development preceding sexual differentiation. A similar pattern of sexual dimorphic effect was demonstrated with regards to the key genes involved in mouse brain development, which suggests that genetic factors may play a key role in differential expression of sex-specific genes (Dewing *et al*., 2003). In conclusion, lower *STAR*, *CYP11A1* and *MR* in females suggests the existence of early androgen sensitive window in the adrenal gland, which permits masculinization of the adrenal via testicular androgens. This then implies existence of a male and female adrenal.

5.5.3 Prenatal androgen excess: male fetal adrenal glands

Direct fetal injection of TP had no effect on any of the GOI's analysed in male adrenal glands assayed at d90 of gestation. The possible reason for such lack of effects, given that androgen receptors are expressed, could be pre-exposure to testicular androgens from d30 (Quirke *et al*., 2001). As described above, testicular steroidogenic enzyme expression was decreased in fetal testis from midgestational androgen–excess manipulated pregnancies (Connolly *et al*., 2013). However, this was likely due to suppression of LH, and thus lack of trophic support for the testes, as at this stage of development there appears to be a functional HPG axis. There is no functional HPA axis during midgestation, since ACTH secretion is not activated until later in gestation (Challis and Brooks, 1989). Hence there is unaltered functional HPA and trophic support during midgestation, which could be the possible reason behind the apparent lack of effects in males.

5.5.4 Prenatal androgen excess: female fetal adrenal glands

MR was the only gene which was significantly decreased by excess prenatal androgen exposure in female fetal adrenal. Since *MR* was also lower in males than females, and also decreased by early androgenisation at d30 in females, this suggests that *MR* remains responsive to androgens throughout gestation. However, the lack of effect of midgestational androgen excess on *STAR* and *CYP11A1* in female fetal adrenal, suggests that these genes were only sensitive to excess androgens earlier in gestation (d30). Hence these data tentatively suggest a window of sensitivity to androgens in female fetal adrenal, which was operant at d30 and ended by d62 of gestation.

5.5.5 Prenatal estrogen excess: male fetal adrenal glands

ACTH-R and *STAR* mRNA were increased by excess prenatal estrogen exposure in male fetal adrenal. Whilst it is known that estrogens can increase ACTH secretion (C. E. Wood and Saoud, 1997), resulting in increased *STAR* (at least in rats) (Lehoux *et al*., 1998), the increased *ACTH-R* in males in this study is novel and may be indicative of increased adrenal ACTH sensitivity. Wood and Saoud (1997) have demonstrated that ovine fetuses exposed to 17-β estradiol in the last month of gestation led to significantly increased fetal plasma ACTH and cortisol concentrations, which in turn led to parturition 4 days prior to term (term ~ 147 days). As ACTH secretion is active in the last month of gestation in sheep (Challis and Brooks., 1989), the above increased fetal plasma cortisol and ACTH concentrations in response to estrogens (C. E. Wood et al., 1997) may play a key role in parturition by altering HPA axis activity near to term. However, given the lack of ACTH during midgestation, the functional significance of increased ACTH sensitivity in the current study is perhaps lesser than it would be at the end of gestation. Nonetheless, in the present study, it can be speculated that, if excess estrogens were to be continued till end of gestation, driving increased ACTH sensitivity, then coupled with the potential for estrogen induced increased ACTH, this could drive towards increased cortisol secretion, which may have implications in

terms of timing of parturition (Wood and Saoud., 1997). Finally, decreased *HSD17B* in response to excess estrogens leads to speculation that there may be a lowered capacity for androgen synthesis, but again, as the effects are during quiescent period, it is difficult to place too much emphasis on this finding.

5.5.6 Prenatal estrogen excess: female fetal adrenal glands

STAR, CYP11B1, GR and ERβ were increased by midgestational excess estrogen exposure in females. Increased *STAR* and *CYP11B1* suggests increased steroidogenic potential, possibly in the direction of glucocorticoid synthesis, however, once again, functional relevance is unknown due to the fact that adrenal remains quiescent at this stage of development, unlike later in gestation where excess estrogens aid in parturition in sheep (Wood and Saoud., 1997). Increased *GR* suggests increased glucocorticoid sensitivity driven by excess estrogens, and in support of this are observations made in breast cancer cells where estrogens are known to regulate *GR* mRNA (Krishnan *et al*., 2001). Increased *ERβ* in female fetal adrenal in this study suggests direct effect of estrogens, hence increased sensitivity to excess prenatal estrogens in female adrenal at this stage of development. *ERβ* is also expressed during both mid and late gestation in primate fetal adrenal gland (Albrecht *et al*., 1999). It is also worth noting here that, during late gestation in sheep, estrogen is produced by the placenta at the expense of progesterone due to 'fetal glucocorticoid surge', which induces placental *CYP17* expression and thus permits forward placental metabolism to estradiol (Anderson *et al*., 1975). The resultant increased estrogens in turn activate placental prostaglandin (PG) synthesis, which leads to myometrial contraction and initiation of parturition (McLaren *et al*., 2000). However, as the fetal adrenal gland is 'quiescent' during midgestation, the functional consequences are less when compared to the effects seen towards the end of gestation.

5.5.7 Prenatal glucocorticoid excess: male fetal adrenal glands

Increased *ACTH-R, STAR, CYP11A1, HSD3B, CYP17, HSD11B1, SRD5A1, SRD5A2,* suggests altered adrenal steroidogenic potential and increased *GR, MR, ERα, ERβ* and *AR,* suggest altered receptivity to glucocorticoids, mineralocorticoids, estrogens and androgens, respectively in male fetal adrenals from glucocorticoid excess exposed pregnancies. Previous studies have demonstrated that gestational (d40) exposure to dexamethasone resulted in elevated *ACTH-R* and *STAR* in male sheep offspring at 7 months old (S. Li et al., 2012) and increased *CYP11A1* at d140 gestation (Braun et al., 2009b). *HSD3B* and *CYP17* were significantly elevated in male offspring from early glucocorticoid manipulated pregnancies (S. Li et al., 2012). Collectively, then, it appears that *ACTH-R*, *STAR, CYP11A1, HSD3B and CYP17* are sensitive to excess prenatal dexamethasone exposure during both early and midgestation. This has important consequences for the current study, as this previous work indicates that alterations in these genes during fetal life by glucocorticoid excess can have postnatal consequences for male offspring (S. Li et al., 2012). Such potential consequences will be examined in the next chapter of this thesis. Elevated *HSD11B1* suggests potential for increased conversion of inactive cortisone to active cortisol (Michael *et al*., 2003), however functional relevance is unknown. Although, given the function of the placenta to deactivate the majority of maternal cortisol to cortisone via 11βHSD-2 (Michael *et al*., 2003), this would appear to work against the placental barrier and hence could potentially, in the presence of inactive glucocorticoids of maternal origins, reverse the placental effects of glucocorticoids inactivation. This could then increase local adrenal cortisol concentrations, which could then 'feed-forward' by mimicking the exogenous dexamethasone effects noted here, amplifying glucocorticoid activity via increased regeneration of maternal glucocorticoids combined with increased sensitivity to glucocorticoids via increased *GR*. This is the first study to demonstrate increased *SRD5A1, SRD5A2, ERα* and *AR* in male adrenals as a result of prenatal glucocorticoid exposure. Increased *MR* by excess

glucocorticoid exposure demonstrates *MR* is sensitive to glucocorticoids during midgestation, however functional relevance is unknown. As *GR* expression is already higher in males compared to females and further exposure to prenatal excess glucocorticoids leading to increased *GR* during midgestation, this clearly shows that *GR* gene expression is sensitive to glucocorticoids throughout gestation.

5.5.8 Prenatal glucocorticoid excess: female fetal adrenal glands

STAR, SRD5A2 and *GR* were significantly increased by excess glucocorticoid exposure in female fetal adrenals. As the same genes were increased in males, this suggests that there were no gender differences in terms of expression of these adrenal genes in response to prenatal glucocorticoid exposure. The major finding of this study was that there were fewer adrenal genes altered in females when compared to males in response to excess prenatal glucocorticoid exposure, indicative of the female adrenals relative insensitivity to glucocorticoids as compared to the male, evidenced by the relatively low GR expression in females as compared to males. Even though *GR* was increased in both male and female fetal adrenal in response to excess glucocorticoids, basal *GR* expression was much higher in males (~3 fold increase) than in females. Similarly, the increased *GR* observed in females, whilst significant was much less than that observed in males in response to dexamethasone. Since female *GR* expression could not be 'masculinised' by early androgenisation, this suggested that in males that high GR expression likely precedes sexual differentiation i.e was not dependent upon testicular androgens. The sex-specific, male predominant *GR* expression may be the possible reason behind the male fetal adrenal being apparently more sensitive to midgestational prenatal glucocorticoid exposure than the female adrenal, and hence the increased adrenal steroidogenic gene expression in male fetal adrenal in response to dexamethasone. A potential mechanism for the differential expression of *GR* could be via gender-specific co-activators and transcription factors, for example gender specific TATA- box binding protein was higher in adult male rat liver compared to females in

response to glucocorticoids (Duma *et al*., 2010). Moreover, the dimorphic expression of RNA splicing, microRNAs, RNA stability, and posttranslational modifications of *GR* (Duma *et al*., 2010) could be also leading to the above gender-specific action of glucocorticoids on adrenal steroidogenic gene expression. *GR* transcript expression and the use of GR promoter region were differently expressed within the placentas of male and female fetuses in women during term of pregnancy (R. F. Johnson et al., 2008). This clearly suggests differential responses to increased cortisol concentrations in the placentas associated with male or female fetuses in women. For example, there was increased cortisol secretion in males sheep fetuses compared to females fetuses from prenatal hypoxic stress induced pregnancies (Giussani *et al*., 2011). It should be noted however that in the current study this effect was only noted in the adrenal glands, since as described in Chapter 3, there was no gender difference as regards to fetal pancreatic *GR* expression. Nonetheless, in this study, if the gender differences in responses to prenatal excess glucocorticoid exposure remained later in gestation, it could have implications for the male fetal adrenal in terms of function. Along with stress responses, glucocorticoids are also known to regulate anti-inflammatory actions (Reynolds, 2010). In support of the latter, genome wide microarray data analysis revealed 84 genes involved in inflammation were sex-specifically altered in response to dexamethasone exposure in liver of adult male rats compared to females, suggesting gender specific anti-inflammatory effects of glucocorticoids (Duma *et al*., 2010). This has clinical relevance where synthetic glucocorticoids prescribed for inflammatory disorders may be more potent in males compared to females, however such speculation clearly needs further investigation in humans.

Collectively, this fetal adrenal study has demonstrated a fetal sex-specific effect of prenatal glucocorticoids, likely due to higher expression of *GR*. This suggests that sex hormones (androgens and estrogens) are not the sole contributors of sexually dimorphic effects in both physiological and pathophysiological conditions; instead there are other signaling pathways independent of sex steroids signaling such as glucocorticoids acting via GR. This may have possible clinical implications on HPA axis and function,

metabolic functions such as glucocorticoid mediated hepatic gluconeogenesis and anti-inflammatory actions of glucocorticoids.

5.5.9 Summary and Conclusions

This chapter gives insight to gender differences in fetal adrenal development, and responses to exogenous steroids. The data collected provides some evidence of apparent windows of sensitivity of adrenal steroidogenic genes to different exogenous steroids during development. Whilst most of the gender-specific responses observed are attributable to masculinization effects, in turn attributable to testicular androgen exposure (phenotypical sexual differentiation) in males during early gestation, *GR* expression in males is apparently higher in expression than in females independent of any androgenisation effect. As the fetal adrenal samples in this chapter were collected during a 'quiescent period', it is hard to speculate any functional and health relevant significances from such altered adrenal gene expression. Furthermore, a proportion of the changes in gene expression seen in response to the steroids applied may be contemporary effects, which resolve upon cessation of the steroid excess, similar to that observed in fetal testis (Connolly *et al*., 2013). Therefore, examination of adrenal glands in terms of function in offspring from these steroid manipulated pregnancies is essential, which should provide insight of understanding possible postnatal implications.

Chapter 6 The effects of direct prenatal steroidal excess on adult adrenal function

6.0 Introduction

In chapter 5, the effects of direct fetal exposure to androgen, estrogen and glucocorticoid excess *in utero* (TP, DES and DEX respectively) on adrenal development were examined. As a result of the fetal DES and DEX treatment, genes involved in fetal adrenal steroidogenic pathway were altered in females and males, respectively. However, it was DEX treatment in males that exhibited profound effects in terms of adrenal steroidogenic gene expression. Given the focus in PCOS research on androgenic programming, it was perhaps a little surprising that there were so few effects of androgenic excess in female fetal adrenal glands. It was concluded that there were sex-specific and steroid specific effects on the developing fetal adrenal. Whilst such studies can invariably lead to speculation as to what the postnatal legacy of such altered *in utero* environments could be, in order to definitively address such speculation a follow up study on postnatal adrenals was necessary to determine any long-term functional, and hence health-relevant legacy of fetal excess steroid exposure. The adrenal gland secretes steroids such as glucocorticoids and androgens in response to ACTH released by the pituitary gland and is a major source of androgen in addition to gonads. Adrenal DHEA, DHEAS, androstenedione and testosterone accounts for approximately about 50% of androgen load in women (Burger, 2002). This is important in clinical conditions such as PCOS, where women suffer from excess androgen secretion from both ovary and adrenal (Azziz *et al*., 1998; Rosenfield, 1999). There is also evidence from animal models that fetal exposure to excess androgens could lead to excess adrenal androgen secretion in terms of increased basal DHEA and ACTH stimulated DHEA and androstenedione during adulthood (Zhou *et al*., 2005) and increased ACTH stimulated corticosterone production is certainly evident in non-human primate PCOS animal models (Zhou *et al*., 2005), suggestive of altered HPA axis activity.

The aim of this chapter was to assess adrenal steroidogenic gene expression and adrenal function in female adult offspring adrenal gland derived from steroid manipulated pregnancies. We also assessed adrenal gene expression

and function in lambs from steroid manipulated pregnancies to determine whether or not any effects seen in adults might develop pre or post puberty. Steroidogenic gene expression was measured using qRT-PCR in lamb and adult offspring. Adrenal function was assessed by measuring ACTH-analogue (Synacthen) stimulated production of cortisol and testosterone in lamb and adult offspring from steroid manipulated pregnancies.

6.1 Materials and methods

6.1.1 Animal Treatment and Husbandry

Animal husbandry is as detailed in section 2.1-2.1.1 and treatment regime of pregnant ewes was followed as explained in section 2.1.2. Table 6.1 details the postnatal animals used in this study.

Analysis	Postnatal Stage	Sample Number (n)
Lamb (pre-pubertal)		
RNA (qRT-PCR)	Female	C=6; TP=6(20mg) DES=5 (50μg); DEX=4 (100μg)
	Male	C=6; TP=6 (20mg) DES=6(50μg); DEX=10(100μg)
Functional Analysis	Female	C=4; TP=11(20mg) DES=7(50μg); DEX=5(100μg)
	Male	C=6;DES=6(50μg); DEX=6(100μg)
Adult (Post-pubertal Adolescence)		
RNA (qRT-PCR)	Females	C=6;TP=6 (20mg) C=4;DES=6(50μg); DEX=6 (100μg)
Functional Analysis	Females	C=7; TP=13(20mg) DES=8(50μg); DEX=10(100μg)

Table 6.1 The experimental animals included in the assessment of postnatal adrenal gland gene expression and function, treatment regime and sample numbers.

6.1.2 Quantitative real time PCR

Genes coding for adrenal steroidogenic enzymes and steroid receptors were assessed in adrenal glands of both lamb and adult offspring from steroid manipulated pregnancies. The SYBR qRT-PCR protocol is detailed in section

2.3.9. The primer details for each gene are as detailed in table 5.2, section 5.2.3.

6.1.3 Synacthen test to measure adrenal function

To assess adrenal function a Synacthen test was performed at approximately 10 weeks and 10.5 months of age on ewes exposed to prenatal steroids via the FI route. Synacthen is a synthetic analogue of endogenous pituitary secreted ACTH and is routinely used to investigate adrenal insufficiency in clinical medicine. Animals were fasted on the day of the test, which was carried out during late morning in groups of 8-10 animals at a time. The animals within treatment cohorts were randomly mixed in these test groups and all tests occurred at the same time of day to avoid any distortion of data due to circadian rhythm. A basal blood sample was drawn from the jugular vein prior to administration of a 2ml intravascular dose of Synacthen (50µg/ml in saline; Alliance Pharmaceuticals Ltd., Wiltshire, UK) and subsequent sampling occurred 15 and 30 min post-stimulation. Blood was decanted into heparin containing test-tubes, spun at 3000 rpm at 4°C for 15 min, and the supernatant (plasma) stored at -20°C. Trained animal husbandry staff and Drs Duncan and Rae performed all blood sampling.

6.1.4 Enzyme Immunoassay

6.1.5 Serum Extraction

This step was identical for both ELISA and RIA experiments. To separate steroids from binding proteins present in plasma samples and thereby to maintain accuracy of the assay, sheep plasma samples were extracted along with standards and controls (high, medium and low) as detailed in section 2.6.1.

6.1.6 Enzyme linked immunosorbent assay (ELISA) – Cortisol

Enzyme linked immunosorbent assay (ELISA) works on the principle of antigen-antibody binding principle to detect the presence of proteins in the samples. The work in this study optimized a cortisol ELISA protocol to measure cortisol in sheep serum. The detailed report of the assay optimization is explained in section 2.7.8.

6.1.7 Radioimmunoassay

The RIA protocol is detailed in section 2.6.2 and was used to assess testosterone concentrations in sheep plasma. Intra and inter assay co-efficient of variation (CVs) were determined by the quality control samples included in each assay and were 8.7% and 7.9% respectively. Hormone measured and specific antibodies used are detailed in Table 6.2.

Hormone	**Primary Antibody**	**Tracer (^{125}I labelled)**	**Standards**	**Intra and Inter assay CVs(%)**
Testosterone	Rabbit anti-testosterone-19; AMS Biotechnology. Oxfordshire, UK	Testosterone ^{125}I,MP Biochemicals, France	Testosterone (T1268), Sigma Aldrich	8.7%, 7.9%

Table 6.2 Antibody information used to measure plasma testosterone by RIA- listed are the primary antibody, tracer and standards.

6.1.8 Statistical Analysis

Graphpad Prism (v.6.0) was utilized for all data analyses and transformations. Student's unpaired two-tailed t-test was performed on gene expression data to analyze the difference between control and treatment groups, with log transformation (log base 10) was carried out where the data showed unequal variances. One- way ANOVA followed by Tukey's post hoc test was performed on values obtained from Synacthen treated plasma samples from 0, 15, 30 time points in control and treatment groups separately and unpaired

t-test was carried out comparing each time point separately between control and treatment groups. Area under the curve (AUC) was calculated to analyse the overall secretion of hormones over the duration of the Synacthcen challenge between control and treatment groups, and an unpaired t-test performed on this data. In all cases, data was considered significant where $P<0.05$.

6.2. Results

In order to examine the effects of direct fetal steroid exposure on adult adrenal steroidogenic gene expression at the molecular level, genes coding for enzymes involved in adrenal steroidogenesis such as *STAR, ACTH-R, CYP11A1, CYP17, CYP21A1, HSD3B, HSD11B1, HSD11B2, HSD17B, CYP11B1, SRD5A1, SRD5A2* and steroid receptors such as *AR* and *GR* were assessed.

6.2.1 FI-TP had different effects compared to FI-DES treatment on female adult adrenal (11 months) steroidogenic gene expression

STAR mRNA expression was significantly increased by FI-TP treatment (P<0.01; Figure 6.1.A) but not by FI-DES (Figure 6.2.A) in female adult animals compared to control animals. *ACTH-R* and *CYP11A1* mRNA expression was unaltered by TP, however *ACTH-R* was significantly decreased by DES treatment (P<0.001; Figure 6.2.B and Figure 6.2.C respectively) compared to controls. *CYP21A* and *HSD3B* mRNA abundance was significantly increased by FI-TP (P<0.05, Figure 6.1.E and Figure 6.1.F respectively) treatment but not by FI-DES treatment compared to controls. *HSD11B1* mRNA expression was significantly increased by FI-TP treatment only (P<0.05; Figure 6.1.G), however *HSD11B2* expression was unaltered by either of the treatment groups. *CYP11B1* mRNA abundance was significantly increased by FI-TP treatment only (P<0.001; Figure 6.1.I) compared to controls. *HSD17B* mRNA abundance was significantly increased by FI-TP treatment (P<0.01; Figure 6.1.J), but was significantly decreased by FI-DES treatment (P<0.01; Figure 6.2.J). Finally, FI-TP treatment had no effect on *SRD5A1* mRNA expression, however it was significantly decreased by FI-DES treatment (P<0.001; Figure 6.2.K) in female adult offspring compared to controls.

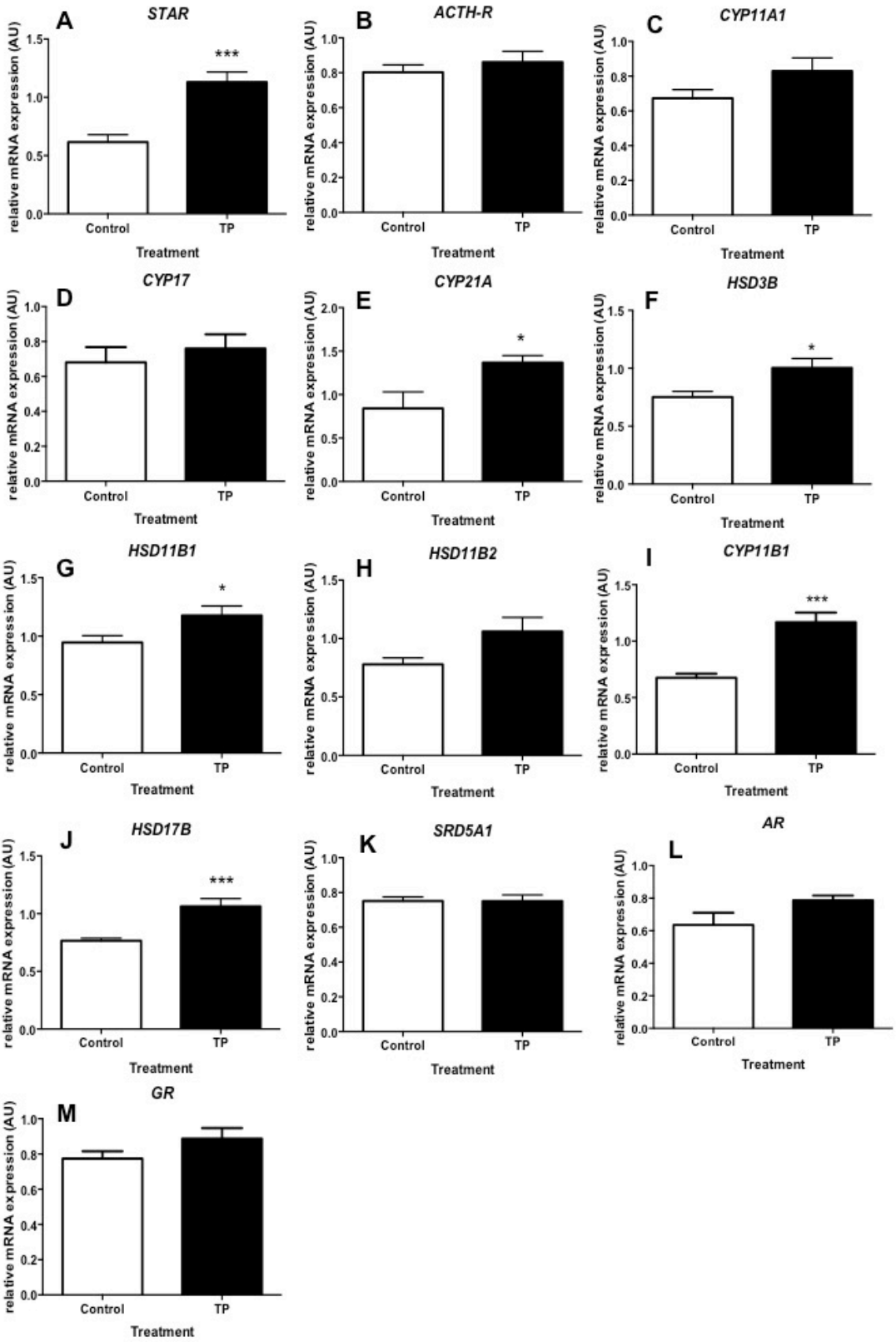

FIGURE 6.1. EFFECT OF DIRECT FETAL EXPOSURE TO TP ON EXPRESSION OF GENES CODING FOR STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, SRD5A1, SRD5A2, CYP11B1, HSD11B1, HSD11B2, HSD17B, AR AND GR IN ADULT FEMALE ADRENAL.
(Control n=6, TP n=6). Values represent mean ±SEM, * $P<0.05$, *** $P<0.01$.

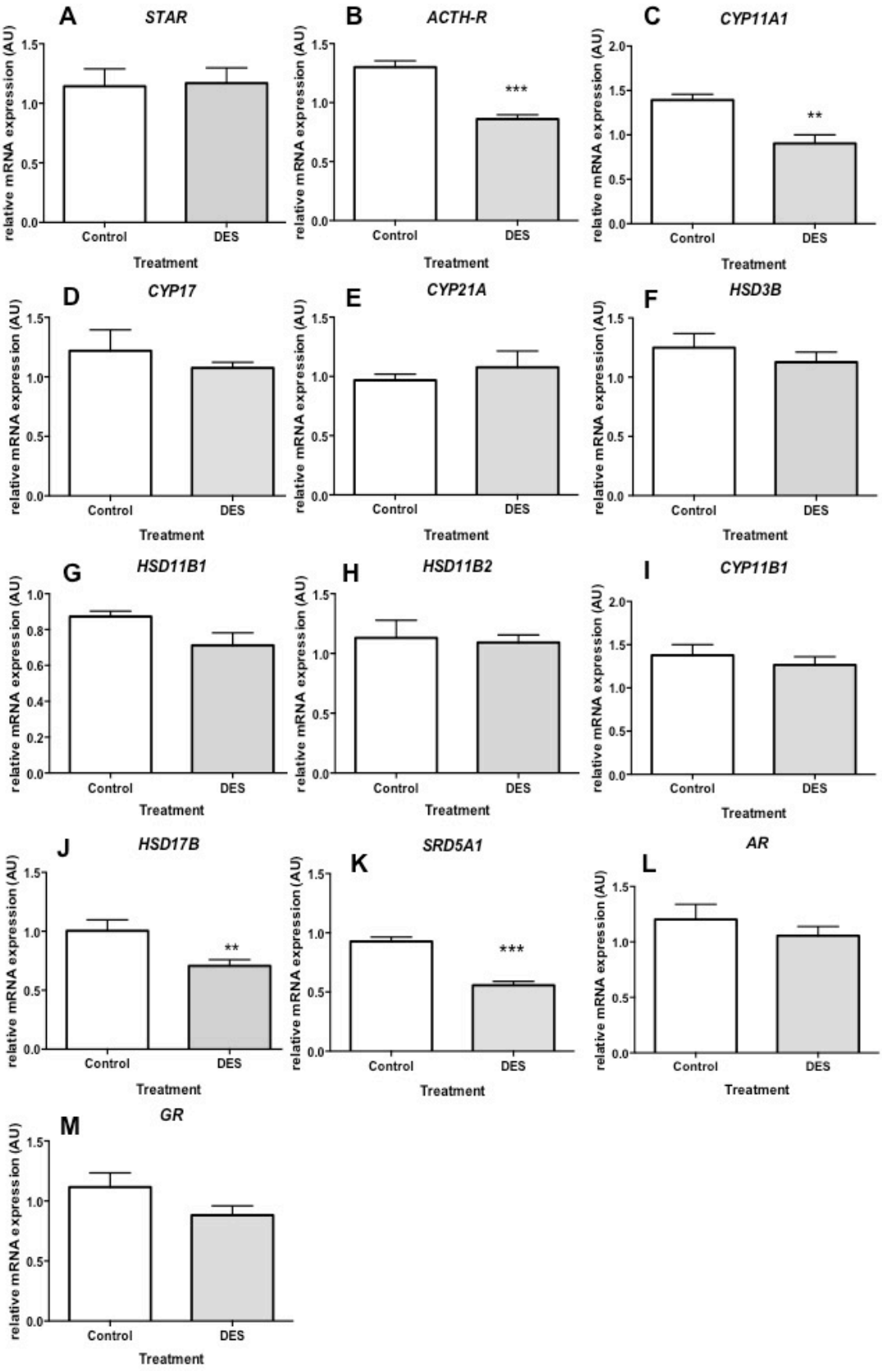

FIGURE 6.2. EFFECT OF DIRECT FETAL EXPOSURE TO DES ON EXPRESSION OF GENES CODING FOR STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, SRD5A1, SRD5A2, CYP11B1, HSD11B1, HSD11B2, HSD17B, AR AND GR IN ADULT FEMALE ADRENAL.

(Control n=4, DES n=6). Values represent mean ±SEM. * *P*<0.05, ***P*<0.01, ****P*<0.001.

6.2.2 Effects of direct prenatal exposure to DEX treatment on female adult sheep (11 months) steroidogenic gene expression

Direct fetal steroid exposure to DEX treatment significantly decreased the mRNA expression of *SRD5A1* (*P<0.01*; Figure 6.3.K) in the female adult adrenal, compared to controls, however, there was no change in any of the other genes assessed between control and FI-DEX treated group.

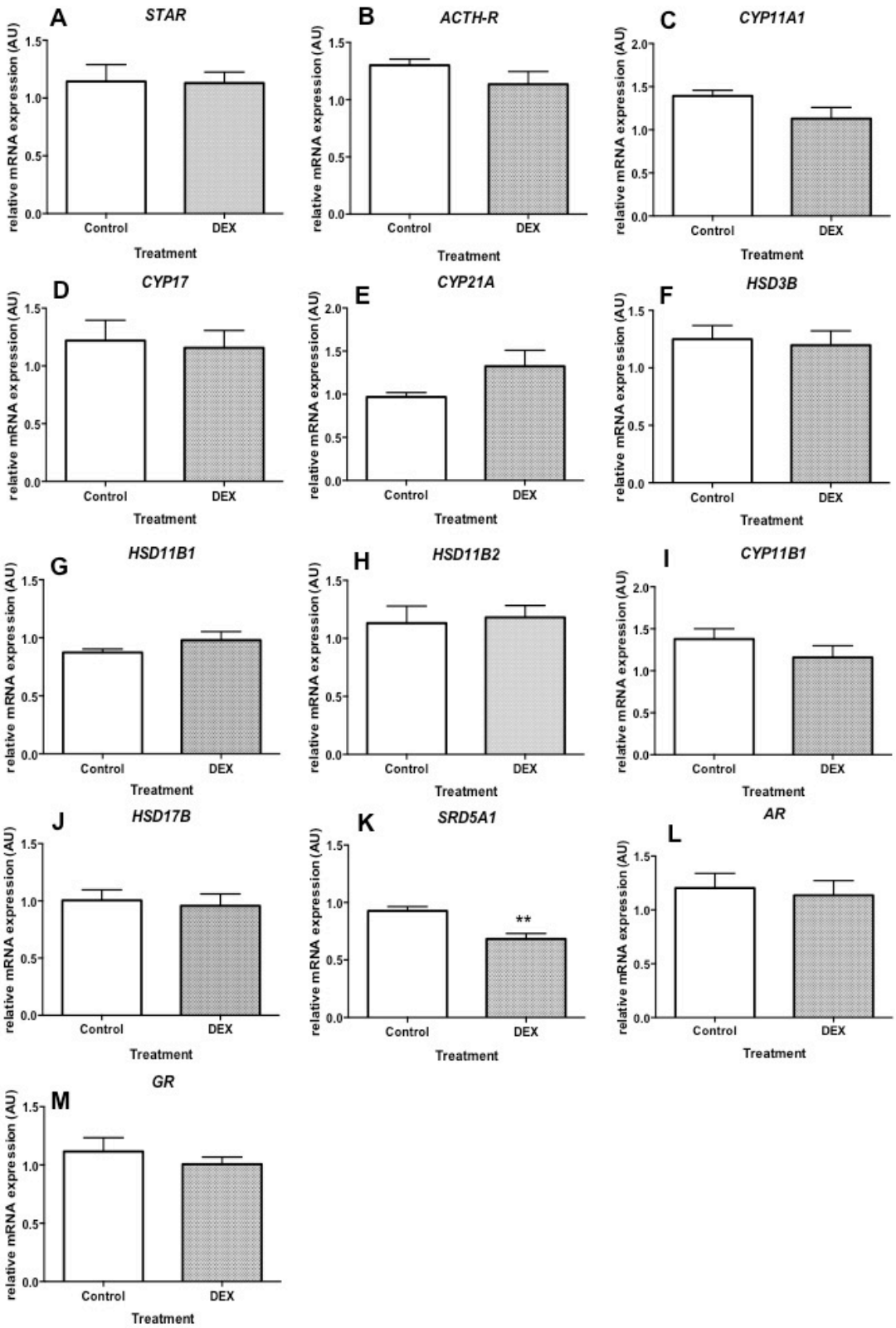

FIGURE 6.3. EFFECT OF DIRECT FETAL EXPOSURE TO DEX ON EXPRESSION OF GENES CODING FOR STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, SRD5A1, SRD5A2, CYP11B1, HSD11B1, HSD11B2, HSD17B, AR AND GR IN ADULT FEMALE ADRENAL.
(Control n=4, DEX n=6). Values represent mean ±SEM, $^{**}P<0.01$.

6.3.1 Cortisol secretion was unaltered in female adult offspring (post-pubertal) (11 months) by FI-TP or FI-DES

Synacthen challenge was performed in order to examine the adrenal gland function in the adult offspring from fetal steroid manipulated pregnancies.

There was a significant increase in cortisol secretion at 30 min (P<0.05) post Synacthen stimulation in control group and both at 15 min (P<0.01) and 30 min (P<0.01) post stimulation in TP treated adult females compared to basal levels (0), however, there was no significant difference in terms of the magnitude of response between control and TP treatment (Figure 6.4.A and B). Similarly, there was a significant increase in cortisol secretion at 30 min (P<0.01) post Synacthen stimulation in DES treated animals compared to 0 time point (Figure 6.4.C), however the difference at each individual time point between control and DES was not significant, and similarly total cortisol ($AUC_{cortisol}$) secretion between control and DES treatment groups was not different (Figure 6.4.D).

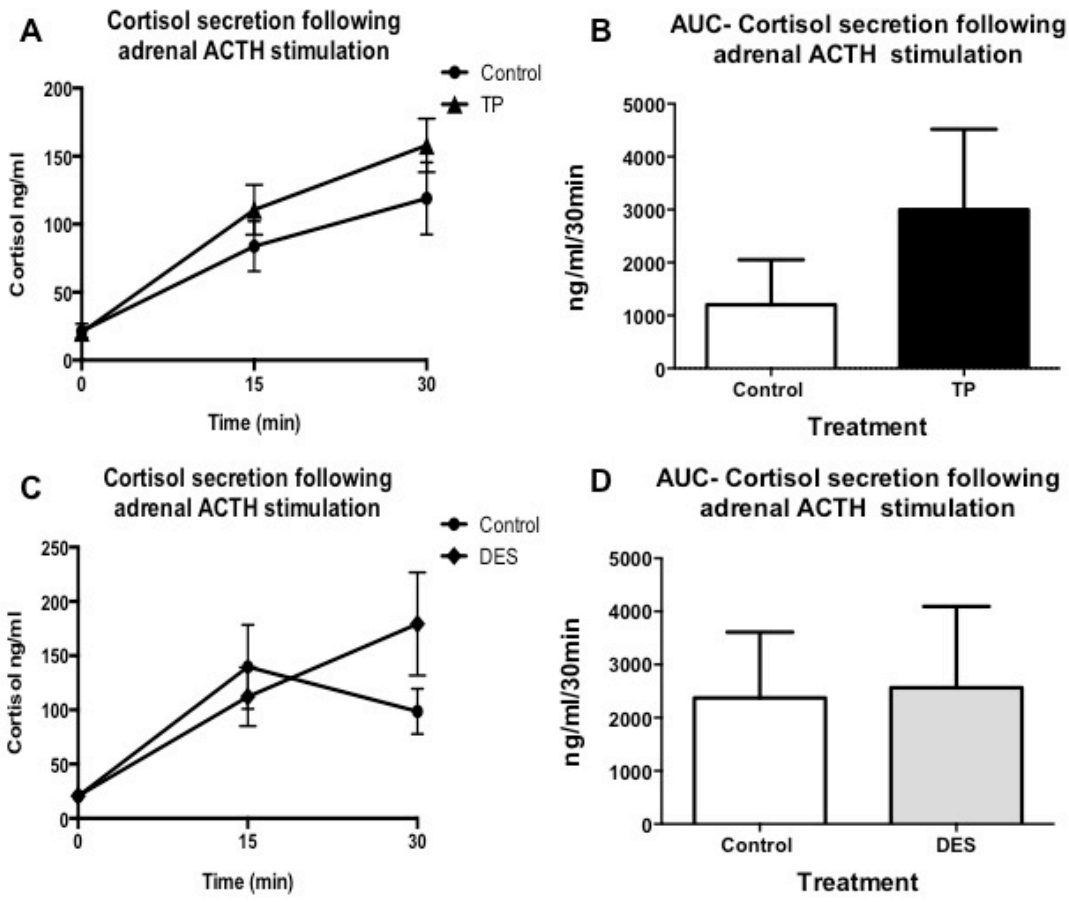

Figure 6.4 Cortisol secretion following adrenal stimulation in female adult exposed prenatally to different steroids.

(A). Control n=7; TP n=13, (C) Control, n=4 vs DES, n=7. Area under the curve (AUC) for total cortisol secreted in female lamb pre and post ACTH stimulation between control and treatment groups. (B). Control vs TP; (D) Control vs DES.

6.3.2 Cortisol secretion was unaltered in female adult offspring (post-pubertal) (11 months) due to FI-DEX

There was an increase in cortisol secretion at 15 min (P<0.05) in control cohort and both at 15 (P<0.01) and 30 (P<0.01) (Figure 6.4.1.A) min post ACTH stimulation in the DEX treated group compared to the basal levels (0), however there was no difference between control and DEX at each individual

time points, which was reflected in the overall cortisol secretion ($AUC_{cortisol}$) between control and DEX treatment being unaltered (Figure 6.4.1.B).

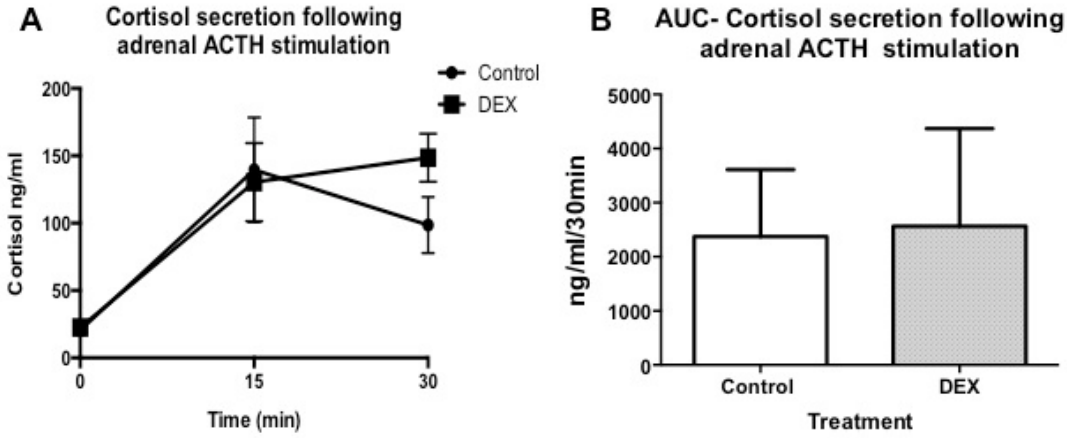

Figure 6.4.1 (A) Cortisol secretion following adrenal stimulation in female adult exposed to Fi-DEX treatment.
Control n=4; DEX n=11. (B) Area under the curve (AUC) for total cortisol secreted in female lamb pre and post ACTH stimulation between control and DEX group.

6.3.3 FI-TP (but not FI-DES) exposure altered testosterone secretion in female adult (11 months)

There was no significant increase in testosterone secretion at 0, 15, 30 min time intervals in response to Synacthen stimulation in the control (vehicle) animals, however there was a trend towards increased secretion at 15 minutes post-stimulation. On the other hand, there was a marked, and prolonged significant increase at 15 (P<0.05) and 30 (P<0.01) (Figure 6.4.2.A) minutes post Synacthen stimulation compared to basal levels (0) in the FI-TP treated animals. This exaggerated increase in testosterone response in the FI-TP treated (P<0.001) (Figure 6.4.2.A) cohort at 30 min post ACTH stimulation compared to controls was reflected in the total area (AUC) measured showing a significant increase (P<0.001; Figure 6.4.2.B) in overall testosterone ($AUC_{testosterone}$) production in the TP treated animals compared to controls.

Synacthen stimulation in prenatally DES treated animals showed a significant increase in testosterone concentrations at 15 (P<0.05) and 30 minutes (P<0.01) (Figure 6.4.2.C) post stimulation compared to basal levels, however there was no difference in the testosterone response at different time intervals (Figure 6.4.2.D) or in overall testosterone production (Figure 6.4.2.D), when compared to control animals.

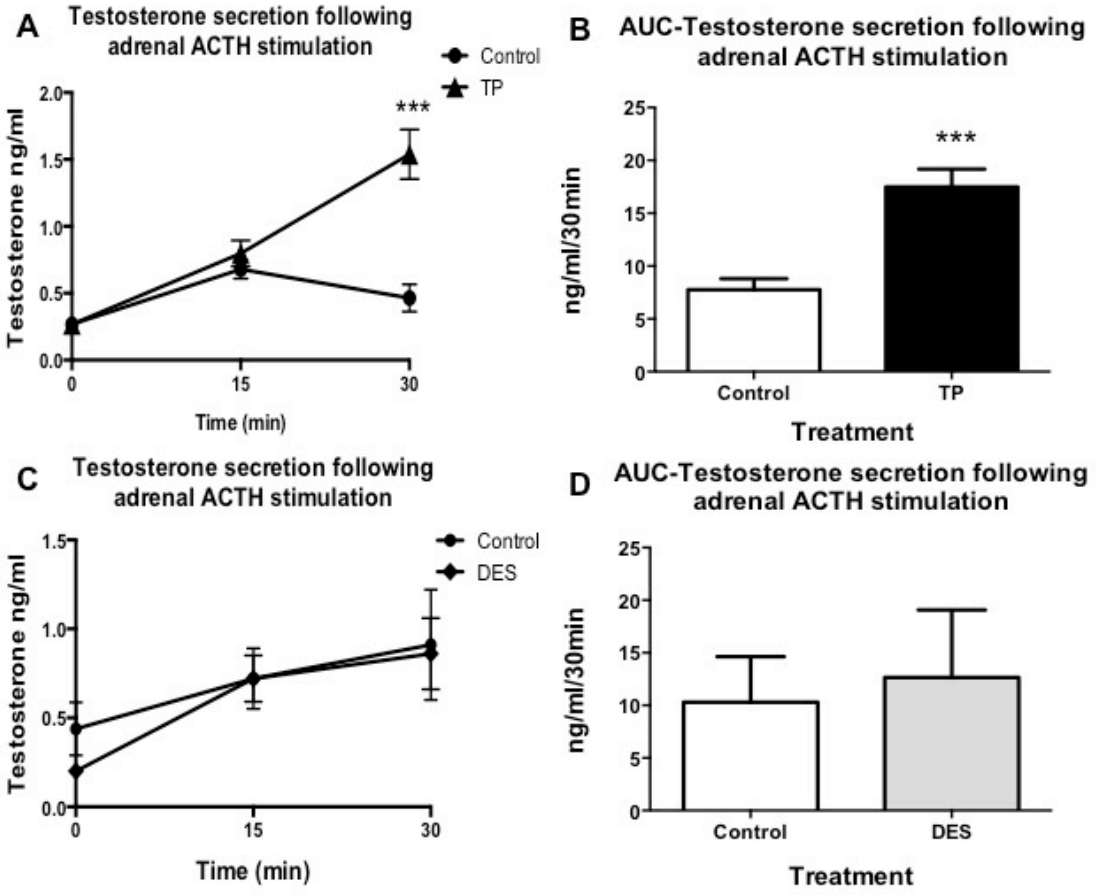

Figure 6.4.2. Testosterone secretion following adrenal stimulation in female adult exposed prenatally to different steroids.

(A). Control n=7; TP n=13, (C) Control, n=4; DES, n=7. Area under the curve (AUC) for total testosterone secreted in female adult pre and post ACTH stimulation between control and treatment groups. (B). Control vs TP; (D) Control vs DES. ***P<0.01

6.3.4 FI-DEX exposure had no effect on adrenal testosterone secretion in female adult offspring

There was no difference in testosterone response at different time intervals in both control and DEX treated animals compared to basal levels, however there was a trend towards increases at 15 and 30 min post-stimulation as compared to basal concentrations. Furthermore, there was an apparent trend towards a decrease in testosterone response in DEX treated animals (Figure 6.4.3.A), but was not statistically significant (Figure 6.4.3.B) when compared to control animals.

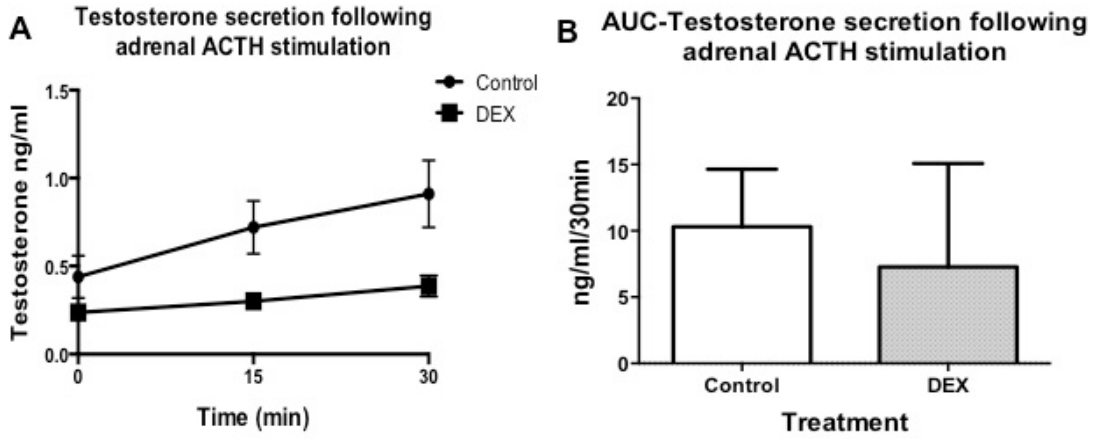

Figure 6.4.3. (A) Testosterone secretion following adrenal stimulation in female adult exposed to FI-DEX treatment.
Control n=4; DEX, n=11. (B) Area under the curve (AUC) for total cortisol secreted in female lamb pre and post ACTH stimulation between control and DEX group.

6.4 Prepubertal lamb adrenal steroidogenic gene expression

We earlier observed in this study that FI-TP treatment, in adult female offspring, was associated with increased expression of key genes encoding for enzymes involved in the adrenal steroidogenic pathway and also elevated testosterone secretion in response to adrenal stimulation. In order to investigate whether there is a developmental period during which the above adrenal phenotype might arise in females due to FI-TP exposure, we studied

only FI-TP treated female lambs (3 months old) by assessing only those genes that were altered by FI-TP in female adults, however functional analysis was assessed in female lambs from FI-TP, FI-DES and FI-DEX exposed pregnancies.

6.4.1 FI-TP exposure had no effect on female lamb adrenal steroidogenic gene expression

Apart from *CYP11B1*, which was significantly decreased by FI-TP exposure (*P*<0.05; Figure 6.5.G), none of the other genes analysed showed any effect of prenatal TP exposure in the female lamb adrenal at 12 weeks postnatal life.

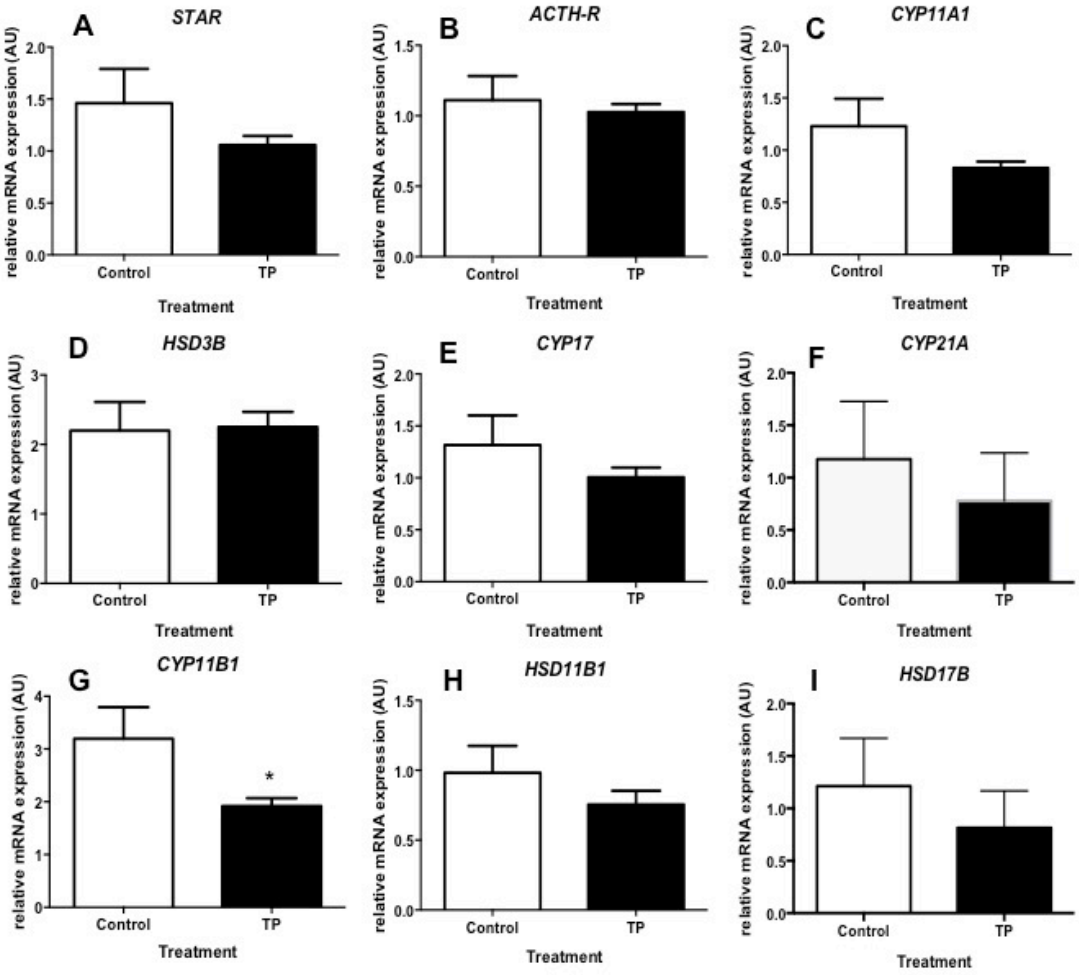

Figure 6.5. Effect of direct fetal exposure to TP on expression of genes coding for STAR, ACTH-R, CYP11A1, HSD3B, CYP17, CYP21A, SRD5A1, SRD5A2, CYP11B1, HSD11B1, HSD17B in female lamb adrenal.
(Control n=6, TP n=6). Values represent mean ±SEM, * *P*<0.05

6.4.2 Effects of direct prenatal exposure to exogenous steroids on cortisol secretion in female lambs (prepubertal) (3 months old)

There was a significant increase in cortisol secretion at 30 min post stimulation (P<0.05) (Figure 6.5.1.A) in the control group and a significant increase at 15 (P<0.05) and 30 min (P<0.01) post stimulation in the TP treated compared to the basal levels (0), but there was no significant difference between treatments at each individual time point (0, 15 and 30 minutes), which was confirmed by the overall cortisol secretion ($AUC_{cortisol}$) (Figure 6.5.1.B).

There was a significant increase in cortisol secretion at 30 min (P<0.05) in the control animals and both at 15 min (P<0.05) and 30 min (P<0.05) in DES treated animals compared to their respective basal levels (0) (Figure 6.5.1.C), but there was no significant difference at each individual time point or in terms of overall cortisol secretion ($AUC_{cortisol}$) between control and *in utero* DES treatment animals (Figure 6.5.1.D).

There was a significant increase in cortisol secretion at 30 min (P<0.05) (Figure 6.5.2.A) post stimulation in both control and DEX treated female offspring, but there was no significant difference between control and DEX treated animals in terms of overall cortisol secretion ($AUC_{cortisol}$) (Figure 6.5.2.B).

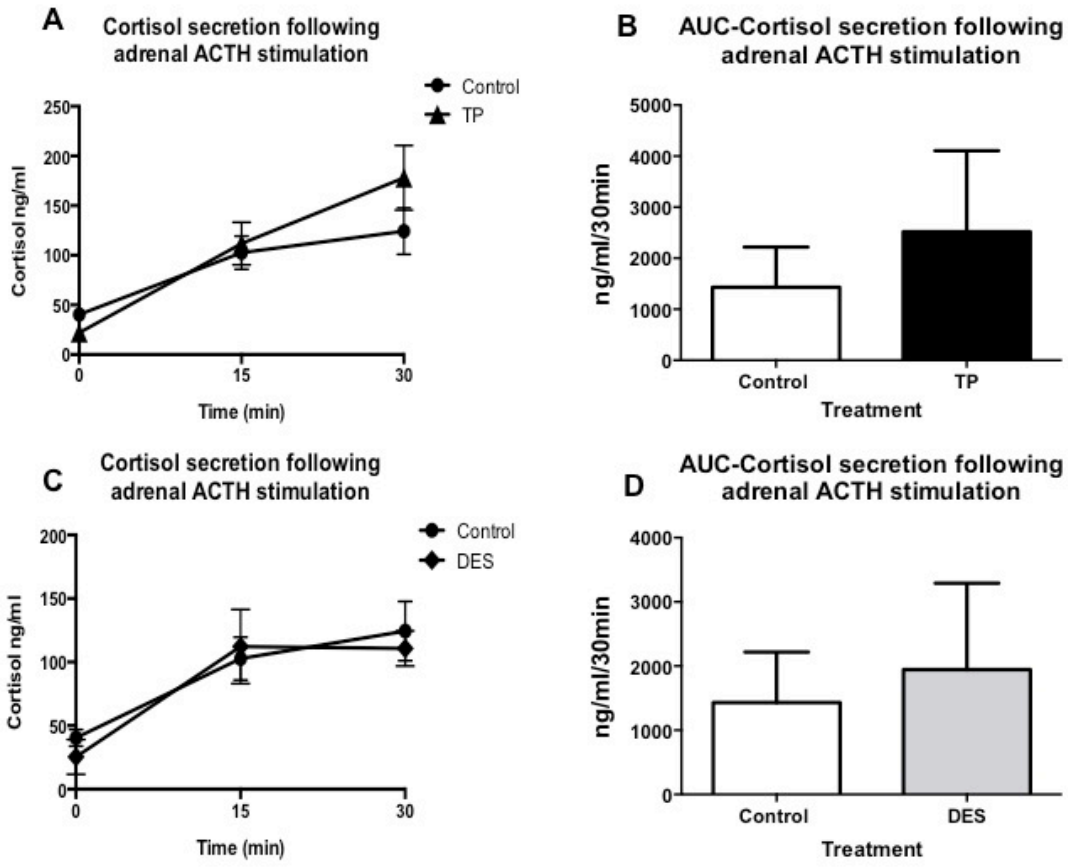

Figure 6.5.1. Cortisol secretion following adrenal stimulation in female lamb exposed prenatally to different steroids.

(A). Control n=4; TP n=10; (C) Control n=4; DES n=7. Area under the curve (AUC) for total cortisol secreted in female lamb pre and post ACTH stimulation between control and treatment groups. (B). Control vs TP; (D) Control vs DES.

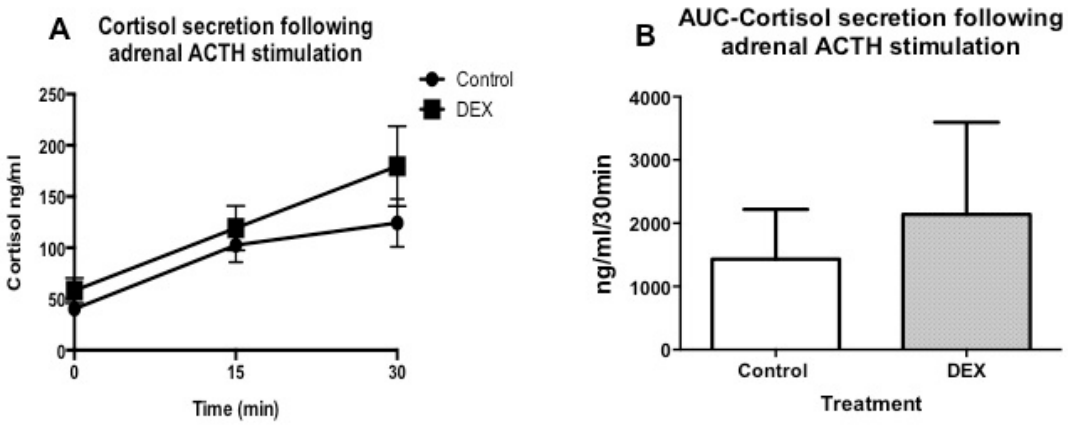

Figure 6.5.2. Cortisol secretion following adrenal stimulation in female lamb exposed to FI-DEX treatment.

(A). Control n=4; DEX n=11; (B) Area under the curve (AUC) for total cortisol secreted in female lamb pre and post ACTH stimulation between control and DEX group.

6.4.3 Effects of direct prenatal exposure to different steroids on testosterone secretion in female lamb (3 month old)

There was no change in testosterone secretion between control and TP or DES treated cohorts post ACTH stimulation (Figure 6.5.3.A, B, C, D).

There was a trend towards a decrease in testosterone response in DEX treated animals, but this was not statistically significant (Figure 6.5.4.A and B).

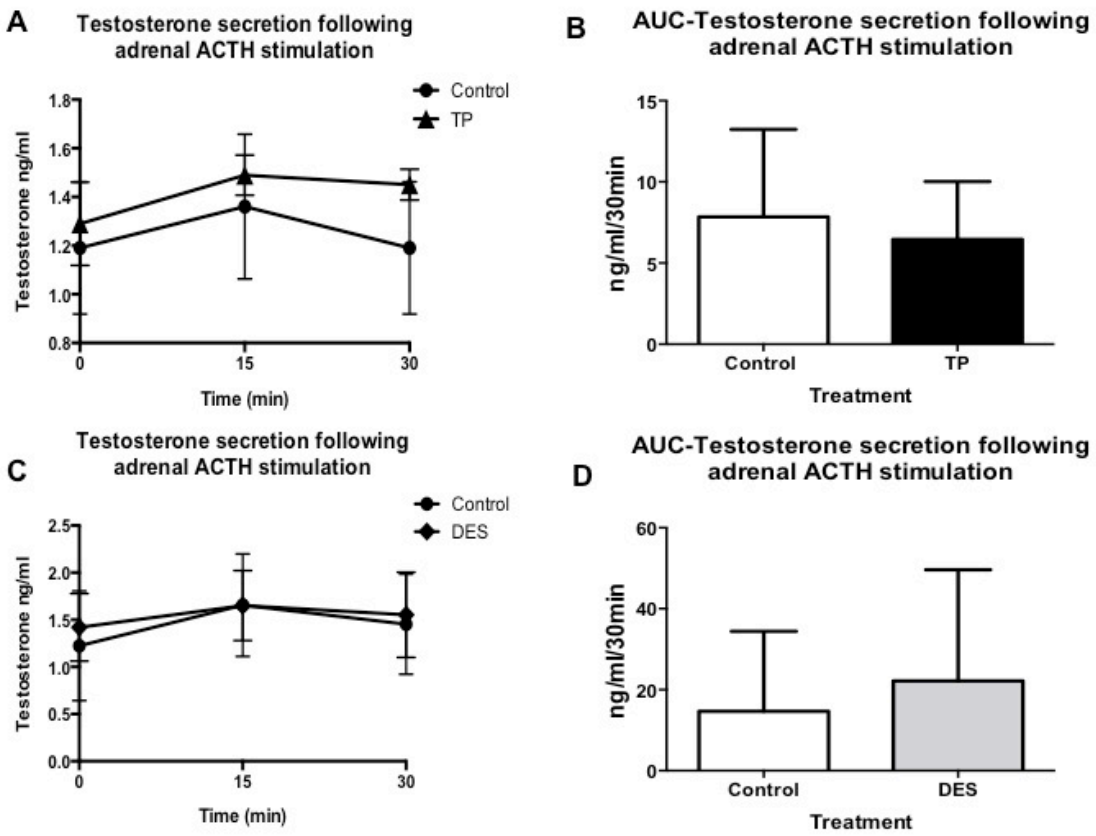

Figure 6.5.3. Testosterone secretion following adrenal stimulation in female lamb exposed prenatally to different steroids.

(A). Adrenal stimulation in FI-TP treated animals, Control n=4; TP n=10, (B). Area under the curve (AUC) for total testosterone secreted in female adult pre and post ACTH stimulation between control and treatment groups. Control vs TP; (C) Adrenal stimulation in FI-DES treated animals Control, n=4; DES, n=7. (D) (D) Area under the curve (AUC) for total testosterone secreted in female adults pre and post ACTH stimulation between control and treatment groups.

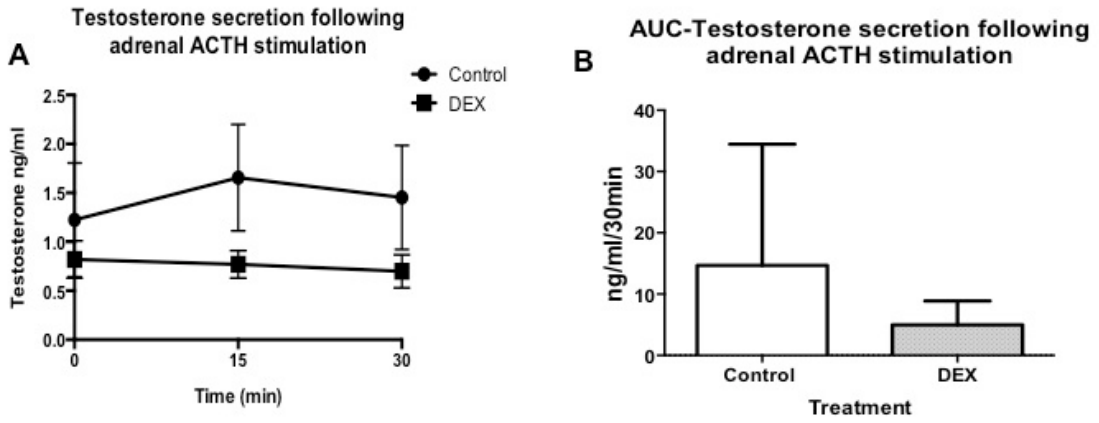

Figure 6.5.4. (A) Testosterone secretion following adrenal stimulation in female lamb exposed to FI-DEX treatment.
Adrenal stimulation in FI-DEX treated animals, Control n=4; DEX, n=11. (B) Area under the curve (AUC) for total testosterone secreted in female lamb pre and post ACTH stimulation between control and DEX group.

6.5 Effects of FI-DES and DEX exposure on male lamb adrenal steroidogenic gene expression

This study had the opportunity to study male lambs (10-12 weeks old), as the male animals from steroid manipulated pregnancies were sacrificed at lamb stage, thereby allowing investigation of any sex specific effects in terms of adrenal gene expression and function. In terms of gene expression, only FI-DES and FI-DEX cohorts were assessed (only tissues available at the time of study), however functional analysis was assessed for all three (FI-TP, FI-DES, FI-DEX) steroidal treatments.

FI-DES treatment resulted in a significant increase in the mRNA abundance levels of *HSD11B1* (P<0.05; Figure 6.6.G), *HSD17B* (*P*<0.05; Figure 6.6.I) and *GR* (*P*<0.05) (Figure 6.6.L) in male lamb adrenals, however, there were no changes in expression levels of the other genes analysed attributable to FI-DES treatment.

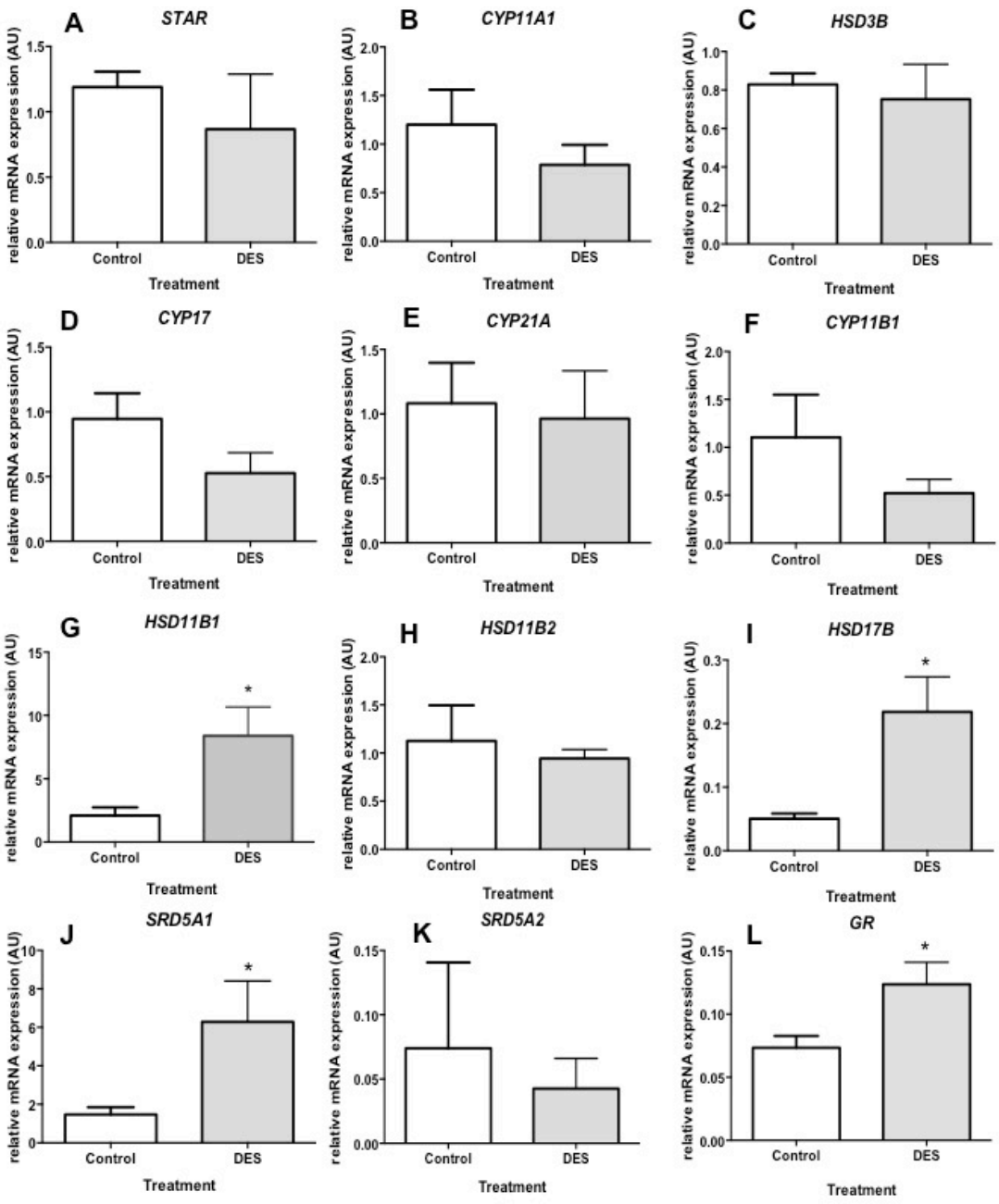

Figure 6.6 Effect of direct fetal exposure to DES on expression of genes coding for STAR, CYP11A1, HSD3B, CYP17, CYP21A, SRD5A1, SRD5A2, CYP11B1, HSD11B2, HSD17B and GR in male lamb adrenal.
(Control n=6, DES n=8). Values represent mean ±SEM. *P<0.05.

In contrast, FI-DEX treatment resulted in increased mRNA abundance levels of genes encoding for *HSD3B* (P<0.05; Figure 6.6.1.C), *HSD11B1 (*P<0.05; Figure 6.6.1.G), *HSD11B2* (P<0.05; Figure 6.6.1.H), *HSD17B* (P<0.05, Figure 6.6.1.I), and a trend towards increased expression of *GR* (P=0.0587; Figure

6.6.1.L), whilst there were no differences for the remainder of the genes analysed.

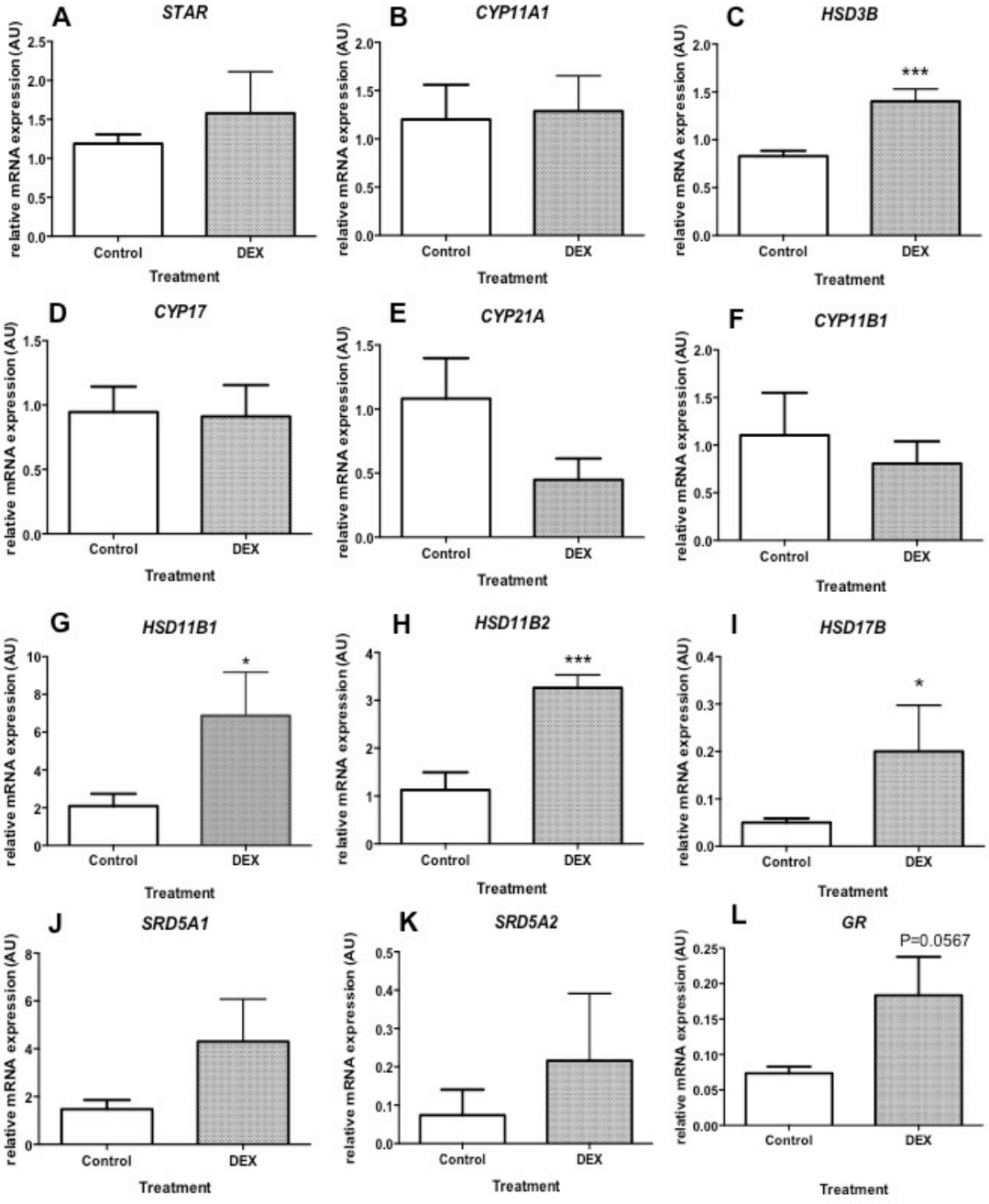

Figure 6.6.1. Effect of direct fetal exposure to DEX on expression of genes coding for STAR. CYP11A1, 3βHSD, CYP17, CYP21A, SRD5A1, SRD5A2, CYP11B1, HSD11B2, HSD17B and GR in male lamb adrenal.
(Control n=6, DEX n=7). Values represent mean ±SEM. **P*<0.01, ****P*<0.001.

6.5.1 Effects of direct prenatal exposure to different steroids on cortisol secretion in male lamb (3 months old)

There was a significant increase in cortisol secretion at 15 and 30 min in both control (15 min (P<0.05; 30 min (P<0.01) respectively) and TP (15 min P<0.01; 30 min P<0.05 respectively) treatment groups compared to their respective basal time point (0) (Figure 6.6.2.A) and also between 15 min and 30 min time points by TP treatment alone. However, there was no difference in the total cortisol secretion between control and TP group (Figure 6.6.2.B).

There was a significant increase only at 15 and 30 min time points compared to basal time points in control animals (P<0.05 and P<0.01, respectively; Figure 6.6.3.C) and only at 30 min post (P<0.01) Synacthen stimulation compared to basal (0) for both FI-DES and FI-DEX treatment, but no differences at individual time points between treatments, which was mirrored in the total cortisol secretion (Figure 6.6.2.D and Figure 6.6.3.B respectively).

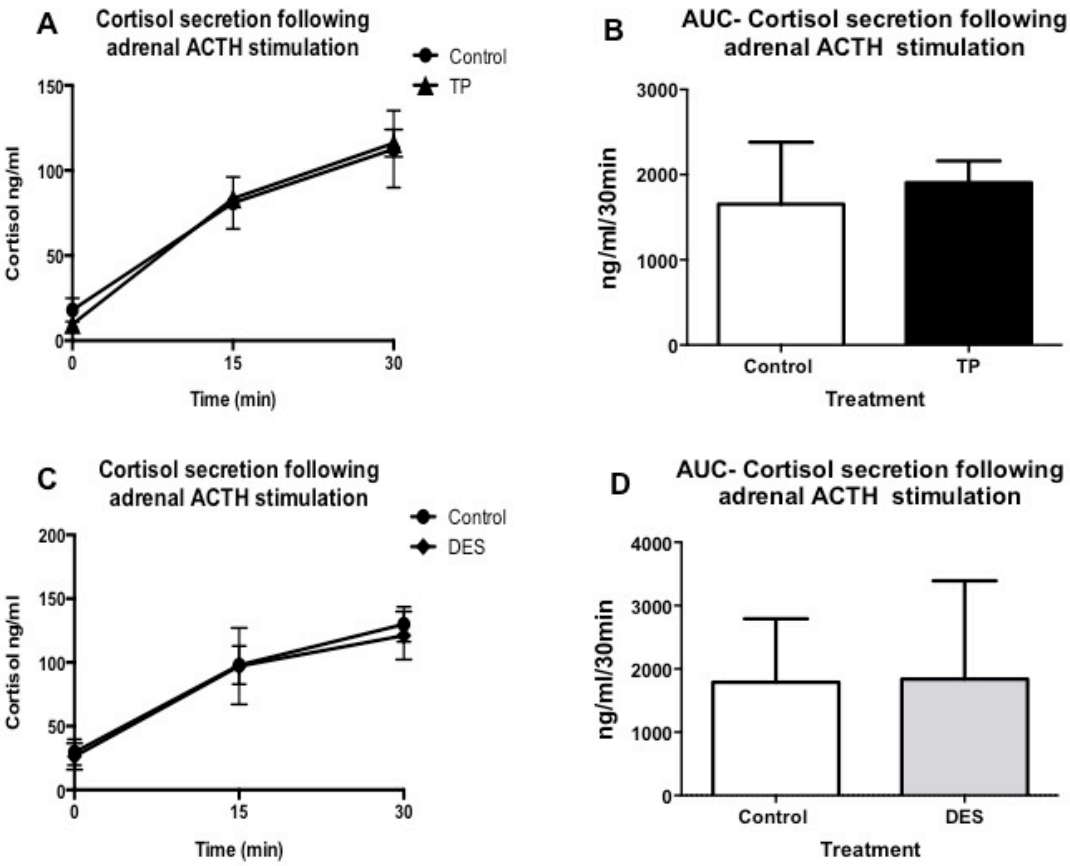

Figure 6.6.2. Cortisol secretion following adrenal stimulation in male lambs exposed prenatally to different steroids.

(A). Control n=6; TP n=5, (C) Control, n=11 vs DES, n=8. Area under the curve (AUC) for total cortisol secreted in male lamb pre and post ACTH stimulation between control and treatment groups. (B). Control vs TP; (D) Control vs DES.

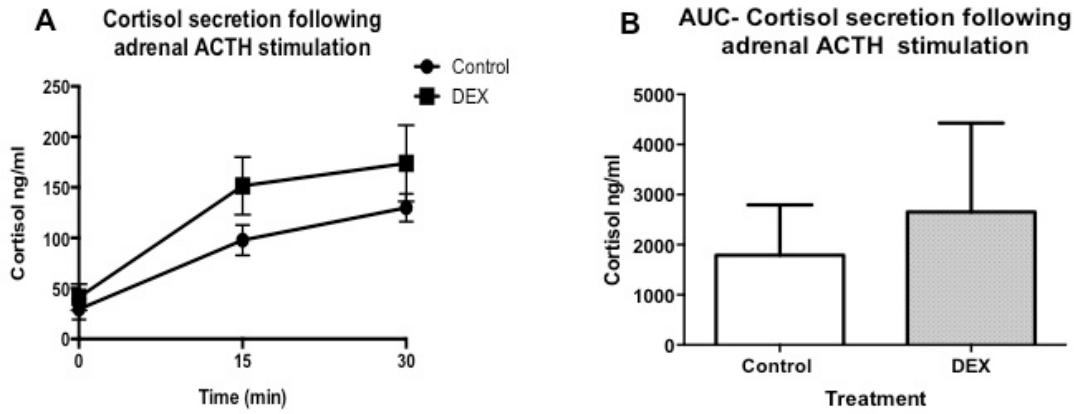

Figure 6.6.3. Cortisol secretion following adrenal stimulation in male lamb exposed to FI-DEX treatment.

(A). Control n=11; DEX n=4; (B) Area under the curve (AUC) for total cortisol secreted in male lamb pre and post ACTH stimulation between control and DEX group.

6.5.2 Effects of direct prenatal exposure to different steroids on testosterone secretion in male lamb (3 months old)

None of the prenatal steroid treatments caused any alterations in testosterone secretion, and in no case there were any effects of such synacthcen stimulation on overall circulating testosterone concentrations.

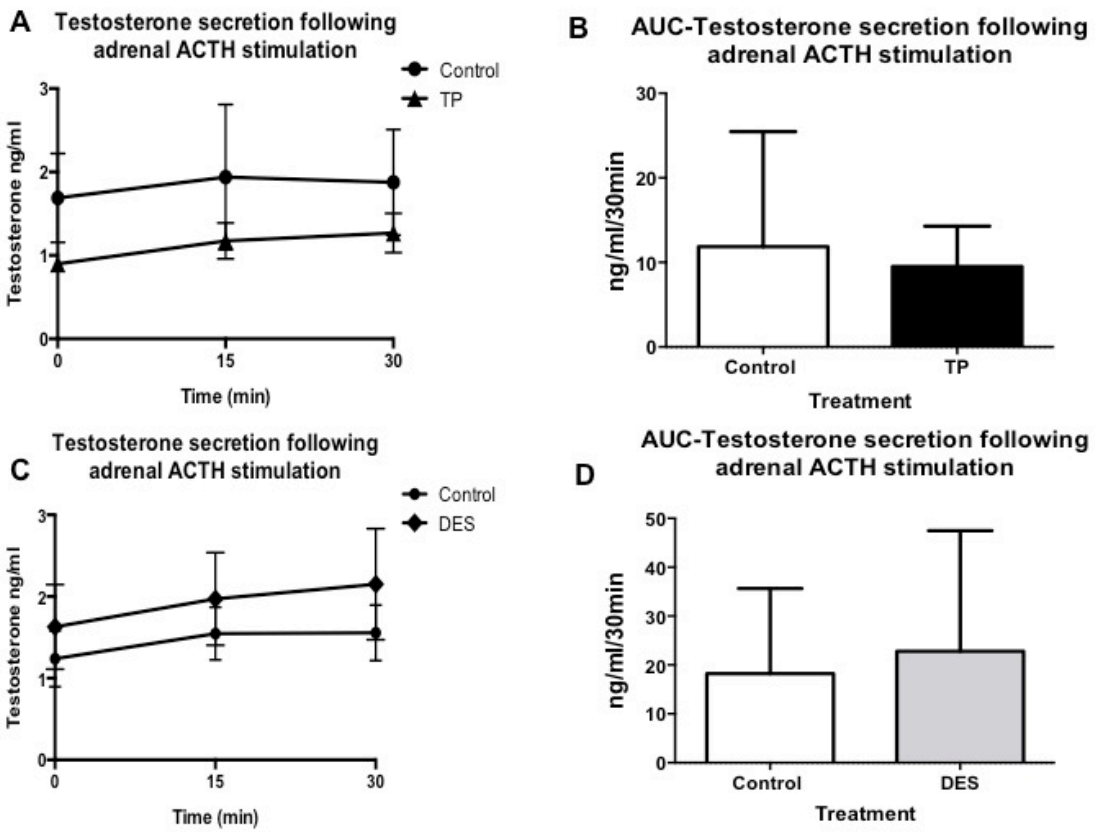

Figure 6.6.4. Testosterone secretion following adrenal stimulation in male lambs exposed prenatally to different steroids.

(A). Control n=6; TP n=5, (C) Control, n=11 vs DES, n=8. Area under the curve (AUC) for total cortisol secreted in male lamb pre and post ACTH stimulation between control and treatment groups. (B). Control vs TP; (D) Control vs DES.

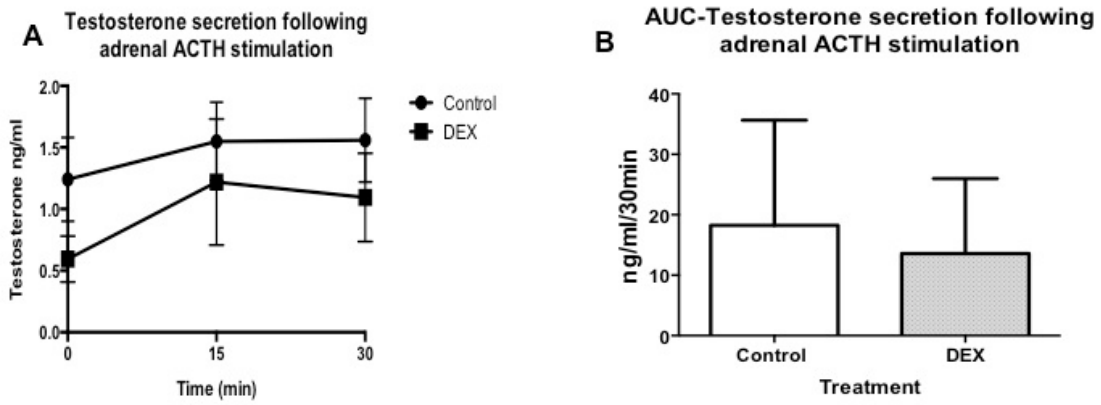

Figure 6.6.5. (A) Testosterone secretion following adrenal stimulation in male lamb exposed to FI-DEX treatment.

Control n=11; DEX, n=4. (B) Area under the curve (AUC) for total cortisol secreted in male lamb pre and post ACTH stimulation between control and DEX group.

6.6. Discussion

In Chapter 5, gene expression data derived from fetal adrenals suggested altered adrenal development in a sex specific manner in response to direct fetal exposure to steroid excesses. FI-DES and FI-DEX treatment showed significant effects on female and male fetal adrenal gene expression associated with steroid synthesis, respectively. However such steroidogenic associated gene expression was largely unaltered by FI-TP during fetal life, and collectively, the data described during fetal life left only speculation as to whether or not the perturbations observed could have any long-term effects extending into postnatal life. Therefore, in this study, we investigated the effects of prenatal steroid excess on both lamb and adult adrenal steroidogenic gene expression and physiological function.

Due to animal handling housing practicalities and economical constraints only female adult offspring from steroid manipulated pregnancies were used in this study, however both male and females were studied during lamb stage, which will be addressed later in this discussion.

6.6.1 Prenatal excesses of TP lead to adrenal hyperandrogenism in female adults

STAR mRNA abundance was significantly increased by FI-TP in adult females. Increased *STAR* mRNA abundance indicates likely increased cholesterol transport for steroid synthesis as StAR is involved in cholesterol transport from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) (Flück *et al*., 2011). It was important therefore to quantify genes encoding for steroidogenic enzymes downstream of StAR in the steroidogenic pathway to see if such increased substrate (cholesterol) availability was associated with increased adrenal steroid synthetic potential. Such analysis may also reveal the directionality of any altered synthetic potential I.e increased glucocorticoid or androgen secretion. To this end, *CYP21A* mRNA abundance was examined and found to be significantly

increased by FI-TP. CYP21 enzyme helps in conversion of progesterone to 11-deoxycorticosterone and 17α-hydroxyprogesterone to 11-deoxycortisol, respectively, thereby providing substrates for both mineralocorticoid and glucocorticoid synthesis (Miller et al., 2011). *HSD3B* mRNA abundance was also significantly increased by FI-TP. The 3β-HSD enzyme converts 17α-hydroxy-pregnenolone to 17α-hydroxyprogesterone in the zona fasiculata and also dehydroepiandrosterone to androstenedione in the zona reticularis region of the adrenal cortex (Miller *et al*., 2011). There was also elevated *CYP11B1* and *HSD11B1* mRNA abundance associated with FI-TP. CYP11B1 catalyses conversion of 11-deoxycortisol to cortisol within the adrenal zona fasiculata (Payne et al., 2004a) whilst 11β-HSD1 converts inactive cortisone to active cortisol (Michael *et al*., 2003). Together, the steroidogenic gene expression data suggested that fetal androgen overexposure might promote excess cortisol secretion in the female adult offspring. It is important to note, however, that such analyses are complicated by the fact that they are an examination of a mixture of all three adrenal cortical zones, and hence cannot be used with certainly to indicate whether or not glucocorticoid or androgenic elevations are likely. Such data, at best, indicates only that there was a likelihood of increased steroid synthesis *per se*. In order to examine adrenal function, and address this issue of lack of zone specificity of mRNA analyses and thus identify any specific excess steroid secretion(s), animals were challenged with an injection of Synacthen, a potent ACTH analogue. There was no significant alteration in serum cortisol levels associated with any of the prenatal steroid treatments. This is in accordance with a previous study, which showed no increase in ACTH-stimulated cortisol concentrations in female rhesus monkeys from androgen manipulated pregnancies (Eisner *et al*., 2002).

17βHSD is exclusively expressed in zona reticularis, the androgen secreting region of the adrenal cortex, where it catalyses reduction of androstendione, a weak androgen, to testosterone, a more potent androgen (Dufort *et al*., 1999; Lin *et al*., 1997). The increase in *HSD17B* and the aforementioned *HSD3B*, suggest the potential for excess testosterone secretion in the adult offspring from androgen- (TP) manipulated pregnancies. This was indeed the case,

since we observed an exaggerated testosterone output from female offspring but only from those pregnancies treated with excess androgens. The lack of such increased testosterone secretion in ewes derived from DES-treated pregnancies suggests this was a purely androgenic effect in altering adrenal function with no estrogenic contribution. Furthermore, this increased testosterone secretion with no increased cortisol secretion defines the gene expression alterations as being functionally only associated with adrenal hyperandrogenism. Since there is no expression of ACTH receptor in the ovine ovarian follicle (Drs Duncan and Rae, personal communication), the adrenal origin of such increased testosterone in response to Synacthen challenge is not in any doubt.

Since there were no alterations in *ACTH-R* expression in these animals, it is unlikely that the increased testosterone output is in any way due to increased adrenal sensitivity to trophic stimulation, and the lack of effects on cortisol secretion also support this, however, this possibility cannot be totally excluded since the *ACTH-R* mRNA measured was not specific to any one cortical zone. Prenatally androgenized female rhesus monkeys exhibited increased basal and ACTH induced DHEA levels (Zhou *et al*., 2005)- criteria used for identification of adrenal hyperandrogenism. Similar results of elevated DHEAS levels were also observed in daughters of women suffering from PCOS (Maliqueo *et al*., 2009). However, even if DHEA and DHEAS levels are elevated as seen in aforementioned studies, they have limited physiological value as they both are weak androgens (or pro-androgens) (Burger, 2002) and their effects depend on metabolism to a potent androgen such as testosterone or DHT (Auchus *et al*., 2004). Therefore, in the current study, the elevated testosterone concentration in the prenatally androgenized female adult offspring is of direct clinical relevance.

There thus appeared to be reasonable linkage between adult gene expression and function in all three steroid treated groups assayed in adult life, in that only animals with increased steroidogenic gene expression were also those with increased adrenal androgen output i.e the FI-TP group. Given a lack of adrenal gene expression alterations in fetal life (Chapter 5) that could

underpin the hyperandrogenaemic adult phenotype, it was important to examine the same genes in younger animals. Twelve week old female FI-TP animals were chosen for this analysis, as they were the only treatment group associated with adult functional perturbations. Female lambs showed no changes with respect to gene expression or physiological function (both cortisol and testosterone concentration) by FI-TP treatment compared to control animals, suggesting that the adult adrenal phenotype is developed as the animals mature and therefore suggesting that the altered adult function may be an indirect consequence of androgenic programming during fetal life.
In response to prenatal estrogen excess, decreased gene expression in terms of *ACTH-R*, *CYP11A1* and *HSD17B* mRNA abundances was noted. This downregulated *CYP11A1* and *HSD17B* by DES along with the elevated *ACTH-R* expression suggests that estrogen might aid in downregulation of adrenal steroidogenic pathway in the female offspring from estrogen manipulated pregnancies, as estrogens are known to influence the ACTH sensitivity on the adrenal cortex (Atkinson and Waddell, 1997; Lo *et al*., 2000). Functionally, however, we observed no decrease in response to Synacthen, a synthetic ACTH analogue, and hence conclude that this decrease in mRNA encoding *ACTH-R* was not associated with any measurable decrease in sensitivity to ACTH. The data are nonetheless important in ruling out any potential for there being any estrogenic contributions to the aforementioned androgenic driven hyperandrogenism. This also serves to highlight that alterations in specific genes may not always marry with altered functional outputs and that care must be taken in extrapolation of mRNA data to function, particularly in the case of mRNA encoding for enzymes, since mature enzymatic activity is dependent upon many other factors, such as co-factor, substrate and indeed product availability.

Whilst it was intriguing to note that *HSD11B1*, *HSD17B* and *GR* were upregulated by DES treatment in male lamb adrenal glands, again, there was no functional significance of these alterations in terms of cortisol or testosterone secretion. However, although the relevance of such changes cannot be determined here, it should be noted that alterations in these genes might not manifest in, for example cortisol or testosterone concentrations.

This is a limitation of utilizing gene expression of multiple enzymes in a synthetic scheme were all have multiple substrates and products to forecast organ function.

6.6.2 The adult legacy of prenatal glucocorticoid excess

In response to FI-DEX (glucocorticoid excess) female adult offspring showed no significant changes in terms of gene expression apart from *SRD5A1,* which was significantly decreased however, functional consequences remain unknown. In male lambs, even though *HSD11B1*, *HDS11B2*, *HSD3B and HSD17B* mRNA abundance were significantly increased by FI-DEX treatment, and there were such profound effects upon gene expression in male fetuses attributable to this treatment (Chapter 5), there was no alteration in serum cortisol or testosterone concentrations, suggesting that prenatal DEX exposure had no long-term effect on adrenal function. It remains unknown if there may be effects in later life in adult male, as no adult males were available for study, but it is also a distinct possibility that the effects noted in fetal life were contemporary responses to elevated fetal glucocorticoids, which resolved once the excess steroid was removed, similar to that observed in the case of testosterone in male fetuses (Connolly *et al*., 2013). Previous studies in sheep have shown sex specific differences in terms of stress responses (Giussani *et al*., 2011), however, such effects are dependent upon timing of prenatal glucocorticoid exposure (Braun *et al*., 2009; Seckl, 2004; Yehuda *et al*., 2005).

Collectively, the increased testosterone output of female adrenal during adulthood noted in the present study suggests that changes induced by exposure to TP by the maternal route of application in various tissue types (Hogg *et al*., 2012; Hogg *et al*., 2011; Rae *et al*., 2013) is due to androgenic excess and not due to placental estrogenic action derived by metabolism of testosterone into estradiol. Only fetal androgen excess in females was associated with any health relevant functional adrenal alterations. Given the lack of fetal effects, and the lack of effects prior to puberty in androgen-excess

female offspring, it is important to consider what could be a driver of adult, post-pubertal hyperandrogenism, specific to prenatal TP treatment.

Although adrenarche has not been described in sheep, it is known to occur in primates and humans, and is marked by increased adrenal androgen secretion, associated with the time of puberty (Conley *et al*., 2011; Nakamura *et al*., 2009). Adrenal changes associated with sexual maturity are also observed in rodents (de Almeida *et al*., 1998). Daughters of women suffering from PCOS exhibit exaggerated adrenarche during puberty, suggesting that adrenal androgens might modulate the progression of puberty (Teresa Sir-Petermann et al., 2009). Hence it may be the case that until the time of puberty altered adrenal function remains 'silent'. However, the current study also throws up an alternative possibility. The previously observed hyperinsulinemia was evident in female offspring exposed to fetal androgen excess prior to adulthood, but was in fact not significantly elevated until adulthood. This pattern is mirrored by adrenal hyperandrogenism in the same animals, and resembles some but not all young girls born to PCOS mothers who demonstrated exaggerated DHEA and DHEAS in response to ACTH stimulation (Maliqueo *et al*., 2009).

It is very well established that gonadal steroidogenesis can be regulated by insulin in both male and female (A. Dunaif and Graf, 1989; Poretsky and Piper, 1994). Insulin along with other growth factors such as IGF1 and IGF2 are known to regulate ovarian thecal and granulosa cells *in vitro* in both humans and animals (Poretsky *et al*., 1999), and enhance the stimulatory effect of LH dependant androgen secretion (Barbieri *et al*., 1986), which provides the basis for insulin regulated excess ovarian androgen secretion in clinical conditions such as PCOS (Poretsky *et al*., 1999). Meanwhile, evidence *in vitro* and in humans also suggests insulin mediated adrenal steroidogenesis.

Metformin induced (insulin sensitizer) decrease in adrenal androgen secretion in hyperinsulinaemic PCOS women in response to ACTH challenge (Marca et al., 1999). Couple with increased adrenal androgen secretion by modulating

fetal adrenal IGF-II (acting via IGFR-1) *in vitro* in humans (Mesiano *et al*., 1997), this suggests a role for insulin action on adrenal steroidogenesis. Moreover, increased adrenal steroid metabolites in response to ACTH challenge in hyperinsulinaemic PCOS women suggests insulin mediated altered adrenal steroidogenic pathway (Tosi *et al*., 2011). Therefore it is possible that insulin might have a key role in adrenal steroidogenesis. Nevertheless, whether adrenal steroidogenesis and the possible mechanisms are directly regulated by insulin is still a matter of debate.

In conclusion, direct fetal androgen exposure predisposes to adrenal hyperandrogenism in adulthood. This work also suggests that such altered adrenal function, whilst a consequence of fetal androgen excess, may be indirectly driven by changes in other organ function such as pancreatic hyperinsulinaemia. Importantly, it adds further evidence to the suggestion that prenatal programming of the adrenal function may well be contributing, along with excess ovarian androgens (Hogg *et al*., 2012), towards excess androgen secretion in clinical conditions such as PCOS (Rosenfield, 1999).

Chapter 7 General Discussion

7.0 General Discussion

PCOS is a common heterogeneous disorder affecting about 6-8% women of reproductive age (Diamanti-Kandarakis and Piperi, 2005), comprising reproductive, endocrine and metabolic abnormalities (Dumesic *et al*., 2007). In addition to these observable, functional issues, PCOS also leads to anxiety, depression and is a frustrating experience for sufferers (Kerchner *et al*., 2009), negatively impacting on their quality of life. Several societies across the world have provided a framework to diagnose PCOS (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group., 2004), but the underlying cause(s) remain unknown. There are genetics links to PCOS with respect to candidate genes in both reproductive and metabolic tissues (Conway *et al*., 1994; Reddy *et al*., 2014; Rosenfield *et al*., 1994; Talbot *et al*., 1996), however these lack reproducibility with respect to the main features of PCOS, therefore the reach of genetics in terms of PCOS development and severity remains unresolved. Prenatal androgen exposure is a widely accepted research paradigm in development of PCOS-like animal models including monkeys, sheep and rodents (Dumesic *et al*., 2005; Padmanabhan and Veiga-Lopez, 2013; Roland and Moenter, 2014), and these have likely clinical implications, namely the concept of early life programmable developmental origins of PCOS.

In addition to reproductive disorders, fetal androgen excess also leads to metabolic disorders that characterize PCOS, such as pancreatic β-cell dysfunction, excess insulin secretion and increased adipose tissue distribution (Dunaif and Finegood, 1996; Hogg *et al*, 2011; Roland *et al*., 2010). This thesis focused upon midgestational, direct fetal exposure to excesses of steroids on the developing pancreas and adrenal gland and their postnatal structures and function.

Due to limitations of maternal applications of steroids, the direct fetal injection employed herein represents a series of refinements designed to:

a. Bypass placental/maternal aromatase activity, since maternal androgen exposure leads to elevated fetal circulating estrogen levels in female fetuses (Rae *et al*., 2013; Veiga-Lopez *et al*., 2011), which may have programming effects in adulthood (Steckler *et al*., 2007; West *et al*., 2001) and thus cloud interpretations based around androgenic actions.
b. Permit control of the dose of steroids to which the fetus was exposed. Maternal exposure of DHT or TP to pregnant ewes to delineate androgenic effects from estrogenic during fetal programming (Steckler *et al*., 2007; West *et al*., 2001), does not give a clear picture due to lack of fetal dosage control given variable maternal clearance and metabolism. In addition, there could be estrogenic effects upon placental function, again, potentially masking the true situation.
c. To avoid any effects of androgens on the pregnant mother, which could indirectly affect the fetus. For example androgens alter maternal glucose (Nicol *et al.*, 2014).

Chapter 3 defined the ovine fetal pancreas as being a target tissue of androgens during development. This work highlighted that the fetal pancreatic tissue responded to androgen excess, resulting in altered pancreatic development and function. This response was specific to female fetuses and equally specific to androgens with no estrogenic contribution. Likely, due to endogenous pre- and contemporary exposure to testicular androgens, mid-gestational androgen excess exposure left males unaffected, whilst in females it led to a detrimental effect in terms of altered pancreatic development and altered postnatal structure and function. Whilst testosterone treatment's lack of effects in males in maternal androgen exposure studies (Rae *et al*., 2013) could also be partially explained by compensatory reductions in fetal testicular testosterone output (Connolly *et al*., 2013), this cannot be the case here since direct fetal injection causes a supraphysiological increase in androgen concentrations. Hence, earlier physiological exposure to endogenous testicular testosterone in males from the time of sexual differentiation (around d30 gestation in sheep) would appear to be perhaps a more plausible reason for lack of effects in males. This led us to examine differences between males

and females in general. An interesting finding of this study was the differences observed between male control fetuses compared to females in terms of gene expression.

Male fetuses showed greater gene expression of *PDX-1* as compared to females. Female fetuses responded to excess androgens with elevated *PDX-1*, increased *in vitro* insulin secretion and increased β-cell numbers. The female fetus does not produce such high concentrations of testosterone as males (Reyes *et al*., 1974; Reyes *et al*., 1973), and therefore the female fetus likely cannot compensate when exposed to such exogenous androgen concentrations, but also, in terms of timing of exposure, females are relatively 'androgen-naïve' when treatments commenced, whereas males have developed for the previous 30 days in an androgen rich environment (Quirke *et al*., 2001). The male pancreas may already have reached a nadir of androgen response, whereas the female pancreas may be being altered down a more male trajectory by our androgen treatments- importantly however this is a trajectory that had been female up until treatment.

Apart from the genetic associations (Florez, 2008), development of type-2 diabetes mellitus also involves exposure to suboptimal *in utero* environments such as intra uterine growth retardation (Simmons, 2001) possibly via epigenetic modifications. This could be in part due to the action of Pdx-1, which is involved in early pancreatic development and also differentiation and function of β-cells during adulthood (Offield et al., 1996). Histone acetylation in the promoter region of the *PDX-1* in IUGR rats, suggests *PDX-1* is likely a susceptible target gene in terms of epigenetic modifications, ultimately affecting gene expression (Park *et al*., 2008). Altered regulation of *PDX-1* due to maternal undernutrition (Gatford *et al*., 2008) and excess androgenic conditions in PCOS animal model during fetal life is also evident postnatally (Gatford *et al*., 2008), and implied in terms of altered function (Rae *et al*., 2013). Collectively then these observations direct attention to *PDX-1* being a possible candidate gene for mediation of a number of the effects observed in these studies and also the work presented in this thesis. Elevated *PDX-1*

mRNA abundance occurring in females during fetal life due to prenatal androgen exposure may be an initial occurrence underpinning a primary pancreatic defect observed in both fetal and adult life, and may be at least in part be attributable to epigenetic modification. The effects of androgen overexposure as noted above drive *PDX-1* expression towards a male profile, and if indeed there is permanent epigenetic modification that maintains this expression profile this would fit with the idea of a male pancreas housed in a largely female environment. This opens up an interesting area of research in future to look into the epigenetic modifications of *PDX-1* in excess steroidal conditions/models of PCOS. A recent study based in human pancreatic islets has demonstrated that female pancreatic islets exhibited decreased DNA methylation patterns on the X chromosome and autosomal chromosome compared to males (Hall *et al*., 2014). This suggests sex differences in epigenetic modifications (DNA methylation patterns) on both sex and autosomal chromosome within human pancreatic islets. Hall *et al*, (2014) study also demonstrated that there was higher insulin secretion *in vitro* in response to glucose stimulation in females compared to males (Hall *et al*., 2014). With this being the case, then it suggests 'female' and 'male' pancreatic tissues being of great interest from the context of stem cell biology and transplantation. Collectively, this serves to highlight that epigenetic modifications remain one of the cause for sex differences in pancreatic insulin secretion.

Insulin secreting β-cell numbers and *in vitro* insulin secretion were increased in TP treated females, giving a functional relevance to the gene expression alterations observed during fetal life. The forward speculation of permanent alteration of pancreatic function in females was addressed in Chapter 4, where female lambs showed a trend towards increased insulin secretion, which by adulthood was a significant increase in insulin secretion in response to controlled glucose stimulation in concert with increased β-cell numbers. Women with PCOS are known to suffer from increased risk of development of type-2 diabetes, the major metabolic link to this heterogeneous disorder (Ovalle et al., 2002). Given the apparent absence of insulin resistance in our

study, this suggested a permanent β-cell defect due to prenatal androgen exposure- perhaps a mismatch in terms of a phenotypically male pancreas in an otherwise phenotypically female physiological environment. This is in accordance with a study by Goodarzi *et al* (2005) who have demonstrated that a correlation exists between bioavailable testosterone and insulin secretion and not insulin resistance in women with PCOS compared to normal, which suggests that there could be a β-cell dysfunction in PCOS women (Goodarzi *et al*., 2005). Our study provides evidence in terms of changes at the β-cell level in terms of altered β-cell specific gene expression, cell numbers and function in response to prenatal androgen exposure, suggesting that there could be a primary β-cell dysfunction and can have implications in understanding the importance of insulin secretion in PCOS. This could again open up directions for therapies, which could directly modulate insulin secretion rather than the presently used insulin-sensitizing therapies indirect modulation, or a combination of both.

Chapter 5 revealed that fetal adrenal gland was altered by exposure to both prenatal estrogen and glucocorticoid excess, however the functional endpoint of such alterations was difficult to speculate upon as the fetal adrenal gland remains quiescent during midgestation. An important finding of this study was that there was sex-specific regulation of *GR* expression in the male fetal adrenal, which likely precedes sexual differentiation (as it could not be recreated by early androgenisation of females), thereby making the male fetal adrenal more responsive to prenatal glucocorticoid exposure as compared to females. To the best of our knowledge, this is the first study to demonstrate altered fetal steroidogenic pathway in male adrenals due to midgestational dexamethasone exposure. Consequences of these alterations remain unknown. Whilst tempting to speculate possible legacies of such changes in terms of adrenal function, there was no clear evidence of health-relevant altered function in males prenatally exposed to excess glucocorticoid.

Postnatal adrenal glands did appear to be affected by prenatal dexamethasone exposure in males, but only in terms of gene expression.

Unfortunately, we could not assess effects during adulthood as males were sacrificed as lambs. In female adult animals, DES did not appear to have functional adult consequences, but prenatal androgen excess did have adult functional consequences. Since during fetal life, androgen excess in females caused very little in the way of altered steroidogenic gene expression changes, then speculation as to what could be inferred from male fetuses treated with dexamethasone must be extremely cautious. Importantly, in female offspring, excess adrenal testosterone secretion in response to ACTH was observed as a legacy of prenatal androgen exposure, suggesting the adrenal hyperandrogenism seen in clinical conditions such as PCOS (C. Moran and Azziz, 2001) may have *in utero* origins.

In the current model presented, it is clear that adrenal gland also contributes towards a hyperandrogenic condition in addition to ovarian excess androgens (Hogg *et al*., 2012) due to prenatal androgen exposure- but the question remains, that even though apparently 'androgens begat androgens' is this a direct effect of androgenic programming or an effect secondary to such programming elsewhere in the endocrine system? Even though the adrenal gland phenotype was sex-specific, it is important to note that this tissue does not exhibit developmental programming effects (since there were no antecendental alterations in fetal life), rather, the adrenal phenotype is noticed as the animal matures, suggesting that adrenal phenotype may be a downstream effect, possibly due to previously observed excess insulin secretion. We saw no change in testosterone concentrations in prepubertal females, but observed a trend towards increased insulin secretion in female lambs- once this increased insulin secretion was more pointed then the adrenal phenotype was also more evident. This is in accordance with clinical studies where metabolic abnormalities arise before the onset of hyperandrogenic dysregulation in pre-pubertal girls (Sir-Petermann *et al*., 2007; Sir-Petermann *et al*.,2009). It is suggested that hyperinsulinemia is the link between premature adrenarche and PCOS in girls born to women suffering from PCOS (Ehrmann *et al*., 2006; Ibáñez *et al*.. 2009). This hypothesis is possible from a mechanistic point of view, as IGFs and insulin are known to regulate adrenal steroidogenesis *in vitro* (Mesiano *et al*., 1997).

The absence of insulin resistance (IR) with the pancreas already hypersecreting insulin in the female adult offspring from androgen manipulated pregnancies suggests the secretory capacity of pancreas may be already be limited towards its maximal, and if subsequently IR develops, it might place the pancreas under unavoidable stress leading ultimately towards a faster development of complete type 2 diabetes mellitus, than would be the case in a non-PCO women as they would have greater plasticity in terms of capacity to increased insulin secretion. Thus current suggestions of insulin perhaps having a predisposition role in terms of development of insulin resistance (Cao *et al*., 2010) fit into this suggestion, and the work in this thesis presents a strong case for further examination of pancreatic dialogues with both peripheral insulin reception and steroidogenic organ function. In conclusion, I suggest that there might be altered communication between the pancreas and adrenal gland, and when such aberrant signalling occurs it may have implications for adult health and metabolism. Given the importance of these systems to lifelong health it is critical that this receives further study.

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