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THE ROLE OF PROSTAGLANDIN E_2 IN THE REGULATION OF THE OVINE FETAL ADRENAL CORTEX

By

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For my family

Ian, Grandpa, Pete, Will, Fi, Rob, Lori, Jack, Dad, Lauri, and most importantly Mum

WE ALL NEED AN END TO JOURNEY TOWARDS, BUT IT IS THE JOURNEY THAT MATTERS IN THE END.

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DECLARATION

I hereby declare that, to the best part of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. No part of this thesis has been submitted to any other university for any degree or diploma.

Penelope Simmonds

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PUBLICATIONS/ABSTRACTS

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2. **Simmonds PJ**, Phillips I.D., Coghill I.C., Poore K.R., Canny B.J. and Young I.R 1999. Effect of a pituitary and ACTH on adrenal steroidogenesis in the ovine fetus in late gestation. Endocrine Society of Australia, Melbourne, Australia.
3. **Simmonds PJ**, Young IR and Canny BJ 2000. Prostaglandin (PG) E_2, but not ACTH, stimulated cortisol production in ovine fetal adrenal tissue *in vitro*. International Congress of Endocrinology, Sydney.
4. **Simmonds PJ**, Phillips ID, Poore KR, Coghill ID, Young IR and Canny BJ 2001. The role of the pituitary gland and ACTH in the regulation of messenger ribonucleic acids encoding proteins essential for adrenal steroidogenesis in the late gestation ovine fetus. *Journal of Endocrinology* **168**, 475-485.
5. **Simmonds PJ**, Young IR and Canny BJ 2001. PGE_2 can directly stimulate cortisol production from the ovine fetal adrenal gland *in vivo*. *Placenta* **22** (7), A.48.
6. **Simmonds PJ**, Young IR and Canny BJ 2001. Prostaglandin (PG) E_2 can directly stimulate cortisol production from the ovine adrenal gland *in vivo*. Endocrine Society of Australia, Gold Coast, Australia.
7. **Simmonds PJ**, Young IR and Canny BJ 2001. PGE_2 can directly stimulate cortisol production from the ovine adrenal gland *in vivo*. International Federation of Placental Associations, Sorrento, Italy.
8. **Simmonds PJ**, Young IR and Canny BJ 2001. Prostaglandin (PG) E_2, directly stimulates cortisol secretion from the fetal adrenal gland. Perinatal Development and Early Origins of Adult Diseases, Melbourne.
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SUMMARY

The rise in cortisol in late gestation that leads to parturition has been well established, yet the trigger for this pre-partum increase in cortisol has so far remained elusive. Early studies showed that interruptions of the hypothalamo-pituitary-adrenal (HPA) axis delayed labour, while infusions of either glucocorticoid or adrenocorticotrophin (ACTH) resulted in premature labour. In the past, the cortisol surge was thought to be explained solely by ACTH action, however several studies have demonstrated that i) an increase in ACTH is not required for a cortisol surge or parturition to occur, ii) increased adrenal responsiveness to ACTH is not sufficient to trigger the cortisol surge, iii) there is a marked discrepancy between the increases in ACTH (2 - 3 fold) and cortisol (20 – 30 fold). This evidence has led to the hypothesis that ACTH plays only a permissive role in the surge in cortisol and that another factor drives the increase in cortisol that is required for maturation of organ systems and extra uterine survival. Prostaglandin E_2 (PGE_2) is one candidate as it has been shown to have many interactions with the HPA axis and has been shown to be a positive stimulator of ACTH and cortisol secretion. PGE_2 in the fetus is derived from the placenta and concentrations have been shown to increase in late gestation, prior to the surge in cortisol. The central aim of this thesis was to determine if PGE_2 may be the factor that drives the surge in cortisol by acting directly at the fetal adrenal gland *in vitro*, *in vivo* and to determine if the PGE_2 receptors were present in the ovine fetal adrenal gland.

In the studies reported in chapter 3 of this thesis, PGE_2, but not ACTH was able to stimulate cortisol secretion from 125 d and 140 d fetal adrenal glands *in vitro*, confirming that PGE_2 was capable of stimulating cortisol secretion directly from the fetal adrenal gland. This study also demonstrated that the fetal adrenal was less responsive to ACTH than the adult adrenal gland.

To determine if PGE_2 could stimulate cortisol directly from the fetal adrenal gland *in vivo*, two experimental groups were used. Intact, saline infused fetuses (INT/SAL) or hypophysectomised fetuses infused from the time of surgery with a continuous, low dose, infusion of ACTH, which has previously been shown to maintain the structure and basal cortisol secretion of the fetal adrenal gland (HX/ACTH). The two groups were further subdivided at 140 d GA, with half receiving an infusion of saline and half receiving a 24 hour infusion of PGE_2. This study showed that infusion of PGE_2 into both INT/SAL and

HX/ACTH fetuses resulted in a significant increase in plasma cortisol concentrations. The increase in the HX/ACTH fetuses occurred without a corresponding increase in ACTH suggesting the PGE_2 can stimulate secretion of cortisol from the ovine fetal adrenal gland directly. This is a novel and potentially important finding as it offers an alternative stimulus for the cortisol surge in late gestation.

In the last experimental chapter presented in this thesis, real time PCR was used to confirm the presence of the mRNA encoding the four PGE_2 receptor subtypes (EP1-4) in the ovine fetal adrenal gland. The abundance of the mRNA encoding the EP receptors were not affected by the 24 hour infusion of PGE_2, and only the mRNA encoding EP3 was increased in the HX/ACTH groups when compared to the INT/SAL fetuses. Three of the key steroidogenic enzymes P450scc, P450c17 and 3βHSD were not affected by the infusion of PGE_2, and only 3βHSD was decreased in the HX/ACTH groups when compared to the INT/SAL fetuses. The mRNA encoding the ACTH Receptor was unaffected by the infusion of PGE_2 but was also decreased in the HX/ACTH fetuses.

The principal findings of this thesis are that exogenous PGE_2 can stimulate cortisol secretion directly from the ovine fetal adrenal gland and may act through any one of the four receptor subtypes, as the mRNAs encoding all four subtypes are present in the fetal adrenal gland. These findings suggest that PGE_2 may play a role in elevating cortisol concentrations in the late gestation ovine fetus and therefore possibly contribute to the initiation of parturition and successful extra uterine survival of the fetus.

LIST OF ABBREVIATIONS

%	percent
±	plus or minus
°C	degrees celsius
β-End	beta-endorphin
µl	microlitre
β-LPH	beta-lipotrophin
<	less than
>	greater than
^{125}I	iodine-125
17α-OH	17-alpha hydroxylase enzyme
3βHSD	3 beta hydroxysteroid dehydrogenase enzyme
^{3}H	tritiated thymidine
A	adenosine
aa	amino acid
AA	arachidonic acid
AC	adenylate cyclase
ACTH	adrenocorticotrophic hormone
ACTH R	adrenocorticotrophin receptor
ADX	adrenalectomy
Ang II	angiotensin II
ANOVA	analysis of variance
ATP	adenosine triphosphate
AVP	arginine vasopressin
bp	base pairs
C	cytosine
c.p.m.	counts per minute
CA	carotid artery
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic AMP
cDNA	complimentary DNA
CLIP	corticotrophin like intermediate peptide
cm	centimeter
COX	cyclooxygenase
CP	crossing point
CRF/CRH	corticotrophin releasing factor/hormone
C_t	crossing threshold
CTP	cytosine triphosphate
CYP11A1	cytochrome side chain cleavage enzyme
d	days
DA	ductus arteriosus
dbl	days before labour
DEX	dexamethasone
dH_2O	distilled water
DHEA	dihydroepiandrosterone
DNA	deoxyribonucleic acid
DOC	deoxycortisol
DsDNA	double stranded DNA
E	efficiency
EP	prostaglandin E receptors
FBM	fetal breathing movement
Fg	fentogram
FSH	follicle-stimulating hormone
g	gram
G	guanidine
GA	gestational age
γ	gamma
G_i	G-protein inhibitory
G_s	G-protein stimulatory
GTP	guanidine triphosphate
h/hr	hour
Hb	hemoglobin
HMW	high molecular weight
HPA	hypothalamo-pituitary-adrenal axis
HPD	hypothalamo-pituitary dissconection
HX	hypophysectomy
i.d.	internal diameter
i.v.	intraveonous
INT	intact
ir	immunoreactive
IRMA	immunoradiometric assay
JP	joining peptide
JV	jugular vein
kb	kilobases
KCl	potassium chloride
KD/ KDa	kiloDalton
kg	kilogram
KH_2PO_4	potassium dihyrogen orthophosphate
L/l	litre
LMW	low molecular weight
LPH	lipotrophin

LSD	least significance difference test
M	molar
MC2R	melanocortin type 2 receptor or ACTH R
Mg	milligram
MgCl	magnesium chloride
μ	micro
mins	minutes
ml	millilitre
mm	millimeter
mM	millimolar
mmol	millimole
mol	mole
mRNA	messenger RNA
MSH	melanocyte stimulating hormone
N	nano
N/ n	number
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium carbonate
ng	nanogram
NIL	neurointermediate lobe
nm	nanometer
nM	nanomolar
NTP	nucleotide triphophate
o.d.	outside diameter
p	pico
P450c11	11-hydroxylase enzyme
P450c17	17-alpha hydroxylase enzyme
P450c21	21-hydroxylase enzyme
P450scc	cytochrome side chain cleavage enzyme
$PaCO_2$	partial pressure of carbon dioxide
PaO_2	partial pressure of oxygen
PBS	phosphate buffered saline
PC	prohormone covertase
PCR	polymerase chain reaction
PE	pituitary extracts
PEG	polyethylene gycol
pg	picogram
PG	prostaglandin
PGE_2	prostaglandin E_2
PGHS	prostaglandin H synthase
pmol	picomole
POMC	proopiomelanocortin
PVN	paraventricular nucleus
r.p.m.	revolutions per minute
RIA	radioimmunoassay
RT	reverse transcription
SAL	saline
SaO_2	oxygen saturation
secs	seconds
StAR	steroidogenic acute regulatory enzyme
T	thymidine
t	time
Tm	melting temperature
TTP	thymidine triphosphate

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

In 1933, Malpas, reported several cases of prolonged pregnancy in the human, all associated with fetal anencephaly. From this Malpas concluded that firstly "the time of the onset of labour is determined by the fetus", and secondly that "the fetal adrenal, pituitary or nervous system, perhaps in combination, are suggested as tissues possibly concerned in the actual excitation of the neuro-muscular expulsive mechanisms". Further circumstantial evidence for the importance of the pituitary gland in the initiation of parturition came in 1963, when a number of sheep fetuses were found to have cyclopean type malformations (Van Kampen & Ellis, 1972). These lambs had abnormal or missing neural connections to the pituitary caused by the ewe ingesting skunk cabbage (*veratrum californicum*) on the 14th day of gestation.

The first experimental evidence for the importance of the pituitary gland in the initiation of parturition came in 1967, when Liggins developed a technique to destroy the fetal pituitary by electrocoagulation. They found that if more than 70% of the pituitary was destroyed, parturition did not occur (Liggins *et al.*, 1967). The importance of adrenocorticotrophic hormone (ACTH) and the fetal adrenal gland was demonstrated the following year when Liggins (1968) continuously infused high doses of ACTH or cortisol into sheep fetuses and labour occurred prematurely (Liggins, 1968). In 1969, Bassett and Thorburn established the importance of cortisol *in vivo*. They chronically catheterised fetal sheep for the first time and were able to demonstrate that plasma corticosteroids begin to increase in the last 15 days of gestation (Bassett & Thorburn, 1969). In addition, parturition did not occur in bilaterally adrenalectomised fetuses, however if the adrenal cortex was left intact and only adrenal medullary tissue was ablated, gestation was not prolonged, conclusively demonstrating the active role of the fetal adrenal cortex in parturition (Drost & Holm,

1968). Furthermore, van Rensburg (1971) reported that fetal adrenal hyperplasia was found in habitually aborting angora goats, suggesting that excessive cortisol secretion leads to premature labour.

Since these pioneering studies in the field of fetal physiology, it has been well established that the activation of the Hypothalamo-Pituitary-Adrenal (HPA) axis is required for the coordination of maturation and parturition in fetuses, which is required for successful extra uterine survival. In the sheep model, some controversy still remains after over 30 years of research, concerning how a large increase in cortisol arises in the presence of negative feedback, and very modest or, more commonly, no increase in plasma ACTH concentrations before the increase in cortisol begins. Among the attempts to explain the cortisol surge are the suggestions that either chronic stress or decreased cortisol negative feedback causes increases in ACTH or that increases in ACTH bioactivity or the responsiveness of the adrenal, to ACTH in late gestation leads to the cortisol surge (Challis & Brooks, 1989; Carr *et al.*, 1995; McMillen *et al.*, 1995a; McMillen *et al.*, 1995b; Schwartz *et al.*, 1995). This review will discuss the main hypotheses that have been proposed to explain the discrepancy between ACTH and cortisol concentrations in the late gestation ovine fetus and discuss the hypothesis that Prostaglandin E_2 (PGE_2) may contribute to the surge in cortisol in the ovine fetus.

1.2. THE HPA AXIS AND PARTURITION

1.2.1. Cortisol

The surge in cortisol in late gestation is critical for postnatal survival. Cortisol has many maturational actions throughout the fetus in late gestation. In the liver it serves the purpose of stimulating glycogen synthesis, in the pancreas it stimulates the maturation of beta cells and in the small intestine cortisol initiates enzyme induction (See Liggins, 1976 for review). In fetal life, cortisol is perhaps best known for its role in the maturation of the fetal lung and the stimulatory effects it has on the production of pulmonary surfactant. Glucocorticoids are used currently to mature the lungs and help alleviate some of the symptoms of respiratory distress syndrome in premature babies (Liggins, 1976). Cortisol also has actions in the brain, placenta, thyroid, adrenal and maternal mammary gland (Liggins, 1976). These actions of cortisol and thus the prepartum increase in cortisol in

late gestation are critical for the successful transition from intra uterine to extra uterine life and survival as a neonate.

1.2.2. *The Hypothalamus*

The hypothalamus and the hypophyseal portal blood vessels develop very early in gestation. The hypothalamus arises from the differentiation of the diencephalon that starts by the fifth week in human development and is complete with all nuclei present in the human fetus by the 15th week of gestation. In the sheep this process is complete by less than 60 days (d) gestational age (GA) (Gluckman, 1982, 1985). The hypophyseal portal blood vessels are functional as early as 45 d GA in the sheep allowing substances from the hypothalamus to reach the pituitary (Levidiotis *et al.*, 1989). The two major hypothalamic peptides that regulate ACTH are corticotrophin releasing factor/hormone (CRF/CRH) and arginine vasopressin (AVP). CRH has been localised to the paraventricular nucleus (PVN) of the hypothalamus and these neurons have been detected by immunohistochemistry in the sheep fetal PVN from 90 d of gestation, and are connected to the external lamina of the median eminence by 105 d (Challis & Brooks, 1989). AVP immunoreactive fibres are present as early as 42 d in the sheep (the earliest age tested) and are connected to the external lamina of the median eminence. Later in fetal development there is a redistribution of AVP fibres towards the internal lamina of the median eminence and this is similar to the innervation seen in adult life. The external lamina of the median eminence is closely associated with the portal blood vessels while the internal lamina is involved with the innervation of the posterior pituitary (Figure 1-1; Levidiotis *et al.*, 1987). In the adult rat, co-localisation of CRH and AVP has been reported in axons in the median eminence although this has not been identified in the fetus (Whitnall *et al.*, 1985; Whitnall *et al.*, 1987).

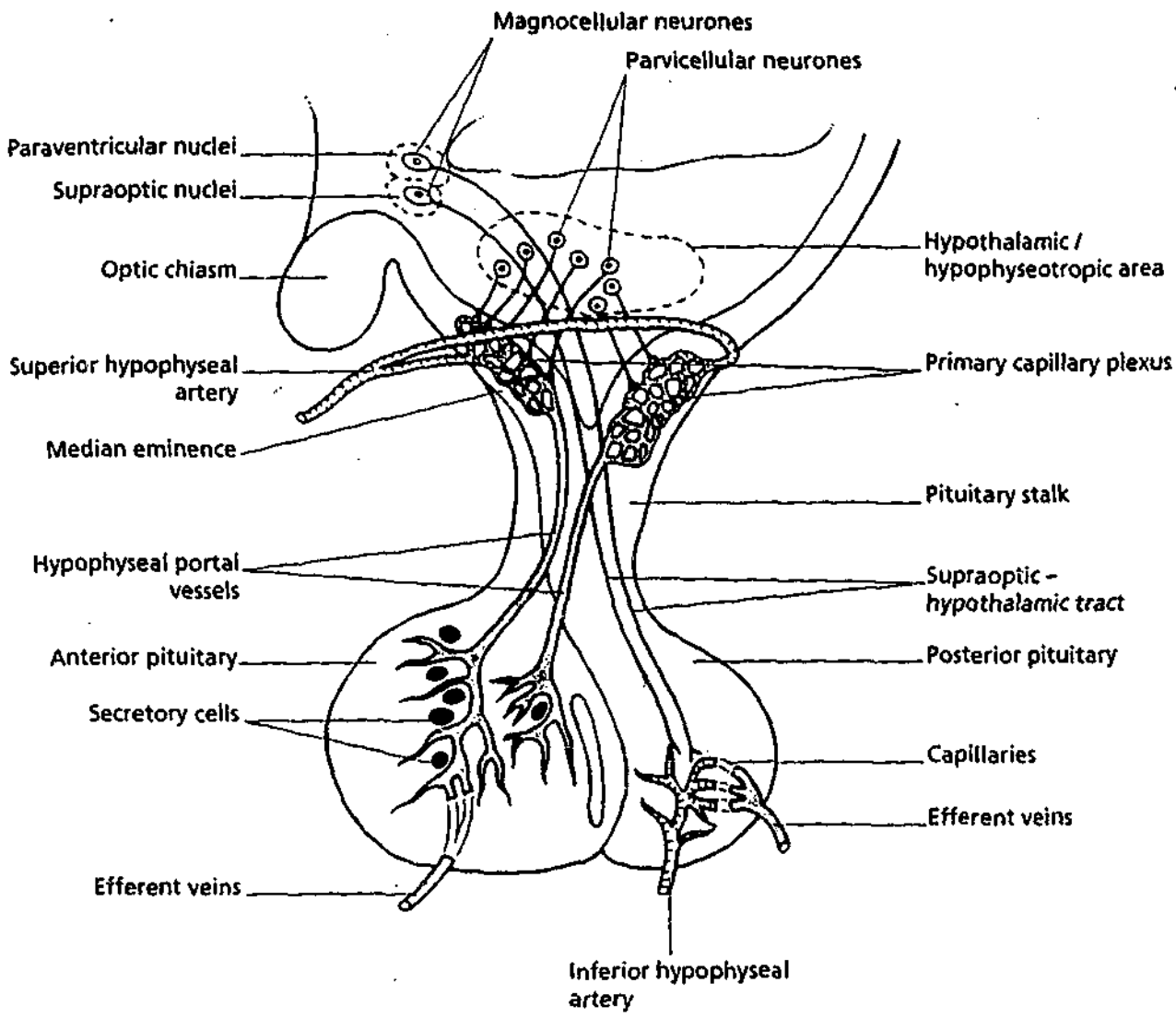

Figure 1-1 Schematic diagram of the neural and humoral connections between the hypothalamus and pituitary glands. From Brook & Marshall, 1996, page 33.

The importance of the fetal PVN and hence CRH and AVP has been demonstrated in a series of studies by McDonald and Myers (1990, 1992a, 1992b) in which they bilaterally lesioned or placed implants of glucocorticoids next to the fetal PVN, and studied the basal and stress responses on the fetal HPA axis. When the fetal PVN was lesioned, it was reported that initiation of parturition was prevented and this procedure blocked the prepartum increases in cortisol and ACTH (McDonald *et al.*, 1990; Myers *et al.*, 1992a). Similarly, placement of dexamethasone adjacent to the PVN in fetal sheep decreased CRF mRNA levels and prevented a fetal ACTH response to the stressors, hypotension and hypoxemia (Myers *et al.*, 1992b). Placement of dexamethasone next to the fetal PVN also significantly reduced basal fetal plasma ACTH concentrations 6-fold when compared with control fetuses (McDonald *et al.*, 1990).

1.2.2.1. CRH

CRH is a 41 amino acid molecule that has been detected by radioimmunoassay (RIA) as early as 63 d of gestation in the sheep (Brieu *et al.*, 1988)]. CRH concentrations in the medial basal hypothalamus are low at d 100, but are significantly increased between 122 – 135 d GA. By d 140, CRH concentrations in this region have declined to the concentration seen at d 100 (Brieu *et al.*, 1988; Brooks & Challis, 1988). Saoud and Wood (1996) measured the amounts of ir-CRH and ir-AVP in hypothalami and pituitaries of fetal sheep from 74 - 101 d gestational age to 3 - 4 week old postnatal lambs. They reported that ir-AVP increased with gestational age in the hypothalamus (highest in postnatal lambs) but there was no difference in the gestational ages tested in the pituitary. CRH also increased with increasing gestation in the hypothalamus, being highest at term (Saoud & Wood, 1996).

Several investigators have infused or injected CRH into the fetus and found differing ACTH secretion rates at varying gestational ages. Wintour *et al.* (1984) administered a variety of doses of CRH (0.1μg – 1 μg) to fetal sheep at gestational ages ranging from 104 –149 d and measured the ACTH released. They observed an increased ACTH response to the bolus dose of CRH at 118 and 130 d and this response increased as gestation progressed. McFarlane *et al.* (1995) also administered graded doses of CRH (0.8, 1.6, 3.8 and 7.6 μg/h for 60 minutes each, given consecutively in ascending order) to fetal sheep at 104 - 108 d and repeated the procedure at 138 - 142 d. They took blood samples hourly for the measurement of plasma ACTH and reported a rightward shift in the log regression line of younger fetuses, which suggested that the younger fetuses were less responsive than the mature fetuses to CRH (McFarlane *et al.*, 1995).

CRH acts through two receptor subtypes, the CRH Receptor 1 (CRHR1) or the alternatively spliced form of the receptor, CRH Receptor 2 (CRHR2). CRHR2 is further subdivided into alpha and beta receptor subtypes (Chen *et al.*, 1993). The CRH receptors exhibit a specific tissue distribution with CRHR1 being expressed in the brain and pituitary (Chen *et al.*, 1993; Webster *et al.*, 1996). In the ovine fetus, the number of CRH-binding sites in the anterior pituitary has been reported to increase progressively from d 65 - 70 to a maximum at 125 - 130 d and then decreases at term (Lu *et al.*, 1991). The authors reported that this binding site expression follows a similar time-course to the responsiveness of the

pituitary to CRH stimulation *in vivo* (Lu *et al.*, 1991). In a more recent study, CRHR1 mRNA and protein expression in the fetal anterior pituitary has been shown to decrease from 102 - 105 d to 137 - 139 d GA and cortisol infusion for 4 days into the fetal circulation resulted in a decrease in CRHR1 mRNA expression (Green *et al.*, 2000).

Infusion of a CRH type 1 receptor antagonist (antalarmin) for 10 days into late gestation fetal sheep resulted in a delay in the timing of parturition. The animals that received vehicle delivered at 142 d while the group that received antalarmin delivered at 149 d. In addition, the animals that were infused with antalarmin had no increases in the plasma concentrations of ACTH or cortisol in the first three days of the infusion unlike the vehicle infused controls, suggesting that the antalarmin did indeed inhibit the HPA axis. The authors concluded that a CRH type 1 receptor antagonist can delay labour and suggested that CRH plays a role in the increase in ACTH and cortisol concentrations in late gestation (Chan *et al.*, 1993). This study was complicated however, as the vehicle used to administer the antalarmin was a significant stimulus that advanced labour in the control animals by approximately 5 days, while the antalarmin treatment delayed labour to normal term (Chan *et al.*, 1998). Further studies using antalarmin have shown that the CRHR1 receptor antagonist inhibits CRH, cortisol and stress-induced ir-ACTH secretion, but not basal ir-ACTH secretion or adrenal responsiveness (Young *et al.*, 2002). Taken together, this data may suggest that CRH has an important role in the cortisol surge prior to term.

1.2.2.2. AVP

AVP is produced by the supraoptic and paraventricular nuclei (magnocellular and parvocellular neurons) of the hypothalamus (Matthews & Challis, 1995). AVP mRNA and peptide concentration, as measured by *in situ* hybridisation are present in the parvocellular neurons of the posterior pituitary by d 60 of gestation and expression has been reported not to alter with increasing gestational age (Matthews & Challis, 1995). Brieu *et al.* (1988) examined the concentration of AVP in the hypothalamus and reported that it increased from d 63 and d 138, and is decreased by d 143. This is consistent with the theory that AVP is more important than CRH early in gestation (Durand *et al.*, 1986; Norman & Challis, 1987a, 1987b). The ACTH response to AVP is very rapid with ACTH returning to basal levels within 30 - 60 minutes (Norman & Challis, 1987b; Brooks & White, 1990).

This short response may be due to the half-life of AVP, which lasts only 2 - 10 minutes in the fetal sheep circulation (Wiriyathian *et al.*, 1983).

CRH and AVP have also been shown to work synergistically *in vitro* and *in vivo* (Durand *et al.*, 1986; Norman & Challis, 1987a; Carr *et al.*, 1995). Brooks *et al.* (1990) administered either CRF (1 μg), AVP (200 ng) or a combination of CRF and AVP, at 4 h intervals for 7 d, to fetuses, starting between 117 and 120 d gestation and reported that CRF alone increased ACTH for at least 240 mins, while AVP alone only increased ACTH transiently (back to basal by 30 mins). CRF in combination with AVP gave a greater ACTH response than either treatment alone (Brooks & White, 1990). Carr *et al.* (1995) performed a similar experiment to Brooks and colleagues by injecting CRF, AVP or a combination of the two hormones, into fetal sheep at a variety of gestational ages. Carr *et al.* (1995) reported that both hormones, when administered individually, significantly increased plasma ACTH at all gestational ages tested in a time frame similar to that reported by Brooks. In addition, Carr *et al.* (1995) reported that the combination of CRF and AVP caused an additive increase in ACTH secretion, except at 120 d when the hormones caused a synergistic increase in plasma ACTH. It is thought that AVP potentiates the CRH response through enhancing the CRH-stimulated cAMP pathway (Bilezikjian & Vale, 1987). CRH and AVP are not the only stimulators of ACTH secretion. Many of the opioid family have been shown to stimulate ACTH secretion (Brooks & Challis, 1988; Taylor *et al.*, 1996a, 1996b). Other agents that have been demonstrated to stimulate ACTH release *in vitro* include oxytocin, noradrenaline, adrenaline and angiotensin II (Vale *et al.*, 1983). PGE_2 can also stimulate the release of ACTH and this is discussed in detail in Section 1.5.4.

1.2.3. The Pituitary

The pituitary gland is located at the base of the brain in the sella turcica and is attached to the hypothalamus by a funnel-shaped structure called the infundibulum (See Figure 1-2 for the location of the pituitary gland). The gland is composed of three lobes, the anterior (adenohypophysis), posterior (neurohypophysis) and intermediate (neurointermediate) lobes (See Figure 1-1). The hormones of the intermediate lobe are thought to be under tonic dopaminergic inhibition from the hypothalamus. The posterior lobe stores and secretes oxytocin and arginine vasopressin that are synthesised in the supraoptic and

paraventricular nuclei of the hypothalamus and then transported axonally to the posterior pituitary.

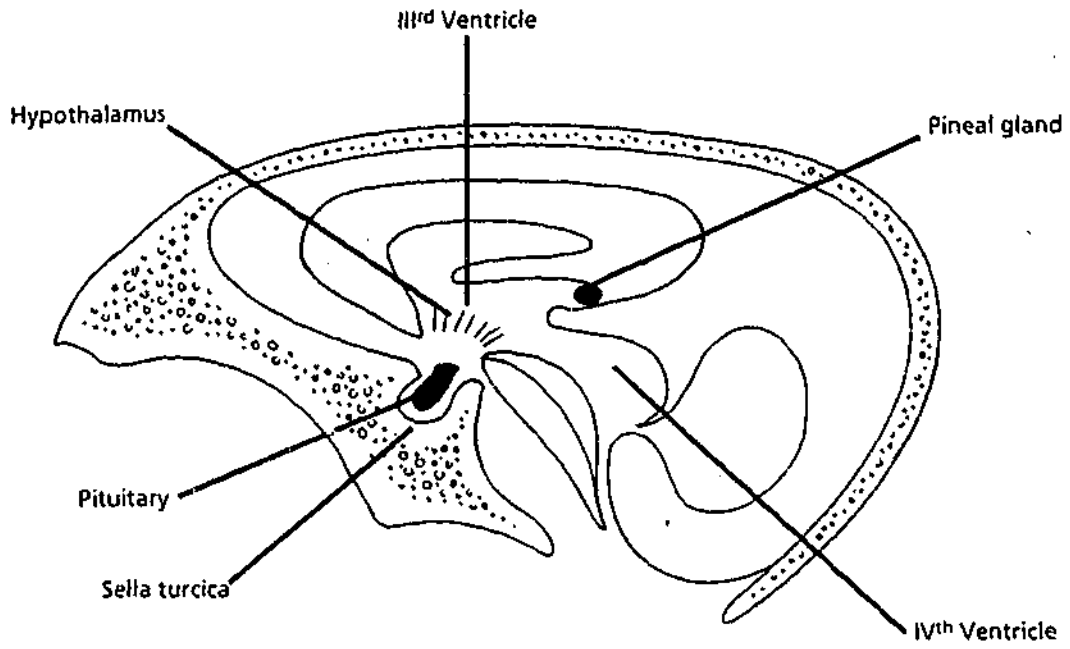

Figure 1-2 A schematic diagram showing the location of the pituitary gland in relation to the rest of the brain. From Brook & Marshall, 1996, page 32.

The anterior pituitary contains six distinct cell types, which in mice, all arise from a common progenitor cell (Simmons *et al.*, 1990; Japon *et al.*, 1994). 1. The somatotrophs synthesise growth hormone, which has roles in growth and metabolism. 2. The thyrotrophs produce thyroid-stimulating hormone, which, as its name suggests acts at the thyroid to stimulate the production of the thyroid hormones, T3 and T4. 3. The lactotrophs synthesise and secrete prolactin that is most well known for its role in lactogenesis. 4. Luteinizing hormone and follicle stimulating hormone are produced in the gonadatrophs and regulate the sex hormones and gametogenesis. 5. Folliculo-stellate cells are star shaped cells that form tiny follicles (Inoue *et al.*, 1999; Inoue *et al.*, 2002). These cells are unlike the other cell types of the pituitary as they do not produce a pituitary hormone as such, but they do produce many cytokines and growth factors (Inoue *et al.*, 2002). The cells have a tendency to surround endocrine cells with long cytoplasmic processes, suggesting that they may play a role in regulating endocrine cells through paracrine intracellular communication (Inoue *et al.*, 2002). 6. The last cell type is the corticotrophs, which are discussed in greater detail in the next section; the corticotrophs synthesise and secrete ACTH

1.2.3.1. Corticotrophs

The corticotrophs are a population of cells in the pituitary that secrete ACTH. This population of cells constitutes approximately 15 - 20% of all pituitary cells in the adult (Perez *et al.*, 1997). ACTH has been identified by immunohistochemical techniques as early as 38 d gestational age (Alexander *et al.*, 1973; Perry *et al.*, 1985).

There has been some suggestion that there are subdivisions within this population in the fetus, with two morphologically different cell types being described. The "fetal-type" corticotrophs are columnar cells that stain for ACTH only weakly, whereas the "adult-type" corticotrophs are small and angular and stain strongly for ACTH (Perry *et al.*, 1985). It has been suggested that there is "maturation" of the corticotrophs in late gestation as the population of fetal corticotrophs declines after 130 d and the number of adult-type cells increase (Mulvogue *et al.*, 1986; McFarlane *et al.*, 1995).

There is also some evidence for the presence of functionally different subpopulations of corticotrophs, in addition to the fetal and adult type cells. These subpopulations are defined by the sensitivity of the individual subtypes to CRF and AVP. Jia *et al.* (1991) used a double reverse hemolytic assay to show that some individual pituitary cells formed plaques in response to CRH but not AVP, some formed plaques in response to either AVP or CRH and some cells formed plaques to a combination of CRH and AVP. No cells formed plaques to AVP alone. The results of this study suggest that different populations of corticotrophs are responsive to the different secretagogues (Jia *et al.*, 1991). Schwartz *et al.* (1988) used a different technique to assess if different populations of corticotrophs were present in the anterior pituitary. This group applied a specific cytotoxic analogue of CRH to bovine anterior pituitary cells and reported that CRH stimulated ACTH secretion was reduced when the cells were pretreated with the cytotoxin but ACTH secretion was unchanged when the pretreated cells were stimulated with AVP. The authors suggested that there are separate subpopulations of corticotrophic cells (Schwartz & Vale, 1988). To extend this study, Butler *et al.* (1999) collected pituitary glands from fetal sheep that had been infused with either saline or cortisol, and then pretreated half of the pituitary cells with the CRH cytotoxin. They demonstrated that approximately 70% of ACTH in the fetal anterior pituitary is stored within corticotrophs that are CRH responsive. In addition, they reported that cortisol acts to inhibit ACTH synthesis in corticotrophs that are CRH

responsive whereas AVP responsive cells are relatively resistant to cortisol (Butler *et al.*, 1999). This apparent resistance to cortisol feedback may be the mechanism that allows ACTH synthesis and secretion to continue in the presence of high circulating concentrations of cortisol in the late gestation ovine fetus. Fora *et al.* (1996) have also reported that although pituitary cells are more responsive to CRH at 108 d GA, they were more sensitive to AVP at 135 d GA and in adulthood. In direct contrast to Fora *et al.* (1996), Carr *et al.* (1995) and Norman & Challis (1987) have reported *in vivo* that the relative role of AVP in stimulating ACTH release decreases with increasing gestational age and suggest that CRH gains in importance as gestation advances.

The switch from fetal to adult type corticotrophs can be manipulated by experimental interventions. Antolovich *et al.* (1990) investigated the effects of adrenalectomy, hypothalamo-pituitary disconnection (HPD) and cortisol infusion on corticotroph populations. Adrenalectomised fetuses had an increased number of "fetal" corticotrophs compared to control fetuses, while cortisol infused fetuses had an increased number of "adult" corticotrophs when compared to control fetuses. This study suggests that the maturation of corticotrophs is dependent on cortisol and possibly on the increase in cortisol in late gestation (Antolovich *et al.*, 1990). In another study, the effects of HPD on corticotroph maturation were examined and it was reported that this treatment also prevented the switch of the "fetal" type corticotrophs to "adult" type corticotrophs. This failure to switch to the mature corticotroph population may be caused by the low cortisol concentrations seen in these fetuses or perhaps suggest that hypothalamic factors may play a role in pituitary maturation (Antolovich *et al.*, 1991). Unfortunately, no attempt has been made to correlate the changing populations of fetal and adult cells with the functionally defined changes of CRH only, AVP only and CRH + AVP responsive corticotrophs.

1.2.3.2. POMC

Proopiomelanocortin (POMC) is synthesised in both the corticotrophs of the anterior pituitary and the melanotrophs of the intermediate lobe of the pituitary. It is a precursor molecule that is cleaved into several structurally related neuromodulators and endocrine peptides including ACTH. The 30 kD POMC molecule is cleaved to form a 22 kD intermediate peptide, pro-ACTH and the 8 kD peptide β-Lipoptrophin (β-LPH). β-LPH is further processed into β-endorphin and γ-LPH, while pro-ACTH is also cleaved into

$ACTH_{1\text{-}39}$ and a 16 kD molecule, pro-γ-MSH. Pro-γ-MSH is cleaved to form N-$POMC_{1\text{-}48/49}$, γ_3-MSH and a joining peptide (Seger & Bennett, 1986). In the intermediate lobe $ACTH_{1\text{-}39}$ is also further cleaved into corticotrophin-like intermediate peptide (CLIP) and α-MSH (Mains *et al.*, 1977; Mains & Eipper, 1979; Figure 1-3).

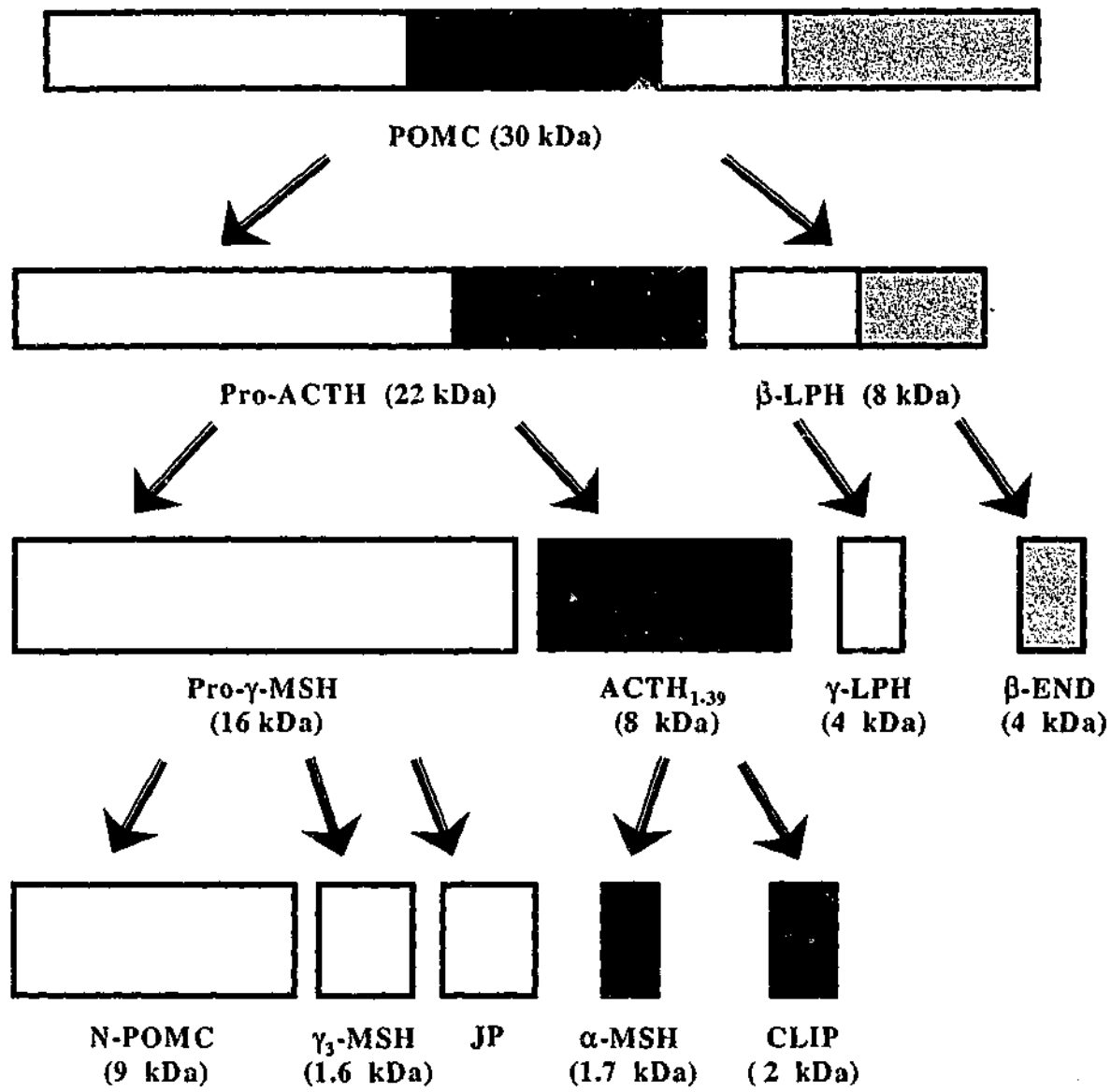

Figure 1-3 Schematic diagram of the processing of the proopiomelanocortin (POMC) molecule. Cleavage of $ACTH_{1\text{-}39}$ and pro-γ-MSH occurs in the intermediate lobe, all other processing occurs in the anterior and the intermediate lobe. ACTH: adrenocortiocotrophin; LPH: lipotrophin; MSH: melanotrophin; End N-POMC: N-terminal $POMC_{1\text{-}48/49}$; JP: joining peptide; CLIP: corticotrophin-like intermediate peptide. Adapted from Orth *et al.*, 1992.

1.2.3.3. POMC Processing

Differential processing of POMC results in different peptides being found in the different lobes of the pituitary. In the anterior pituitary, ACTH, β-endorphin and β-lipotrophin are the main products of post-translational processing, while in the intermediate lobe, the main products are corticotrophin-like-intermediate peptide (CLIP), α-melanocyte stimulating hormone (MSH) and acetylated β-endorphin (Eipper & Mains, 1980). Much of this differential processing has been attributed to the actions of two enzymes, prohormone convertase 1 (PC1) and 2 (PC2). Using *in situ* hybridisation studies and more recently northern blots, PC1 has been localised to the anterior and intermediate lobes of the pituitary while PC2 has only been identified in the intermediate lobe (Seidah *et al.*, 1991; Bell *et al.*, 1998). PC1 cleaves POMC into ACTH and β-LPH, while PC2 cleaves POMC into β-endorphin, an N-terminally extended ACTH containing the joining peptide, and either α-MSH or deascetyl-α-MSH (Benjannet *et al.*, 1991).

Yang *et al.* (1991) demonstrated the presence of POMC mRNA in the fetal sheep pituitary at 60 d gestational age bynorthern blot analysis. However in the late gestation fetus there is some conflict over the ontogenic profile of POMC mRNA in the pituitary. Matthews *et al.* (1994) used *in situ* hybridisation to examine the regional distribution of POMC mRNA in the pituitary at different times in gestation. They reported that POMC mRNA expression increased from 60 - 80 d gestation to 0 - 7 d postpartum in the inferior region at the base of the pars distalis (anterior lobe) (Matthews *et al.*, 1994). There was no change in the mRNA encoding POMC in the superior region on the pars distalis (the region nearest to the NIL) (Matthews *et al.*, 1994). Two other studies however, found a decrease in POMC mRNA abundance in late gestation before birth. McMillen *et al.* (1988) used dot blots to show that POMC mRNA abundance was lower at 141 – 144 d than at 100 - 135 d, while Merei *et al.* (1993) used northern blotting, to also show a decrease in POMC mRNA between 130 - 136 d to 141 - 143. POMC mRNA was more abundant in the NIL than the anterior pituitary at all time points measured (Merei *et al.*, 1993). However, these two studies do not take into account the differences in distinct regions of the pituitary. Yang *et al.* (1991) found that POMC levels did not change in the anterior pituitary from 60 – 130 d but then increased by 138 - 143 d while Myers *et al.* (1993) reported that POMC mRNA levels increased between days 105 – 107 and 138 –140 d gestational age. Bell *et al.* (1997) reported no difference in the superior region of the anterior pituitary between 100 – 147 d

but found an increase in POMC mRNA in the inferior region of the anterior pituitary after 144 –147 d.

1.2.3.3.1. Regulation of POMC

POMC mRNA appears to be regulated, at least in part, by glucocorticoids as Myers *et al.* (2000) reported a decrease in POMC mRNA abundance in dispersed pituitary cells from fetuses aged 140 - 144 d GA that had been incubated with dexamethasone (DEX), a synthetic glucocorticoid. However, pretreatment of these cells with CRH actually prevented the decrease in POMC mRNA seen when DEX was added, suggesting that cortisol acts by negative feedback at the hypothalamus (Myers & Myers, 2000). Jeffray and co-workers (1998) infused cortisol into fetuses from 126 d GA for 96 h and caused a decrease in POMC mRNA abundance in both the anterior and intermediate lobes of the pituitary. POMC mRNA was also measured in the anterior pituitary from fetuses that had undergone bilateral adrenalectomy and it was reported that POMC mRNA was elevated in these animals when compared to control fetuses. In this study the authors concluded that the fetal adrenals were acting to suppress the expression of POMC mRNA. If this is the case and glucocorticoids suppress POMC mRNA, one would expect with increasing cortisol levels in late gestation fetuses, POMC mRNA abundance would decrease. This is consistent with the studies of McMillen *et al.* (1988) and Merei *et al.* (1993) who reported decreasing POMC abundance in late gestation.

Bell *et al.* (1997) reported a differential regulation between the inferior and superior regions of the anterior pituitary after lesion of the fetal PVN. The authors reported a decrease in POMC mRNA after lesion of the PVN, in the inferior, but not the superior anterior pituitary (Bell *et al.*, 1997).

1.2.3.3.2. POMC-Derived Molecules and Steroidogenesis

Silman *et al.* (1979) investigated the profiles of POMC derivatives in the fetal and adult pituitary and reported that in the adult pituitary, $ACTH_{1-39}$ predominated, but β-LPH and γ-LPH were also present. In the fetus however, there was a large proportion of high molecular weight material when compared with the adult (Silman *et al.*, 1979). The authors suggested that these high molecular weight peptides were "stem" hormones. Jones (1976a) examined the secretion of ACTH from dispersed fetal pituitary cells and reported

that fractionation gave four biologically active ACTH species of molecular weights, 50 000, 10 000, 5 000 and 3000 kDa. After addition of CRF to the dispersed fetal pituitary cells, the 5000 kDa peak was predominately secreted. The author suggested that the HMW forms of ACTH are the storage forms and the conversion of the HMW forms to the 5000 molecular weight species causes an increase in the biological activity of ACTH 10 – 20 fold (Jones, 1976a). Saphier *et al.* (1993) measured the content of cortisol, ACTH and N-terminal POMC (1-77) (N-POMC) in the ovine fetal circulation. They reported an 880% increase in plasma cortisol between 110 - 119 d GA and 139 - 147 d GA. In comparison, plasma ACTH concentrations only increased 137% during this time and N-POMC concentrations actually decreased by 33%. There was however, an increase in the concentration of MSH suggesting that there was an increase in the cleaving of POMC (Saphier *et al.*, 1992).

There has been some controversy about the effects of the high molecular weight fragments of ACTH on steroidogenesis in the fetal adrenal gland. Using dispersed ovine adrenal cells, Roebuck *et al.* (1980) showed that HMW forms of ACTH block the action of $ACTH_{1\text{-}39}$. The authors suggested that this might be the reason that fetal adrenal cells are less responsive *in vivo* than *in vitro* (Roebuck *et al.*, 1980). The steroidogenic capacity of adult and fetal adrenal cells to POMC and pro-ACTH was also investigated by Schwartz *et al.* (1995), who reported that the ACTH precursors were able to neither stimulate nor inhibit cortisol production. However, POMC and pro-ACTH did inhibit the stimulatory effect of $ACTH_{1\text{-}24}$ on fetal adrenal cells but the effect was concentration dependent (POMC at 2.6 nM, pro-ACTH at 0.7 - 0.23 nM when ACTH is present at 0.01 nM) (Schwartz *et al.*, 1995). In a similar study, where cultured adrenal cells were used to test the effects of non-ACTH POMC derived peptides on steroidogenesis, it was reported that there was only an increase in cortisol production after at least three days in culture using these peptides (Durand *et al.*, 1984b).

An additional report used perifused rat adrenal glands from late gestation rat fetuses and added either, "big" (30 000 Da; POMC), "intermediate" (13 000 Da; ACTH + LPH) or "little" (4 500 Da; ACTH) ACTH from pituitary extracts that had been fractionated via chromatography. This study reported that, "big ACTH" was not as potent as "intermediate" or "little ACTH" at any of the gestational ages tested, although it did become more potent in the last few days before labour. "Big ACTH" when placed in

combination with "little ACTH" had no effect on the adrenal response to "little ACTH" (i.e. did not inhibit). On d 17 and 19 of pregnancy (term = 21 d) "intermediate" and "little ACTH" had the same corticosteroidogenic response although by d 21 "little ACTH" was more potent than the "intermediate" type. In addition, "intermediate ACTH" also increased 4 fold in potency from d 17 to d 19 and then remained stable while, "little ACTH" increased 10 fold in potency from d 17 to d 21 (Chatelain & Cheong, 1987). These studies suggest that the LMW ACTH species are the most biologically active and that as gestation progresses there are trends towards an increase in the proportion of these LMW forms of ACTH. However, it still remains unclear if the HMW species inhibit steroidogenesis *in vivo* in the sheep as in the rat at least, it would appear that HMW ACTH does not inhibit the steroidogenic action of LMW ACTH.

Durand *et al.* (1985) stimulated cultured adrenal cells from 113 d ovine fetuses with either ACTH, fetal pituitary extracts (FPE) or newborn pituitary extracts (NPE) and reported that even after repeated stimulation, no difference between cAMP output and cortisol production was observed. The authors suggested as there was no difference between FPE and ACTH stimulated cortisol production, big forms of ACTH do not have an inhibitory effect on steroidogenesis (Durand *et al.*, 1985).

Estivariz and co-workers (1982) reported that the N-terminal region of POMC (non MSH portion) can stimulate adrenal DNA synthesis *in vitro* and mitosis *in vivo* and the authors suggested this might be the factor that controls adrenal growth. In a separate study, N-$POMC_{1-77}$ or N-$POMC_{1-49}$ was infused into fetal sheep from 136 - 138 d gestational age and the infusion of $POMC_{1-77}$ was found to increase the abundance of the mRNA encoding 17α-hydroxylase enzyme and, although the infusion of either peptide failed to increase the concentration of ACTH and cortisol, the infusion of these N-terminal POMC peptides did stimulate adrenal growth, as was previously reported by Estivariz *et al.* (1982) (Ross *et al.*, 2000). In a recent study, Coulter and colleagues (2000) also infused either $POMC_{1-49}$ or $POMC_{1-77}$ into fetal sheep for 48 h at 136 d gestational age and reported that $POMC_{1-77}$, but not $POMC_{1-49}$ suppressed StAR mRNA, which could have the downstream effect of decreasing cortisol synthesis.

1.2.3.4. ACTH

There has been some debate about the plasma concentrations of ACTH achieved in late gestation. Firstly, there are those that argue that ACTH concentrations increase from approximately 120 d gestation, prior to the increase in cortisol (Challis & Brooks, 1989). Others propose that there is a switch in the processing of the POMC molecule from HMW precursors to LMW ACTH and that this switch is responsible for the increase in cortisol concentrations (Jones & Roebuck, 1980; Roebuck *et al.*, 1980). In addition, some argue that this switch may be due to a maturation of the pituitary corticotrophs (Antolovich *et al.*, 1988). Some groups argue that ACTH concentrations do not increase until the very last days before labour, well after the basal cortisol concentrations have started to increase (Jones, 1983; Jacobs *et al.*, 1994; Hollingworth *et al.*, 1995; Poore *et al.*, 1998a).

A great deal of this controversy may arise from differing specificities in the methods of measuring ACTH. Early radioimmunoassays (RIA) measured not only ACTH but also its precursors POMC and pro-ACTH and other smaller fragments of the POMC molecule. This probably accounted for the high measurements of ACTH obtained in the late 1970's. Since then the development of more specific antisera have led to a decrease in the amount of ACTH being measured. Two-site immunoradiometric assays (IRMA) that measure the concentration of ACTH in the plasma utilise specific antisera and may be more accurate than assays, which employ non-specific antisera. The antibodies for the two site IRMA's do not take into account the first 10 residues of the ACTH molecule and thus will still measure peptides that extend past these first 10 residues (Crosby *et al.*, 1990). One method to obtain a relatively accurate measurement of ACTH appears to be an RIA after peptide separation by gel filtration. Using this method several investigators have reported no increases in plasma ACTH concentrations until after the increase in cortisol has commenced in the fetal sheep (Jones, 1983; Jacobs *et al.*, 1994; Hollingworth *et al.*, 1995; Poore *et al.*, 1998a).

Castro *et al.* (1992, 1993) measured plasma ACTH from fetal sheep by both an RIA (measured ir-ACTH) and a bioactivity assay (measured bioactive ACTH-like activity) where the treated plasma was added to acutely dispersed rat adrenal cells. This group reported that there was less bioactive ACTH in immature fetuses (less than 95 d GA) than mature fetuses (greater than 130 d GA) suggesting that the increasing cortisol response in

late gestation could be due in part to an increase in the bioactivity of ACTH (Castro *et al.*, 1992). Furthermore, this group reported that in ovine fetuses in the last 2 weeks before birth, the amount of bioactive ACTH secreted was low under non-stress and mild-stress conditions, but increased after marked stress (Castro *et al.*, 1993). Zehnder *et al.* (1998) extended these findings and reported that in adrenalectomised (ADX) fetuses, basal ir-ACTH, bioactive ACTH and $ACTH_{1-39}$ (as measured by a two-site IRMA) increased from 126 - 130 d to 136 - 140 d. In addition, this study showed that cortisol infusion decreased ir-ACTH, bioactive ACTH and $ACTH_{1-39}$ in ADX fetuses at both age groups (Zehnder *et al.*, 1998).

Hollingworth *et al.* (1995) examined the chromatographic profile of ir-ACTH in the ovine fetus and reported an increase in basal LMW ACTH between 136 - 140 d and 140 - 145 d GA, well after the cortisol surge had commenced. The authors also reported that there was no change in the proportion of LMW immunoreactive material between 119 and 140 d GA (Hollingworth *et al.*, 1995). Poore *et al.* (1998a) extended the findings of Hollingworth and colleagues (1995), reporting that in the ovine fetus, ir-ACTH increased only in the last 10 d of gestation, as measured by two independent radioimmunoassays, a commercially available kit assay (ICN) and a locally developed RIA. In addition, the LMW portion of ir-ACTH was measured, showing that LMW ACTH only increased in the last 9 d of gestation (Poore *et al.*, 1998a). Similarly, in the goat fetus, Ford *et al.* (1998) showed that bioactive ACTH and the ratio between HMW and $ACTH_{1-39}$ did not differ, from 18 days before labour (dbl) until 4 dbl, while cortisol showed the same profile as in the sheep, with concentrations increasing 10 – 14 dbl. Furthermore, Young *et al.* (1996b) reported in the ovine fetus that ir-ACTH only increased in the last week before labour and that $ACTH_{1-39}$ (as a proportion of total ir-ACTH) increased from 10% to 25%, only in the last 5 dbl. These studies suggest that ACTH does not increase before the cortisol surge begins, and furthermore, that the proportion of LMW ACTH may not increase until the last week before labour. In addition, analysis of the concentration profiles of ACTH and cortisol show a clear correlation at 126 d GA but not at 145 d GA after the cortisol surge is well established. This suggests that changes in cortisol secretion may not be explained by changes in ACTH during the pre-partum period (Canny *et al.*, 1998).

Two experimental models, hypophysectomy (HX) and hypothalamo-pituitary disconnection (HPD) have been employed to further define the role of ACTH in the timing

of the cortisol surge. In the HPD model, fetuses do not initiate parturition, but have normal or elevated basal plasma ACTH concentrations and an apparently normal increase in ir-ACTH concentrations, in comparison to intact fetuses (Deayton *et al.*, 1994). This may be because the ACTH secreted by the fetal pituitary is not biologically active at the adrenal. In the HX model, there is no fetal pituitary and the adrenal cortex becomes hypotrophic and hyporesponsive, secreting very low concentrations of cortisol (Liggins *et al.*, 1968; Mesiano *et al.*, 1987; Jacobs *et al.* 1994; Poore *et al.* 1998). Parturition is not initiated in these fetuses. Whereas in HX fetuses that are maintained on a continuous steady-state infusion of $ACTH_{1-24}$ and thus do not have an increase in ACTH, have normal adrenal growth, normal increases in plasma cortisol and an appropriately timed labour (Jacobs *et al.*, 1994). These studies suggest that an increase in ACTH is not required for the increase in cortisol concentrations in late gestation and brings into question the role that ACTH plays in the cortisol surge.

1.3. THE ADRENAL

1.3.1. Development

The adrenal gland is divided structurally and functionally into two regions, the medulla and the cortex, which arise from different primordia and in some species, are separate organs (Baird (ed.),1975). The medulla is neural in origin and forms the innermost region of the gland. It synthesises and secretes adrenaline and noradrenaline in response to either sympathetic or parasympathetic neural inputs (Brook & Marshall, 1996).

The cortex arises from the mesenchymal cells adjacent to the urogenital ridge. It is further subdivided into three regions in the adult, the zona glomerulosa that produces aldosterone, the zona fasiculata that produces glucocorticoids and the zona reticularis, which produces glucocorticoids and androgens. In the sheep fetus the main glucocorticoid produced is cortisol, while the main androgen produced in the zona reticularis is dehydroepiandrosterone (DHEA), a precursor that is processed further in other steroidogenic tissues to estrogen and testosterone (Brook & Marshall, 1996).

In the sheep fetus, the adrenal gland can be identified from 28 days of gestation. Morphologically, between 40 and 50 days gestation, one cortical cell type predominates,

which is characterised by free ribosomes in the cytoplasm, oval or round mitochondria with lamellar and vesicular cristae and a moderate amount of endoplasmic reticulum (Robinson *et al.*, 1979). The adrenal gland at this stage of development is able to secrete more cortisol per milligram of tissue than at any other time during gestation (Robinson *et al.*, 1979). After 60 d GA, the two zones of the adrenal can be identified and the outer zone begins to resemble the adrenal cortex and undergoes increases in width, while the inner zone remains disorganised and does not undergo any growth. From 120 d GA onwards, the inner zone becomes reorganised to form the adrenal medulla, while the adrenal cortex undergoes rapid maturation (Robinson *et al.*, 1979).

1.3.2. Roles of ACTH on the Fetal Adrenal Gland

1.3.2.1. ACTH Receptor

The binding of ACTH to its receptor stimulates a second messenger cascade that activates adrenocortical steroidogenesis both directly, via protein kinases, which stimulate enzyme activity, and also indirectly, via transcription factors, that modulate mRNA expression (Mountjoy *et al.*, 1992). The ACTH receptor is part of the melanocortin receptor family and has been designated the melanocortin-2 receptor (MC2R). The melanocortin receptors have several unusual characteristics in that they are encoded by intronless genes, lack several amino acids present in most G-protein coupled receptors, and have some short intracellular, extracellular and transmembrane domains (Mountjoy *et al.*, 1992).

Y1 cells (mouse adrenocortical cell line that expresses the ACTH receptor) were used to measure the binding of radioactively labelled ACTH and MSH to the melanocortin receptors (1 - 5). ACTH bound only to ACTH-receptor and was unable to bind the other melanocortin receptors, while labelled MSH peptides were unable to bind to the ACTH receptor. The authors concluded that MSH peptides may not be able to induce steroidogenesis *in vivo*, as this peptide was unable to bind to the MC2R receptor (Schioth *et al.*, 1996).

The number of ACTH receptors in the late gestation adrenal gland has been studied *in vitro* in a number of studies. In 1979, Durand measured the number of ACTH receptors, by investigating the binding of radioactively-labelled ACTH to subcellular preparations of

ovine fetal adrenal glands and reported that the number of receptors increased 5-fold from 123 days to birth, and continued to increase for at least 3 days after birth. In addition, he reported a correlation between plasma cortisol levels and the number of ACTH receptors (r = 0.57) in late gestation (Durand, 1979). Durand *et al.* (1981a) expanding on these earlier studies, used crude fetal adrenal membrane preparations to measure the binding of iodinated $ACTH_{1-24}$. They reported no change in the number of ACTH binding sites from 124 –140 d GA but a 3 fold increase from 140 d to birth (Durand *et al.*, 1981a). In a recent study, the abundance of ACTH receptor (ACTH R) mRNA was measured in the ovine fetus *in vivo* and no change was observed between 132 d and term in the ovine fetal adrenal (Simmonds *et al.*, 2001). Fraser *et al.* (2001) reported a significant increase in the abundance of ACTH R mRNA in the ovine fetal adrenal between days 126 - 8 and 140 - 1 with further increases with the onset of spontaneous labour. The results of these studies are inconsistent and the number of ACTH binding sites in the adrenal requires further investigation.

Several *in vitro* studies have indicated that the ACTH receptor is regulated by its own ligand, as the pre-treatment of fetal adrenal cells in culture with ACTH, stimulated a two-fold increase in ^{125}I-ACTH binding to adrenal cell membranes (Morita *et al.*, 1995). In addition, perfusion *in utero* for 5 days with $ACTH_{1-24}$ increased the number of ACTH receptors as measured by binding assays, but did not change the binding affinity (Durand *et al.*, 1981b). *In vivo* studies measuring the mRNA abundance of the ACTH receptor have failed to detect any increases with exogenous infusion of ACTH. ACTH infusion to the fetal sheep from 115 d GA till either 132 d GA or term did not increase the abundance of ACTH receptor mRNA from the levels obtained in saline infused controls, while in a separate study, a 24 hour infusion of ACTH at 127 d GA also did not increase the abundance of ACTH R mRNA, suggesting at least at the mRNA level, the ACTH R *in vivo* is not regulated by its own ligand (Simmonds *et al.*, 2001; Carter *et al.*, 2002).

1.3.3. Steroidogenic Enzymes

The enzymes of the cortisol synthetic pathway are important regulators of cortisol production. In the human adult, the adrenal cortex produces three steroid hormones, aldosterone, cortisol and dehydroepiandrosterone (DHEA). ACTH stimulates adrenocortical steroidogenesis by binding to the ACTH R and acting via a cAMP

dependent pathway to stimulate cortisol synthesis. cAMP activation results in low-density lipoproteins being mobilised from lipid stores in the cell, which are transported with the aid of steroidogenic acute regulatory protein (StAR), to the inner membrane of the mitochondria. Cholesterol side chain cleavage enzyme ($P450_{scc}$ or CYP11A1) is located in the mitochondria and is the initial enzyme in the production of all physiological steroids and is responsible for the cleaving of cholesterol into pregnenolone. There is then a cascade of reactions in the endoplasmic reticulum and the mitochondria, using enzymes such as 17α-hydroxylase ($P450_{c17}$; 17α-OH) and 3β-hydroxysteroid dehydrogenase (3βHSD) that are involved in the production of cortisol. See Figure 1-4 for a schematic diagram of the cortisol synthetic pathway.

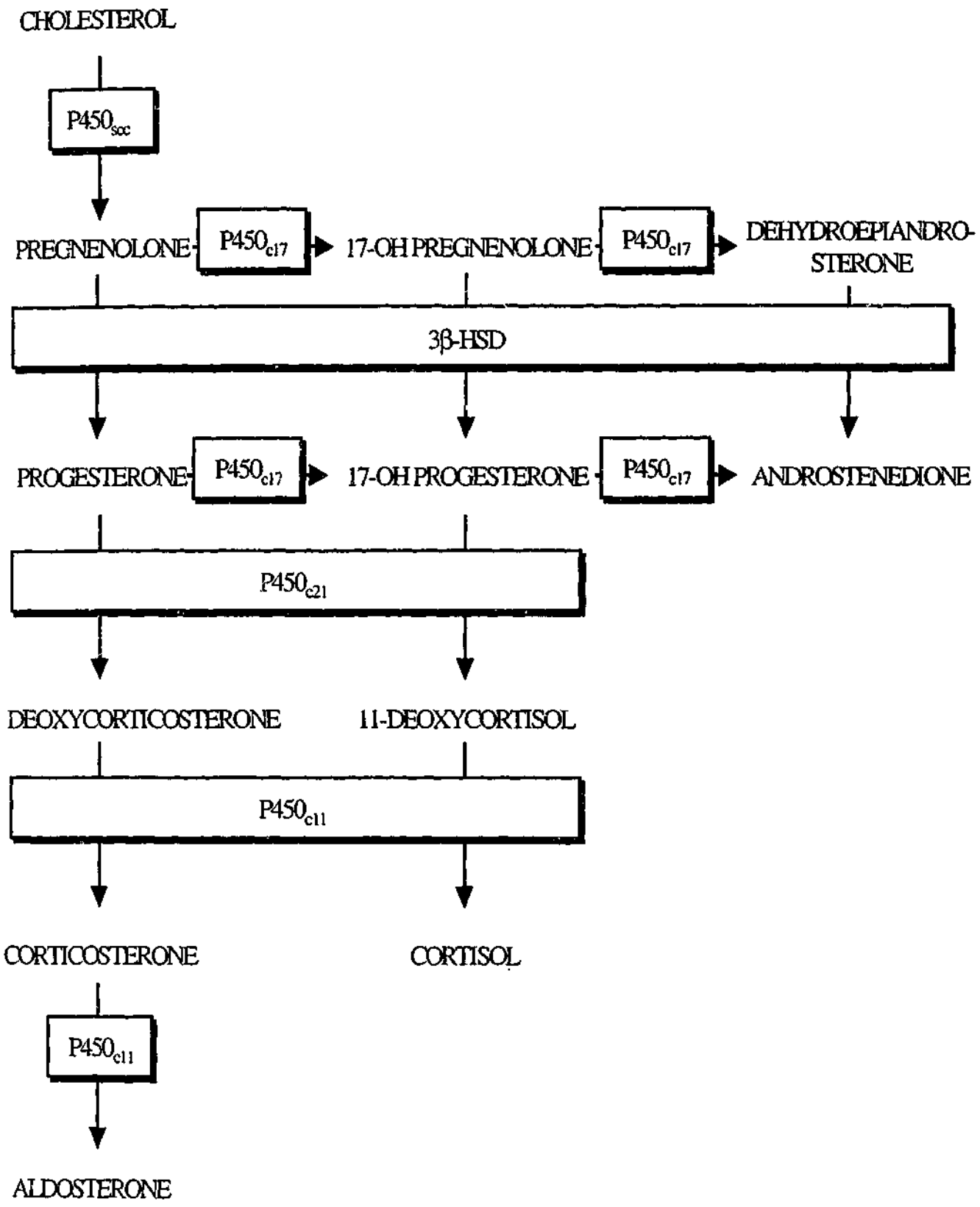

Figure 1-4 Schematic diagram of the cortisol steroidogenic pathway. Cholesterol side chain cleavage enzyme ($P450_{scc}$ or CYP11A1); 17α-hydroxylase (17α-OH, $P450_{c17}$ and CYP_{c17}); 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD); 21-hydroxylase ($P450_{c21}$, CYP_{c21}); 11-hydroxylase ($P450_{c11}$, CYP_{c11}).

Durand & Cathiard (1982) examined the time course of steroidogenesis using isolated adrenal cortical cells from ovine fetuses that had received either ACTH or vehicle (daily dose 0.1 mg) for 5 days. The cells were incubated with radiolabelled ^{14}C-pregnenolone and the disappearance of pregnenolone, the formation of labelled progesterone, 11-DOC, corticosterone, dihydroxypregnenolone, dihydroxyprogesterone, and cortisol was measured. They reported that pregnenolone metabolism was more rapid after treatment with ACTH, with all of the pregnenolone metabolised after 2 hours, compared with only 45% in controls. The rate-limiting enzymes (according to the time course of metabolites) were 3β-HSD and 17α-OH in the control cells, while the ACTH treatment increased the activities of these enzymes so they were no longer rate-limiting (Durand *et al.*, 1982).

There is some evidence that the rate limiting enzyme in the cortisol synthetic pathway may vary across gestation, as immunohistochemical studies have shown that by 90 d GA $P450_{C21}$ and $P450_{scc}$ are both present in the zona glomerulosa and fasiculata, while $P450_{c17}$ was confined the zona fasiculata. Between 90 - 120 d GA $P450_{scc}$ and $P450_{c17}$ staining was weak when compared with $P450_{C21}$ but from 120 d onwards until term $P450_{scc}$ labeling became more intense and an increase in $P450_{c17}$ was reported and this was in similar amounts to $P450_{C21}$, perhaps suggesting that these enzymes may no longer be rate limiting (Tangalakis *et al.*, 1989).

A number of investigators have examined the abundance of steroidogenic enzymes after interventions to the HPA axis, including lesions of the PVN, HPD and HX. Myers *et al.* (1992b) reported a 3-fold decline in the abundance of $P450_{c17}$ and $P450_{scc}$ mRNA from 105 - 120 d GA however, these mRNA increased from 126 d to term. In addition, $P450_{c21}$ increased from 126 d to term, while $P450_{c11}$ decreased with increasing gestational age (i.e. from 105 d to term). In this study, the placement of dexamethasone next to PVN prevented the re-emergence of $P450_{c17}$ and $P450_{scc}$ at 126 - 128 d but had no effect on $P450_{c21}$ and $P450_{c11}$ (Myers *et al.*, 1992b). In another study by Myers *et al.* (1992a), bilateral lesions of the PVN caused a decrease in the mRNA encoding $P450_{scc}$ and $P450_{C17}$ but not $P450_{c11}$, $P450_{C21}$ or 3β-HSD in the fetal adrenal at 157 d GA when compared to sham fetuses at term. Phillips *et al.* (1996) examined the role of the HPA axis in adrenocortical steroidogenesis using intact or hypothalamo-pituitary-disconnected fetuses and measured the abundance of several steroidogenic enzymes. In an ontogeny study they reported an

increase in $P450_{scc}$ mRNA between 130 and 140 d GA and showed that this mRNA remained elevated after 141 d. 3β-HSD mRNA did not change between 130 and 145 d GA while $P450_{C17}$ mRNA increased after 141 d GA. $P450_{C21}$ mRNA increased between 130 - 135 and 136 - 140 d and remained high after 140 d (Phillips *et al.*, 1996). HPD caused a decrease in the expression of $P450_{scc}$, 3βHSD and $P450_{c17}$ at the mRNA level. Simmonds *et al.* (2001) expanded the findings of Phillips *et al.* (1996), showing that $P450_{scc}$, $P450_{c17}$ and 3β-HSD mRNA abundance increased between 132 d and term, and a low dose continuous infusion of ACTH could further increase the abundance of $P450_{scc}$ and 3β-HSD mRNA at 132 d GA but not at term, suggesting that these enzymes are maximally induced at term. Furthermore, hypophysectomy (HX) caused a significant reduction in the mRNA encoding $P450_{scc}$, $P450_{c17}$ and 3β-HSD mRNA and ACTH infusion to the HX fetuses restored the mRNA level back to that seen in control intact fetuses. Tangalakis *et al.* (1990) also infused ACTH into sheep fetuses and reported that ACTH infusion increased the abundance of the mRNA encoding $P450_{scc}$ and $P450_{c17}$, but not $P450_{c21}$. These studies all demonstrate that both ACTH and hence, the presence of an intact HPA axis closely regulate the steroidogenic enzymes.

1.4. *Proposed Mechanisms of the Cortisol Surge*

1.4.1. *Chronic Stress Leading to Increased ACTH Secretion*

As the fetus nears term, the physiological demands of the fetus and placenta on the mother increase. Stressors such as hypoglycemia and hypoxemia may act to cause marked stimulation of the fetal HPA axis. Insulin-induced hypoglycemia has been demonstrated to cause secretion of ACTH and cortisol in intact fetal sheep and the increases in these hormones may increase as gestation progresses (Jones, 1976b; Ozolins *et al.*, 1992). If placental blood flow is restricted for long enough, there is a resulting hypoglycemia and hypoxemia, which also causes an increase in fetal CRF, AVP, ACTH and cortisol concentrations (Hooper *et al.*, 1990; Sue-Tang *et al.*, 1992). This theory has been reviewed extensively (Thorburn, 1994; McMillen *et al.*, 1995b) but this model does not match the chronic stress model in adults, where ACTH concentrations are generally not elevated and glucocorticoid concentrations have been reported to be increased, decreased or not changed (Harbuz & Lightman, 1992).

1.4.2. Decreased Negative Feedback on the Fetal HPA Axis

In the late gestation sheep fetus, cortisol and perhaps ACTH concentrations are elevated in the weeks preceding parturition, suggesting that there maybe a decrease in glucocorticoid negative feedback that allows the increase in cortisol concentrations to occur. This theory however, is not supported by experimental studies which show that infusion of cortisol into either the fetus or the ewe impairs basal, stress-induced and CRF-induced ACTH secretion, suggesting the negative feedback is not impaired in the late gestation sheep fetus (Wood & Rudolph, 1983; Rose *et al.*, 1985; Wood, 1987; Ozolins, 1992).

1.4.3. Withdrawal of an Inhibitor Allowing the Cortisol Surge

The trigger for the surge in cortisol in late gestation still remains elusive after 30 years of research. It is possible that prior to the surge in cortisol, a putative inhibitor of steroidogenesis may be removed, that then drives the increase in cortisol. Durand *et al.* (1984a) reported that if adrenal glands were removed and cultured *in vitro*, the cortisol response to ACTH increased the longer the cells remained in culture, suggesting this was indicative of the presence of a inhibitor *in vivo*. The HMW precursors of ACTH have also been proposed as inhibitors of steroidogenesis and this is discussed in more detail in Section 1.2.3.3.2.

1.4.4. Increased Bioactivity of ACTH

In Section 1.2.3.3.2, the increase in the bioactivity of ACTH from HMW precursors to the more steroidogenically-potent LMW ACTH species was discussed. An increase in the bioactivity of ACTH that results in an increase in the responsiveness of the adrenal cortex to ACTH could possibly explain the increase in cortisol seen in late gestation. It has been shown that there is a 2-fold change in the ratio of HMW to LMW ACTH species and approximately a 2-fold increase in biologically active ACTH as opposed to ir-ACTH (Carr *et al.*, 1995; McMillen *et al.*, 1995a; Schwartz *et al.*, 1995). Although there is a modest increase in ACTH bioactivity, Poore *et al.* (1998a) demonstrated that changes in the responsiveness of the adrenal to ACTH, did not affect the timing of the cortisol surge.

1.4.5. Increased Adrenal Responsiveness

The cortisol surge could be explained, even without major increases in ACTH by an increase in the responsiveness of the fetal adrenal gland to ACTH in late gestation. A number of studies both *in vitro* and *in vivo* have demonstrated that adrenal responsiveness does increase in the late gestation ovine fetus, or with ACTH treatment (Madill & Bassett, 1973; Durand *et al.*, 1981b; Rose *et al.*, 1982; Manchester *et al.*, 1983; Poore *et al.*, 1998a; Poore *et al.*, 1999). Poore *et al.* (1998a) continuously infused a low dose of $ACTH_{1-24}$ into both intact (INT) and HX fetuses and reported that compared to saline-infused controls the ACTH-infused groups had dramatically enhanced adrenal responsiveness to a standard ACTH challenge at 120, 130 and 140 d GA. To extend this study, Poore *et al.* (1999) further examined the effect of a low dose, continuous ACTH infusion into HPD fetuses and a saline infusion to intact and HPD fetuses. Ir-ACTH concentrations were indistinguishable between the three groups but only the HPD/ACTH and INT/SAL fetuses had a cortisol surge and initiated labour at the normal time. HPD/SAL fetuses did not have a cortisol surge or initiate labour, reflecting the importance of a replacement infusion of ACTH or an intact hypothalamo-pituitary connection. Adrenal responsiveness to a bolus dose of ACTH was greatest in the HPD/ACTH fetuses, but in spite of this enhanced adrenal responsiveness in the HPD/ACTH group there was no advancement of the cortisol surge or the timing of labour in association with this increased adrenal responsiveness. The authors concluded that cortisol concentrations and the timing of labour were unrelated to adrenal responsiveness (Poore *et al.*, 1999). These two studies suggest that while adrenal responsiveness does increase in late gestation it does not appear to regulate the timing of the cortisol surge or labour.

The evidence summarised in the previous discussion is consistent with the hypothesis that increases in ACTH are not a pre-requisite for the cortisol surge and ACTH only plays a priming, maintenance role to keep the adrenal gland patent, but is not actually involved in the regulation of the cortisol surge. The suggestion that other factors are involved in adrenal corticosteroidogenesis is not a new one, as several groups have suggested that POMC-derived products are involved. Angiotensin II (Ang II) has also been suggested as a negative regulator of fetal adrenal function. Rainey *et al.* (1991b) reported that ACTH-stimulated cortisol production in primary cultures of ovine fetal adrenal cells was inhibited by approximately 80% when Ang II was added to the culture. In contrast to the

results of Rainey *et al.* (1991b), Poore *et al.* (1998b) infused Ang II into late gestation fetal sheep for either 2 or 48 hours and demonstrated that there was no change in basal cortisol or ACTH secretion in response to Ang II, suggesting that this hormone does not modulate cortisol secretion *in vivo*. Endogenous opioids have been demonstrated to increase plasma ACTH and cortisol *in vivo* in the ovine fetus although the exact mechanism by which these opioids act to increase ACTH is unclear, but it has been suggested that kappa opioid agonists act at receptors in the hypothalamus to stimulate release of CRH and/or AVP (Taylor, 1996a, 1996b).

Another possible candidate is Prostaglandin E_2, which has been demonstrated to have effects at multiple levels of the HPA axis. In the next section of this discussion, the role of prostaglandins, particularly PGE_2 in pregnancy and the HPA axis will be addressed.

1.5. PROSTAGLANDINS

1.5.1. Prostaglandin Synthesis

Prostaglandins (PG) play important roles in both the fetus and the adult. They play roles in the inflammatory response to infection, the initiation and maintenance of pregnancy and they are critical for parturition to occur. Prostaglandins, thromboxanes, leukotrienes and epoxyeicosatrienoic acids are synthesised from arachidonic acid (AA) albeit through different pathways. Prostaglandins and thromboxanes are synthesised through the cyclooxygenase (COX) pathway.

Arachidonic acid, a 20 carbon polyunsaturated fatty acid, is released from glycerophospholipids by phospholipases and can then by metabolised by either the COX, lipoxygenase or the epoxygenase pathways. The COX pathway utilises a microsomal enzyme that converts AA into PGG_2, which is then reduced to form PGH_2 (Lands & Hanel, 1983). COX-1 and COX-2 (PGHS-1 and PGHS-2) are differentially regulated with COX-1 being constitutively expressed in most tissues, while COX-2 is undetectable in most tissues but can be induced, particularly in cases of inflammation (Simmons *et al.*, 1991; Sirois *et al.*, 1992). COX-1 has an mRNA transcript size of 2.8 kB while COX-2 has a much larger transcript size of 4 – 4.5 kB (Simmons *et al.*, 1991; Sirois *et al.*, 1992). In cultured human amnion cells, glucocorticoid treatment has been reported to increase

COX-2 mRNA (Zakar *et al.*, 1995). PGH_2 is converted into specific prostaglandins and thromboxanes via specific terminal synthase enzymes. Prostaglandin E synthase is responsible for the conversion of PGH_2 into PGE_2 (Figure 1-5).

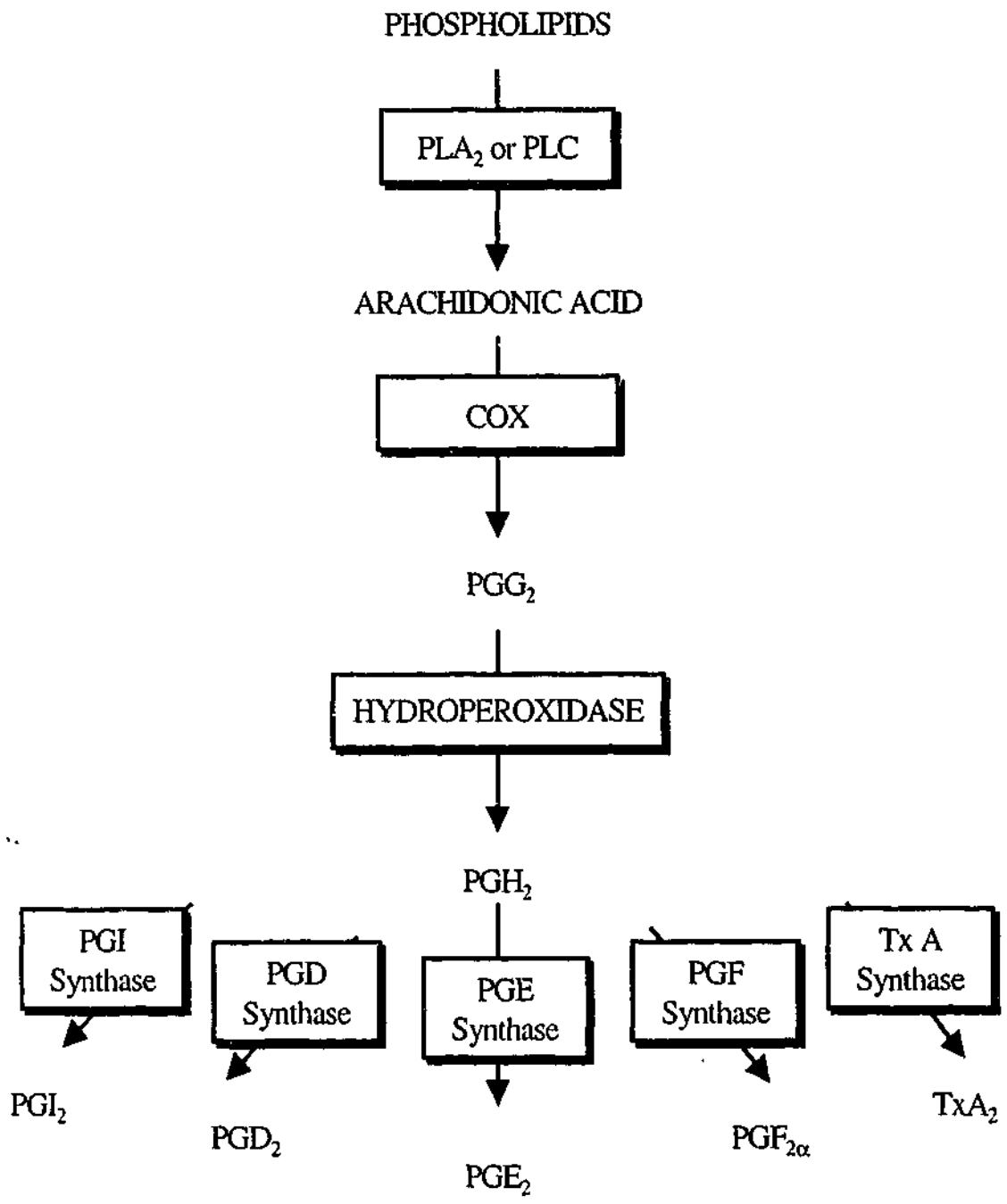

Figure 1-5 Schematic diagram of the biosynthetic pathway for prostaglandin formation. Arachidonic acid (AA) is liberated from phospholipid stores by phospholipid A_2 or phospholipase C. AA is then converted to PGH_2 by the two prostaglandin GH Synthase enzymes cyclooxygenase (COX) and hydroperoxidase. COX activity catalyses the oxygenation of AA into PGG_2. A hydroperoxidase activity catalyses the reduction of PGG_2 to PGH_2. The conversion of PGH_2 into the various prostaglandins is mediated by the action of specific enzymes.

1.5.2. PGE_2 Receptors

PGE_2 can act through a number of receptors designated EP1 through to EP4. These receptors have only been partially cloned in the sheep so the following discussion refers to the human, rat and mouse EP receptors. The EP receptors act through different second messenger systems to mediate differential effects in diverse tissues. The EP receptors belong to the rhodopsin-type receptor family, which are G-protein-coupled receptors that contain seven transmembrane spanning proteins (Narumiya *et al.*, 1999). Most of the EP receptors are located on the plasma membrane however EP1, EP3 and EP4 have also been localised to the nuclear envelope (Bhattacharya *et al.*, 1998; Bhattacharya *et al.*, 1999).

EP2 and EP4 are thought to be "relaxant" receptors as they act to relax smooth muscle, by signaling through G_s – adenylate cyclase mediated increases in cAMP (An *et al.*, 1993; Honda *et al.*, 1993; Bastien *et al.*, 1994; Coleman *et al.*, 1994; Regan *et al.*, 1994b; Katsuyama *et al.*, 1995; Breyer *et al.*, 1996). The EP2 receptor is 358 amino acids long in the human, 362 in the mouse and 357 in the rat (Bastien *et al.*, 1994; Katsuyama *et al.*, 1995; Boie *et al.*, 1997). While most EP receptors are expressed in all tissues, the EP2 receptor has only been identified in the lung, spleen, thymus, ileum, stomach, testis, kidney, uterus, brain and placenta (Bastien *et al.*, 1994; Regan *et al.*, 1994b; Katsuyama *et al.*, 1995; Boie *et al.*, 1997). However, there is some evidence that the expression of EP2 can be induced, as it has been demonstrated that administration of lipopolysaccharide to a mouse macrophage-like cell line increased EP2 mRNA abundance in a dose-dependant manner (Katsuyama *et al.*, 1998). In mouse uterine epithelial cells, EP2 can be induced by gonadotrophins such as human chorionic gonadotrophin and pregnant mare's serum gonadotrophin and because of this the EP2 receptor has been implicated in the implantation process of mouse pregnancy (Katsuyama *et al.*, 1997; Lim & Dey, 1997).

EP4 is 488 amino acids long in the human, rabbit and rat and 513 in the mouse and has been identified in most adult tissues (An *et al.*, 1993; Honda *et al.*, 1993; Breyer *et al.*, 1996; Boie *et al.*, 1997). EP4 was originally cloned by Honda *et al.* (1993) in the mouse and An *et al.* (1993) in the human and because of the similarity in second messenger activation with EP2, was thought to be EP2. Pharmacologically, EP2 receptors are sensitive to butaprost but these cloned EP2 receptors were found to be insensitive to this

compound. These butaprost-insensitive receptors were later identified as a separate isoform of the EP receptors and designated EP4 (Coleman *et al.*, 1990).

EP1 differs from both EP2 and EP4 in that it is considered a "contractile" receptor that acts to contract smooth muscle. EP1 signals through a G_q - mediated increase in phosphatidyl inositol turnover that elevates intracellular free Ca^{2+} (Funk *et al.*, 1993; Watabe *et al.*, 1993). EP1 is 402 amino acids long in the human and 405 in the rat and mouse and has been identified in the kidney, lung, spleen, skeletal muscle, iris sphincter muscle, vas deferens, ovary and myometrium (Funk *et al.*, 1993; Watabe *et al.*, 1993).

The EP3 receptor generally couples to G_i and is thus regarded as inhibitory (Narumiya *et al.*, 1999). There are a number of splice variants of the EP3 receptor, which differ by alternate splicing at the C-terminal tail and thus the number of amino acids varies depending on the isoform but ranges from approximately 365 – 425 amino acids long in the human and 361 – 365 in the mouse (Regan *et al.*, 1994a; Narumiya *et al.*, 1999). Namba *et al.* (1993) showed that the variation in the C-terminal end of the genes did not change ligand specificity of the receptor but did affect which G-protein the receptor couples to and hence the second messenger cascade that is activated (Namba *et al.*, 1993). In the human, 6 EP3 isoforms have been identified, while 4 splice variants have been identified in the bovine and rabbit models. 3 isoforms have been recognised in the mouse and only two have been identified in the rat. These isoforms have been named alphabetically, such as $EP3_A$ – $EP3_F$ in the human and $EP3_A$ – $EP3_D$ in the bovine model. It is interesting to note that although the alternate splicing of the human and bovine isoforms of EP3 start at the same amino acid in the carboxyl terminus, only one of the splice variants ($EP3_D$) in the cow is homologous to the human and none of the bovine variants are homologous to the mouse (Namba *et al.*, 1993; Regan *et al.*, 1994a). The various isoforms have different binding capabilities but EP3 generally acts to inhibit adenylate cyclase induced cAMP formation, although some variants appear to act via an increase in Ca^{2+} levels and other variants act to cause increases in cAMP (Narumiya *et al.*, 1999).

1.5.3. PGE_2 Concentrations During Pregnancy

PGE_2 is undetectable in the plasma of non-pregnant ewes, but is present in both the maternal and fetal circulations during pregnancy. The fetal cotyledon has been

demonstrated to be the main site of synthesis of PGE_2 during pregnancy and the capacity for production of PGE_2 increases in late gestation (Mitchell & Flint, 1977, 1978; Risbridger *et al.*, 1985; Rice *et al.*, 1988).

Fowden *et al.* (1987) demonstrated that PGE_2 concentrations increased over the last third of gestation in the maternal and fetal carotid artery and the uterine vein. Rice *et al.* (1990) also commented that this increase in plasma PGE_2 concentrations in the fetus closely paralleled fetal cortisol concentrations at that time in gestation (Figure 1-6).

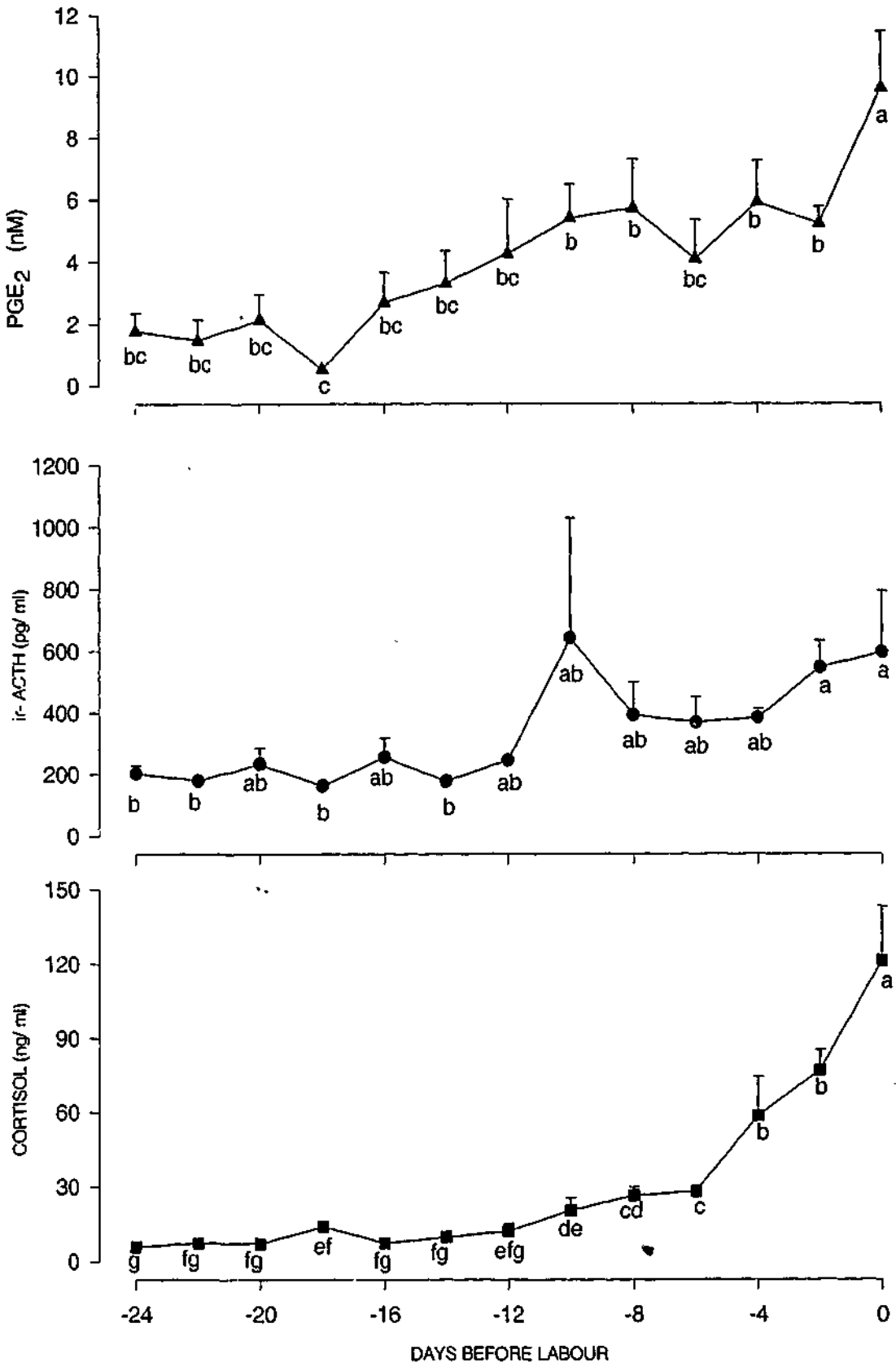

Figure 1-6 Hormonal profiles of ACTH, cortisol and PGE_2 in the ovine sheep fetus in the days approaching labour. Data obtained from Hollingworth (1993).

1.5.4. PGE_2 Functions During Pregnancy

Fetal breathing movements (FBM) are essential for the development of a normal functioning lung after birth. It has been demonstrated that PGE_2 inhibits FBM and administration of indomethacin, a COX inhibitor, reverses this inhibition (Kitterman *et al.*, 1979; Kitterman *et al.*, 1983). At times of high voltage, slow electrocortical activity, FBM are inhibited by PGE_2 (Wallen *et al.*, 1986). In addition, at times of stress, such as approaching labour, or during infusion of ACTH, the incidences of FBM are decreased, which may suggest some protective mechanism (Patrick *et al.*, 1987).

The ductus arteriosus (DA) in fetal life plays the important role of shunting blood away from the pulmonary vasculature of the fetus and directing blood to the systemic circulation, serving to reduce pulmonary vascular resistance, and to make more oxygenated blood available to developing peripheral tissues. The patency of the DA is maintained by high levels of PGE_2, which decrease rapidly after birth to assist in the closure of the DA and aid in the switch of the newborn to breathing air (Heymann & Rudolph, 1975; Coceani *et al.*, 1978; Coceani *et al.*, 1988). Furthermore, it has recently been demonstrated that PGE_2 acts through EP2, EP3 or EP4 receptors to maintain the patency of the DA, and the loss of EP3 and EP4 receptors in the newborn accounts for the decrease in responsiveness of the DA to PGE_2 (Bouayad *et al.*, 2001).

PGE_2 has been demonstrated to also play a role in regulating the resistance of the uterine vasculature, fetal and placental hemodynamics and umbilical blood flow (Novy *et al.*, 1974). Prostaglandins of the E series usually act as vasodilators and depress adrenergic vasoconstriction which allows adequate blood flow for implantation and fetal development (Clark *et al.*, 1976; Clark *et al.*, 1977).

PGE_2 has also been proposed to have a regulatory role in modulating the fetal plasma levels of insulin. Philipps *et al.* (1984) administered indomethacin to the fetal sheep and demonstrated an inhibition of the release of fetal pancreatic insulin in response to a glucose challenge (Philipps *et al.*, 1984). Fowden *et al.* (1987) demonstrated that insulin-induced fetal hypoglycemia resulted in increases in fetal plasma PGE_2 concentrations suggesting that the concentrations of PGE_2 are closely correlated to the nutritional status of the fetus. Furthermore, Hooper *et al.* (1992) reported that PGE_2 is a potent stimulator of insulin

secretion in the late gestation fetal sheep. Fasting late gestation ewes for 48 h results in a 60% increase in fetal plasma PGE_2 and the increases in plasma PGE_2 are inversely correlated with the fetal plasma glucose concentration (Fowden *et al.*, 1987; Fowden *et al.*, 1994). The increase in plasma PGE_2 in response to fasting also reduced the incidence of fetal respiratory activity, presumably to spare glucose for more essential fetal tissues (Fowden *et al.*, 1989). As PGE_2 can also evoke increases in cortisol secretion, Fowden *et al.* (1994) suggested that the increase in cortisol may accelerate the maturational changes of the fetus during fasting, to maximise the survival of the fetus should delivery occur. These observations also provide an essential link to the nutritional-stress related hypothesis of parturition (Thorburn, 1994).

1.5.5. PGE_2 Actions at the Hypothalamus and Pituitary

PGE_2 has a number of interactions with the HPA axis at various levels. Administration of exogenous PGE_2 infusions to intact fetal sheep results in robust increases in ACTH and cortisol secretion. However, the sites of action of PGE_2 within the HPA axis have not clearly been determined.

Early studies in the adult rat involved pharmacological blockade of endogenous CRF activity and then exogenous administration of agents to break through the blockade. PGE_2 was one of the substances that was able to stimulate ACTH secretion (de Wied *et al.*, 1969). In the 1990's, several studies in the adult rat were conducted that examined the ability of PGE_2 to stimulate ACTH secretion *in vivo*, with and without pretreatment with indomethacin. Watanabe *et al.* (1990) investigated the ability of intravenous and intrahypothalamic injections of PGE_2 to stimulate ACTH secretion from the pituitary. This group reported that both routes of PGE_2 administration could stimulate the secretion of ACTH and pretreatment with indomethacin suppressed PGE_2-stimulated ACTH release. Furthermore, the authors systemically pretreated the rats with an anti-CRF antibody that also suppressed the ACTH response to intrahypothalamic PGE_2, suggesting that PGE_2 stimulates ACTH release via CRF (Watanabe *et al.*, 1990). The findings of Watanabe *et al.* (1990) were extended in several other studies using stress models of ACTH secretion. PGE_2 was involved in the release of ACTH in response to fever, swimming stress, cage-switch psychological stress and noradrenaline and these mechanisms of ACTH release were all inhibited when the rat was pre-treated with indomethacin (Morimoto *et al.*,

1991; Watanabe *et al.*, 1991a; Watanabe *et al.*, 1991b). Furthermore the increases in ACTH secretion depended on the dose of PGE_2 administered (Watanabe *et al.*, 1991b).

In the fetal sheep, PGE_2 has also been shown to stimulate ACTH release, although it is unknown if this is a direct effect at the fetal pituitary or at the hypothalamus. Brooks and Gibson (1992) cultured anterior pituitary cells from fetal sheep between 130 and 140 d GA and reported that PGE_2 can have a direct effect in the pituitary gland by enhancing AVP-stimulated but not CRH-stimulated ACTH secretion.

Louis *et al.* (1976) infused PGE_2 (1.6 μg/min) into ovine fetuses for 60 minutes and reported a significant increase in plasma corticosteroids within 30 minutes of the commencement of the infusion and these concentrations remained elevated for a subsequent hour after the infusion was switched off, but had returned to control levels by the following day. The authors suggested that the increase in corticosteroids was not likely to be mediated via ACTH as the infusions were administered at a time when the adrenal was relatively unresponsive to exogenous ACTH. Hollingworth *et al.* (1995) gave 2 h infusions of PGE_2 (2 μg/min) from 119 - 125 d GA and found that it increased ir-ACTH (from 104 to 3219 pg/ml) and increased plasma cortisol concentrations from 9.6 – 23 ng/ml. This PGE_2 infusion regime resulted in a significant increase in ir-ACTH and cortisol at all ages tested. PGE_2 infusion also increased the proportion of LMW ACTH being secreted (Hollingworth *et al.*, 1995). Extending this study, Young *et al.* (1996a) infused PGE_2 continuously from 121 d gestation until labour and demonstrated a dramatic increase in ir-ACTH with the commencement of the PGE_2 infusion but ir-ACTH had returned to control levels by 24 h. However, the proportion of LMW ACTH was also higher with the continuous infusion of PGE_2 throughout the study and the fetuses that had received the infusion delivered 10 d earlier than saline infused controls (Young *et al.*, 1996a).

Brooks (1992) administered PGE_2 to fetal sheep via intravenous (i.v.) or intracerebroventricular (i.c.v.) routes to determine if PGE_2 can stimulate ACTH and cortisol secretion via a hypothalamic site of action. Brooks (1992) reported increases in ACTH secretion after both routes of administration, however cortisol secretion was only stimulated after the highest dose of PGE_2 administered intravenously, whereas PGE_2

administered via the i.c.v. route did not increase cortisol secretion above basal concentrations. The author concluded that there was greater release of ACTH after i.v. administration compared to i.c.v. PGE_2 administration, suggesting that a site of action other than the brain may be more important. In contrast, Young *et al.* (1996b) infused PGE_2 into intact and HPD fetuses. There was no increase in plasma ACTH or cortisol concentrations in response to the infusion in the HPD group of fetuses however intact fetuses had robust increases in ACTH and cortisol in response to the PGE_2 infusion, suggesting that the effects of PGE_2 on the fetal HPA axis are exerted above the level of the pituitary gland. From the above studies it would appear that PGE_2 might mediate ACTH secretion at the level of the hypothalamus and the pituitary gland in the fetal sheep.

1.5.6. Inhibition of Prostaglandin Synthesis and ACTH and Cortisol Release

Early studies using the COX inhibitor indomethacin in rats, demonstrated that systemic administration of indomethacin resulted in an increase in plasma ACTH concentrations (Hedge, 1977; Thompson & Hedge, 1981). In contrast, implantation of indomethacin into the rat hypothalamus reduced the normal corticosterone response to stressors such as hemorrhage, laparotomy or intestinal manipulation (Thompson & Hedge, 1978). In the rat and human adrenal gland *in vitro*, the presence of indomethacin reduced the corticosterone or cortisol response to ACTH or PGE_2 (Honn & Chavin, 1976; Spat *et al.*, 1977).

Since these early studies using non-selective COX inhibitors, some inhibitors have been developed that target COX-2, the pro-inflammatory inducible form of the enzyme. The inhibition of COX-2 has been suggested as a tocolytic treatment for preterm labour and McKeown *et al.* (2000) tested the COX-2 inhibitor meloxicam with this purpose. The authors induced labor in pregnant ewes with the progesterone receptor antagonist RU486, and when active labor was reached, meloxicam was infused. RU486 treatment increased the levels of PGE_2, ACTH and cortisol in the fetal circulation and the infusion of meloxicam significantly reduced the concentrations of these hormones in the fetal circulation (McKeown *et al.*, 2000). In a study of spontaneous labor, nimesulide, another specific COX-2 inhibitor has been infused, and fetal concentrations of PGE_2, ACTH and cortisol were measures. These hormones were decreased by the infusion, however, cortisol

concentrations returned to baseline 5 hours after the commencement of the infusion (Unno *et al.*, 1998). Together these two studies suggest that PGE_2 or another prostanoid and the HPA axis are closely linked and that inhibition of prostaglandin synthesis plays a role in the regulation of the fetal HPA axis.

1.6. PGE_2 AND THE ADRENAL

PGE_2 has been investigated both *in vitro* and *in vivo* for its effects on the fetal and adult adrenal gland in a variety of species. PGE_2 has been found to simulate cortisol or corticosterone release from rat, bovine, cat and avian adult adrenocortical cells *in vitro* (Warner & Rubin, 1975; Ellis *et al.*, 1978; Rainey *et al.*, 1991a; Tominaga *et al.*, 1991). The interaction between PGE_2 and the adrenal is discussed in greater detail in chapter 3.

Infusion of PGE_2 has also been shown to stimulate secretion of cortisol in the fetal sheep *in vivo* (Jones *et al.*, 1975; Louis *et al.*, 1976; Challis *et al.*, 1978; Ratter *et al.*, 1979; Liggins *et al.*, 1982). A 2 hour infusion of PGE_2 was administered by Hollingworth *et al.* (1995), who reported that it increased plasma cortisol levels from 9.6 mg/ml to 23 ng/ml. This increase in cortisol was accompanied by an increase in plasma ACTH. In another report, PGE_2 was also infused continuously from 121 d until labour was initiated, and the authors reported an initial increase in ir-ACTH plasma concentrations, but these levels returned to control levels by 24 h after the commencement of the infusion. However, the concentration of cortisol in the plasma continued to increase even after ir-ACTH concentrations had returned to control levels, suggesting a dissociation between ACTH and cortisol that may indicate that PGE_2 can stimulate cortisol directly (Young *et al.*, 1996a).

Liggins *et al.* (1982) infused PGE_2 into HX fetuses and reported an increase in plasma cortisol concentrations. Unfortunately, the authors did not report ACTH concentrations, but the results suggest that PGE_2 may act directly at the level of the adrenal to cause an increase in cortisol concentrations (Liggins *et al.*, 1982).

The idea that PGE_2 can directly stimulate cortisol secretion from the fetal adrenal gland is discussed in greater detail in chapters three and four, where this hypothesis is directly tested.

1.7. SUMMARY

From the preceding discussion it is apparent that there is still uncertainty as to how the cortisol surge occurs. Many studies have now shown that ACTH plays an important role in the cortisol surge, as perturbation of secretion of ACTH delays the cortisol surge and an increase in secretion of ACTH causes a premature increase in cortisol concentrations. Jacobs *et al.* (1994) noticed that hypothalamo-pituitary compromised fetuses such as HX, HPD or even intact ovine fetuses that are continuously infused with a maintenance dose of ACTH (34 pmol/hr/kg), deliver at normal term and exhibit a normal cortisol profile prior to labour (Jacobs *et al.*, 1994). These data suggests that ACTH is required for the cortisol surge, but concentrations do not need to increase for labour to occur and also supports the hypothesis that ACTH plays a permissive, maintenance type role in the cortisol surge. Similarly, further studies using HX, HPD and intact fetal models that received an $ACTH_{1-24}$ infusion were also shown to have an enhanced cortisol response to a bolus dose of ACTH, but in spite of this enhanced adrenal responsiveness their cortisol surge was not prematurely activated (Poore *et al.*, 1998a; Poore *et al.*, 1999). The authors concluded that ACTH can induce adrenal responsiveness but increased adrenal responsiveness does not regulate the cortisol surge nor prematurely trigger parturition. In addition, infusion of $ACTH_{1-24}$ into intact fetuses blocks their ability to mount an ACTH response to a physiological stressor without affecting their cortisol response, i.e. the animals still have a cortisol response to the stressor in the absence of any increase in ACTH, suggesting a stimulator of cortisol secretion other than ACTH (I.R. Young, unpublished observations).

From this evidence it can be argued that increases in ACTH are not a pre-requisite for the cortisol surge, but that ACTH only plays a priming or maintenance role to keep the adrenal gland active and functioning. The central hypothesis of this thesis is that another factor drives the cortisol surge. PGE_2 is one candidate, it has been shown to have many interactions with the HPA axis and has been shown to be a positive stimulator of both ACTH and cortisol secretion. Further, the cortisol surge is a phenomenon unique to fetal life and PGE_2 is only increased in fetal life and pregnancy. If PGE_2 does play a role in the cortisol surge, it would need to be able to directly stimulate cortisol secretion from the fetal adrenal gland without an increase in ACTH. In chapter 3, I have investigated whether PGE_2 can directly stimulate the secretion of cortisol from fetal and adult adrenal slices using a perifusion system. In chapter 4, I have further pursued the idea that PGE_2 can

stimulate cortisol secretion directly, using an *in vivo* animal model. Intact fetuses infused with saline and hypophysectomised fetuses infused with a continuous, maintenance dose of ACTH have been employed to address this question. In chapter 5, I have investigated through which receptor PGE_2 acts at the adrenal using real-time PCR.

1.8. AIMS

The central aim of this thesis was to determine if PGE_2 could act directly at the late gestation ovine fetal adrenal gland to cause cortisol secretion *in vitro* (chapter 3), *in vivo* (chapter 4) and to investigate which of the PGE_2 receptor subtypes PGE_2 acts via to stimulate cortisol secretion (chapter 6; See Figure 1-7).

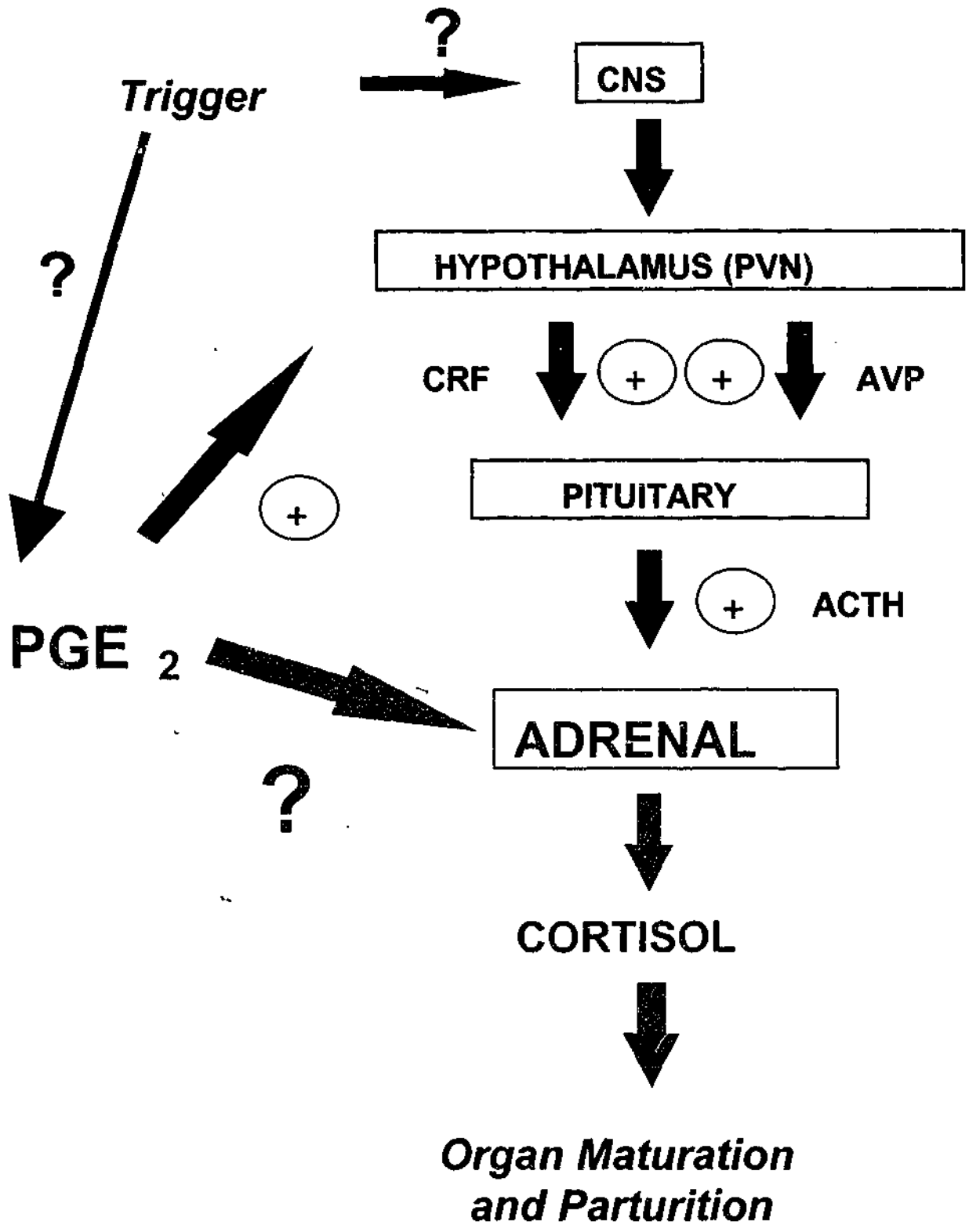

Figure 1-7 Schematic diagram of Hypothalamo-Pituitary-Adrenal Axis and the central question of the thesis – Can PGE_2 directly stimulate the ovine fetal adrenal gland to secrete cortisol?

Chapter 2 GENERAL METHODS

2.1. HORMONE ASSAYS

2.1.1. Cortisol Radioimmunoassay (RIA)

Cortisol concentrations in fetal plasma (chapter 4) were measured as described by Bocking *et al.* (1986). Plasma (50 – 200 µl) was extracted in dichloromethane (2 ml; Merck, Darmstadt, Germany) and 100 µl dH_2O in glass tubes. After vortexing and allowing the layers to settle, 1 ml of the solvent layer was transferred to polypropylene tubes and samples were evaporated under air at 37 °C. Samples of fraction eluent (chapter 3; 50 - 200 µl) did not need to be extracted, so samples were evaporated under air at 37 °C. After evaporation of both plasma and fraction eluent samples, 100 µl of bovine γ-globulin (8 mg/ml; Calbiochem, San Diego, California, USA), cortisol antiserum (initial dilution 1:8000, final dilution 1:24 000) and ^{3}H-cortisol (10 000 c.p.m./0.1 ml; Amersham Pty Ltd., Australia) were added to the residue in the standard and sample tubes and allowed to incubate overnight at 4 °C. Bound and free hormone were separated by the addition of 1 ml of a 22% polyethylene glycol 6000 (PEG; BDH Chemicals) precipitating solution and centrifuged at 3000 rpm for 15 minutes at 4 °C. The supernatant was aspirated and the precipitate resuspended in 200 µl of assay buffer. ACS Scintillation fluid (1 ml; Amersham Pty Ltd, Australia) was added to each tube and the beta radiation determined with a Beckman LS 3801 liquid scintillation counter (Beckman Instruments Inc, Irvine, California, USA).

Cortisol standard (Hydrocortisone H-4001, Sigma Chemical Company, St Louis, Missouri, USA) was used at concentrations of 0.0195 – 5 ng/100 µl in absolute ethanol, evaporated under air at 37 °C.

Cortisol antiserum (#3368) was raised in sheep and supplied by Bioquest Limited (Bioquest Ltd. North Ryde, New South Wales, Australia). Cross-reactivity of the cortisol

antiserum is summarised in Table 2-1. The intra- and inter-assay coefficients of variation were 9.9% and 13.8% respectively. The sensitivity of the assay was 0.41 ± 0.001 ng/ml. The average recovery for the cortisol extraction procedure was 95 ± 1%.

Steroid	% Cross-reactivity
Cortisol	100.00
Cortisone	20.5
Corticosterone	1.00
Progesterone	0.57
17α-OH-Progesterone	13.90
Testosterone	0.02
4-Androstene-3, 17-dione	0.03

Table 2-1 Percent cross-reactivity of cortisol antiserum with related compounds.

2.1.2. ACTH RIA

In chapter 3, the determination of ACTH in the fraction eluent was performed using a locally developed method, while in chapter 4 a commercially available kit was employed for the measurement of ir-ACTH in the fetal plasma.

2.1.2.1. Locally Developed ACTH RIA

The concentration of ACTH was determined in the samples of fraction eluent from the perifusion experiment. The ACTH antiserum was raised against synthetic human $ACTH_{1-24}$ in rabbits and has 100% cross-reactivity with $ACTH_{1-24}$ and $ACTH_{1-39}$. ^{125}I-ACTH was iodinated in the Department of Physiology, Monash University as previously described (Salacinski *et al.*, 1981). Synthetic human $ACTH_{1-39}$ (Peninsula Laboratories, California, USA) was used as standards. Samples of fraction eluent (100 µl) and standards (0.97 – 500 pg/100 µl) were incubated overnight at 4 °C with ACTH antiserum (100 µl) and assay buffer (to make tube volume total 400 µl). ^{125}I-ACTH (100 µl; 10 000 c.p.m./100 µl) was added and the tubes were again incubated overnight at 4 °C, after which a second antibody precipitating solution (500 µl) containing goat anti-rabbit serum (1/150 dilution) and PEG (2% Solution) was added and again tubes were incubated overnight at 4 °C. After centrifugation at 3500 rpm for 30 minutes at 4°C, the supernatant was aspirated and the precipitate counted for gamma irradiation on a Multidetector RIA system (Packard Gamma

Counter, USA). The intra-assay coefficient of variation was 7% and the sensitivity of the assay was 16.8 pg/ml.

2.1.2.2. Diasorin ACTH RIA Kit

The concentration of immunoreactive ACTH in fetal plasma was measured using a commercially available ACTH RIA kit (Diasorin Inc., Stillwater, Minnesota, USA). Fetal plasma (100 µl) or ACTH standards (20 – 500 pg/ml) were incubated overnight at 4°C with 100 µl of dH_2O and 200 µl of ACTH antiserum and ^{125}I-ACTH. Bound and free hormone were separated by the addition of 500 µl of a second antibody (goat-anti-rabbit) precipitating (PEG) solution which was then incubated at room temperature for 20 minutes before centrifuging for another 20 minutes. The supernatant was decanted and the precipitate counted for gamma radiation on a Multidetector RIA System. The cross-reactivity of this antiserum with HMW ACTH-containing peptides is unknown, however the cross-reactivity of this antiserum with a number of other peptides is listed in Table 2-2.

The inter-assay and intra-assay coefficients of variation were 12.5% and 6.0% respectively and the sensitivity of the assay was 15 pg/ml.

Peptide	**% Cross-reactivity**
α-MSH	< 0.01
β-Endorphin	< 0.01
β-Lipotrophin	< 0.01
Leucine Enkephalin	< 0.01
Methionine Enkephalin	< 0.01
Bombesin	< 0.01
Calcitonin	< 0.01
Parathyroid Hormone	< 0.01
FSH	< 0.01
Human Growth Hormone	< 0.01
Vasopressin	< 0.01
Oxytocin	< 0.01
Substance P	< 0.01
Porcine $ACTH_{1-39}$	100.00
Human $ACTH_{1-24}$	100.00

Table 2-2 Comparison of the cross-reactivity of the ACTH antiserum with various peptides

2.1.3. PGE_2 Radioimmunoassay

Direct RIA measured fetal plasma concentrations or fraction eluent of PGE_2 methyloxime from methyl-oximated plasma or fraction eluent as previously described by Fowden *et al.* (1987). [2, 6, 8, 11, 12, 14 -^{3}H (n)]-prostaglandin E_2 (^{3}H-PGE_2; Dupont NEN Products, Boston, Massachusetts, USA) and unlabelled PGE_2 (0.5 mg; Sigma Chemical Company, USA) were methyl-oximated by overnight incubation at room temperature with methoxyamine hydrochloride (Sigma Chemical Company, USA) at a final concentration of 0.12 mol/l in sodium acetate buffer (0.1 mmol/l, pH 5.6). Methyl-oximated samples of ^{3}H-PGE_2 and unlabelled PGE_2 were extracted with ether:ethyl acetate (3:1 v/v) and the extracts evaporated under air at 37 °C. The residues were resuspended in absolute ethanol and stored at –20°C until required.

Labelled PGE_2-methyloxime was diluted in 0.1 M phosphate buffer to a final concentration of 5000 c.p.m./100 µl. Methyl-oximated PGE_2 standards in absolute ethanol ranged from 0.02 – 2.0 pmol/tube and methyloximated plasma samples or samples of fraction eluent ranging from 50 – 200 µl were assayed in duplicate directly. Antiserum (#9183) was supplied by Dr R.I Cox (CSIRO, Division of Animal Production, Prospect, New South Wales, Australia). The antiserum was used at a final dilution of 1:12 000. The cross-reactivity of the antiserum with various compounds is summarised in Table 2-3. Although the cross-reactivity of the antiserum to PGE_1 is 270% (refer to Table 2-3), it is considered that the PGE_1 precursor is far less abundant than the Prostaglandin E series precursor, arachidonate, and thus PGE_1 is far less abundant than PGE_2 (Lands, 1989).

Standards and samples were incubated with 100 µl of methyloximated ^{3}H-PGE_2 and PGE_2 antiserum overnight at 4 °C. Free and bound hormone were separated by precipitation with 50 µl of bovine-γ-globulin and 0.8 ml of 22% PEG 6000 and centrifugation at 4000 rpm for 15 minutes at 4 °C. The supernatant was aspirated and the pellet resuspended in 50 µl of absolute ethanol. After dislodging the pellet by vortexing, 1 ml of Ecoscint A (National Diagnostics, Atlanta, Georgia, USA) was added to all tubes and then sonicated for 30 minutes. Radioactivity (beta) was determined with a Beckman LS 3801 liquid scintillation counter (Beckman Instruments Inc, Irvine, California, USA).

The intra- and inter-assay coefficients of variation were 5.06 and 10.01% respectively and the sensitivity of the assay was 0.69 ± 0.14 nM.

Methyl-oximated Prostaglandin	% Cross-reactivity
PGB_2	< 0.10
PGD_1	< 0.10
PGD_2	< 0.10
PGE_1	270.00
PGE_2	100.00
15-keto-PGE_2	0.30
$PGF_{2\alpha}$	<0.10
15-keto- $PGF_{2\alpha}$	<0.10
13,14-dihydro- $PGF_{2\alpha}$	<0.10
$PGF_{2\beta}$	<0.10
6-keto- $PGF_{1\alpha}$	<0.10
TXB_2	<0.10

Table 2-3 Percent cross-reactivity of the PGE_2 antiserum with other closely related compounds

Chapter 3 CAN EXOGENOUS PGE_2 OR ACTH STIMULATE CORTISOL PRODUCTION FROM FETAL OR ADULT ADRENAL SLICES *IN VITRO*?

3.1. INTRODUCTION

In late gestation, in all mammals, there is an increase in plasma glucocorticoid concentrations that drives the maturation of organ systems that are essential for the postnatal survival of the animal. In the sheep fetus, this increase in plasma cortisol also initiates parturition. Since the infusion of ACTH or dexamethasone (a synthetic glucocorticoid), into fetal sheep was demonstrated to cause premature labour and the report that corticosteroids increase in the last weeks of gestation in the ovine fetus, the importance of cortisol in parturition has been well defined (Liggins, 1968; Bassett & Thorburn, 1969). However, over 30 years later there is still controversy over whether the concentrations of ACTH actually increase before this late gestation increase in cortisol, with many studies providing little convincing evidence for an absolute increase in ACTH in the fetal circulation until the rise in cortisol has begun (For review see Jones & Roebuck, 1980).

Basal plasma PGE_2 concentrations have also been reported to rise in late gestation in a relatively linear manner until labour has been established, this linear increase resembles the profile of ACTH. Once labour has been established there is a rapid increase increase in PGE_2. The PGE_2 profile also resembles that of cortisol as the basal concentrations rise over the same time period (Thorburn & Challis, 1979; Norman *et al.*, 1985; Fowden *et al.*, 1987).

PGE_2 has been administered into the ovine fetal circulation in a number of studies and an increase in fetal plasma cortisol concentrations was reported (Louis *et al.*, 1976; Ratter *et al.*, 1979; Liggins *et al.*, 1982; Hollingworth *et al.*, 1995). Many of these authors have

suggested that PGE_2 may have a trophic effect on steroidogenesis in the fetal adrenal although, with the exception of Liggins *et al.* (1982), the design of these studies did not permit any definitive conclusions to be drawn.

Liggins *et al.* (1982) reported that PGE_2 could stimulate cortisol secretion in HX fetuses however a subsequent study, where PGE_2 failed to increase cortisol concentrations in HPD fetuses didn't support this, leaving the issue of PGE_2's ability to directly stimulate cortisol production unresolved (Young *et al.*, 1996b). Liggins *et al.* (1982) infused PGE_2 into HX fetuses and found that PGE_2 increased cortisol concentrations, however, this group had similar basal levels of cortisol in both the intact and HX animals, when it would be expected that the hypophysectomised fetuses would have minimal cortisol in the plasma. Young *et al.* (1996b) also infused PGE_2 into the fetal circulation of fetuses with a disconnected hypothalamus and pituitary (HPD). This group found that there was no increase in either plasma ACTH or cortisol in response to an exogenous 2 hour infusion of PGE_2 but the authors suggested that this may be due to the pituitary or adrenal not being capable of secreting ACTH or cortisol respectively. In light of the HPD model not responding to a PGE_2 infusion, it is quite surprising that a cortisol response was observed in the HX fetuses in the study by Liggins *et al.* (1982).

Many studies have administered PGE_2 to adrenocortical cells *in vitro*. The results have been inconsistent, with some studies showing no effects of prostaglandin E_2 on corticosteroidogenesis, while numerous studies have reported a positive effect in a number of species. Flack *et al.* (1969) demonstrated that Prostaglandins of the E series could stimulate corticosteroidogenesis in superfused decapsulated adrenal tissue from intact and HX rats *in vitro*, while Laychock and Rubin (1976), also reported a positive effect of PGE_2 in dispersed cat adrenocortical cells. In the frog intrarenal gland, Perroteau *et al.* (1984) reported that PGE_2 and other prostaglandins could stimulate corticosteroid production. In avian adrenocortical cells, Kocsis *et al.* (1999) also found a positive effect of prostaglandins on cortisteroidogenesis. In the fetal sheep, several investigators have administered PGE_2 to dispersed adrenal cells *in vitro* and reported no cortisol response to PGE_2. However, Dazord *et al.* (1974) showed that incubation of adrenal cells with trypsin destroyed the ability of PGE_2 to bind to the Prostaglandin E receptors, rendering PGE_2 unable to stimulate cortisol secretion (Dazord *et al.*, 1974; Durand *et al.*, 1981a; Chen & Nathanielsz, 1997). Zambrano *et al.* (2001) reported that PGE_2 could stimulate H^+ release

from fetal adrenal cells, which they described as representing cellular activation. However, these studies all examine the role of prostaglandins either on the post-partum adult adrenal gland or on dispersed fetal or adult adrenal cells. Therefore, it remains unclear if the non-dispersed fetal adrenal gland can secrete cortisol in response to PGE_2 *in vitro*. In the present study, 125 d and 140 d fetal and adult adrenal tissue were collected to determine if any differences could be observed at the beginning of the cortisol surge (125 d) or when the cortisol surge is well established (140 d).

In the adult sheep, circulating concentrations of prostaglandin E_2 differ markedly between the late pregnant and post-partum ewe. Plasma concentrations of PGE_2 were measured in the uterine ovarian vein (UOV) of post-partum and day 14 pregnant ewes and the PGE_2 levels were undetectable i.e. below the sensitivity of the assay in the plasma from the UOV in both of these groups (Burgess *et al.*, 1990). However, in a study by Deayton *et al.* (1993), PGE_2 concentrations were readily detectable in the plasma of pregnant ewes at 70 d GA. In fact in this study, PGE_2 concentrations stayed at approximately 3 nM until around 95 d GA and then increased until 130 d when the study ended. In a subsequent study by Deayton *et al.* (1994), PGE_2 was also measured in the late gestation ewe. Plasma concentrations of PGE_2 continued to increase in the ewe from 130 d until term when they reached approximately 10 nM.

Thurley and McNatty (1973) examined the cortisol concentrations of pregnant and post-partum sheep in response to stressful stimuli, such as herding with dogs and reported that pregnant sheep had a larger increase in cortisol than ewes that had given birth three weeks earlier. Similarly, in the mouse model, Barlow *et al.* (1975) showed that pregnant mice on day 14 had a far higher resting plasma corticosterone concentration (80 µg/100 ml) than their post-partum counterparts (2.3 µg/100 ml). In addition, the pregnant mouse had a far greater increase in corticosterone in response to a surgical insult than the post-partum mouse (Barlow *et al.*, 1975). In a subsequent study by Barlow *et al.* (1976), the authors demonstrated that the increase in corticosterone in the mouse in response to stressful stimuli is greater during the second half of pregnancy, compared with early pregnant and post-partum mice. Glickman *et al.* (1980) examined the question of cortisol secretion from the ewe adrenal gland *in vitro* using isolated maternal adrenal cells from post-partum (anestrous) sheep and ewes at 50, 100 and 130 days and term of pregnancy.

Glickman *et al.* (1980) reported that the basal cortisol output from adrenals of post-partum ewes was slightly lower (3.34 pg/ml/4 hr) than pregnant sheep adrenal output (3.97 pg/ml/4 hr). They also reported that pregnant sheep adrenal slices had a larger cortisol response to ACTH than the adrenal cells from post-partum counterparts (Glickman & Challis, 1980).

It is quite difficult to compare the concentrations of cortisol from different studies in adult animals as the method of taking the sample can prove quite stressful to some animals, especially if the animal is not handled by humans routinely, so these values need to be interpreted with care. If remote sampling is employed to measure plasma cortisol concentrations, the concentration in the pregnant ewe in late gestation range from approximately 5 –10 ng/ml (Hughan *et al.*, 2001). The cortisol concentration in an ovine wether (a castrated sheep) that is also remotely sampled falls in the same range (I.R Young, personal communication). Basal cortisol concentrations in sheep that are gonad intact or gonadectomised also fall in the same 5 – 10 ng/ml range (Turner *et al.*, 2002). Simonetta *et al.* (1991) did not remotely sample late gestation pregnant sheep and reported basal cortisol concentrations of approximately 15 - 25 ng/ml, which may suggest that the animals were slightly stressed by the experimental procedure.

Very few studies have examined the steroidogenic capacity of the adrenal of post-partum and pregnant ewes. The present study examines whether adrenal slices from pregnant and post-partum sheep respond differently to ACTH or PGE_2, considering the differences of the endogenous concentrations of PGE_2 and cortisol in the whole animal. The aim of this study was to examine the ability of adrenal gland slices of post-partum and late gestation pregnant ewes at 125 d and 140 d GA to respond to stimulation by ACTH and PGE_2 using an *in vitro* perifusion system . In addition, one of the main aims of this chapter was to elucidate if PGE_2 could stimulate the secretion of cortisol directly from the ovine fetal adrenal gland at either 125 d or 140 d GA.

3.2. MATERIALS AND METHODS

3.2.1. Experimental procedures

Pregnant Corriedale ewes of known gestational age (either 125 days (d) or 140 d; n = 10), or post-partum ewes (n = 7) were killed by pentobarbitone overdose. The fetus was removed from the uterus and the adrenal glands of the ewes and fetuses were quickly dissected out, trimmed of connective tissue and fat, weighed and then placed in ice cold phosphate buffered saline (PBS). Adrenal glands were cut longitudinally into quarters. Using a McIlwain tissue chopper the adrenals were further sliced into 800 µm thick slices. These slices were weighed and approximately 200 –300 mg of tissue was swathed in glass wool. The slices were placed in a continuous flow incubation system, set at 37 °C, through which Yamamoto's Buffer (1L contains the following 1M solutions: 117 ml NaCl, 5 ml KCl, 2 ml $MgCl_2$, 1.8 ml $CaCl_2$, 0.5 ml KH_2PO_4, 2.6 g/ L Hepes, 1.8 g / L Glucose, 420 mg /L $NaHCO_3$ and 1 g/ L Bovine Serum Albumin; pH 7.4) was pumped using a peristaltic pump at a rate of 100 µl/ min (1 ml/ 10 min). The time lapse from the removal of the adrenal glands to the start of perifusion never exceeded 40 minutes.

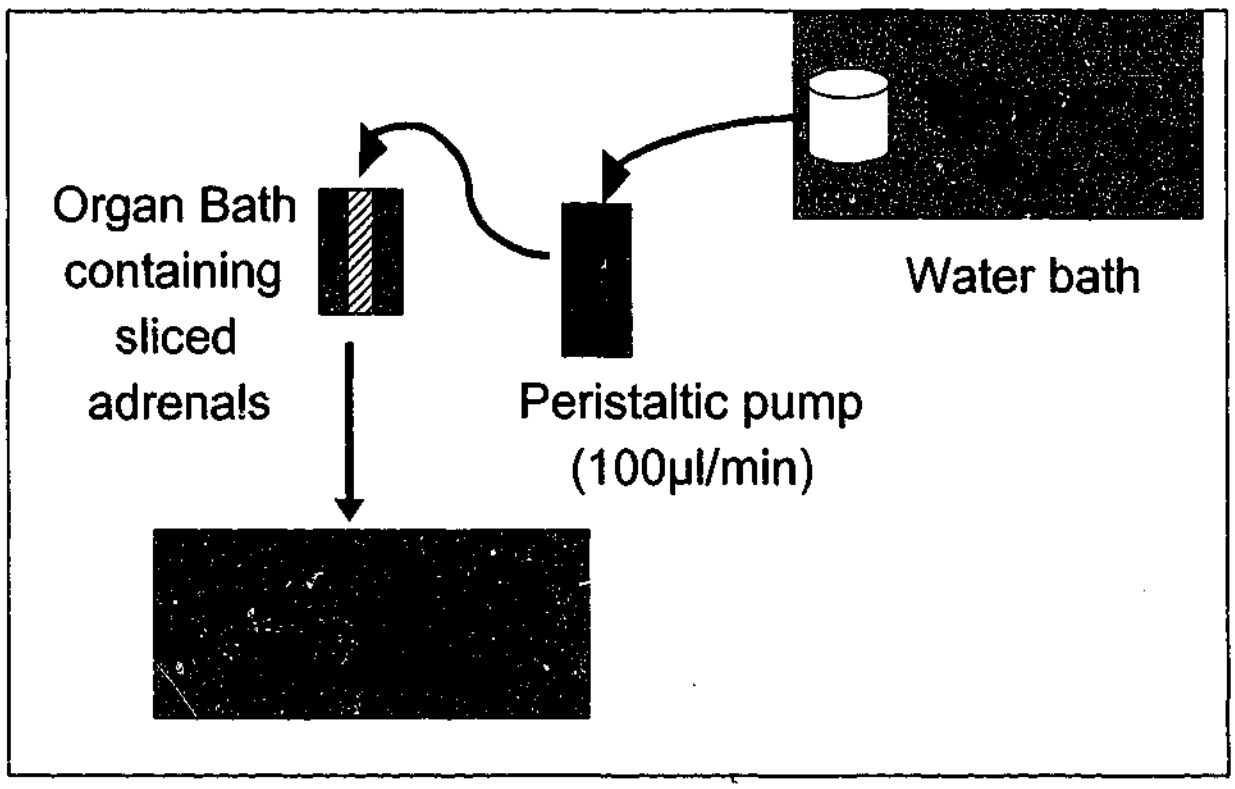

Figure 3-1 Schematic diagram of the perifusion system used. Sliced adrenals were swathed in glass wool to avoid blockage of the tubing. Siliconised tubing was used to prevent peptide build-up. Water and organ baths were kept at 37 °C.

3.2.2. Perifusion System

3.2.2.1. Characterisation and Validation of System

3.2.2.1.1. Determination of the Temporal Flow Rate Profile

The flow rate of the system was determined using Evans blue dye. It was perifused through the system for 10 minutes and the time taken for it to reach the tissue and fraction collector was visually determined. The time profile for elution of the dye was also determined by putting each five-minute fraction in a spectrophotometer. The dye started to elute 10 minutes after the dye was administered to the system (Figure 3-2). The dye when eluted into the fraction collector was approximately half the original starting concentration.

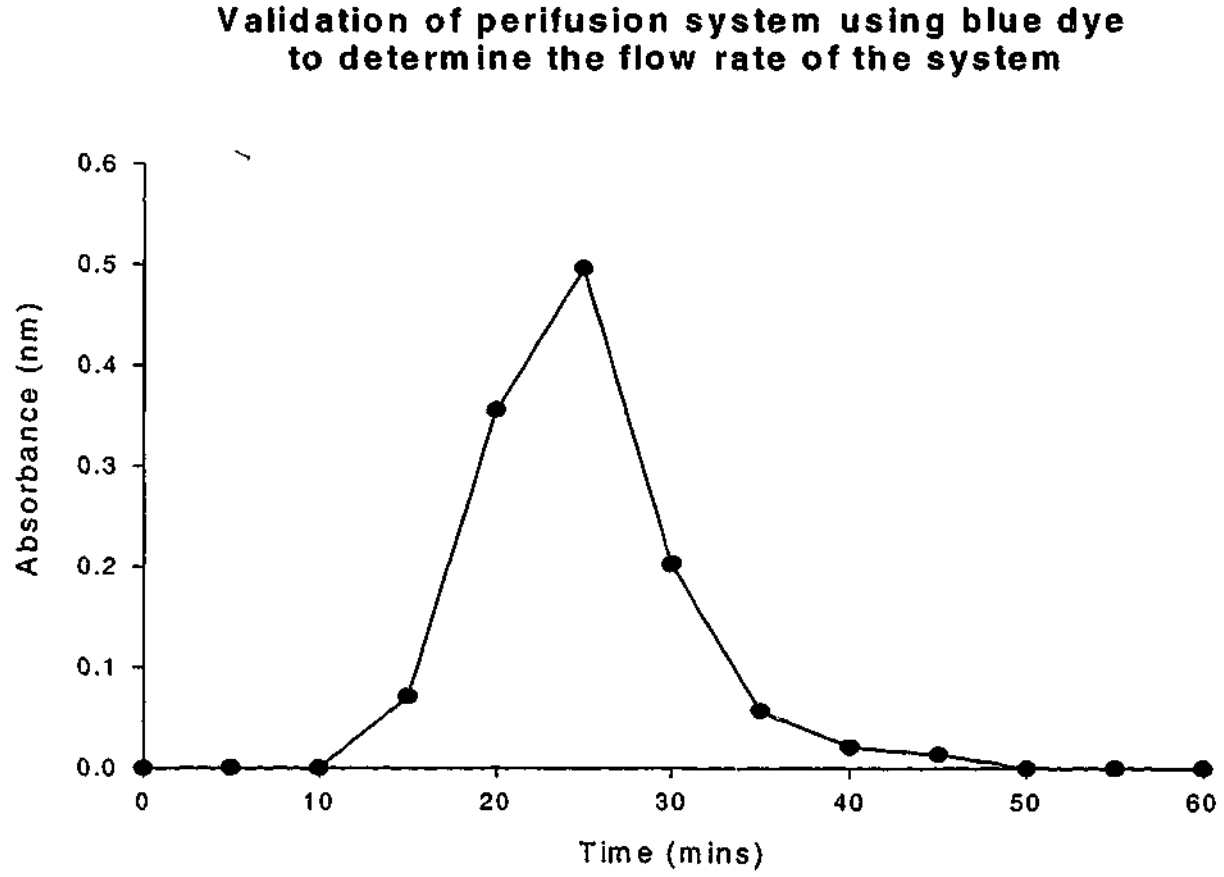

Figure 3-2 The temporal profile of Evans blue dye secreted from the perifusion system. Dye was administered for 10 minutes to the system, starting at time 0 and took between 10 and 15 minutes to reach the fraction collector. At approximately 50 minutes all the dye had been washed out of the system.

3.2.2.1.2. Time for Tissue Slices to Reach a Basal Secretion Rate

The secretion of adrenal slices was studied to determine how long the slices would take to reach a basal secretion rate. Fresh adult tissue from abattoirs was used in this procedure and it was shown that after 4 hours in the system the adrenal slices had reached a basal level of cortisol output (Figure 3-3).

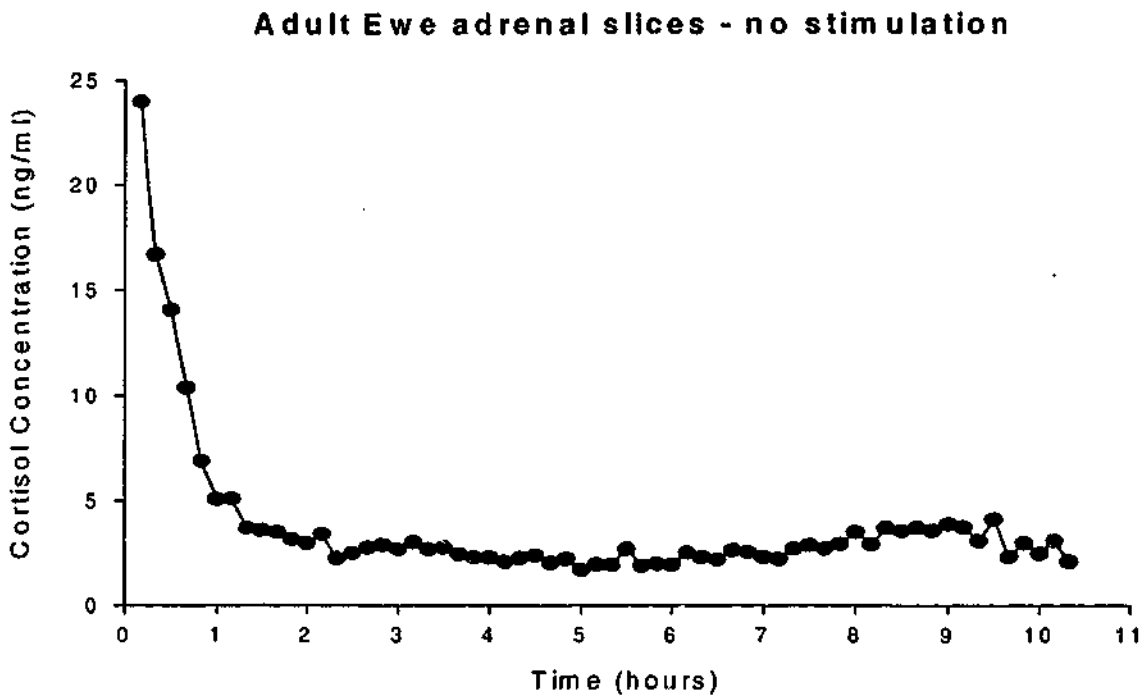

Figure 3-3 An example of the cortisol output of adrenal slices from an adult pregnant ewe over 11 hours. Adrenal slices reached a basal cortisol secretion rate by approximately 4 hours.

3.2.2.1.3. Determination of Doses of PGE_2 and ACTH

The doses of ACTH and PGE_2 to be administered were tested on adult adrenal glands. The concentrations of PGE_2 and ACTH that were able to double cortisol output in adult adrenal slices were determined. The doses of PGE_2 at 1×10^{-6} M and ACTH at 1×10^{-7} M were able to double cortisol output in adult post-partum sheep adrenal slices (Figure 3-4).

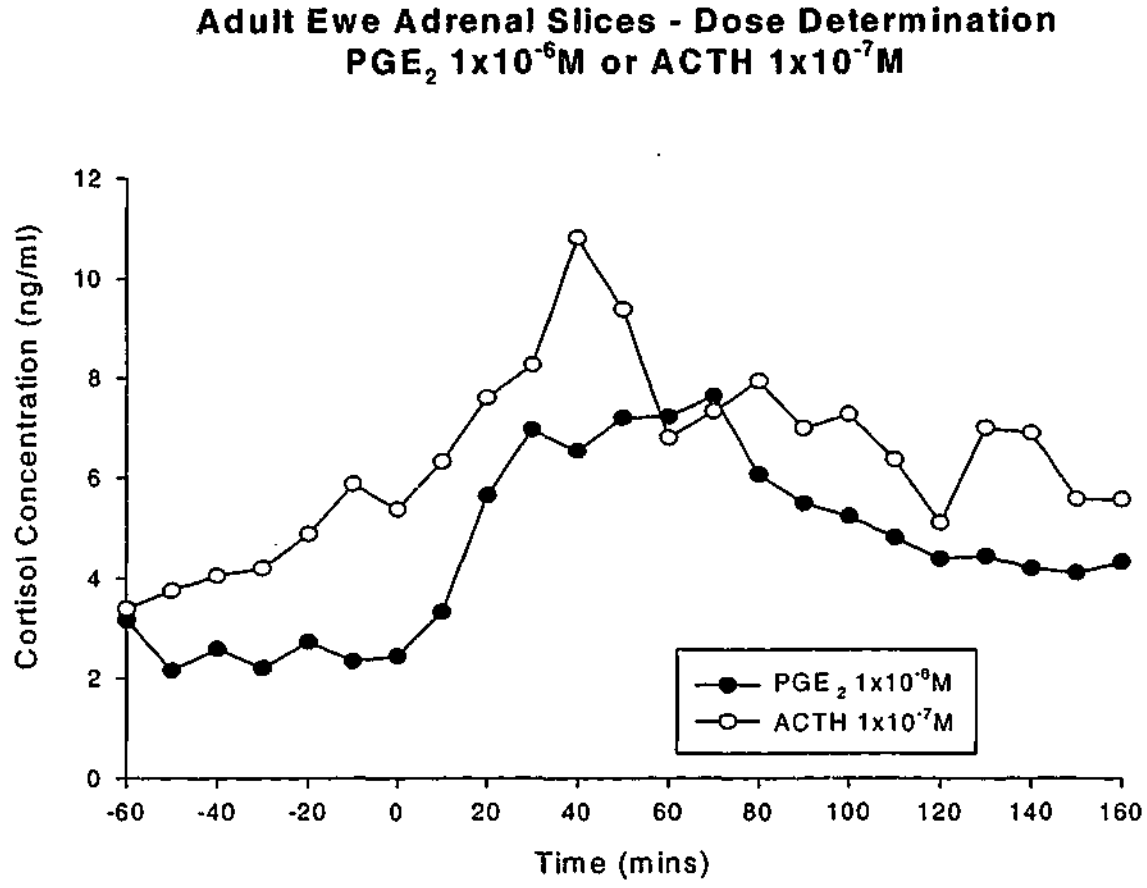

Figure 3-4 The dose of ACTH ($1x10^{-7}$ M) and PGE_2 ($1x10^{-6}$ M) that gave an approximate doubling in the cortisol output of adult adrenal slices when administered for 10 minutes. Time 0 is the time when test substance first reached the tissue i.e. agonist was administered at time point –20.

3.2.2.1.4. Dose Administered Versus Dose of ACTH and PGE_2 Recovered

To determine the recovery of ACTH and PGE_2 administered to the adrenal slices, radioactively labelled PGE_2 or ACTH was added to the system in the absence of adrenal slices and the amount of radioactivity collected in the first hour after the administration of the labelled agonist was determined. The average recovery of tritiated-PGE_2 that was available to the adrenal slices was 100 ± 1.81%. The amount of ACTH that was available to the adrenal slices was measured using iodinated ACTH. The mean recovery of iodinated-ACTH that reached the adrenal slices was 34.87 ± 2.04%. In the text, the reported concentrations are those that were administered, not values that have been corrected for recovery.

3.2.2.2. Experimental Protocol

Experiment One - To measure cortisol output from fetal adrenal glands from 125 d and 140 dfetuses' adrenal glands, slices were incubated in a continuous flow incubation system *in*

vitro. The perifusion chambers were maintained at 39°C with water running directly from a water bath into an external compartment of the tissue chamber. Fractions of eluent were collected in 10-minute blocks for 10 hours. At 4 hours the first treatment, either PGE_2 (final concentration administered $1x10^{-6}$ M) or $ACTH_{1-24}$ (final concentration administered $1x10^{-7}$ M) was infused into the system for 10 minutes at a rate of 1 ml/10 minutes. At 6 hours the second treatment, a combination of PGE_2 ($1x10^{-6}$ M) and $ACTH_{1-24}$ ($1x10^{-7}$ M) was infused into the system. Fractions were assayed for the determination of cortisol concentrations (See Section 2.1.1).

Experiment Two – Adrenal gland slices from fetuses at approximately 140 d GA were used to examine the effect of ACTH and PGE_2 at a different dose. The experimental protocol was performed the same way as in the first experiment, with PGE_2 being administered at a final concentration of 1×10^{-7} M and $ACTH_{1-24}$ was administered at 1×10^{-6} M. PGE_2 and ACTH were not administered in combination.

Experiment Three – To examine the responsiveness of the female adult adrenal gland to ACTH and PGE_2 in the perifusion system, pregnant and post-partum ewe adrenal glands were tested. Pregnant ewe adrenals were collected at 125 d GA and 140d GA using the mothers of the fetuses used in experiment one. Post-partum ewe adrenals were obtained at elective postmortem examination with the criteria that the ewe must have given birth at least one week prior to collection and that the ewe was not lactating. The protocol was conducted in the same manner and performed in parallel with experiment one.

3.2.3. Statistical Analyses

The cortisol concentration of the fractions of eluent for 30 minutes prior and for one hour following the treatment was used. Data were corrected for the mass of tissue in each chamber. For treatment one (single agonists at two ages), the data were analysed by 3-way ANOVA with repeated measures, where Gestational Age, Agonist and Time were the 3 factors tested. The combination treatment was also analysed by 3-way ANOVA with repeated measures, where Age, Order of Agonists and Time were the 3 factors. Experiment two (fetal dose reversal experiment) was analysed by two-way ANOVA with repeated measures with Time being the within subjects variable and Agonist the between subjects variable. If a significant effect was observed, least significant difference (LSD) tests were

used post-hoc to determine at which time point differences occurred. Data is expressed as the mean ± s.e.m. Data are presented as the 30 minutes before the agonists reach the tissue slices and 60 minutes post treatment.

As there were no significant differences between ages and treatment in basal secretion all data are presented as percentage change from basal secretion. In addition, the same analyses were also undertaken using raw secretion data, and, in no case, was a different statistical conclusion reached when compared with those obtained using percentage data.

3.3. RESULTS

3.3.1. Pre-Mortem Plasma Concentrations

Ewe/Fetus	Gestational Age	Cortisol (ng/ml)	PGE_2 (nM)
Fetus	125 d	16.45 ± 1.14	7.6 ± 1.05
	140 d	34.12 ± 4.19	10.38 ± 1.40
Ewe	Post-partum (Luteal phase of reproductive cycle)	35.5 ± 4.16	1.27 ± 0.27
	125 d	37.24 ± 3.66	8.17 ± 0.88
	140 d	28.03 ± 4.53	12.56 ± 1.17

Table 3-1 The mean plasma hormone concentrations of ewes and fetuses, taken at the time of postmortem before adrenals were collected and used for perifusion analysis.

3.3.2. Groups, Treatments and Adrenal Weights

Experiment Number	Experimental Group	Treatment	Average combined Adrenal Weight
1	Fetal 125 d (n = 5)	PGE_2 1 x 10^{-6} M ACTH 1 x 10^{-7} M Individually and in combination	220.6 ± 20 mg *
1	Fetal 140 d (n = 5)	PGE_2 1 x 10^{-6} M ACTH 1 x 10^{-7} M Individually and in combination	369.0 ± 25 mg
2	Fetal 140 d (n = 8)	PGE_2 1 x 10^{-7} M ACTH 1 x 10^{-6} M Individually only	341 ± 28 mg
3	Pregnant Ewe 125 d GA (n = 5)	PGE_2 1 x 10^{-6} M ACTH 1 x 10^{-7} M Individually and in combination	4963 ± 620 mg
3	Pregnant Ewe 140 d GA (n = 5)	PGE_2 1 x 10^{-6} M ACTH 1 x 10^{-7} M Individually and in combination	4564 ± 477 mg
3	Non- Pregnant Ewe (n = 7)	PGE_2 1 x 10^{-6} M ACTH 1 x 10^{-7} M Individually and in combination	3750 ± 222 mg

Table 3-2 Summary of the experimental groups, treatments and adrenal weights. The 125 d fetal combined adrenal weight was significantly less than the 140 d fetal adrenal weights.

3.3.3. Experiments 1 & 2 - Cortisol Responses of the Fetal Adrenal Cortex

3.3.3.1. PGE_2 or ACTH Treatment

Experiment One - There was a non-significant trend for the adrenal slices from 140 d fetuses to secrete more cortisol *in vitro* than the adrenal slices from the younger 125 d fetuses ($P = 0.056$). Administration of PGE_2 (1×10^{-6} M) to adrenal slices from fetuses at both 125 d and 140 d resulted in increased cortisol output. The 125 d fetal adrenals were increased 30 minutes after PGE_2 treatment, while the 140 d fetal adrenals were significantly higher 20 and 30 minutes after PGE_2 treatment (125 d, $P = 0.004$; 140 d, $P = 0.001$). There was no effect of ACTH treatment (1×10^{-7} M) on the secretion of cortisol from the fetal adrenal slices ($P = 0.307$; Figure 3-5; Figure 3-6).

Experiment Two – Administration of ACTH at a higher dose (1×10^{-6} M) than in experiment one had no effect on the secretion of cortisol from the 140 d adrenal slices ($P = 0.180$). Similarly, administration of PGE_2 at the lower dose of 1×10^{-7} M had no significant effects on the output of cortisol from the 140 d fetal adrenal slices ($P = 0.150$; Figure 3-6).

3.3.3.2. PGE_2 and ACTH Combination Treatment

Experiment One - There was a highly significant difference between the 125 d and 140 d adrenal slices in terms of their basal cortisol output with 140 d slices secreting more cortisol than their 125 d counterparts ($P = 0.012$). This differs with experiment one but suggests that the more time the adrenals spent in the perifusion system either the 125 d adrenal slices became less responsive or more 125 d cells died than the 140 d cells.

There was no effect of treatment one on the response to the combination treatment; i.e. the cortisol response to the combined treatment was not affected by the order of agonist treatment, therefore the groups were combined. There was an effect of the combination treatment 10 and 20 minutes after agonist treatment in both age groups ($P = 0.001$; Figure 3-7).

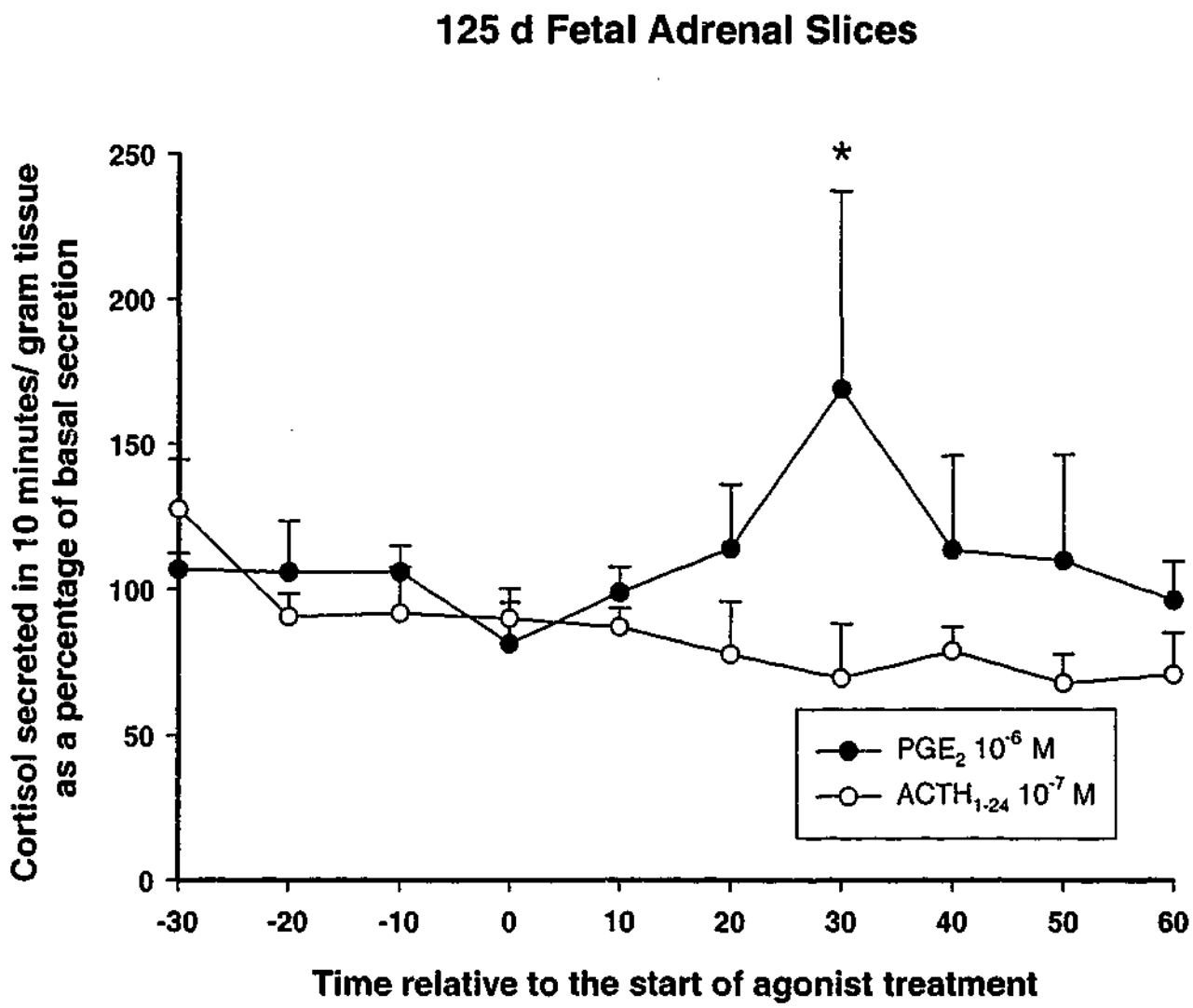

Figure 3-5 The cortisol response of adrenal slices from 125 d fetuses to PGE_2 (1×10^{-6} M) or ACTH (1×10^{-7} M). There was a significant increase in the cortisol output in response to PGE_2 ($P = 0.001$), but ACTH had no effect on the cortisol output from the adrenal slices. * indicates significant differences within the groups.

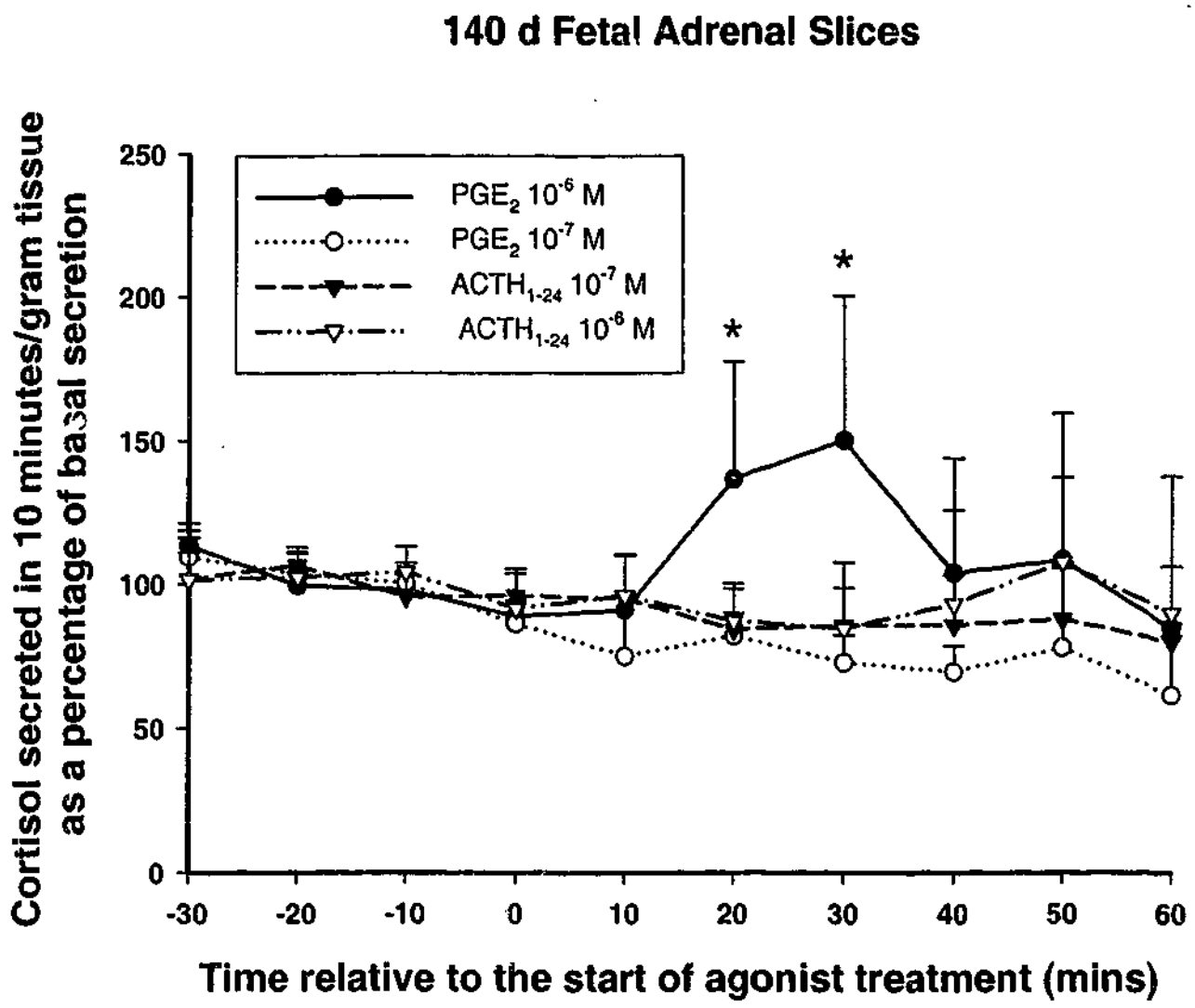

Figure 3-6 The cortisol response of 140 d fetal adrenal slices to PGE_2 (10^{-7} M or 10^{-6}M) or ACTH (10^{-6} or 10^{-7} M). There was no effect of ACTH at either dose or PGE_2 at 10^{-7} M on the cortisol output from the adrenal slices. PGE_2 administration at 10^{-6} M did result in a significant increase in the amount of cortisol secreted from the adrenal slices ($P = 0.001$).

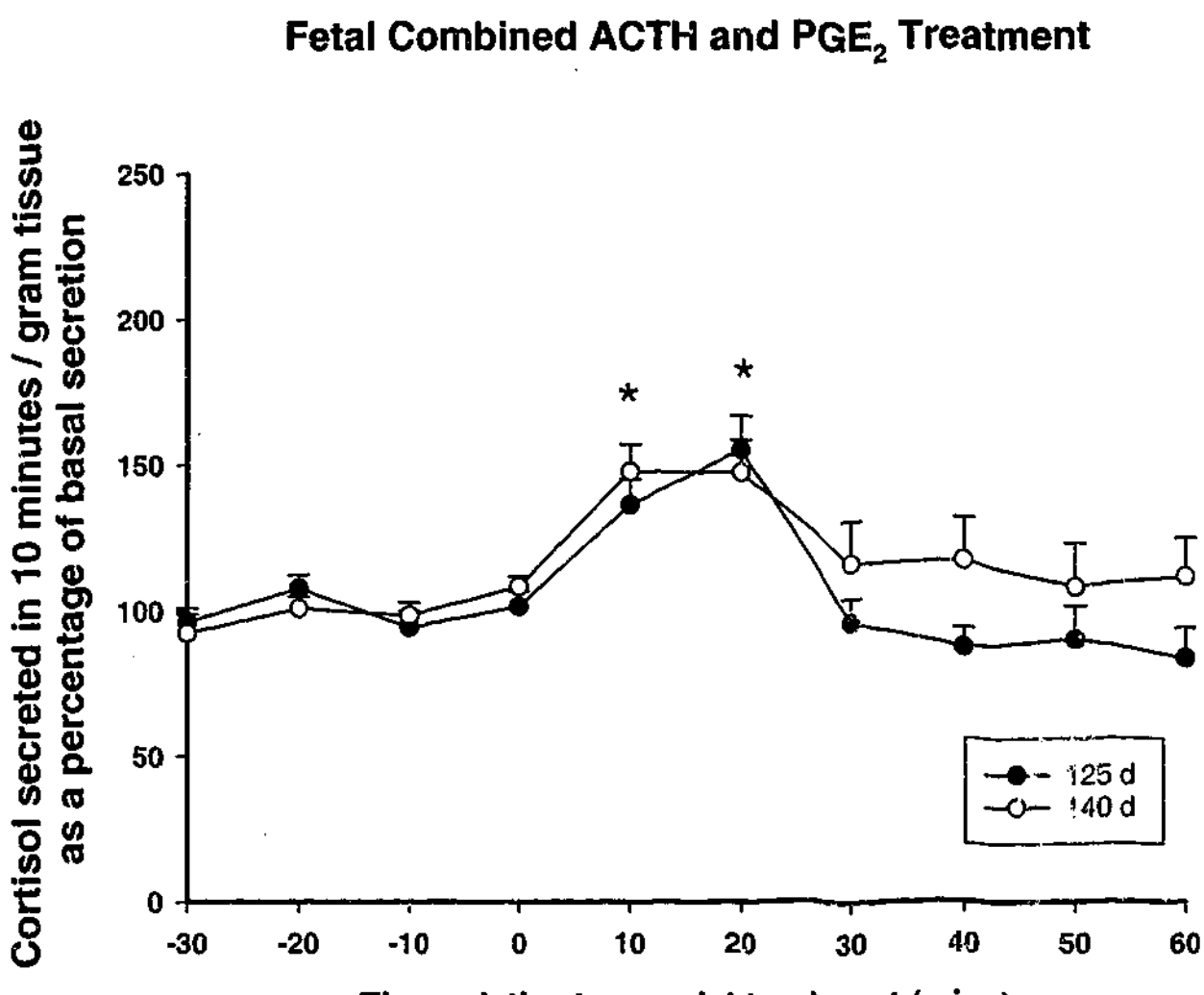

Figure 3-7 The response of fetal 125 d and 140 d GA adrenal slices to a combination of ACTH (1×10^{-7} M) and PGE_2 (1×10^{-6} M). 140 d adrenal slices had a significantly higher basal output of cortisol than the 125 d fetuses. Both age groups had a significant increase in cortisol in response to the combination treatment. * indicates significant differences within the groups.

3.3.4. Experiment 3 - Cortisol Responses of the Adult Adrenal Cortex

3.3.4.1. PGE_2 or ACTH Treatment

Experiment Three - There was no effect of age or pregnancy status on the output of cortisol, thus the groups were combined for analysis. Ewe adrenal slices exhibited a significant increase in the output of cortisol in response to ACTH ($P = 0.001$) and PGE_2 ($P = 0.001$) alone at the doses of 1×10^{-7} M and 1×10^{-6} M respectively, 10 and 20 minutes after treatment with the agonist (Figure 3-8).

3.3.4.2. PGE_2 and ACTH Combination Treatment

Experiment Three - There was a significant effect of combination treatment on ewe adrenal slices. In the 125 d ewe adrenal slices, the order in which the agonists were used before the combination treatment had a significant effect i.e. the slices that had seen PGE_2 first had a higher baseline cortisol output than those slices that had seen ACTH first ($P = 0.046$). The 125 d pregnant ewe slices did not respond to the combination treatment irrespective of which treatment the slices had been exposed to first ($P = 0.72$). There was no effect of the order of the agonist solution observed in the 140 d or the post-partum ewe adrenal slices therefore these two groups were combined for statistical analysis. The 140 d and post-partum adrenal slices did significantly respond to the combination treatment of ACTH and PGE_2, regardless of which agonist solution had been used in the first experiment ($P = 0.001$; Figure 3-9).

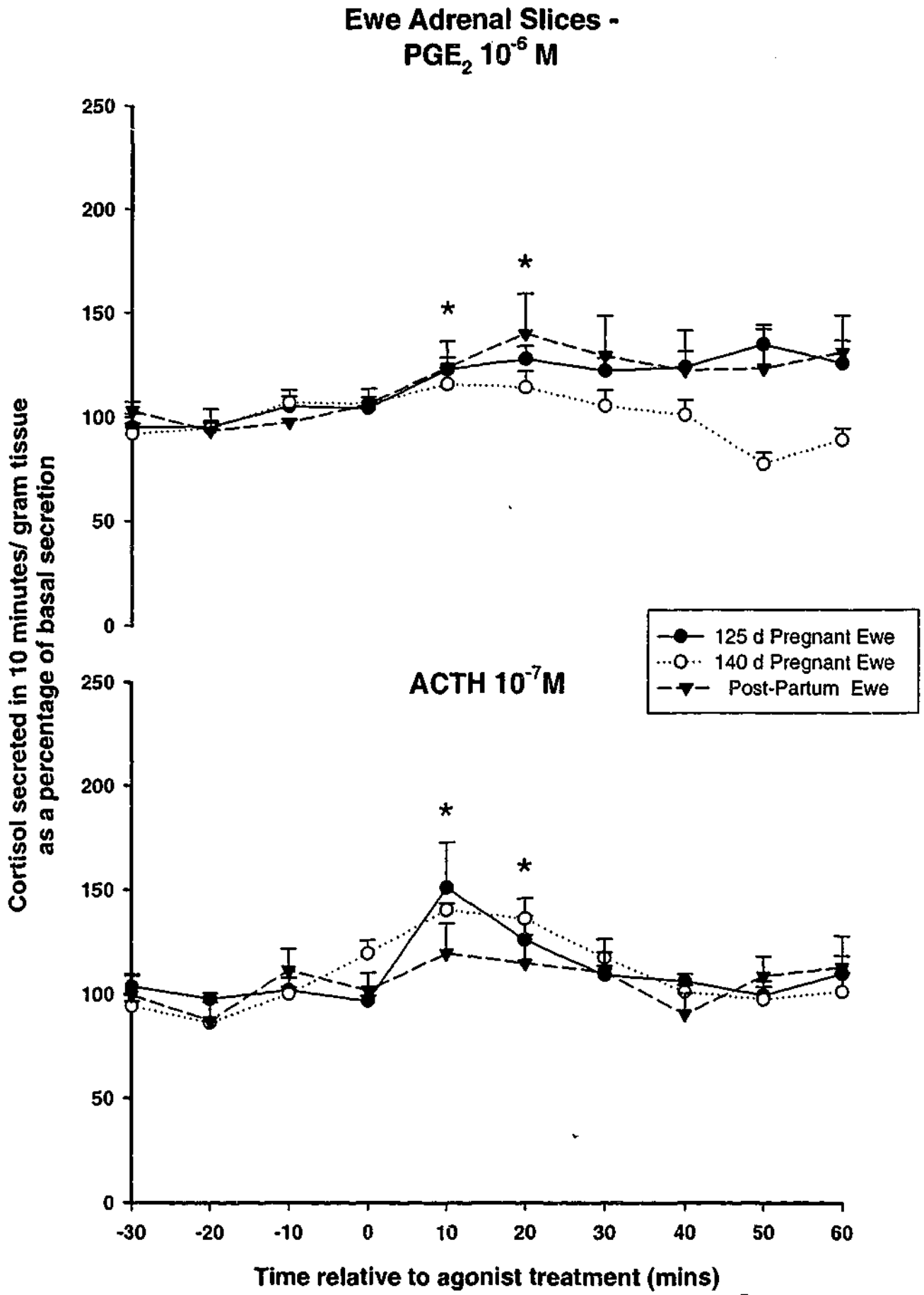

Figure 3-8 The ewe adrenal slices response to either ACTH (1 x 10^{-7} M) or PGE_2 (1 x 10^{-6} M). There was a significant increase in cortisol output in response to both of the agonist treatments at all ages. There was no difference in the basal cortisol output or the response of the slices to the agonist treatments at any of the ages tested. * indicates significant differences within the groups.

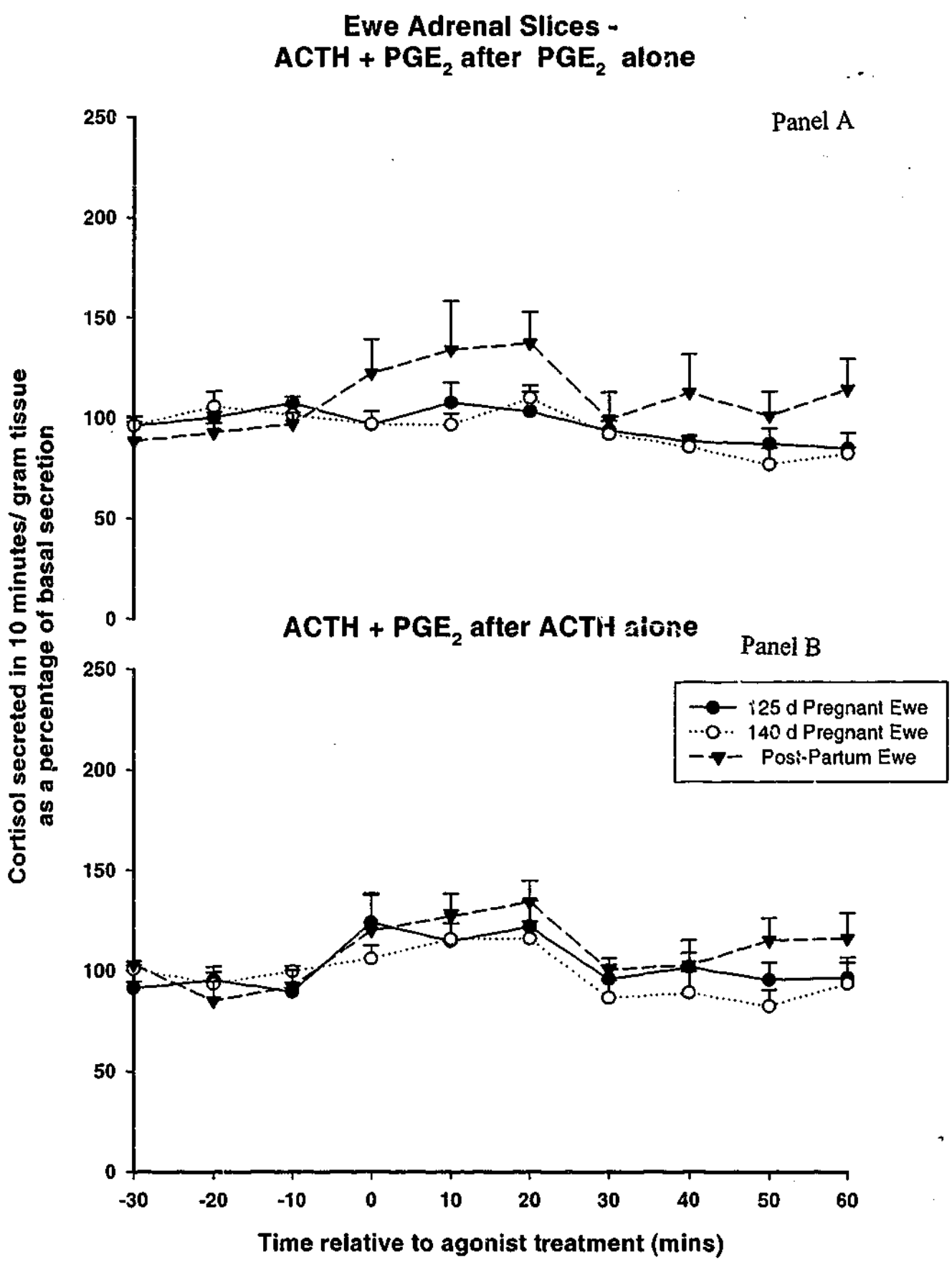

Figure 3-9 Ewe adrenal slice response to a combination of ACTH and PGE_2 after receiving either PGE_2 (Panel A) or ACTH (Panel B) two hours earlier. There was no effect of the earlier treatment in the 140 d or post-partum ewe adrenals but the 125 d slices that had been exposed to PGE_2 first had a higher basal cortisol secretion than the slices that had seen ACTH first. The 125 d ewe adrenal slices did not respond to the combination treatment ($P = 0.072$). The 140 d and post-partum adrenal slices ($P = 0.04$) had a significant increase in cortisol output in response to the combination treatment

3.4. DISCUSSION

The principal finding of this study is that PGE_2 at the dose of 1 x 10^{-6} M is able to act directly at both fetal and female adult adrenal slices *in vitro* to stimulate cortisol secretion. ACTH could not stimulate secretion of cortisol from fetal slices at either 1 x 10^{-6} or 1 x 10^{-7} M but could stimulate the secretion of cortisol in adult pregnant and post-partum ewes.

3.4.1. Fetal Basal Responses

The finding that the fetal adrenal slices from the 140 d fetuses had a non-significant trend towards higher basal output of cortisol than the slices from the 125 d fetuses was expected, as it has been reported that adrenals from fetuses between 90 and 125 d go through a refractory period and thus secrete less cortisol (Wintour *et al.*, 1975). Further, the 140 d fetal slices were collected at a time when the cortisol surge is well established resulting in a higher output of cortisol (Magyar *et al.*, 1980; Poore *et al.*, 1998a). Before the combination treatment the adrenal sliced of the 125 d fetuses had a significantly lower basal secretion rate than their 140 d counterparts. This suggests that the basal secretion rate of the 125 d adrenal glands decreased faster than the 140 d adrenal slices or perhaps the cells of the 125 slices died quicker than the 140 d cells, reducing the basal secretion rate.

3.4.2. Fetal PGE_2 Stimulation

The administration of PGE_2 to fetal adrenals *in vitro* has yielded variable responses in the past. Chen *et al.* (1997) found that administration of PGE_2 to adrenocortical cells *in vitro* did not stimulate cortisol secretion in dispersed fetal, neonatal and adult cells. Similarly, Durand *et al.* (1981a) also found that PGE_2 could not stimulate adenylate cyclase in adrenal cells. However, Zambrano *et al.* (2001) found that both prenatal and postnatal ovine adrenal cells did respond to PGE_2. In that report, the authors found using microphysiometric techniques, that PGE_2 could stimulate H^+ release, which they described as representing cellular activation from dispersed adrenal cells. The results of Zambrano *et al.* (2001) support the findings of this study in that PGE_2 could activate adrenal cells at 1 x 10^{-6} M, but had very little response to PGE_2 administered at 1 x 10^{-7} M.

3.4.3. ***Fetal ACTH Stimulation***

Unlike the response of the fetal adrenal cells to PGE_2, there was no increase in cortisol secretion in response to ACTH at either of the ages or the doses examined. This finding was unexpected and raised a number of questions about the efficacy of the experimental design. The ACTH was biologically active as the same ACTH solution contemporaneously stimulated cortisol secretion in the ewe adrenal slices. The fetal adrenal cells were not incapable of secreting cortisol as these adrenal slices responded to both PGE_2 alone and to a combination of ACTH and PGE_2. The finding that the 125 d fetuses did not respond to ACTH is consistent with previous studies. Durand *et al.* (1981a) found that adding ACTH at 1×10^{-6} M to crude adrenal membrane preparations from 124 d GA fetuses, did not stimulate an increase in adenylate cyclase (AC) activity. However, this dose of ACTH did stimulate AC activity when tested on 140 d GA and adrenal preparations from fetuses at birth (Durand *et al.*, 1981a). Madill and Bassett (1973) also reported a cortisol response to 3 M $ACTH_{1\text{-}24}$ in adrenal slices from 100 d to 145d GA, with the response increasing with increasing gestational age.

Consistent with the results of the present study, Zambrano *et al.* (2001) observed no effect of ACTH on dispersed 125 d ovine fetal adrenals at any dose from 10^{-13} to 10^{-6} M, although this group did not report a dose response to ACTH at a later gestational age (Zambrano *et al.*, 2001). Using 145 d GA fetal adrenals, this group reported a 20% increase over baseline in H^+ in response to ACTH at 1×10^{-7} M, but this response was not statistically different from the response of the 125 d fetal cells.

The results of this study bring into question the sensitivity of the technique, as in many previous reports adrenal cells particularly at 140 d GA have responded to this dose of ACTH. Some possible explanations are that (i) the slices may have been too thick to be perfused completely by the agonists, or (ii) the receptors may have been damaged by the collection and slicing processes and this may have had a dulling effect. However, the adult adrenal cells did respond to the same doses of ACTH and the fetal cells responded to PGE_2. The amount of ACTH administered was considerably more than was recovered (approximately 35% recovery rate), however the adult adrenal slices were still able to respond to this dose, suggesting that the maternal adrenal is more responsive to ACTH than the fetal adrenal.

3.4.4. Ewe PGE_2 Stimulation

It has been shown in the adult mammalian, avian and amphibian species that PGE_2 can stimulate corticosteroidogenesis (Dazord *et al.*, 1974; Warner & Rubin, 1975; Rainey *et al.*, 1991a; Tominaga *et al.*, 1991; Kocsis *et al.*, 1999). The present study also found that prostaglandins could stimulate cortisol secretion *in vitro* and that gestational age or pregnancy status did not affect the magnitude of the adult cortisol response to PGE_2. At the present time, to the best of my knowledge, this is the first study that has investigated the role of prostaglandins on cortisol secretion in adrenal slices from pregnant and post-partum ewes.

In the adult ewe, pregnancy status and gestational age had no affect on the cortisol response to PGE_2 *in vitro*, which is quite surprising given that the circulating concentration of PGE_2 in each of these models differs quite markedly. Post-partum ewes have undetectable levels of prostaglandin E_2, while the circulating concentration of PGE_2 in the plasma of the pregnant ewe increases from approximately 90 d GA until term (Burgess *et al.*, 1990; Deayton *et al.*, 1993; Deayton *et al.*, 1994). However, as PGE_2 also acts in a paracrine manner it may be that the tissue concentration of PGE_2 that is more important than the circulating concentration for the responsiveness of the tissue.

The importance of the effects of paracrine PGE_2 is discussed in further detail in section 6.7.

3.4.5. Ewe ACTH Stimulation

Unlike the fetal adrenal slices, the ewe adrenal glands responded to ACTH and this response did not differ between the gestational ages in either the amount secreted or the timing of the response. In the study by Zambrano *et al.* (2001), the response of the dispersed adult cells to ACTH was a doubling of the basal concentration. The response of the adult adrenal in both Zambrano *et al.* (2001) and the present study suggests that the fetal adrenal is relatively unresponsive and the adrenal is far more sensitive to ACTH in adulthood than in fetal life. The finding that the fetal adrenal is relatively insensitive to ACTH is consistent with the idea that ACTH plays a permissive role in the cortisol surge and that other factors drive the increase in cortisol in late gestation.

Glickman & Challis (1980) cultured adrenal cells from ewes at 50, 100 and 130 d and term during pregnancy, 1 - 5 d postpartum and in post-partum adult sheep. The cells were

examined for their response to $ACTH_{1-24}$. The endogenous secretion of cortisol in these adrenal cells increased from 50 to 100 d GA but did not increase any further from 130 d to term and in the postpartum period, however these values were higher than the post-partum group (Glickman & Challis, 1980). The results of the current study are in concordance with Glickman & Challis (1980), with no significant difference between the secretion of cortisol at 125 d GA, 140 d GA or the post-partum group. Glickman & Challis (1980) reported that ACTH stimulated cortisol secretion in the adult at all stages of pregnancy.

ACTH acts at the adrenal gland via its receptor (the ACTH R/MC2R) in the adrenal cortex, however the mechanism of action of PGE_2 at this gland has not been fully investigated. In cultured bovine adrenocortical (BAC) cells, one group of researchers gave PGE_2 (10^{-5} μM) for 3 h and found that it stimulated steroidogenesis and cAMP production over 100-fold. In that study, the authors also employed a cAMP antagonist and found that this agent blocked up to 60% of PGE_2 stimulated steroid production. The authors concluded that PGE_2 acts in part through the cAMP 2nd messenger pathway (Rainey *et al.*, 1991a). In a separate study, preparations of adult human or sheep adrenal cortex or membranes were used to look at the binding of radiolabelled PGE_1 and PGE_2. They reported that both prostaglandins bound to the adrenal glands with an affinity of 10^{-8}M and both stimulated adenylate cyclase activity (Dazord *et al.*, 1974). Karaplis and Powell (1981a) examined the ovine fetal adrenal gland for the presence of PGE_2 receptors and found they were most abundant in the adrenal medulla. Ma *et al.* (1999) looked at the various PGE_2 receptor subtypes and found that the mRNA encoding the receptor subtypes EP2, 3 and 4 were present in the ovine fetal adrenal (Ma *et al.*, 1999).

In this chapter, I have shown that PGE_2, but not ACTH was capable of stimulating fetal adrenal cells to secrete cortisol and both agonists were able to stimulate the corticosteroidogenesis in adult adrenal slices. The next chapter addresses if PGE_2 can stimulate cortisol secretion *in vivo*.

Chapter 4 PGE_2 CAN STIMULATE CORTISOL SECRETION FROM THE OVINE FETAL ADRENAL GLAND *IN VIVO*

4.1. INTRODUCTION

Parturition in the sheep is dependent on an increase in plasma cortisol, which in turn depends on an intact hypothalamo-pituitary-adrenal axis. The mechanism by which cortisol concentrations increase, in spite of negative feedback and the differences between the secretory dynamics of ACTH and cortisol is still under investigation. Plasma PGE_2 concentrations appear to parallel the increase in plasma cortisol concentrations in the late gestation ovine fetus, matching as well as, if not better than, ACTH concentrations (Deayton *et al.*, 1994). Intrafetal PGE_2 infusion in late gestation has been demonstrated to be a potent stimulus for the release of both ACTH and cortisol, while the infusion of a prostaglandin H synthase inhibitor to the pregnant ewe acts to decrease fetal PGE_2, ACTH and cortisol (Hollingworth *et al.*, 1995; Young *et al.*, 1996a; Young *et al.*, 1996b; Unno *et al.*, 1998). In addition to the ACTH-mediated effect of PGE_2 on cortisol secretion, the possibility exists that a direct effect of PGE_2 on cortisol secretion may provide a mechanism that can increase plasma cortisol concentrations in late gestation, that is not itself suppressed by glucocorticoids.

Young *et al.* (1996a) infused PGE_2 into fetuses continuously from 121 d until labour and reported that ir-ACTH concentrations increased for the first 11 hours of the infusion but then returned to baseline levels. Cortisol concentrations remained high throughout the infusion, suggesting a dissociation between ACTH and cortisol that may indicate the ability of PGE_2 to stimulate cortisol directly (Young *et al.*, 1996a). However, in a separate study by Young *et al.* (1996b) where PGE_2 was infused into intact and HPD fetuses, it was reported that intact fetuses had an increase in ACTH and cortisol in response to PGE_2 while HPD fetuses did not. The adrenals of these HPD fetuses were not very responsive to

a bolus dose of ACTH, a finding confirmed by Poore *et al.* (1999), who observed a small response to a bolus dose of ACTH in HPD/SAL fetuses. Young *et al.* (1996b) concluded from their study that the effects of PGE_2 on the HPA axis are exerted predominantly above the level of the pituitary gland. In light of the subsequent study by Poore *et al.* (1999) showing that HPD fetuses are hyporesponsive to ACTH, it now appears that the HPD fetus is not an appropriate model to assess the effectiveness of PGE_2 as a direct corticotrophin. Liggins, Scroop and Haughey (1982) however, infused PGE_2 into hypophysectomised fetuses and reported an increase in cortisol, suggesting that PGE_2 can act to increase cortisol directly at the adrenal gland without an increase in ACTH. However, as that study only measured the concentrations of cortisol and did not measure the concentrations of ACTH or PGE_2, conclusions on the site of action of PGE_2 cannot be determined.

In the previous chapter, it was demonstrated that PGE_2 at supraphysiological concentrations was capable of stimulating cortisol secretion directly from the ovine fetal adrenal gland *in vitro*. The findings summarised above suggest that PGE_2 may play an important role in positively modulating the ovine fetal HPA axis to initiate labour. One question that remains is if PGE_2 at physiological concentrations is able to increase cortisol secretion directly from the ovine fetal adrenal gland *in vivo*.

In this chapter, the interaction between PGE_2 and the HPA axis was further investigated using intact, and hypophysectomised fetuses that were maintained on a low dose infusion of ACTH (HX/ACTH). The effect of a 24 hour infusion of PGE_2 was investigated to further delineate the site of action of PGE_2 within the HPA axis. In addition, a hypoglycemia test was performed, as previous studies have demonstrated that a decrease in fetal glucose concentrations causes a significant increase in PGE_2 (Fowden *et al.*, 1987). In this study, the cortisol concentration in response to this hypoglycemia-driven plasma PGE_2 concentration increase was investigated.

4.2. MATERIALS AND METHODS

4.2.1. Animals

4.2.1.1. Animal Welfare

The animals were housed at least 3 days prior to surgery in standard individual metabolism cages, with feeding (1 kg Lucerne Chaff) occurring between 9 am and 12 noon daily. The animals had free access to water. There was a constant 12 hour light/dark cycle (light phase 0700 h – 1900 h) and the temperature was maintained at 22 °C. Surgery was performed between 123 - 127 days gestational age. The ewes were starved for 24 hours prior to surgery. The animal experiments were conducted over several breeding seasons and had the approval of the Monash University Standing Committee for Animal Experimentation. All procedures were in accordance with the requirements of the Victorian Prevention of Cruelty to Animals Act (1986) and the Code of Practice for the Care and Use of Animals for Scientific Purposes of the National Health and Medical Research Council.

4.2.1.2. Surgery

4.2.1.2.1. Pre-Operative Preparation

An intravenous injection of 20 mg/kg thiopentone sodium in water (Pentothal, Bomac Laboratories Ltd, Asquith, New South Wales, Australia) was administered to induce general anesthesia in both the mother and fetus. Anesthesia was maintained by inhalation through an endotracheal tube (cuffed 9 mm tube, Portex Laboratoire, Berck Sur Mer, France) with 0.5 - 2.0% halothane (Fluothane, ICI, Villawood, Australia) and a nitrous oxide/oxygen mixture (50/50 v/v) contained in a closed circuit apparatus (CIG Midget 3 Medishield, Alexandria, New South Wales, Australia). An automatic ventilator (Campbell anesthetic ventilator, Ulco Engineering, Marrickville, New South Wales, Australia) was used to ventilate the animal while surgery took place. The ewes were prepared for surgeries by clipping the abdomen, right flank, groin and anterior neck with a fine, small animal clipper and washing with povidone-iodine scrub before painting with povidone-iodine solution (Betadine Surgical Scrub Antiseptic, F.H. Faulding & Co Ltd, South Australia, Australia). On the operating table, the disinfected area was sponged with 5% Chlorhexidine Hibitane (ICI, Villawood, New South Wales, Australia) in 70% alcohol

and the ewes were covered in sterile surgical drapes. Surgery was performed under aseptic conditions.

4.2.1.2.2. Surgical Procedures

A 10 - 15 cm midline incision was made in the skin followed by an incision through the linea alba. Once any bleeding had been controlled, the abdominal incision was isolated with abdominal sponges and an incision was made through the uterine wall between cotyledons to exteriorise the fetal head and neck. Babcock clamps were used to prevent leakage of amniotic fluid during the operation. Carotid artery (CA) and jugular vein (JV) catheters were surgically implanted to enable infusions (JV) and routine blood sampling (CA). Saline filled medical grade polyvinyl catheters (i.d. 0.81 mm, o.d. 1.52 mm, 155 cm in length, Dural Plastics and Engineering, Silverwater B.C., New South Wales, Australia) were inserted 8 - 10 cm into each vessel from an incision at mid-neck level and secured in place. A double lumen catheter was implanted if two jugular vein cannulae were required. Fetal catheters were exteriorised through a small incision in the right flank of the ewe. All fetuses received an intramuscular injection of procaine penicillin/dihydrostreptomycin (Troy Laboratories Pty Ltd, Smithfield, New South Wales, Australia). The uterus was closed with a simple continuous suture and then a continuous Cushing stitch using 2/0 chromic catgut (Ethicon, Ethnor Pty Ltd, Sydney, New South Wales, Australia). The linea alba and subcutaneous fat were closed using size 2 chromic catgut suture material while the skin was closed using Vetafil (WDT, Hannover, West Germany).

Maternal carotid artery and jugular vein catheters were implanted via an incision in the jugular groove of the neck. Polyvinyl catheters (i.d. 1.50 mm, o.d. 2.70 mm, Dural Plastics and Engineering) were inserted 10 - 15 cm into the vessels. All catheters were fitted with three way stopcocks and stored in plastic bags on the ewe's flank under tubular elastic net bandage (size 7.0 setonet, FRA Production, Italy).

4.2.1.2.3. Fetal Hypophysectomy

Fetal Hypophysectomy (HX) was performed as described by Mesiano *et al.*, (1987). The fetal head was exposed by hysterotomy and held in extension with the ventral surface uppermost. A midline incision was made in the skin in the area of the basihyoid bone. The basisphenoid bone was exposed and the cranial floor was reached by blunt dissection. Under magnification, the dura covering the base of the pituitary was exposed with a 3 mm

diamond burr drill. The dura mater was opened in the midline and microscopic dissection was used to free the pituitary from the walls of the cavernous sinuses, which surround it. The pituitary was removed with a five french gauge suction instrument and the space where the pituitary had been (the sella turcica) was visually checked for any remaining fragments of pituitary tissue. When certain no pituitary tissue remained, the space was packed with gelatin sponge (Gelfoam, The Upjohn Company, Kalamazoo, Michigan, USA), soaked in thrombin (Human (dried) Thrombin, Commonwealth Serum Laboratories, Parkville, Victoria, Australia) containing a small quantity of procaine penicillin/dihydrostreptomycin (Troy Laboratories Pty Ltd, Smithfield, New South Wales, Australia). The skin incision was sutured using size 3/0 silk horizontal mattress suture. The sella turcica was subsequently examined at post mortem to ensure complete removal of the pituitary.

4.2 1.2.4. Post-Operative Care

While recovering from anesthesia the ewe was ventilated until independent breathing was established, when the endotracheal tube was removed. The ewe was returned to an individual metabolism cage and when the ewe had recovered sufficiently from the effects of anesthetic, food and water was provided. All cannulae were flushed with sterile saline containing heparin (50,000 IU/1 L Saline, Fisons Pty Ltd, Sydney, Australia) daily for 3 days postoperatively and then every second day. A recovery period of at least 5 days was allowed before experiments began.

4.2.2. Experimental Protocols

4.2.2.1. Blood Sampling

Blood samples were collected every 2 - 3 days via the fetal CA catheter. This procedure began 3 days after surgery. Blood samples (4 ml) were collected into chilled sterile tubes containing EDTA (18.6 mg/ml; 100 µl/ml blood; BDH chemicals) for analysis of ACTH and cortisol by radioimmunoassay. Blood samples for analysis of PGE_2 were collected into chilled tubes containing EDTA (as above) and indomethacin (20 mg/ml; final concentration 10 µM). All blood samples were centrifuged (Beckman Instruments Pty Ltd, Australia) at 3000 rpm at 4°C for 10 minutes. The plasma was removed from the red blood

cells. The plasma used for measurement of immunoreactive-ACTH was stored in an ACTH degradation inhibitor mix (20 µl/ml; contains aprotonin (1000 kallikrein inhibitor units/ml), N-ethyl-maleimide (25 mg/ml, Sigma Chemical Company, St. Louis, USA) and EDTA (18.6 mg/ml, BDH Chemicals) in 0.9% NaCl). Plasma for the measurement of PGE_2 was diluted 1:1 with methoximating reagent (containing 0.12 M methoxyamine hydrochloride (Sigma Chemical Company, St. Louis, Missouri, USA) in sodium acetate buffer (1 M, pH 5.6) and 10% absolute ethanol) for 24 h at room temperature. All plasma was stored at –20°C until assays were performed.

4.2.2.2. Fetal Well Being

Fetal arterial blood gas levels were monitored as a measure of fetal well being. Blood (0.4 ml) was collected from the fetal carotid artery catheter into 1 ml syringes rinsed with heparinised saline. Samples were collected to monitor fetal well being with each routine blood sampling and at the start and end of any blood sampling protocol. Fetal blood was measured for the partial pressure of oxygen (PaO_2), carbon dioxide ($PaCO_2$), hemoglobin concentration (Hb), oxygen saturation (SaO_2) and pH. The gas measurements were determined using an ABL30 acid-base analyser and OSM2 hemoximeter (Radiometer, Copenhagen, Denmark). Measurements were corrected for estimated fetal body temperature (39 °C) and barometric pressure.

4.2.2.3. Continuous Infusions

Continuous infusions of saline, ACTH or PGE_2 were administered using a syringe pump (Terufusion syringe pump TE-311, Terumo Corporation, Tokyo, Japan). $ACTH_{1-24}$ (Synacthen, Ciba Geigy Australia Ltd, Pendle Hill, New South Wales, Australia) in heparinised saline (0.9% NaCl; 50 000 IU heparin/L (Fisons Pty Ltd, Sydney, Australia) was administered via the fetal jugular vein catheter at a dose of 13.23 pmol/hr/kg, estimated body weight. The infusion ran continuously from the time of surgery until euthanasia and elective post-mortem. The dose of ACTH administered increased every five days with estimated increases in fetal body weight determined by fetal growth charts in order to keep the concentration of exogenous $ACTH_{1-24}$ in the fetal plasma relatively constant (Cloete, 1939). Saline was administered through the fetal jugular vein catheter at a dose of 1ml/hr. PGE_2 (Cayman Chemical Company, Ann Arbor, Michigan, USA) was infused at a rate of 1 µg/min/kg of fetal body weight at 140 d GA for 24 hours.

4.2.2.4. *Hypoglycemia Challenge*

At 134 ± 2 d GA a hypoglycemia challenge was performed. This challenge was performed to investigate the ability of the fetus to respond to a physiological stressor by integrating inputs at the CNS level and activate the HPA axis. This involved injecting the ewe via the maternal JV catheter with 2 IU/kg body weight of insulin (Actrapid 10 ml vial, Novo Nordisk Pharmaceuticals, Denmark). This depletes the ewe's plasma glucose concentration and the hypoglycemia is therefore translated to the fetus. Plasma was collected for measurement of ACTH, cortisol, PGE_2, glucose and lactate concentrations at –30, 0, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after the insulin injection. At the end of 4 hours the hypoglycemia was reversed by an injection of 12.5 ml of a 50% glucose solution into the ewe's JV catheter. Fetal and maternal blood gases were also collected at each time point to monitor fetal and maternal well being.

4.2.2.5. *24 hour PGE_2 or Saline Infusion*

At approximately 140 d GA fetuses received a 24 hour infusion of either Saline (1 ml/hr) (INT/SAL + SAL; n = 5 or HX/ACTH + SAL; n = 5) or PGE_2 (1 µg/kg/min) (INT/SAL + PGE_2; n = 5 or HX/ACTH + PGE_2; n = 5). Basal samples were taken hourly from 9 am (t = -3) until midday (t = 0) when the Saline or PGE_2 infusion was started. Blood samples were then taken hourly until midnight (t = 12) and then three hourly until midday the next day (t = 24), when the animals were humanely killed by barbiturate overdose and a postmortem examination was performed. Plasma was then analysed by radioimmunoassay for ACTH, cortisol and PGE_2 (See Section 2.1.2).

4.2.2.6. *Postmortem Examination and Tissue Collection*

All ewes and fetuses were killed with an intravenous overdose (20 ml) of Lethabarb (active constituent pentobarbitone sodium 325 mg/ml; Virbac Pty Ltd, Peakhurst, New South Wales, Australia). The fetus was removed by hysterotomy and weighed. The fetal adrenals were collected, weighed and immediately frozen in liquid nitrogen and stored at -80°C for further analysis (See chapter 5).

4.2.3. Statistical Analyses

Analysis of the data was performed using SPSS for Windows (Version 10, SPSS Inc, Illinois, USA). Data were first analysed for homogeneity of variance using Levene's Test. Data found heterogeneous were rendered homogeneous by square root or logarithmic transformation.

Tissue weights were analysed by one way analysis of variance (ANOVA) to examine for differences between the treatment groups at the time of post-mortem. Hormone concentrations for both the basal samples and the hypoglycemia challenge were analysed by 2-way ANOVA with repeated measures with Time and Operation (INT or HX) as the factors. The 24 hour infusion experiment was analysed by 3-way ANOVA with Time, Operation and Treatment as the three factors. If a significant effect of Time was observed, least significant difference tests were used post-hoc to determine when the differences occurred. Data are expressed as mean ± s.e.m.

4.3. RESULTS

4.3.1. Animal Outcome and Welfare

All fetuses were considered healthy at the time of surgery and at the beginning of all subsequent challenges, as measured by fetal blood gases (See Table 4-1). In the INT/SAL group there were a number of sheep used that contained more than one fetus, however only one experimental animal was used from each ewe. All ewes and fetuses were electively killed at the end of the 24 hour infusion experiment at 139 - 141 d GA. At the time of postmortem, all HX fetuses were re-examined to confirm that HX was complete and all traces of pituitary gland had been removed at surgery.

4.3.2. Organ and Body Weights

There was no difference between the bodyweights (P = 0.169; Figure 4-1Top Panel), combined adrenal weights (P = 0.312; Figure 4-1 Bottom Panel) or the adrenal:body weight ratio (P = 0.15; Figure 4-2) between any of the four groups (INT/SAL + SAL, INT/SAL + PGE_2, HX/ACTH + SAL and HX/ACTH + PGE_2). There was also no difference in the pituitary weights of the two intact groups.

Treatment Group	Experiment	Hemoglobin (g/dL)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)
INT/SAL	Basal	9.71 ± 0.41	7.40 ± 0.01	41.58 ± 3.45	20.91 ± 3.49
	Hypoglycemia Challenge	9.50 ± 0.32	7.40 ± 0.01	38.76 ± 5.11	23.2 ± 5.21
INT/SAL + SAL	24 h Saline Infusion	8.54 ± 0.59	7.40 ± 0.01	39.72 ± 5.65	20.88 ± 5.94
INT/SAL + PGE_2	24 h PGE_2 Infusion	8.09 ± 0.23	7.39 ± 0.01	38.92 ± 5.55	23.98 ± 5.85
HX/ACTH	Basal	9.31 ± 0.45	7.39 ± 0.01	43.74 ± 1.72	22.57 ± 2.03
	Hypoglycemia Challenge	9.10 ± 0.57	7.41 ± 0.01	42.01 ± 2.51	24.61 ± 2.50
HX/ACTH + SAL	24 h Saline Infusion	7.82 ± 0.75	7.38 ± 0.01	44.4 ± 1.45	19.63 ± 1.63
HX/ACTH + PGE_2	24 h PGE_2 Infusion	8.49 ± 0.30	7.40 ± 0.01	39.86 ± 4.04	27.4 ± 4.58

Table 4-1 Summary of the blood gas parameters for the experimental groups at each of the challenges undertaken. The INT/SAL group reflects all the intact experimental animals, while the HX/ACTH group reflects all the animals that received this treatment. The INT/SAL and HX/ACTH groups were each separated into two groups, those that received saline and those that received PGE_2. There were no differences in any of the blood gases between the groups.

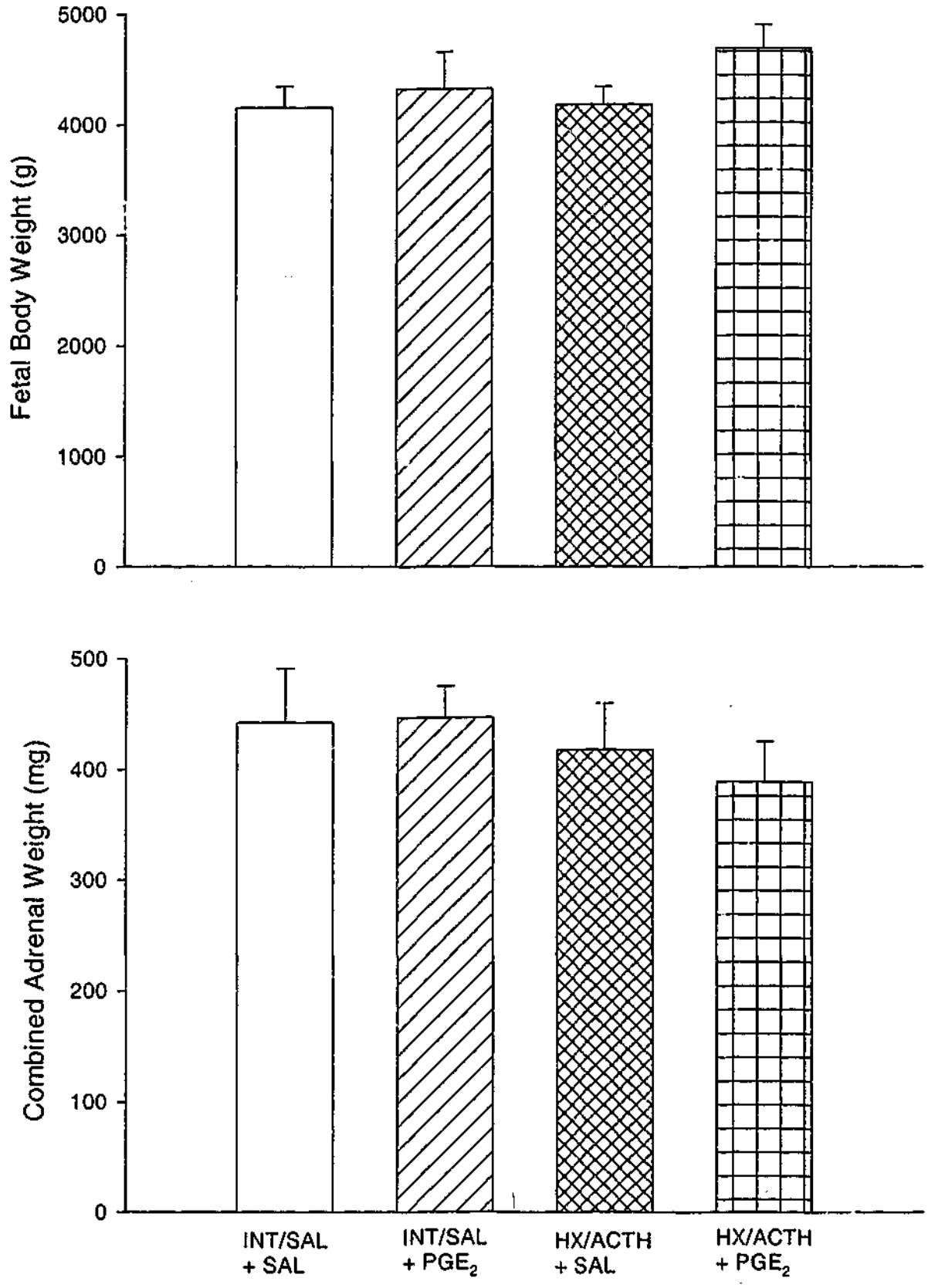

Figure 4-1 Mean fetal body weight (Top Panel) and the combined adrenal weights (Bottom Panel) for each of the experimental groups. There were no differences between any of the groups for either parameter.

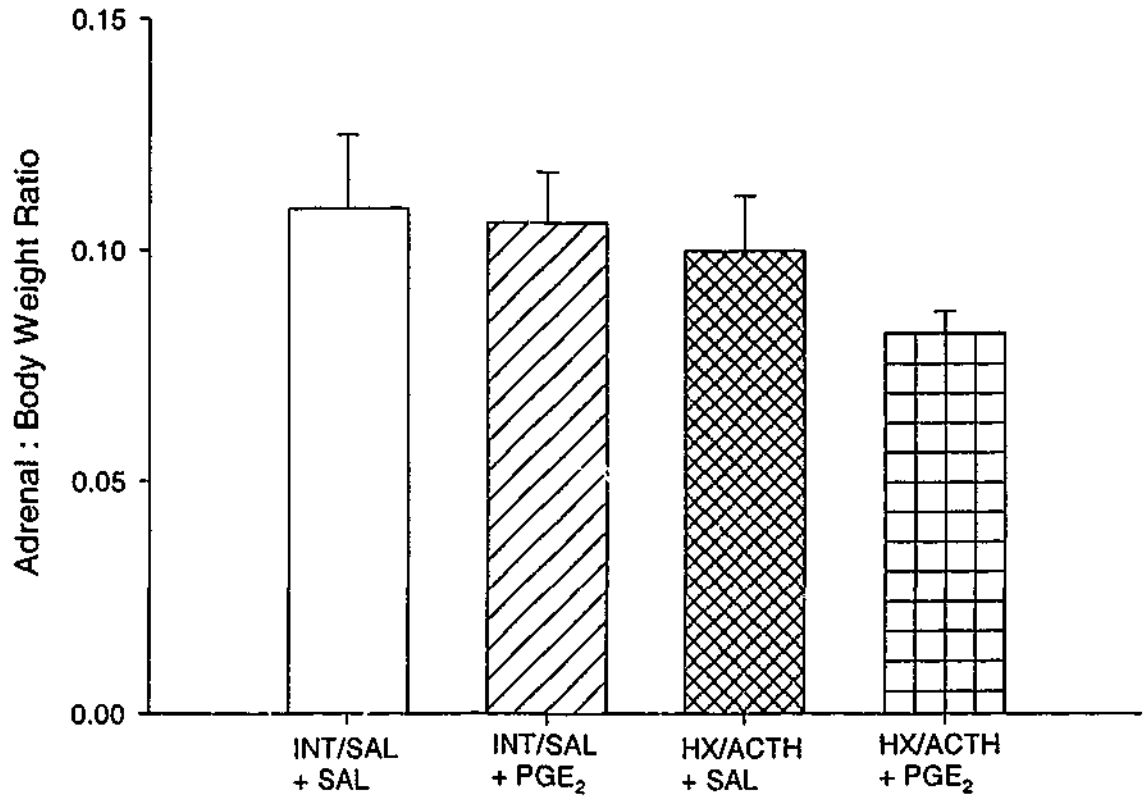

Figure 4-2 Mean adrenal to body weight ratio of the four experimental groups. There were no differences between the groups.

4.3.3. Basal Hormone Concentrations

Data were combined into gestational age blocks for analysis to account for collection of samples on different gestational ages. The gestational age blocks were 125 - 129 d, 130 – 134 d and 135 - 140 d.

There was no difference in the plasma concentration of ACTH between the INT/SAL or HX/ACTH groups at any of the ages tested ($P = 0.231$), nor were there any changes in the groups across the gestational ages tested (group $P = 0.231$, time $P = 0.868$; Top Panel, Figure 4-3). There was a significant increase in the cortisol concentrations in the INT/SAL group at each of the ages as gestation progressed ($P = 0.004$) but there was no change in plasma cortisol concentrations across gestation in the HX/ACTH group ($P = 0.983$; Middle Panel, Figure 4-3). For basal PGE_2 concentrations, there were no differences between the groups or across the gestational ages tested (group $P = 0.528$, time $P = 0.365$; Bottom Panel, Figure 4-3).

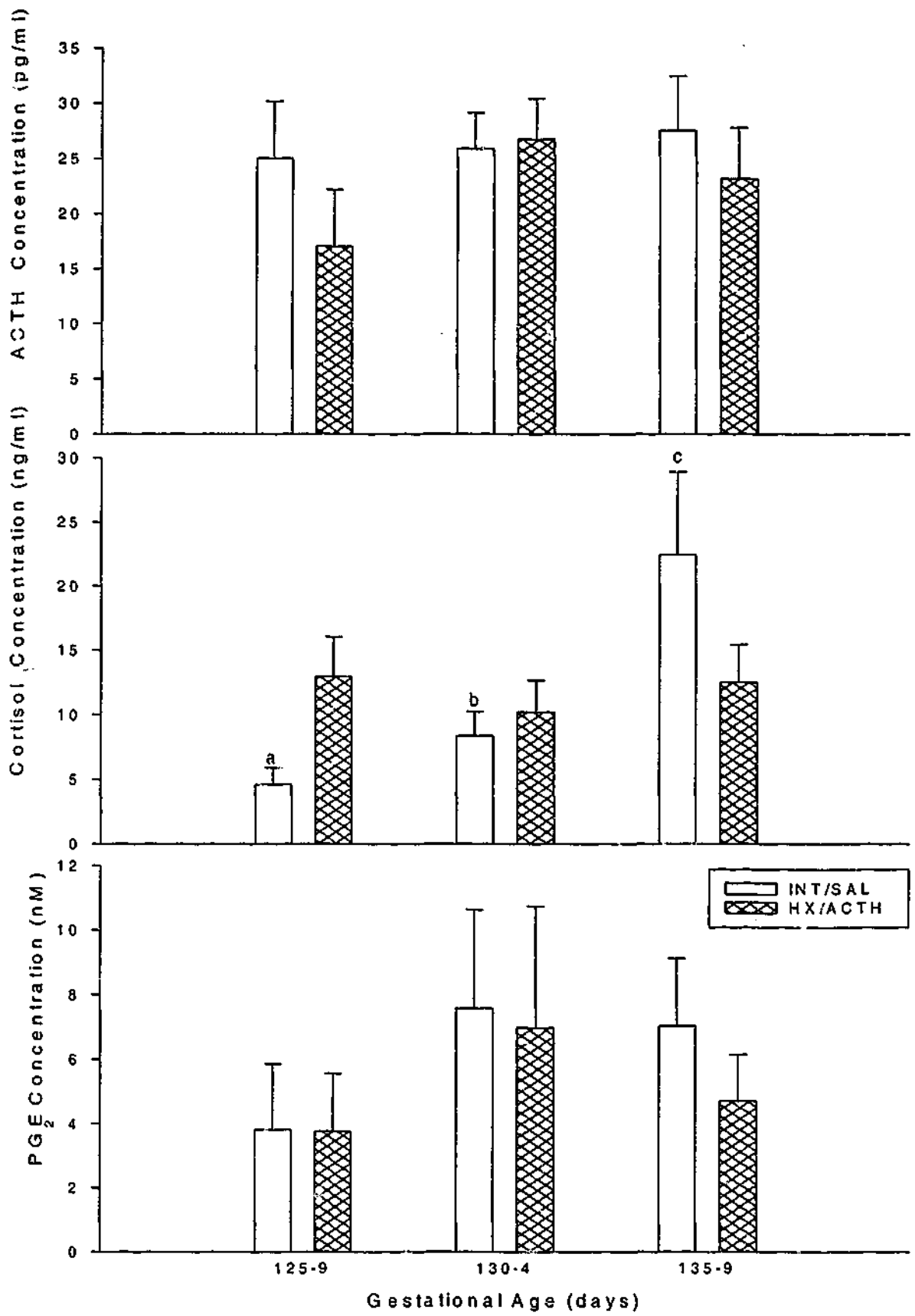

Figure 4-3 The INT/SAL and HX/ACTH fetuses mean basal plasma hormone concentrations for ACTH (Top Panel), cortisol (Middle Panel) and PGE_2 (Bottom Panel) over the entire experimental period. There were no differences between the INT/SAL or the HX/ACTH groups across the ages tested for ACTH and PGE_2 concentrations. There was a significant difference in the cortisol concentration of the INT/SAL group, which increased significantly at each age as gestation progressed. Different letters indicate the different ages that are significantly different from each other within the INT/SAL group.

4.3.4. Hypoglycemia Challenge

To examine the effect of a physiological stressor on the HPA axis in intact and HX/ACTH fetuses, a hypoglycemia challenge was performed. After the injection of insulin to the ewe there was a significant decrease in the fetal plasma glucose concentrations from approximately 0.8 mmol/L to 0.4 mmol/L in both INT/SAL and HX/ACTH groups (group $P = 0.63$, time $P = 0.001$; Figure 4-4, Top Panel). The lactate concentrations of both the INT/SAL and HX/ACTH groups changed over the period of the hypoglycemia challenge. In both the INT/SAL fetuses and the HX/ACTH fetuses plasma lactate concentrations increased, however the increases were different between the two groups (group $P = 0.001$, group by time interaction $P = 0.021$). The INT/SAL group was significantly different 120 minutes after the insulin injection when compared to the two basal samples, while the HX/ACTH group only differed significantly from the pre-insulin samples at 240 minutes after the insulin injection (INT/SAL $P = 0.0001$, HX/ACTH $P = 0.036$; Figure 4-4, Bottom Panel).

This challenge did not increase the plasma ACTH concentrations in either the INT/SAL or the HX/ACTH groups, but the two groups had different ACTH concentrations throughout the challenge, with the INT/SAL group having higher ACTH concentrations at all time points in this challenge (Time $P = 0.316$, Group $P = 0.003$; Figure 4-5, Top Panel). Although no increase in ACTH concentrations occurred, there was a significant increase in the cortisol concentrations in the INT/SAL but not the HX/ACTH fetuses (INT/SAL $P = 0.001$, HX/ACTH $P = 0.966$; Figure 4-5, Middle Panel). PGE_2 concentrations significantly increased in both the INT/SAL and the HX/ACTH fetuses in response to this challenge (group P = 0.274, time P = 0.001; Figure 4-5, Bottom Panel).

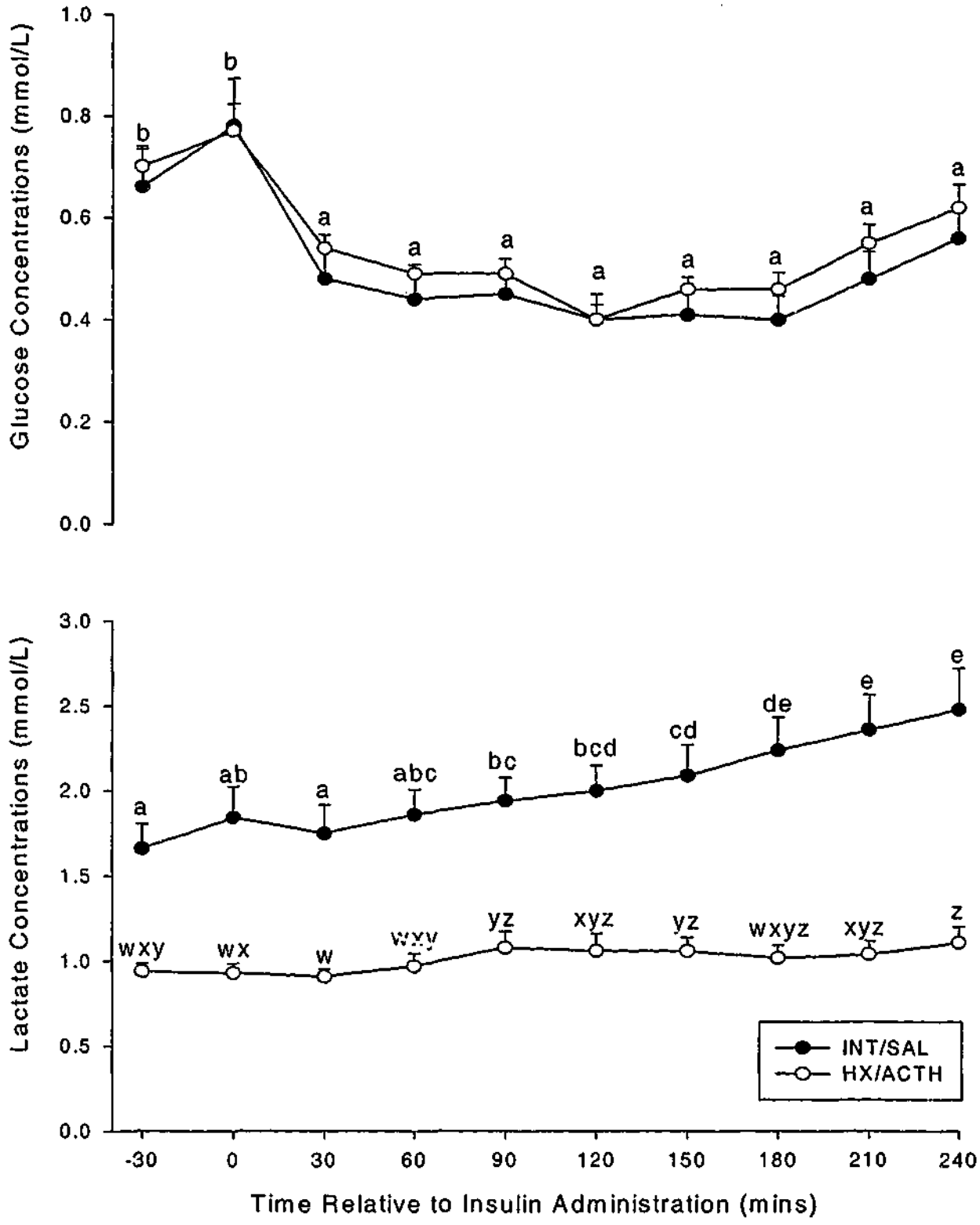

Figure 4-4 The glucose (Top Panel) and lactate (Bottom Panel) concentrations of INT/SAL (n=10) and HX/ACTH (n=10) fetuses during the hypoglycemia challenge. In response to the insulin injection, there was a significant reduction in the plasma glucose concentrations in both groups of fetuses. Lactate concentrations increased in both the INT/SAL and the HX/ACTH groups but the increase was more marked in the INT/SAL group. The lactate responses of the two groups were different. Different letters indicate values that are significantly different from each other.

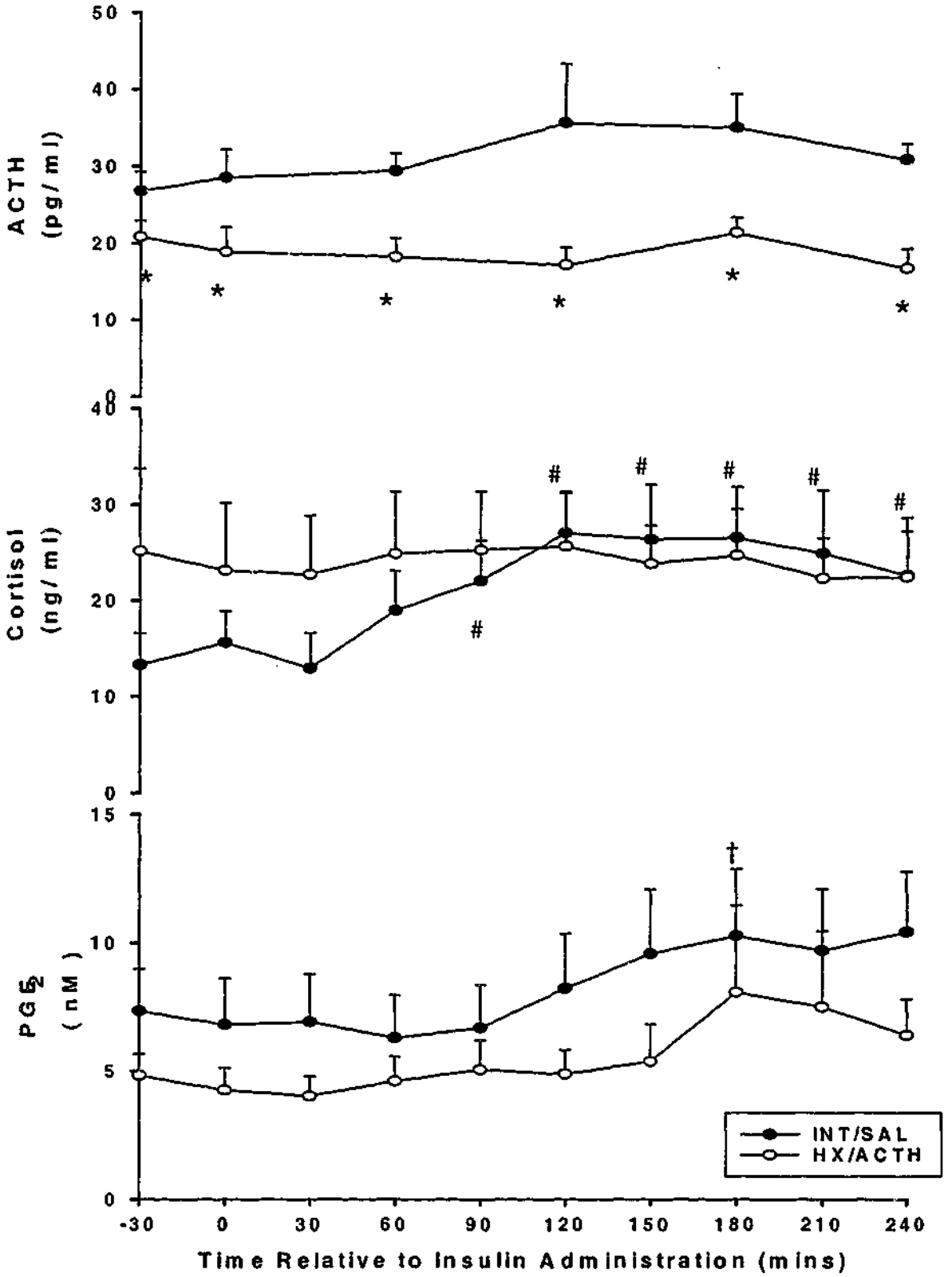

Figure 4-5 ACTH, cortisol and PGE_2 plasma concentrations during the hypoglycemia challenge. The plasma concentrations of ACTH did not change in either experimental group in response to the hypoglycemia challenge (Top Panel). Cortisol concentrations did not change in the HX/ACTH group but significantly increased in the INT/SAL cohort (Middle Panel). PGE_2 concentrations increased significantly in both groups of fetuses (Bottom Panel). * indicate differences between HX/ACTH and corresponding INT/SAL value. # indicate significant differences within the group. † indicate significant differences of both the INT/SAL and HX/ACTH groups from the basal samples.

Data is presented as the mean ± s.e.m.

4.3.5. 24 hour PGE_2 or Saline Infusion

4.3.5.1. PGE_2 concentrations

PGE_2 infusion caused a significant increase in the plasma PGE_2 concentration in both the INT/SAL and HX/ACTH fetuses ($P = 0.004$) that received this infusion. This increase was not observed in those animals that received an infusion of saline ($P = 0.081$). There were no differences in the way the INT/SAL or HX/ACTH fetuses responded to either the PGE_2 or saline infusion ($P = 0.196$; Figure 4-6).

4.3.5.2. ACTH concentrations

ACTH concentrations did not change significantly in response to the PGE_2 infusion in the INT/SAL + SAL ($P = 0.431$), HX/ACTH + SAL ($P = 0.061$) or the HX/ACTH + PGE_2 ($P = 0.954$) groups. However, infusing PGE_2 into an INT/SAL fetus caused a significant increase in plasma ACTH concentrations ($P = 0.049$; Figure 4-8).

4.3.5.3. Cortisol Concentrations

Saline infusion into either INT/SAL or HX/ACTH fetuses caused no significant change in the plasma cortisol concentrations over the length of the infusion (group $P = 0.888$, time $P = 0.212$). Infusion of PGE_2 however, resulted in a highly significant increase in the concentration of cortisol in both the INT and HX fetuses ($P = 0.0001$). There was a difference between the responses of the INT/SAL and the HX/ACTH fetuses to the PGE_2 infusion with the INT/SAL fetuses responding faster and the increase was greater to the PGE_2 infusion than the HX/ACTH group ($P = 0.012$; Figure 4-7).

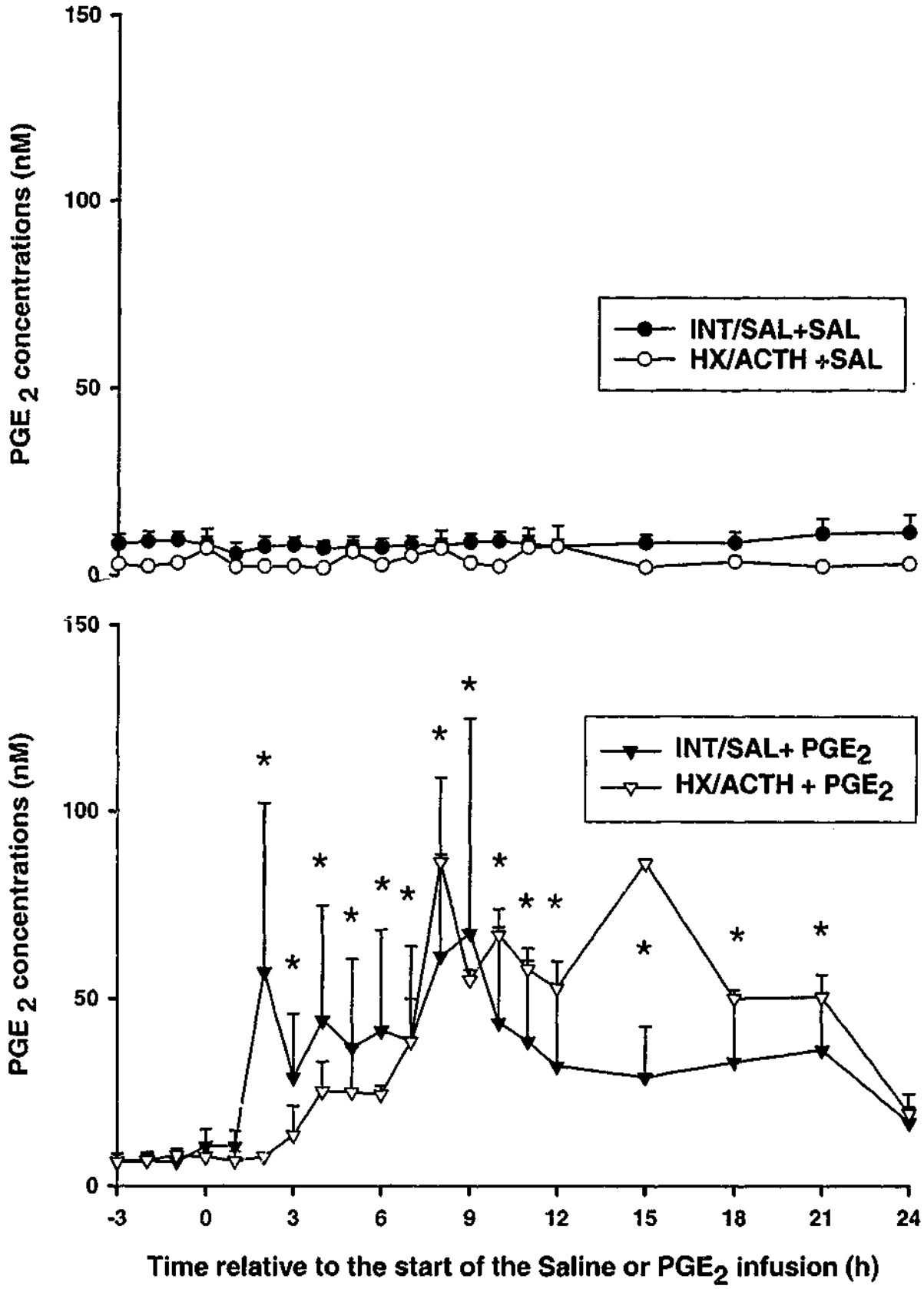

Figure 4-6 Mean plasma PGE_2 concentrations of each of the groups during the 24 infusion of either saline (Top Panel) or PGE_2 (Bottom Panel). Concentrations of PGE_2 were significantly elevated in the groups that received a PGE_2 infusion.

* indicates significant differences from basal samples. Data is presented as the mean ± s.e.m.

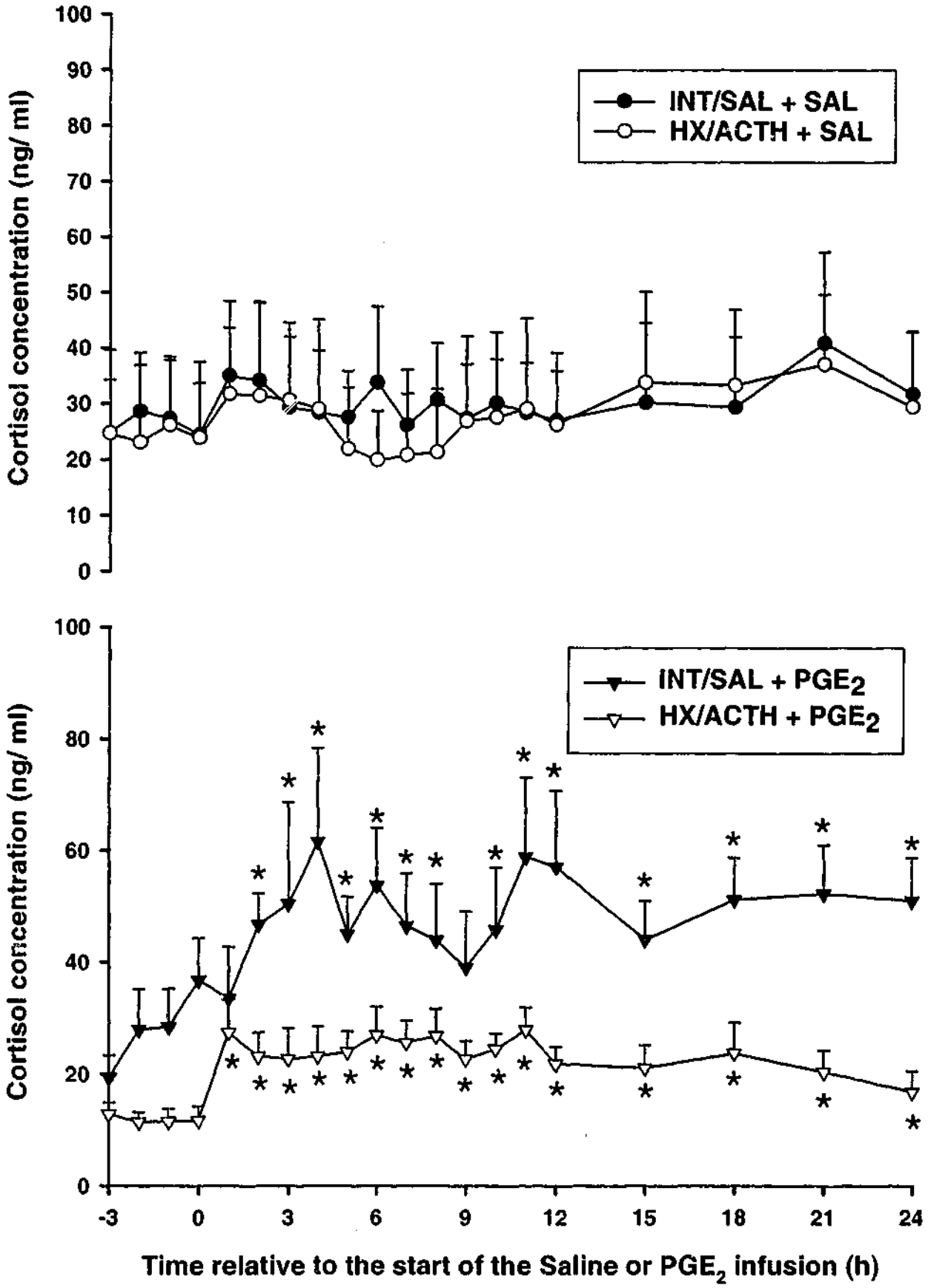

Figure 4-7 Mean cortisol concentrations of each of the 4 groups that received either a 24 hour Saline (Top Panel) or PGE_2 (Bottom Panel) infusion. Cortisol concentrations were significantly elevated in both groups that received a PGE_2 infusion.
* indicates significant differences from basal samples. Data is presented as the mean ± s.e.m.

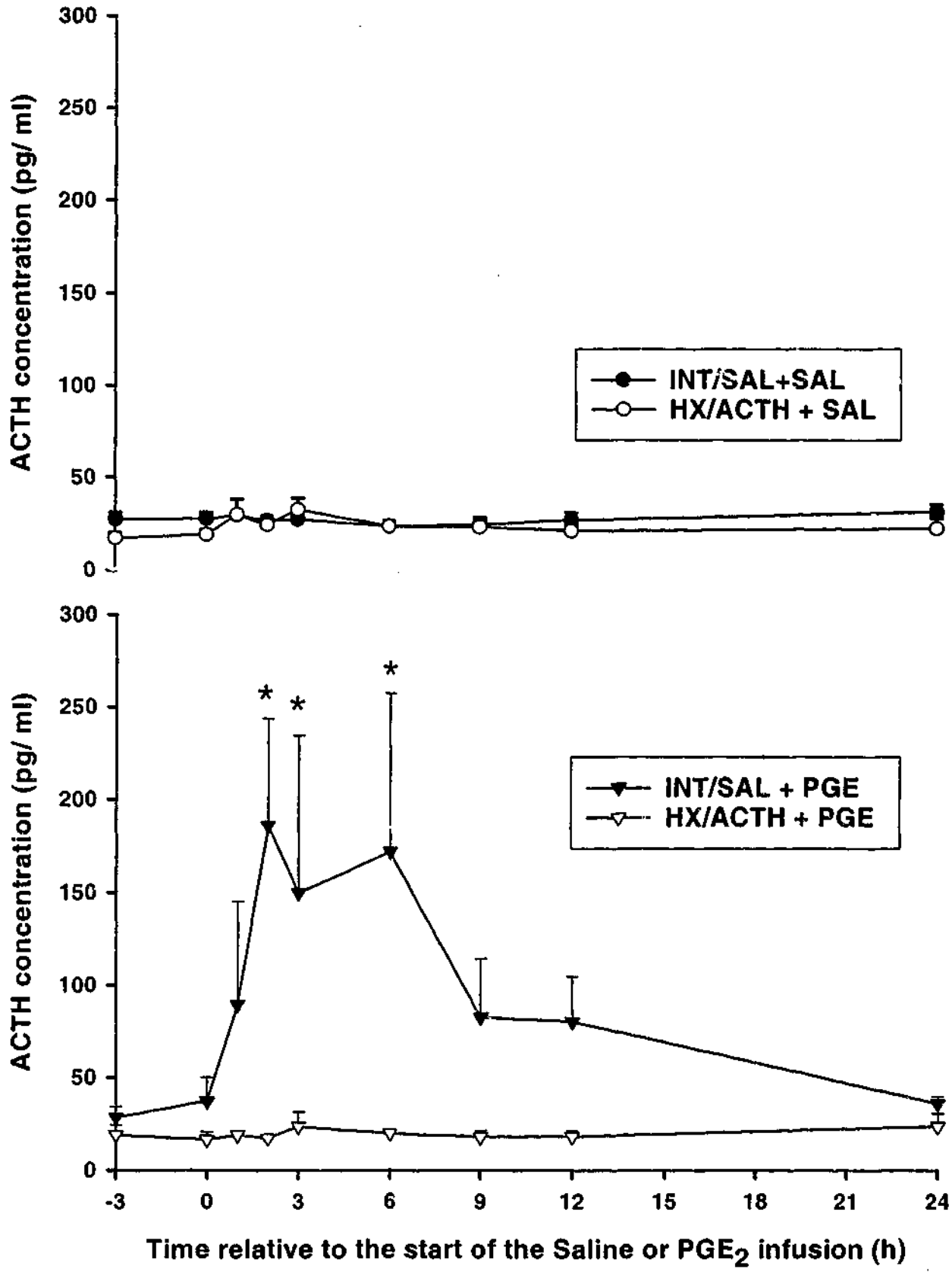

Figure 4-8 Mean ACTH concentration achieved for each experimental group during either a 24 hour infusion of Saline (Top Panel) or PGE_2 (Bottom Panel). There was no difference in the concentration of ACTH between the INT/SAL + SAL or the two HX/ACTH groups. Only the INT/SAL group that received a PGE_2 infusion had a significant increase in ACTH concentrations in response to this challenge.

* indicates significant differences from basal samples. Data is presented as the mean ± s.e.m.

4.4. DISCUSSION

In this chapter, it has been demonstrated that a 24 hour infusion of PGE_2 that significantly increases plasma PGE_2 concentrations, is capable of increasing both plasma ACTH and cortisol concentrations in an intact fetus. The HX fetus that has received a continuous, low dose infusion of ACTH, to maintain the patency of the fetal adrenals, is also able to secrete cortisol in response to an infusion of PGE_2 but unlike intact fetuses, the HX/ACTH fetus had a significant increase in cortisol, without a corresponding increase in ACTH. The implications of these findings and the results of the hypoglycemia challenge are considered in this discussion.

4.4.1. Basal Hormone Concentrations, Body and Organ Weights

There was no difference in the ACTH concentrations between the INT/SAL and HX/ACTH groups, which may suggest that the infusion of ACTH into the HX animals returned ACTH concentrations to the normal range. This finding agrees with the findings of Poore *et al.* (1998a) who also infused ACTH into HX fetuses and found no differences in the ACTH concentrations between INT/SAL and HX/ACTH fetuses. When Poore *et al.* (1998a) interrupted the $ACTH_{1-24}$ infusion for 4 hours there was a significant reduction in the plasma ACTH and cortisol concentrations in HX/ACTH fetuses. The recommencement of the ACTH infusion restored plasma ACTH and cortisol concentrations in these fetuses (Poore *et al.*, 1998a).

In this study, cortisol concentrations did not change in the HX/ACTH group. This differed from the INT/SAL group, whose cortisol concentrations displayed the normal increase in cortisol as gestation progresses toward term. In contrast to the present study, Poore *et al.* (1998a) found that plasma cortisol concentration increased in the HX/ACTH group in exactly the same manner as the INT/SAL fetuses. However, the animals in that study were taken to term and the data analysed with hormone concentrations being aligned retrospectively to the day of labour (Poore *et al.*, 1998a).

There was no difference in any of the body weights, adrenal weights or adrenal:body weight ratio suggesting that the HX procedure with ACTH replacement did maintain adrenal gland growth and did not adversely affect the growth and development of the fetus.

This finding did not replicate the findings of Poore *et al.* (1998a) who reported a decrease in the body weight of the HX/ACTH fetuses when compared with the INT/SAL fetuses. Jacobs *et al.* (1994) who also infused ACTH continuously into HX fetuses, observed no differences in the body weight of ACTH infused HX fetuses. Hypophysectomy is associated with growth retardation over longer periods, but this effect was not apparent in the present study after 15 days post-surgery.

4.4.2. Hypoglycemia Challenge

The response of the INT/SAL and HX/ACTH groups to a physiological hypoglycemic stress differed. In response to a hypoglycemia challenge that halved glucose concentrations in both experimental groups of fetuses, neither group had a change in ACTH concentrations. The INT/SAL fetuses however, had a significant increase in plasma cortisol concentrations, but the HX/ACTH group did not and both groups had a significant increase in plasma PGE_2 concentrations in response to this challenge.

There are a number of interesting observations arising from this challenge. There was no change in ACTH concentrations in the INT/SAL fetuses, yet increases in plasma cortisol concentrations were observed. This may suggest that the increase in cortisol may have been driven by an increase in bioactive ACTH, which was not measurable in the ACTH assay. The assay used in this study measures ir-ACTH and not just bioactive ACTH so a small increase in the plasma ACTH concentrations in the INT/SAL group may have been obscured against a high background of non-bioactive ACTH-containing peptides. Alternatively, it is possible that some other factor may have driven the increase in plasma cortisol observed in this group. This factor is unlikely to be PGE_2 however, as although PGE_2 concentrations did increase in this challenge, the increase was small and did not occur until cortisol concentrations were already elevated. In addition, the increase in PGE_2 in the HX/ACTH group did not stimulate a corresponding increase in cortisol concentrations suggesting that the regulation of cortisol may involve additional factors to ACTH and PGE_2.

Previous findings have demonstrated that the plasma PGE_2 concentrations of the fetus are regulated by the nutritional state of the ewe. Fowden *et al.* (1987) showed that fasting the ewe or an intrafetal infusion of insulin caused increases in fetal PGE_2 concentrations.

These increases in PGE_2 levels were closely associated with the fall in plasma glucose, a finding consistent with the results of this study (Fowden *et al.*, 1987). The increase in the cortisol concentration in the INT/SAL group preceded the increase in plasma PGE_2 so it is unlikely that the increase in cortisol drives the increase in PGE_2, as we observed the same increase in PGE_2 in the HX/ACTH fetuses without any increase in cortisol.

Lactate concentrations increased in both the INT/SAL and the HX/ACTH fetuses, however the response differed between the two groups, being more marked in the INT/SAL group. It is unclear whether this increase is causally related to the increase in cortisol also observed in this group, or may be some feedback mechanism to maintain blood homeostasis.

4.4.3. 24 hour Saline or PGE_2 Infusion

This study is the first demonstration that in HX/ACTH fetuses an exogenous PGE_2 infusion can increase plasma cortisol concentrations without a concomitant increase in ACTH. This finding is potentially significant as it raises the possibility that endogenous PGE_2 may modulate the surge in cortisol in late gestation by an action at the adrenal as well as at the pituitary.

The response of the INT/SAL fetuses that received the PGE_2 infusion is consistent with those reported by others, with an increase in both plasma ACTH and cortisol concentrations (Hollingworth *et al.*, 1995; Young *et al.*, 1996a). Our finding supports those of Liggins *et al.* (1982), who also demonstrated in HX fetuses an increase in plasma cortisol concentrations in response to exogenous PGE_2. This result also suggests that PGE_2 can act directly at the fetal adrenal gland, as suggested by the *in vitro* results in chapter three.

It is interesting to compare the level of increase in the concentration of cortisol reported by Liggins *et al.* (1982) and the results of the present study, in light of the background of ACTH that the HX fetuses in this study received. Liggins *et al.* (1982) unfortunately, did not report the ACTH concentrations of the fetuses in their study, so as a result only cortisol concentrations can be compared between studies (See Table 4-2).
Liggins *et al.* (1982) carried out infusions between 120 and 130 days GA, thus the GA at the time of the PGE_2

infusion would have been earlier than the 140 d used in this study. Thus, it could be expected that cortisol concentrations would be somewhat lower than in the present study.

Experimental Conditions		**Liggins *et al.* (1982) Cortisol concentrations (ng/ml)**	**Present Study Cortisol concentrations (ng/ml)**
Basal	**Intact**	6	5 – 12
	HX	6	5 - 12
PGE_2 Infusion (4 μg/min)	**Intact**	14	50
	HX	21	50

Table 4-2 Comparison of the findings of the present study with those reported by Liggins *et al.* (1982).

It is surprising to note that the adrenals of the HX fetuses in the Liggins study did not become completely unresponsive, as Liggins & Kennedy, (1968) and Jacobs *et al.* (1994) demonstrated that fetal HX without ACTH replacement causes atrophy of the adrenal gland. Poore *et al.* (1999) investigated the adrenal responsiveness of HPD fetuses and reported that the adrenals of these fetuses were very unresponsive. Unlike HX fetuses, HPD fetuses still have pituitary-derived ACTH in their circulation, so if HPD fetuses have unresponsive adrenals, it is interesting that the HX fetuses in the study by Liggins *et al.* (1982) could respond to PGE_2. In addition, Young *et al.* (1996b) infused PGE_2 for 2 hours into HPD fetuses and found them to have no increase in cortisol concentrations at 121, 131 or 141 d GA. The adrenals of these HPD fetuses were also relatively unresponsive to a bolus dose of ACTH. However, Liggins *et al.* (1982) reported that the PGE_2 infusion took place 5 days after the HX operation so it is possible that the adrenals may take longer than 5 days to stop responding to exogenous stimuli. Liggins *et al.* (1982) performed the hypophysectomy by a cryosurgical technique and performed tests to ensure hypophysectomy was complete. The authors of that study were satisfied that hypophysectomy was complete if there was no increase in fetal prolactin levels in response to an intravenous injection of thyrotrophin releasing factor and that more than 90% of the pituitary tissue in the pituitary fossa was destroyed on subsequent histological examination.

The cortisol concentrations achieved by the INT/SAL fetuses that received PGE_2 in this study were higher than those achieved by the HX/ACTH group, which can be accounted

for by the increase in ACTH, observed in the INT/SAL fetuses. This may suggest an additive effect of ACTH and PGE_2 in this group.

The infusion of PGE_2 into the fetuses, resulted in plasma concentrations of PGE_2 that were similar to that seen by Young *et al.* (1996b) which is not surprising considering the same dose of PGE_2 was administered in both of these studies. The doses of PGE_2 achieved in this study were supraphysiological. The average concentration of PGE_2 in an intact animal at 140 d GA is approximately 5 nM, and an intact animal in labour has PGE_2 concentrations of approximately 10 - 15 nM (Deayton *et al.*, 1994; Jacobs *et al.*, 1994; McLaren *et al.*, 1996). The concentrations achieved in this study when PGE_2 was infused were approximately 40 – 50 nM. In this study, the INT/SAL fetuses showed a statistically significant difference in PGE_2 concentrations after 2 hours of infusion while the HX/ACTH did not show a significant increase until 3 hours after the commencement of the infusion. This may suggest that there is some mechanism that is driving the increase in PGE_2 concentrations in the INT/SAL fetuses. Hollingworth *et al.* (1995) infused PGE_2 for only 2 hours and reported no change in plasma PGE_2 concentrations. The findings of this study may explain the lack of an increase in plasma PGE_2 concentrations, as concentrations in the present study were not significantly elevated until after 2 hours of the infusion (Hollingworth *et al.*, 1995).

Although cortisol concentrations were significantly elevated in both the hypoglycemia study and the PGE_2 infusion, it would appear that the physiologically generated PGE_2 failed to elicit the cortisol response in the hypoglycemia challenge. The increase in PGE_2 in this challenge was only very small however, and may not have been sufficient to increase plasma cortisol. This suggests that although PGE_2 may play a role in the amplification of cortisol secretion in late gestation, it may not be the only factor that drives cortisol secretion.

In chapter 3, I investigated the role of prostaglandin E_2 actions on the fetal adrenal *in vitro* and found that PGE_2 had the ability to directly stimulate the ovine fetal adrenal in primary culture. In this chapter, the ability of PGE_2 to act directly at the ovine fetal adrenal gland *in vivo* was examined and was determined that PGE_2 has the ability to increase plasma cortisol concentrations without an increase in ACTH concentrations as demonstrated in the

HX/ACTH group. In the next chapter, I will investigate which PGE_2 receptors are present in the fetal adrenal gland and if these receptors are regulated by a PGE_2 infusion.

Chapter 5 OPTIMISATION OF REAL-TIME PCR CONDITIONS FOR GENES ENCODING THE PGE_2 RECEPTORS, ACTH RECEPTOR & KEY STEROIDOGENIC ENZYMES IN THE OVINE FETAL ADRENAL

In the next two chapters I will be discussing the theory, optimisation and results obtained, using real-time PCR for the following genes: the PGE_2 receptors, the ACTH receptor and the steroidogenic enzymes, P450scc, P450c17 and 3βHSD in the fetal adrenal gland. In this chapter, I discuss the theory behind the real-time PCR technique and go through the design of the primers for each of the genes of interest and describe how the conditions for the real-time technique were optimised for each of these primer pairs. In chapter 6, the importance of investigating the expression of these genes in the adrenal gland will be discussed, as will the results of the real-time PCR.

5.1. INTRODUCTION

The quantification of mRNA can be performed using several techniques such as northern blotting, RNA Protection Assays or Real-Time PCR. Real-Time PCR is a sensitive, rapid, accurate and reproducible method for quantifying very small quantities of specific mRNA. The LightCycler Instrument (Roche Molecular Biochemicals, Manhheim, Germany) is a rapid thermal cycler, combined with a micro-volume fluorescence detector. The LightCycler reaction method we chose utilises the double stranded DNA (dsDNA) binding dye, SYBR Green for fluorescence measurement. SYBR Green preferentially binds to dsDNA and emits a fluorescent signal when bound, giving a signal that is

proportional to the amount of DNA present. SYBR Green is also released upon denaturation of the DNA, which also allows melting curves for each of the amplified products to be obtained.

For each PCR cycle, the DNA is denatured and SYBR Green is released. During annealing the PCR primers hybridise and small amounts of dsDNA are formed to which SYBR Green can bind resulting in an increase in the fluorescence intensity. During the elongation phase, the PCR primers are extended and more SYBR Green dye can bind. At the end of this phase all DNA has become double stranded and a maximum amount of dye is bound. The fluorescence is recorded at the end of the elongation phase before denaturation occurs and the cycle is then repeated. The point where the amplification of a PCR product is first detected i.e. when the fluorescence signal first increases over background fluorescence is called the threshold cycle (C_t) and always occurs in the exponential phase of the amplification. A value for C_t is obtained for each sample and can be translated into a quantitative result by comparing to a number of samples of known quantity i.e. a standard curve. Alternatively, a relative quantification method can be used, in which the PCR signal of the target transcript in a treatment group is compared to the signal of an untreated control group

A number of conditions need to be optimised before quantitation of PCR products can be performed such as (i) annealing temperature, (ii) magnesium chloride ($MgCl_2$) concentration, (iii) melting curve analysis, (iv) concentration of standards and (v) the real-time protocol.

i. The optimal annealing temperature is determined for each primer pair by conventional PCR and varies depending on the content of GC base pairs and the primers used.
ii. Magnesium Chloride ($MgCl_2$) concentration affects the *Taq* DNA Polymerase enzyme activity and increases the melting temperature of dsDNA. As the entire LightCycler reaction is dependent on $MgCl_2$ concentration, the optimal $MgCl_2$ concentration for each of the primer pairs was determined.
iii. At the end of the PCR phase of the LightCycler reaction, a melting curve analysis is performed to determine the melting temperature (T_m) of the PCR product. The melting temperature is defined as the temperature where half of the DNA helical

structure is lost (i.e. half the DNA is double stranded and half single stranded) and varies with nucleotide composition, as structures rich in GC base pairs have a higher melting temperature than those rich in AT base pairs. The LightCycler starts at low temperatures and slowly increases the temperature, while measuring the fluorescence in each sample at frequent intervals (one measurement every 0.2 °C). The fluorescence decreases markedly as the fragment is denatured and the T_m of a PCR fragment can be visualised by taking the first negative derivative of the melting curve. Melting curve analysis allows for the identification of non-specific target sequences that can also be formed during PCR. Primer dimers are products of non-specific annealing and primer elongation events, and are produced when there is no DNA template (no reverse transcribed RNA) or DNA template is low. Specific PCR products produce a single, sharply defined melting curve with a narrow peak, while primer dimers and by-products melt at relatively low temperatures and have broader peaks. The melting temperature of PCR by-products can thus be determined and the temperature at which the LightCycler fluorescence is recorded can be elevated above the melting temperature of the by-products, allowing only the recording of the product of interest.

iv. The concentration of standards was determined to reflect the range of the samples to be validated. The samples were required to reflect the standards in both the amplification efficiency and the melting curve analysis to ensure that the correct PCR product was amplified in the unknown samples.

v. The protocol for each of the genes of interest varies with the size of PCR product expected, as larger products require longer elongation times. The number of cycles for each gene also varies as the more template DNA (abundance of gene of interest) the more rapidly the amplification will occur.

In this chapter, I will discuss the optimisation and validation of the LightCycler for the quantitation of mRNA concentrations of the Prostaglandin E_2 receptors, ACTH Receptor, $P450_{scc}$, 3βHSD and $P450_{c17}$ in the ovine fetal adrenal gland.

5.2. MATERIALS AND METHODS

5.2.1. Experimental procedures

5.2.1.1. Total RNA extraction

Total RNA was extracted from fetal adrenal glands obtained from the animals described in chapter 4 (n = 20), using a Qiagen RNeasy Midi Kit (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia). All reagents below are supplied in the RNeasy kit unless otherwise specified. In brief, the left fetal adrenal gland was weighed and homogenised (VitaTurrax T25, Janke and Kunkel IKA Laboratories, 24 000 rpm) in 4.0 ml Buffer RLT. The tissue lysate was then centrifuged for 10 minutes at 5000 g (Beckman Instruments Pty Ltd, Australia) and the supernatant was collected and 70% ethanol (4 ml) added. The ethanol-supernatant mixture was mixed thoroughly and the samples were added to an RNeasy midi column before centrifuging for 5 minutes at 5000 g. The flow-through was discarded and the column washed with 4.0 ml Buffer RW1, before again centrifuging for 5 minutes at 5000 g. The column flow-through was again discarded and the column again washed with Buffer RPE (2.5 ml) before centrifuging for 2 minutes at 5000 g. The Buffer RPE wash was repeated, however the column was centrifuged for 5 minutes at 5000 g to dry the silica-gel membrane. The column was eluted by twice adding RNase-free water (300 µl) directly onto the silica-gel membrane and centrifuging for 3 minutes at 5000 g. The RNA was treated to remove any possible DNA contamination using a DNA-free kit (Ambion Inc., Austin, Texas, USA).

5.2.1.1.1. Quantitation of Total RNA Extracted

To check the quality and quantity of the total RNA samples, spectrophotometry at 260 and 280 nm and electrophoresis of sample (2 µl) on a 1% agarose gel were performed. The quality of the RNA was visually determined on an agarose gel to confirm that the RNA had not degraded and that there was no DNA contamination. A ratio of the 260/280 nm spectrophotometer results indicates the purity of the RNA. The 260 nm wavelength detects nucleic acids, while at 280 nm both nucleic acids and proteins are detected. All RNA samples had a ratio above 1.8, indicating a relatively pure sample.

RNA was normalised at 0.25 µg/ul by both spectrophotometry results and quantitation of 2 µg of RNA on an agarose gel using Quantity One software (Version 4.1.0, Bio-Rad, California, USA). This normalisation step was performed to reduce differences in the amount of RNA that was reverse transcribed. The difference between the concentration of samples was not allowed to vary by more than 10%.

5.2.1.2. Oligonucleotide Primer Design

mRNA sequences were obtained from Genebank (National Library of Medicine, Bethesda, Maryland, USA; (www.ncbi.nlm.gov) for the following sheep genes of interest (accession numbers in brackets)– EP1 (AF035415), EP2 (AF035416), EP3 (AF035417), EP4 (AF035418), MC2R (AF116874), P450scc (D50057), 3β-HSD (human – M38180; bovine – X17614) and P450c17 (AF251388). If multiple isoforms of a gene existed, the primers were designed to bind to all isoforms. Using these sequences multiple primer pairs were generated with the following primer characteristics: length 18 - 25 base pairs long, product size between 90 - 150 base pairs, melting temperature of primers to be between 55 and 80°C and the GC content to be 50 – 70%. Primers were generated using the software "Genefisher" (http://bibiserv.techfak.uni-bielefeld.de/genefisher). Primer pairs were then checked for stability (no hairpins, dimers within and between primer pairs) and entropy using "NetPrimer" (www.premierbiosoft.com). Primer pairs then underwent electronic amplification using "Amplify" (www.wisc.edu/genetics/CATG/amplify/index.html). Finally, primer pairs were screened for the ability to bind exclusively to the gene of interest and not other genes within the sheep (cow or human) using "BLAST" (www.ncbi.nlm.gov). When all these criteria were met, primer pairs were custom made (Sigma-Genosys, Texas, USA). Primer pairs were diluted and combined to make a 50 pmol/µl solution.

5.2.1.3. Reverse Transcription

RNA cannot serve as a template for either conventional or real-time PCR, thus the first step in the amplification process is to reverse transcribe the RNA template into complementary DNA (cDNA). Reverse transcription (RT) is performed using RNA-dependent DNA polymerases (reverse transcriptase enzymes) and can be primed using specific, random or oligo-dT primers. Oligo-dT primers are oligonucleotides that

bind the endogenous poly $(A)^+$ tail at the 3' end of all mammalian RNA, while random hexanucleotides can randomly bind to any complementary mRNA site. Oligo-dT and random primers maximise the number of RNA molecules reverse transcribed, therefore allowing numerous genes of interest to be analysed from the one RT reaction. Specific primers only reverse transcribe the RNA of interest.

For these studies, total RNA (2 μg; 20 μl final reaction volume) was reverse transcribed using oligo-dT primers (Oligo $(dT)_{15}$, Roche Molecular Biochemicals, Mannheim, Germany) in a PCR Express Machine (Thermo Hybaid, Middlesex, United Kingdom). Synthesis of cDNA was performed using the Expand Reverse Transcriptase Enzyme (Roche Molecular Biochemicals, Mannheim, Germany) as described by the manufacturer. Expand Reverse transcriptase is a genetically engineered version of the Moloney Murine Leukemia Virus reverse transcriptase. Briefly, 1 μl of RNase Inhibitor (protects RNA from degradation during the RT reaction by inhibiting RNases; Roche Molecular Biochemicals, Mannheim, Germany), 2 μg RNA and 0.125 μg Oligo dT were aliquoted into a 0.2 ml nuclease-free PCR tube (Axygen Scientific Inc, California, USA) and made up to a total volume of 12 μl with sterile DEPC-treated water. In the PCR apparatus the samples were heated to 70°C for 10 minutes, before being chilled on ice. Eight μl of the following master mix was then added (4 μl Expand 5x Buffer, 2 μl 0.1M DTT, 1 μl 10 mM dNTP mix (10 mM each dATP, dTTP, dGTP and dCTP). The samples were then returned to the PCR machine for 2 minutes at 42 °C, before again being placed on ice where 1 μl of the Expand reverse transcriptase enzyme was added to each tube. The contents were mixed and incubated in the PCR machine for 50 minutes at 42 °C, followed by 15 minutes at 70 °C, when the cDNA was removed and frozen at –20°C until required.

5.2.1.4. Conventional Polymerase Chain Reaction

5.2.1.4.1. Temperature Optimisation

To determine the optimum temperature for each of the primer pairs, conventional PCR was employed. The DNA Polymerase, *Taq* from the thermophilic eubacterium *Thermus aquaticus BM* was used as it maximises yield, is a highly processive 5' – 3' DNA polymerase and lacks exonuclease activity. The PCR reaction was performed using a PCR Express Machine containing a temperature gradient tube block (Thermo Hybaid, Middlesex, United Kingdom) in sterile 0.5 ml PCR grade tubes (ABgene, Surrey, United

Kingdom). To each tube the following was added; 5 µl of 10 x *Taq* Polymerase Buffer, 0.3 µl *Taq* enzyme, 10 µl of 1.25 mM dNTP's, 50 pmole of specific forward and reverse primer mix, 1 or 2 µl of DNA template (RT) and the volume was made up to 50 µl with sterile H_2O.

The mixture was heated to 95°C for 5 minutes, and then the target DNA was amplified for 40 cycles (30 seconds at 95°C to denature, 30 seconds of different annealing temperatures (47 – 61 °C), followed by 30 seconds at 72°C to allow for extension of the PCR product). At the end of 40 cycles the PCR reaction was heated to 72°C for 7 minutes and then chilled to 4 °C. The contents of the PCR tubes were run on a 1.5% agarose gel with a 1 kB DNA ladder to check for the presence of PCR product, and to determine the optimal temperature for each of the PCR products. If only a small amount of product was recovered annealing and extension times were extended to 1 minute. Once optimal temperatures and PCR conditions had been established for each of the different genes of interest, a PCR reaction was performed to amplify DNA, which was subsequently cut out of the agarose gel, purified (See Section 5.2.1.4.2), sequenced (See Section 5.2.1.4.3) and used as standards (See Section 5.2.1.5.5) for real-time PCR.

5.2.1.4.2. Purification of DNA Fragments from Agarose Gels

PCR products were extracted from agarose gels using a QIAEX II Gel Extraction Kit (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia). This procedure dissolves the agarose gel, and absorbs nucleic acids onto Qiaex II silica-gel particles in the presence of salt, which can then be eluted by removing the salt. The procedure involves excising the appropriate DNA band from the agarose gel, weighing the fragment and adding dissolving Buffer QX1 (3 x the volume of the agarose fragment, supplied in kit), and 30 µl Qiaex II (supplied). The mixture is thenincubated at 50°C for 10 minutes to melt the agarose, with mixing every two minutes to keep the Qiaex particles in suspension. The solution was centrifuged for 30 seconds at 9000 rpm before the supernatant was removed. The pellet was resuspended in Buffer QX1 (500 µl) and again centrifuged for 30 seconds at 9000 rpm. The pellet was then washed in Buffer PE (500 µl; supplied) and centrifuged for 30 seconds at 9000 rpm twice before the pellet was dried for 10 minutes at 37 °C. The DNA was eluted from the Qiaex particles by adding 20 µl sterile H_2O, incubating at room temperature for 5 minutes before centrifuging and transferring the supernatant containing the purified DNA product

into a sterile tube. An additional elution step was performed by adding 10 μl sterile H_2O, incubating at room temperature for 5 minutes before centrifuging (30 seconds at 9000 rpm) and adding the supernatant to the already eluted DNA. The purified DNA was then either diluted for sequencing (Section 5.2.1.4.3), used for the creation of real-time PCR standards (Section 5.2.1.5.5) or run on an agarose gel to confirm size of product.

5.2.1.4.3. PCR Product Sequencing

The purified DNA PCR product (30 ng) was sequenced with 3.2 pmole of either the forward or reverse primer solution, to confirm that the product obtained by the PCR reaction was the desired product. Mrs. Vivian Vasic at the Wellcome Trust Sequencing Centre (a joint facility of Prince Henry's Institute of Medical Research and The Monash Institute of Reproduction and Development) performed the sequencing, using the ABI Prism™ 377 DNA Sequencer (Perkin-Elmer Biosystems, Foster City, California, USA). After sequencing, the sequenced PCR product for each of the forward and reverse primers was confirmed as the gene of interest using "BLAST" (www.ncbi.nlm.gov). It was also checked that if multiple isoforms of any of the genes existed, that the sequenced result bound to all isoforms.

5.2.1.5. Real-Time PCR

5.2.1.5.1. Magnesium Chloride Optimisation

$MgCl_2$ concentrations between 1 and 5 mM were titrated using real-time PCR and the optimum concentration was determined by running on a 1.5% agarose gel. This was necessary as high concentrations of $MgCl_2$ sometimes produce more than one PCR product. The optimal $MgCl_2$ concentration for each of the genes of interest is summarised in Table 5-2.

5.2.1.5.2. Melting Curve Analysis

Primer dimers were present in real-time PCR reactions for EP1 and EP4. The temperature at which the fluorescence was measured for each of the genes of interest is summarised in Table 5-1.

5.2.1.5.3. Standard Curves and Control Samples

Standards for each gene of interest were obtained from purified conventional PCR products (See Section 5.2.1.4.2). The DNA template (RT) added to each of the unknown sample tubes was diluted to obtain similar amplification efficiency (as indicated by parallelism between standard and sample curve), as the standards and ranged from a 1:10 to 1:40 dilution. A blank control (no DNA template added) was also included in each PCR run which assessed the sensitivity of each PCR reaction and also showed the presence of non-specific by-products. The optimal concentration for the DNA template for each of the genes of interest is summarised in Table 5-2.

5.2.1.5.4. Real-Time PCR Protocol Optimisation

The real-time PCR protocol consists of three stages, (i) 95°C for 10 minutes to allow initial denaturing of template DNA and activation of DNA Polymerase, (ii) Amplification of target DNA for set number of cycles and (iii) Melting curve analysis. The optimal PCR conditions for each of the genes of interest is summarised in Table 5-1.

5.2.1.5.5. Negative Control Reaction

PCR Negative controls were used to monitor for DNA contamination in the extracted RNA, as DNA can interfere with the amplification. An RT reaction was performed in which four samples had no reverse transcriptase enzyme added to the reaction. These samples were then amplified using real-time PCR to confirm that no DNA was present in the samples. The real-time results of this reaction are shown in Figure 5-35.

5.3. RESULTS

The optimisation of each of the primer pairs for the genes of interest is described below.

5.3.1. EP1 Optimisation

5.3.1.1. EP1 Temperature Optimisation

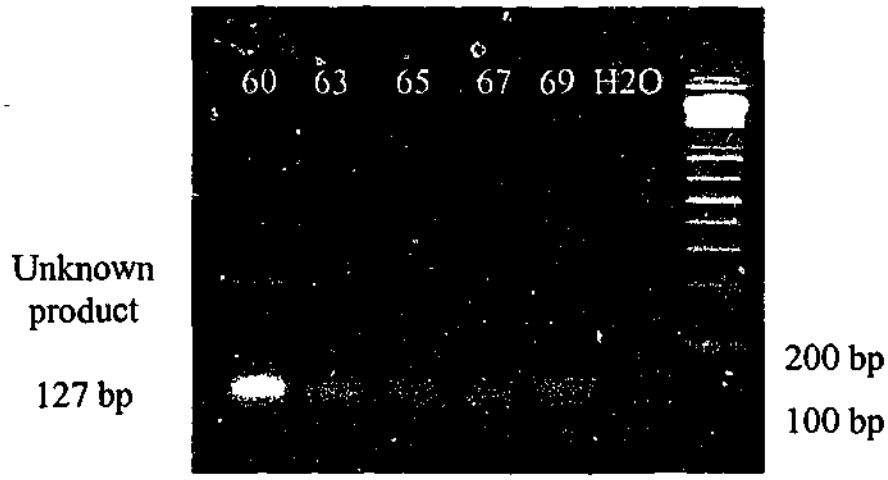

Figure 5-1 Effect of different annealing temperatures (60 – 68 °C) on EP1 amplification. The EP1 product was identified at 127 bp as expected. Products were also found above and below the desired product, which are unwanted amplicons. The optimum PCR temperature chosen was 60°C.

5.3.1.2. Optimal PCR Conditions

i. Magnesium Gradient – To determine the optimal magnesium concentration for the EP1 real-time PCR reaction, a serial magnesium gradient was performed and run on an agarose gel to determine which concentration allowed the best amplification. See Figure 5-2.
ii. Melting Temperature – Figure 5-3 shows the melting temperature of both the H_2O and standards.
iii. RT dilution – Figure 5-4 shows the RT dilution that amplifies at the same efficiency as the standard samples.

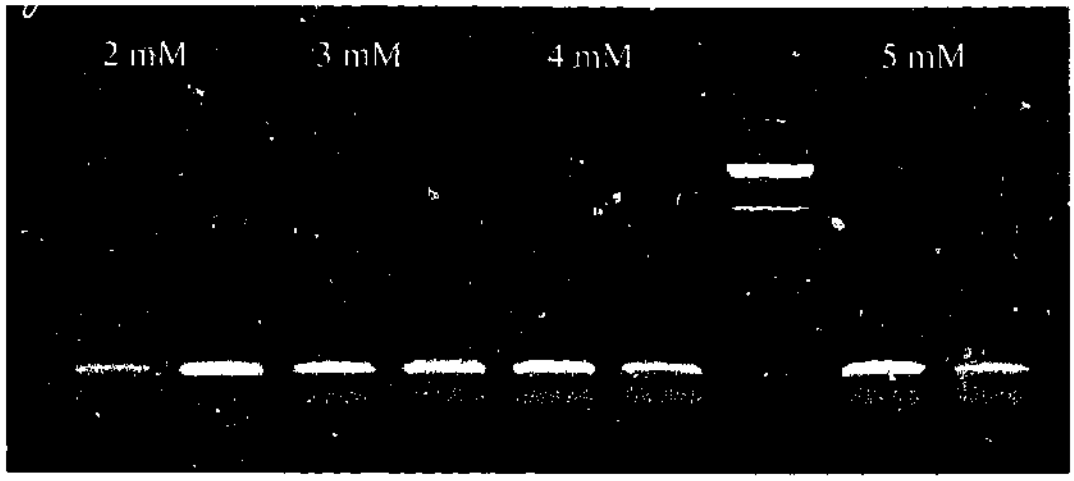

Figure 5-2 Effect of magnesium on the amplification of EP1. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for EP1 was 3 mM.

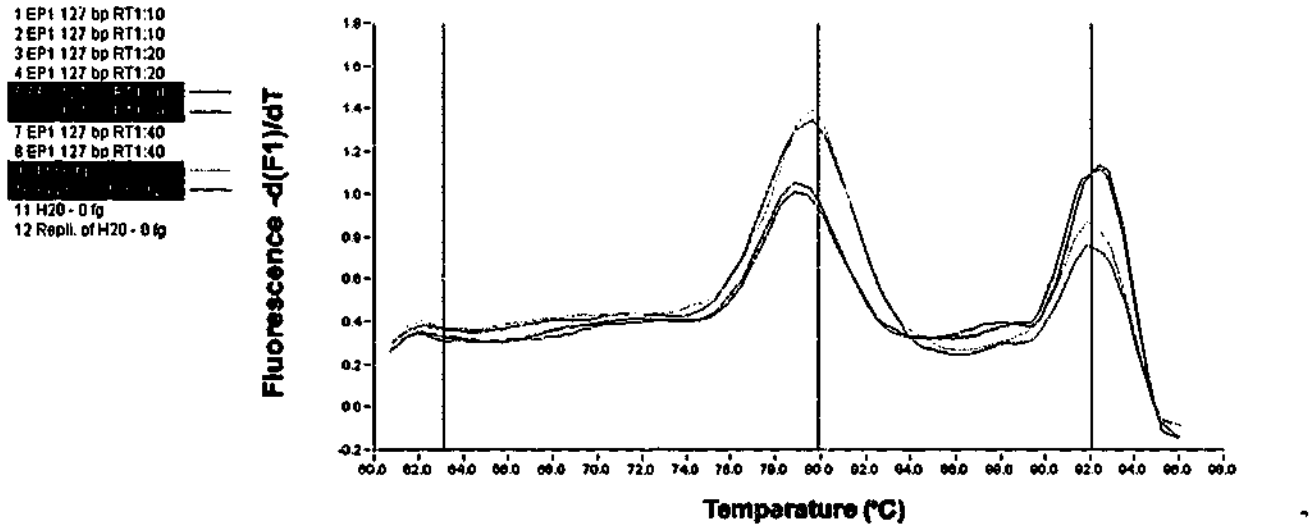

Figure 5-3 Melting curve analysis of the EP1 amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 86 °C.

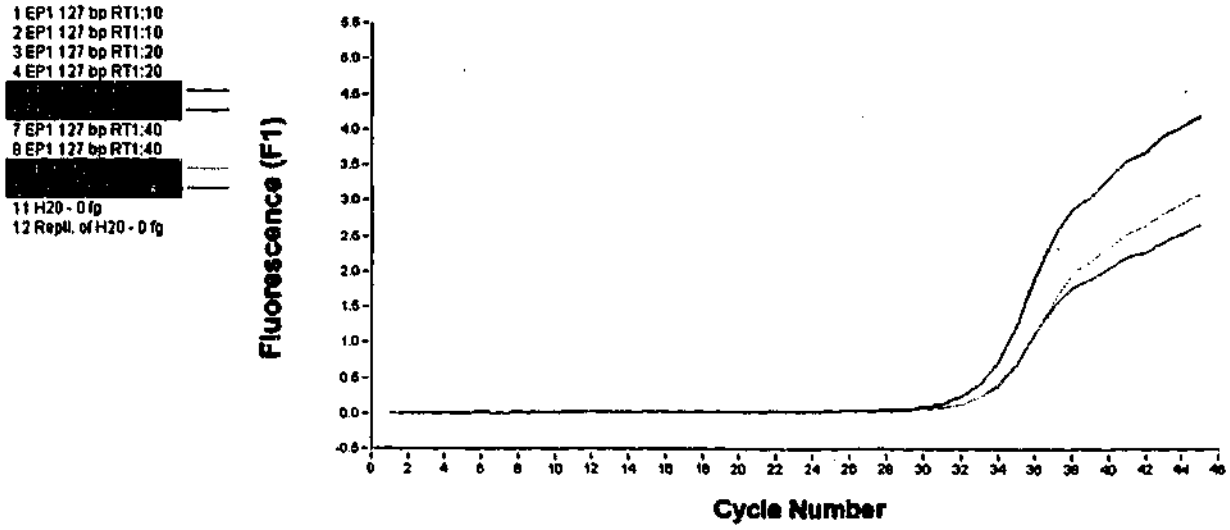

Figure 5-4 Effect of dilution of template DNA on the amplification of EP1. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the EP1 standards was a 1:30 dilution.

5.3.2. EP2 Optimisation

5.3.2.1. EP2 Temperature Optimisation

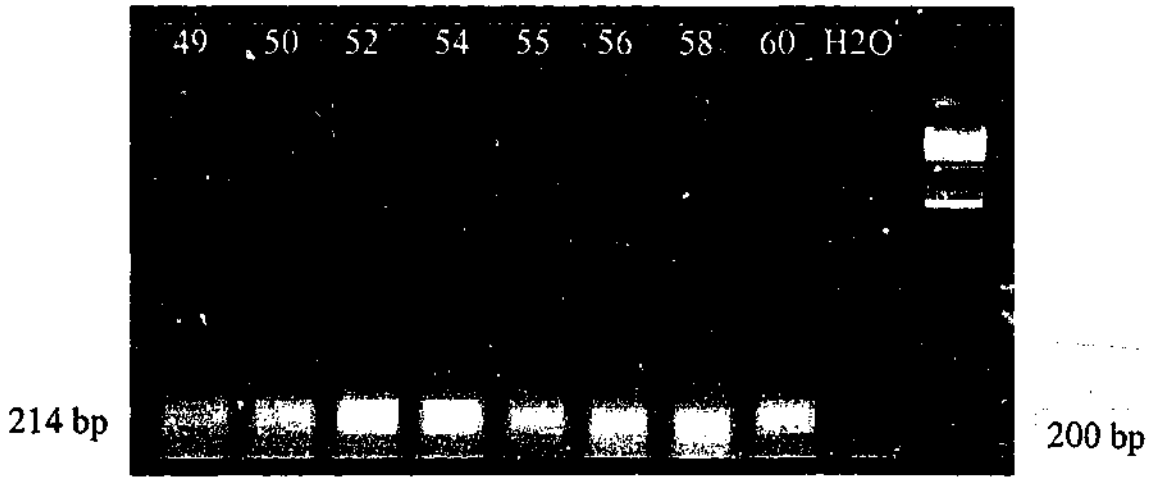

Figure 5-5 Effect of different annealing temperatures (49 – 60 °C) on EP2 amplification. The EP2 product was identified at 214 bp as expected. Products were also found below the desired product, which are unwanted amplicons. The optimum PCR temperature chosen was 60°C.

5.3.2.2. EP2 Sequencing

The standards from the PCR reaction were sequenced with either the forward or reverse primer. The sequence obtained corresponds with known sequences for the EP2 gene in a number of species.

5.3.2.3. EP2 Optimal PCR Conditions

i. Magnesium Gradient – To determine the optimal magnesium concentration for the EP2 real time PCR reaction, a serial magnesium gradient was performed and run on an agarose gel to determine which concentration allowed the best amplification. See figure 5-6 for the gel of the EP2 magnesium gradient.
ii. Melting Temperature – Figure 5-7 shows the melting temperature of both the H_2O and standards.
iii. RT dilution – Figure 5-8 shows the RT dilution that amplifies at the same efficiency as the standard samples.

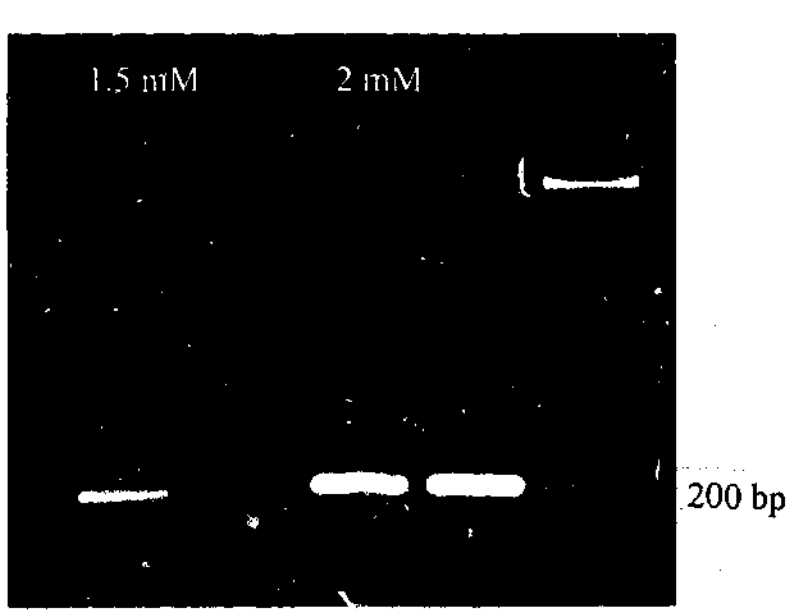

Figure 5-6 Effect of magnesium on the amplification of EP2. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for EP2 was 1.5 mM, as 2 mM produced an unwanted amplicon.

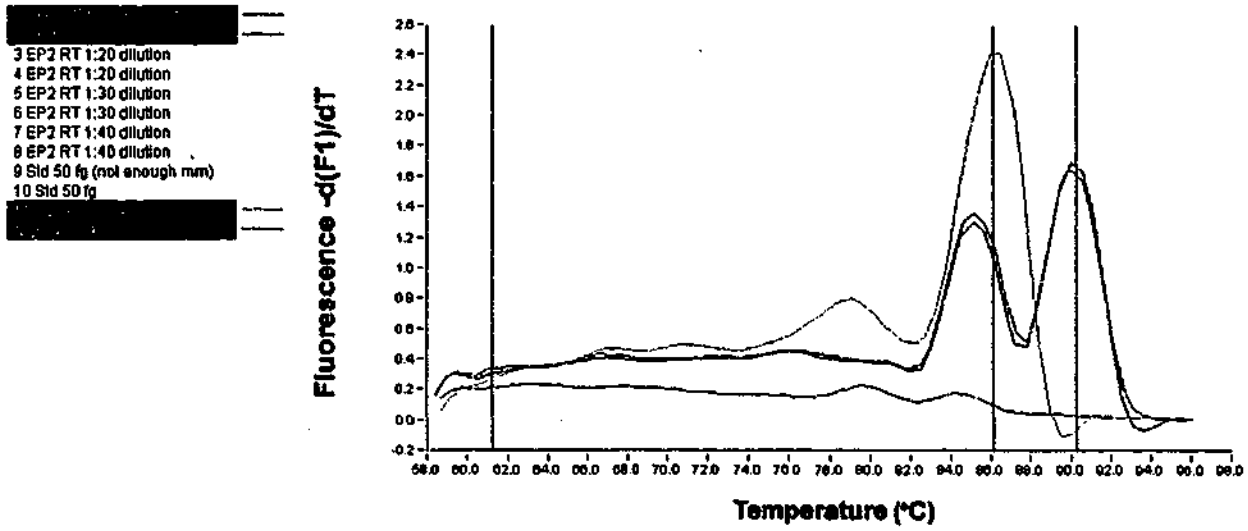

Figure 5-7 Melting curve analysis of the EP2 amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 84 °C.

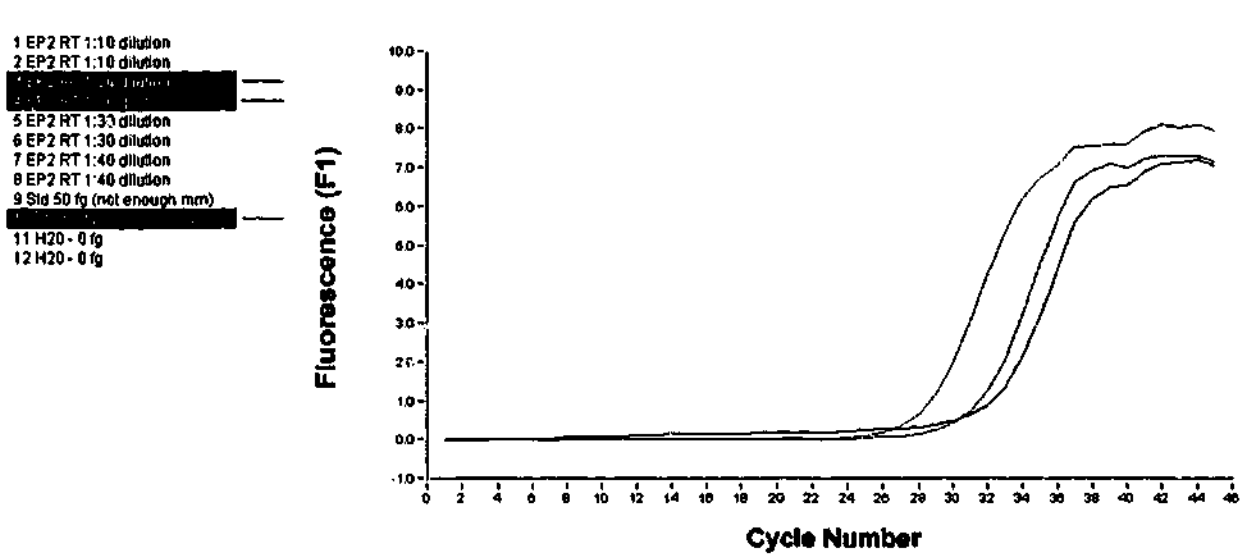

Figure 5-8 Effect of dilution of template DNA on the amplification of EP2. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the EP2 standards was a 1:20 dilution.

5.3.3. EP3 Optimisation

EP3 is known to have at least 4 isoforms in the sheep. The primers designed bound to the conserved region of EP3 and therefore bound to all of the isoforms. This was confirmed at the time of sequencing as all isoforms were retrieved when the sequence if the product was run in BLAST.

5.3.3.1. EP3 Temperature Optimisation

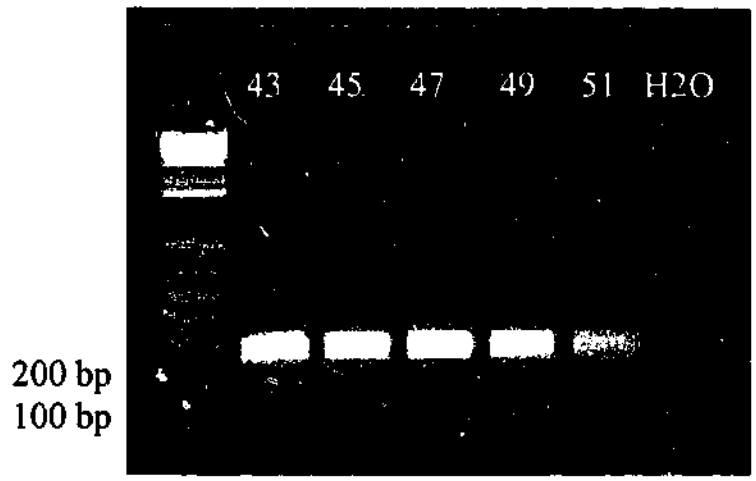

Figure 5.9 Effect of different annealing temperatures (43 – 51 °C) on EP3 amplification. The EP3 product was identified at 264 bp as expected. The optimum PCR temperature chosen was 51 °C.

5.3.3.2. EP3 Optimal PCR Conditions

i. Magnesium Gradient – To determine the optimal magnesium concentration for the EP3 real time PCR reaction, a serial magnesium gradient was performed and run on an agarose gel to determine which concentration allowed the best amplification. See Figure 5-10 for the gel of the EP3 magnesium gradient.
ii. Melting Temperature – Figure 5-11 shows the melting temperature of both the H_2O and standards.
iii. RT dilution – Figure 5-12 shows the RT dilution that amplifies at the same efficiency as the standard samples.

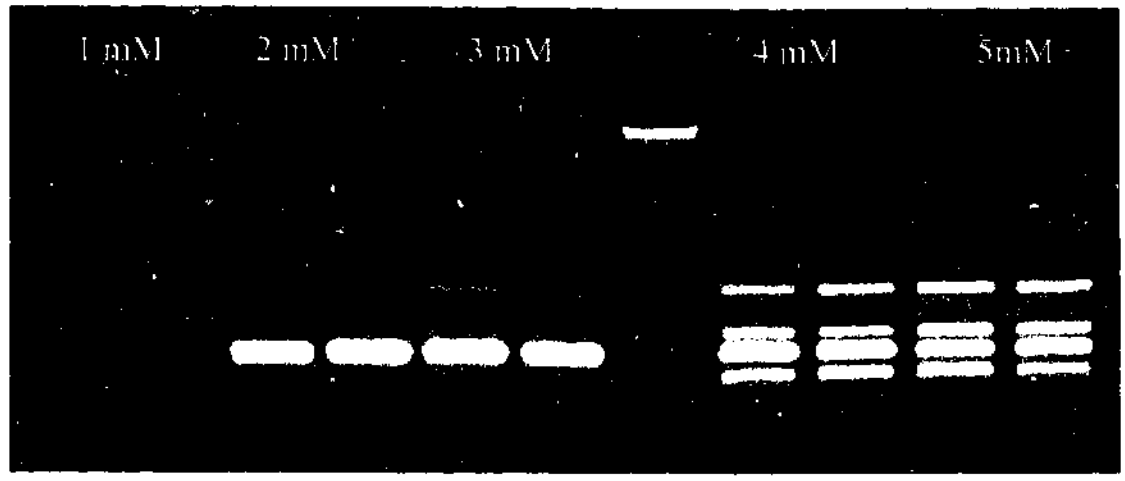

Figure 5-10 Effect of magnesium on the amplification of EP3. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for EP3 was 2 mM as higher concentrations of magnesium produced many unwanted amplicons.

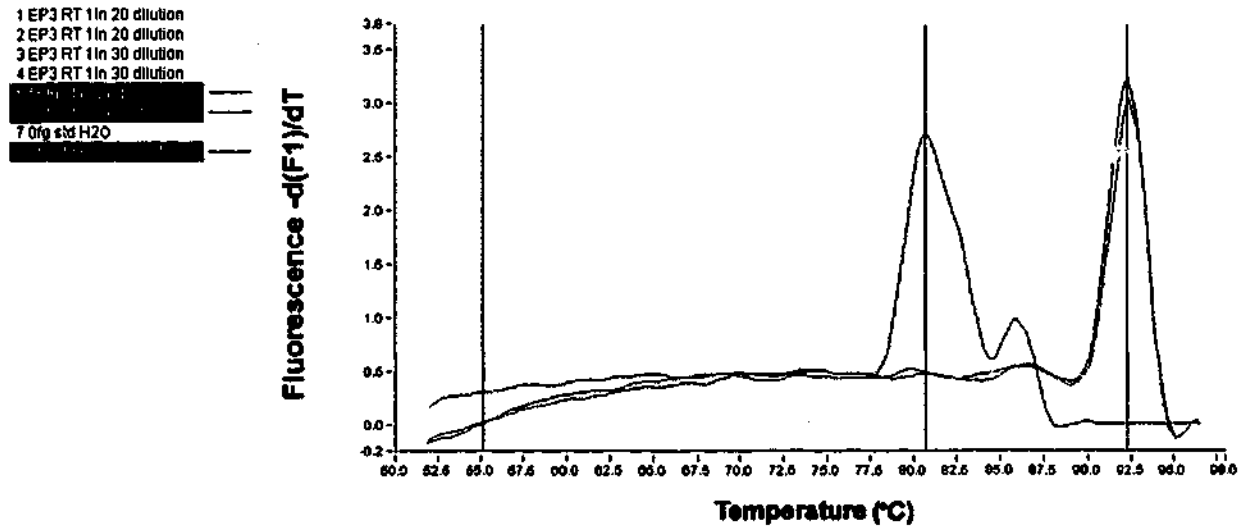

Figure 5-11 Melting curve analysis of the EP3 amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 88 °C.

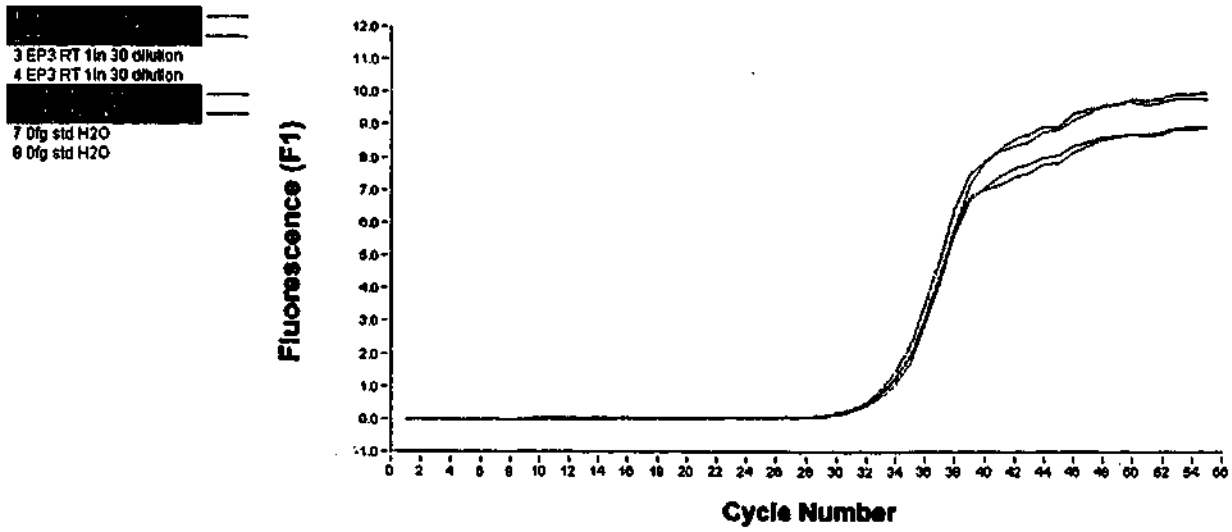

Figure 5-12 Effect of dilution of template DNA on the amplification of EP3. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the EP3 standards was a 1:20 dilution.

5.3.4. EP4 Optimisation

5.3.4.1. EP4 Temperature Optimisation

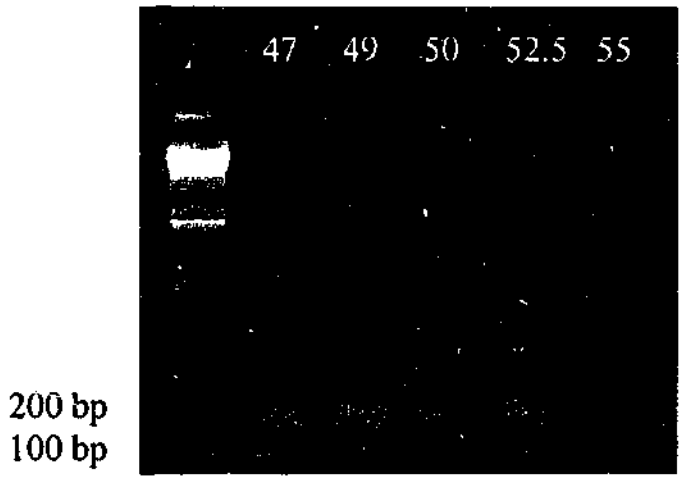

Figure 5-13 Effect of different annealing temperatures (47 – 55 °C) on EP4 amplification. The EP4 product was identified at 178 bp as expected. Products were also found below the desired product, which are unwanted amplicons. The optimum PCR temperature chosen was 55 °C.

5.3.4.2. EP4 Optimal PCR Conditions

i. Magnesium Gradient – To determine the optimal magnesium concentration for the EP4 real time PCR reaction, a serial magnesium gradient was performed and run on

an agarose gel to determine which concentration allowed the best amplification. See Figure 5-14 for the gel of the EP4 magnesium gradient.

ii. Melting Temperature – Figure 5-15 shows the melting temperature of both the H_2O and standards.

iii. RT dilution – Figure 5-16 shows the RT dilution that amplifies at the same efficiency as the standard samples.

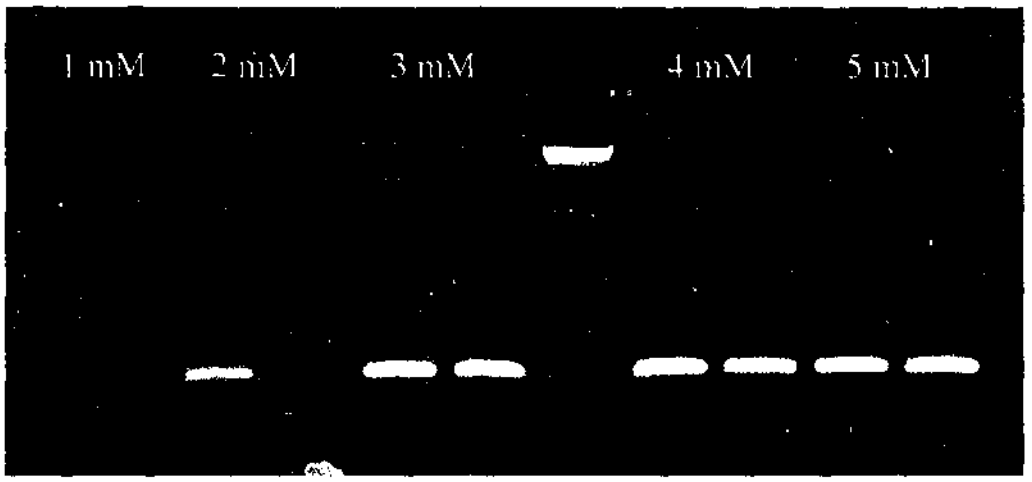

Figure 5-14 Effect of magnesium on the amplification of EP4. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for EP4 was 3 mM.

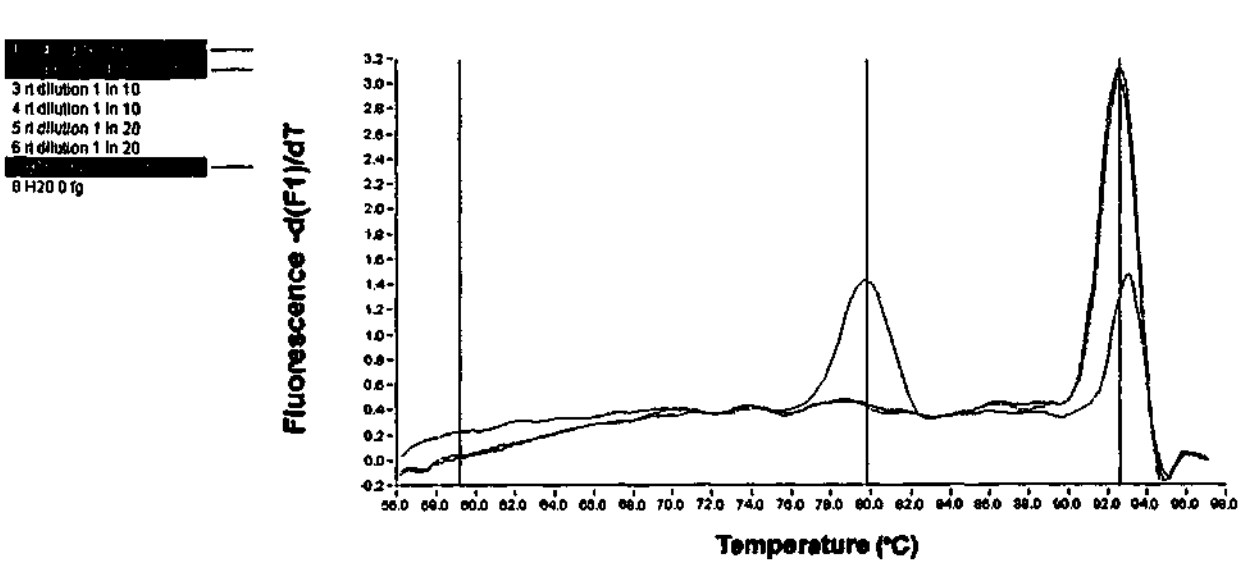

Figure 5-15 Melting curve analysis of the EP4 amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 85 °C.

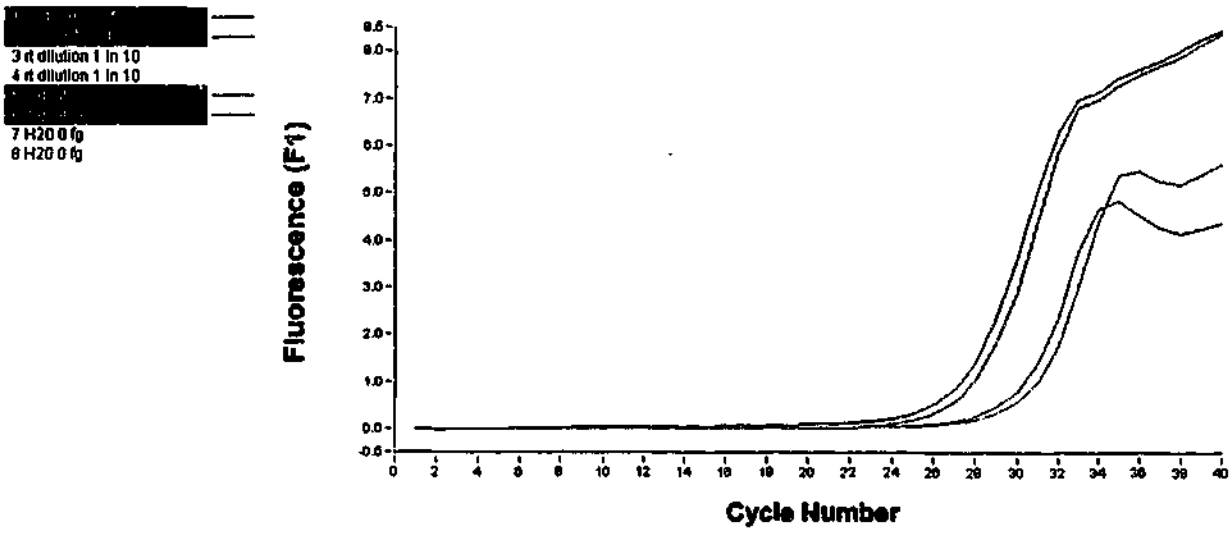

Figure 5-16 Effect of dilution of template DNA on the amplification of EP4. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the EP4 standards was a 1:20 dilution.

5.3.5. *ACTH Receptor Optimisation*

5.3.5.1. *ACTH Receptor Temperature Optimisation*

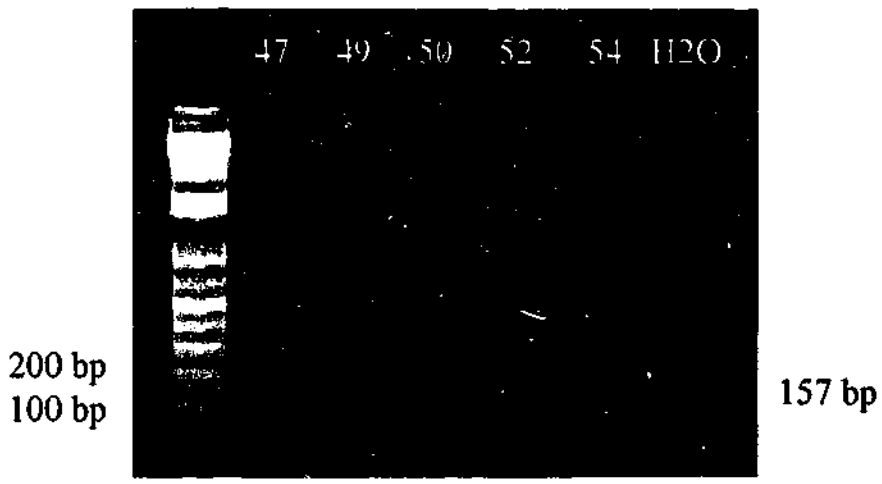

Figure 5-17 Effect of different annealing temperatures (47 – 54 °C) on ACTHR amplification. The ACTHR product was identified at 157 bp as expected. The optimum PCR temperature chosen was 49 °C.

5.3.5.2. *ACTH Receptor Optimal PCR Conditions*

i. Magnesium Gradient – To determine the optimal magnesium concentration for the ACTHR real time PCR reaction, a serial magnesium gradient was performed and

run on an agarose gel to determine which concentration allowed the best amplification. See Figure 5-18 for the gel of the ACTHR magnesium gradient.

ii. Melting Temperature – Figure 5-19 shows the melting temperature of both the H_2O and standards.

iii. RT dilution – Figure 5-20 shows the RT dilution that amplifies at the same efficiency as the standard samples.

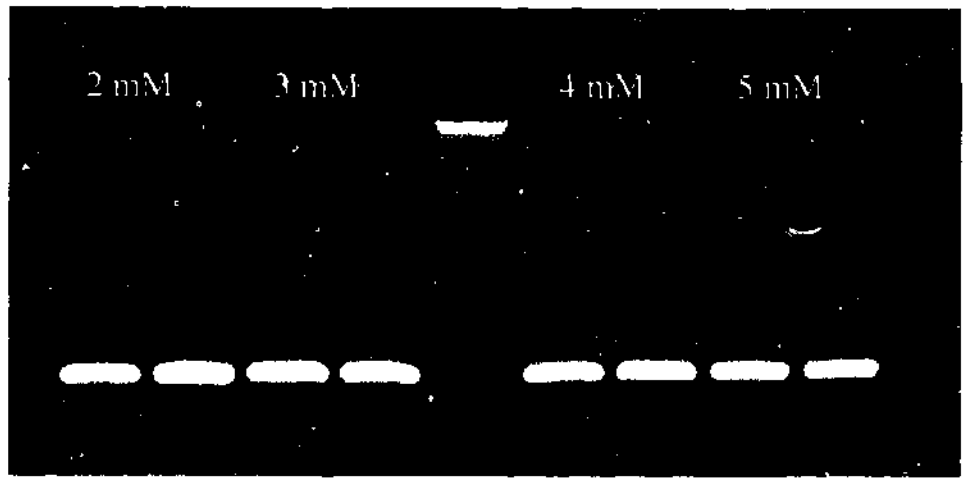

Figure 5-18 Effect of magnesium on the amplification of ACTHR. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for ACTHR was 3 mM.

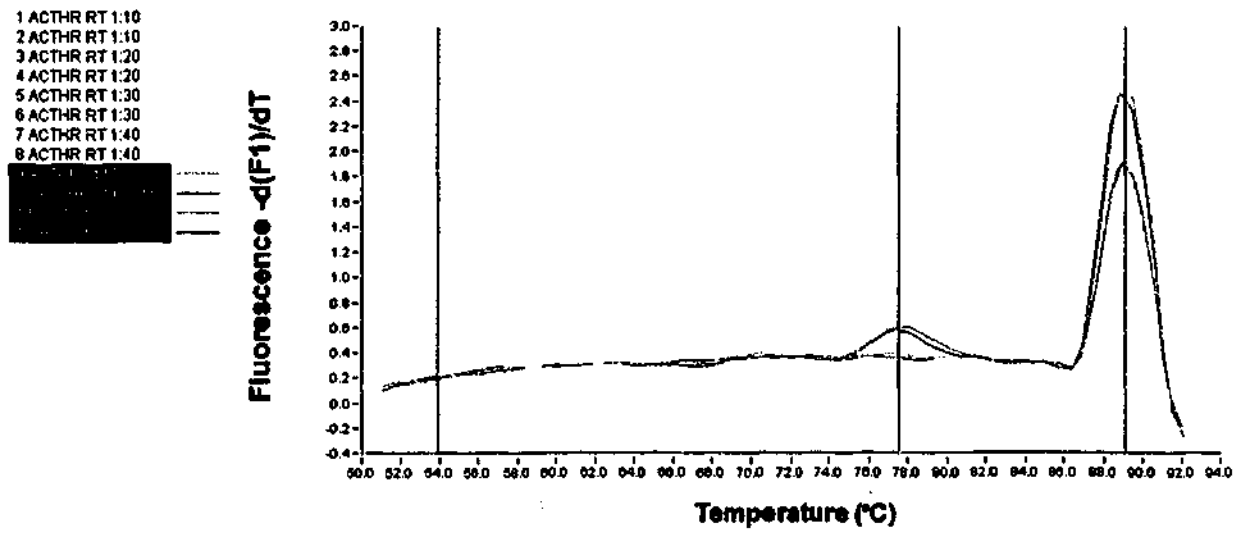

Figure 5-19 Melting curve analysis of the ACTHR amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 85 °C.

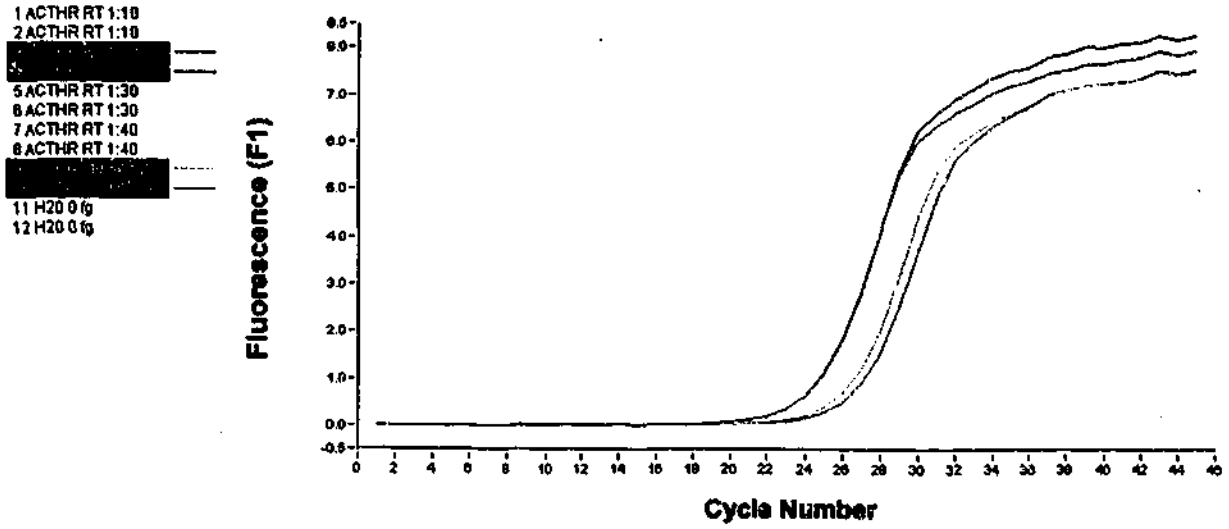

Figure 5-20 Effect of dilution of template DNA on the amplification of ACTHR. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the ACTHR standards was a 1:20 dilution.

5.3.6. *P450scc Optimisation*

5.3.6.1. *P450scc Temperature Optimisation*

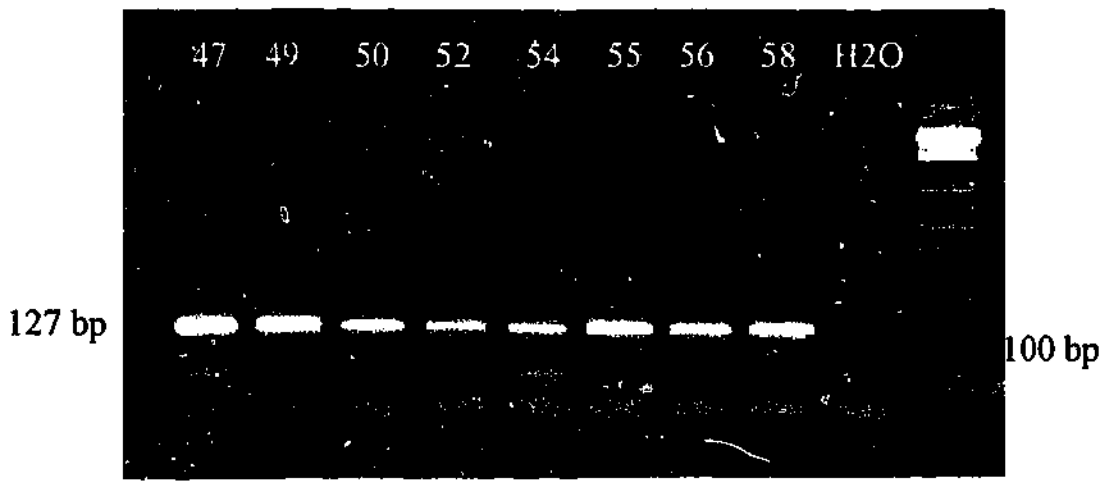

Figure 5-21 Effect of different annealing temperatures (47 – 58 °C) on P450scc amplification. The P450scc product was identified at 127 bp as expected. Products were also found below the desired product, which are unwanted amplicons. The optimum PCR temperature chosen was 52 °C.

5.3.6.2. *P450scc Optimal PCR Conditions*

i. Magnesium Gradient – To determine the optimal magnesium concentration for the P450scc real time PCR reaction, a serial magnesium gradient was performed and

run on an agarose gel to determine which concentration allowed the best amplification. See figure 5-22 for the gel of the P450scc magnesium gradient.

ii. Melting Temperature – Figure 5-23 shows the melting temperature of both the H_2O and standards.

iii. RT dilution – Figure 5-24 shows the RT dilution that amplifies at the same efficiency as the standard samples.

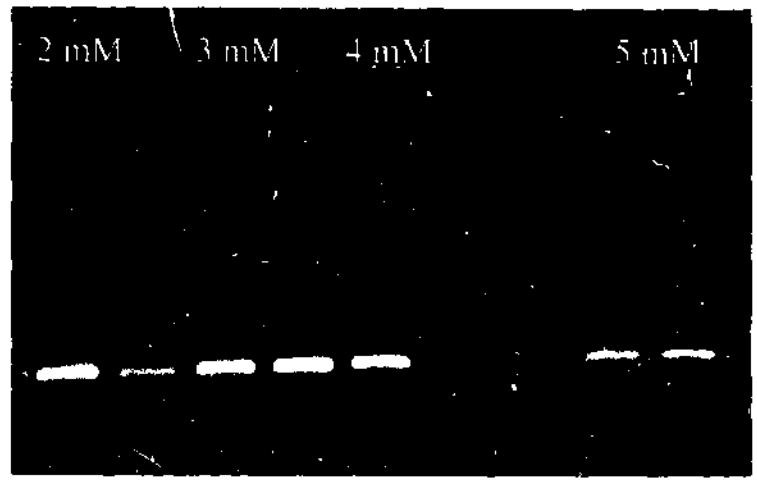

Figure 5-22 Effect of magnesium on the amplification of P450scc. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for P450scc was 3 mM.

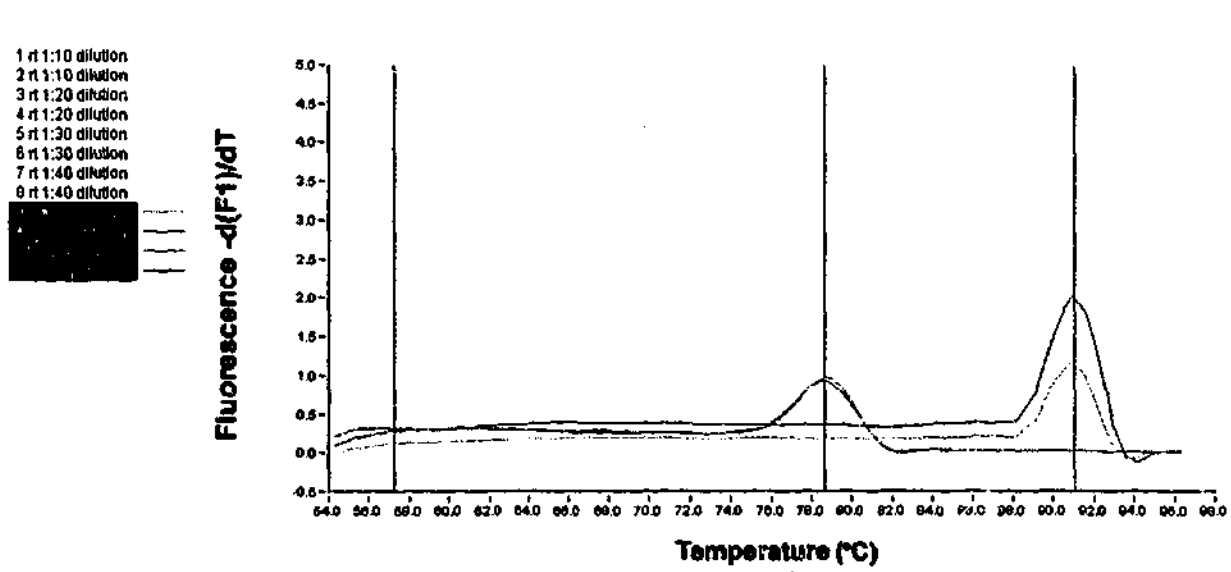

Figure 5-23 Melting curve analysis of the P450scc amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 85 °C.

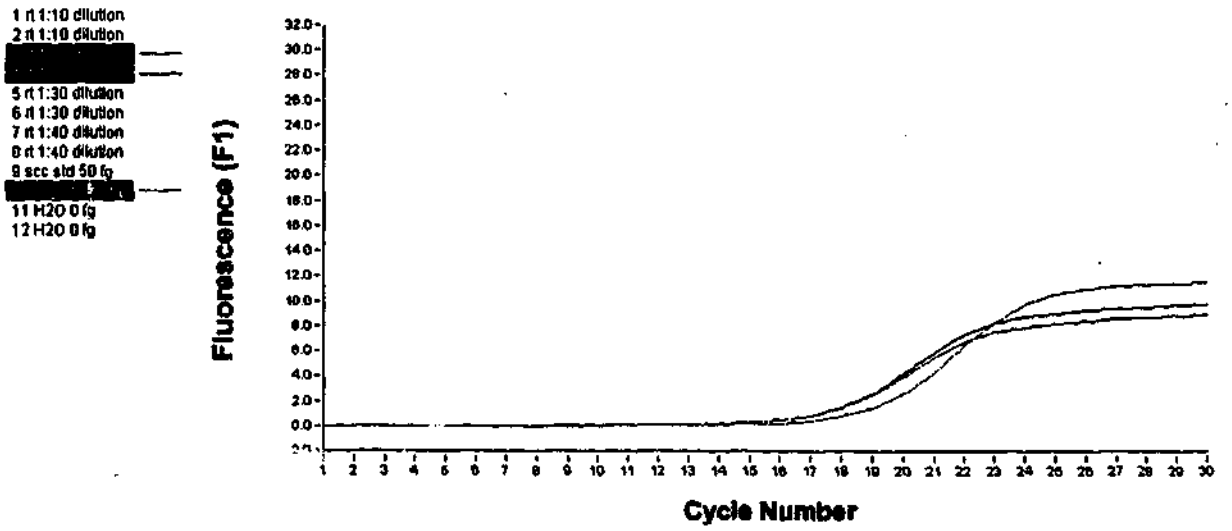

Figure 5-24 Effect of dilution of template DNA on the amplification of P450scc. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the P450scc standards was a 1:20 dilution.

5.3.7. 3βHSD Optimisation

5.3.7.1. 3βHSD Temperature Optimisation

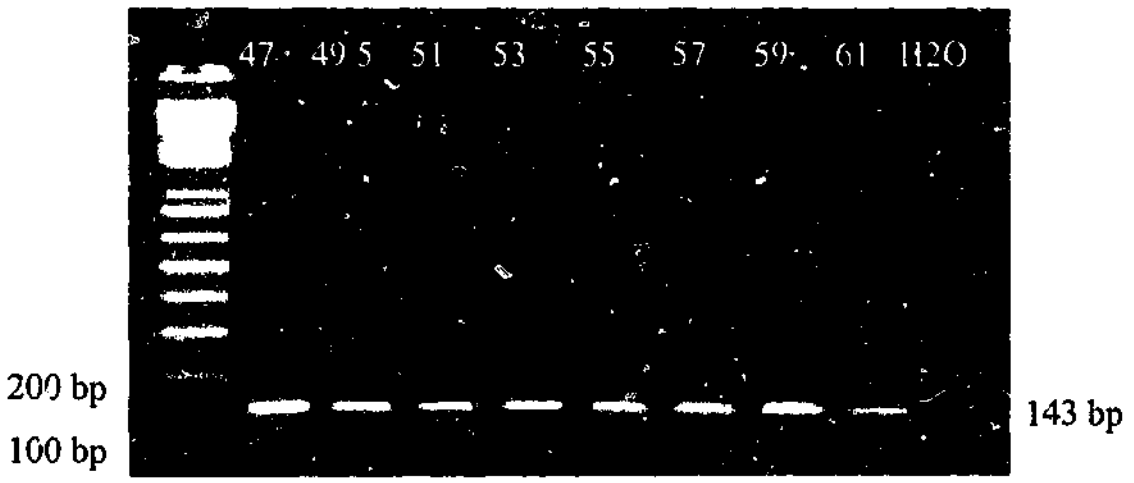

Figure 5-25 Effect of different annealing temperatures (47 – 61 °C) on 3β HSD amplification. The 3βHSD product was identified at 143 bp as expected. The optimum PCR temperature chosen was 57 °C.

5.3.7.2. 3βHSD Optimal PCR Conditions

i. Magnesium Gradient – To determine the optimal magnesium concentration for the 3βHSD real-time PCR reaction, a serial magnesium gradient was performed and

run on an agarose gel to determine which concentration allowed the best amplification. See figure 5-26 for the gel of the 3βHSD magnesium gradient.

ii. Melting Temperature – Figure 5-27 shows the melting temperature of both the H_2O and standards.

iii. RT dilution – Figure 5-28 shows the RT dilution that amplifies at the same efficiency as the standard samples.

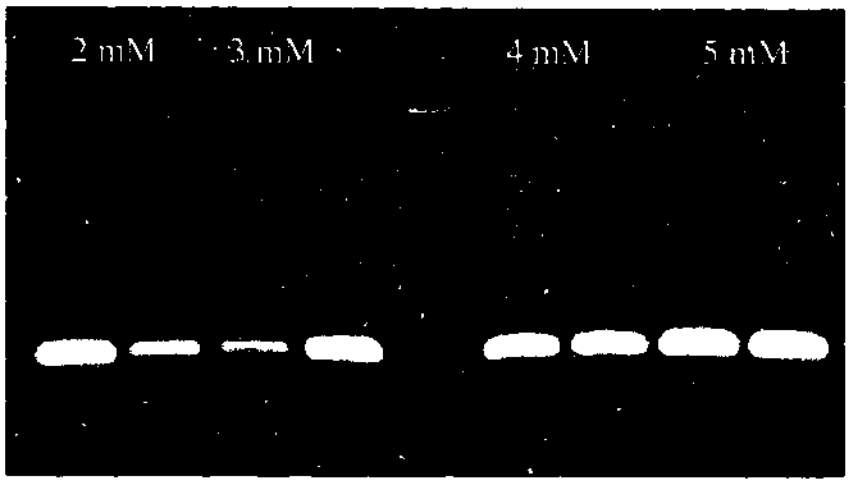

Figure 5-26 Effect of magnesium on the amplification of 3βHSD. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for 3βHSD was 4 mM.

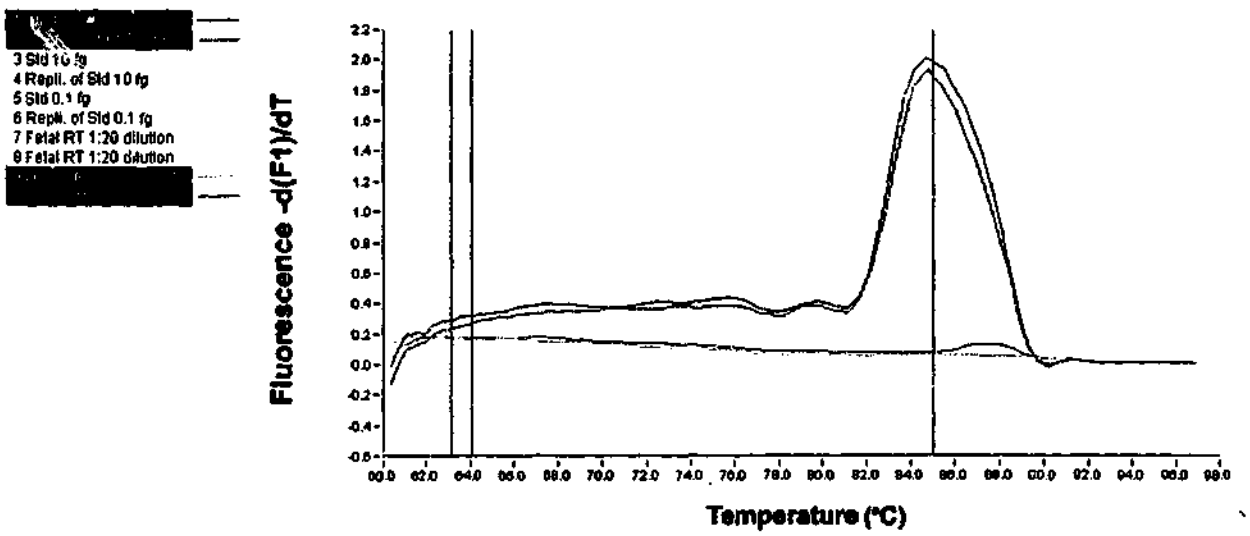

Figure 5-27 Melting curve analysis of the 3βHSD amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 82 °C.

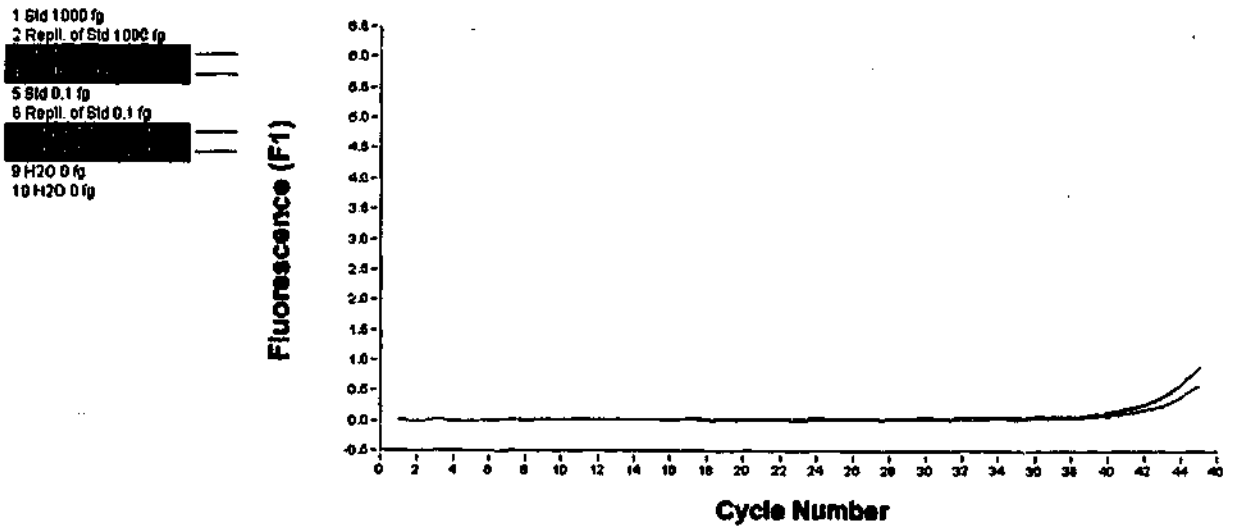

Figure 5-28 Effect of dilution of template DNA on the amplification of 3βHSD. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the 3βHSD standards was a 1:20 dilution.

5.3.8. P450c17 Optimisation

5.3.8.1. P450c17 Temperature Optimisation

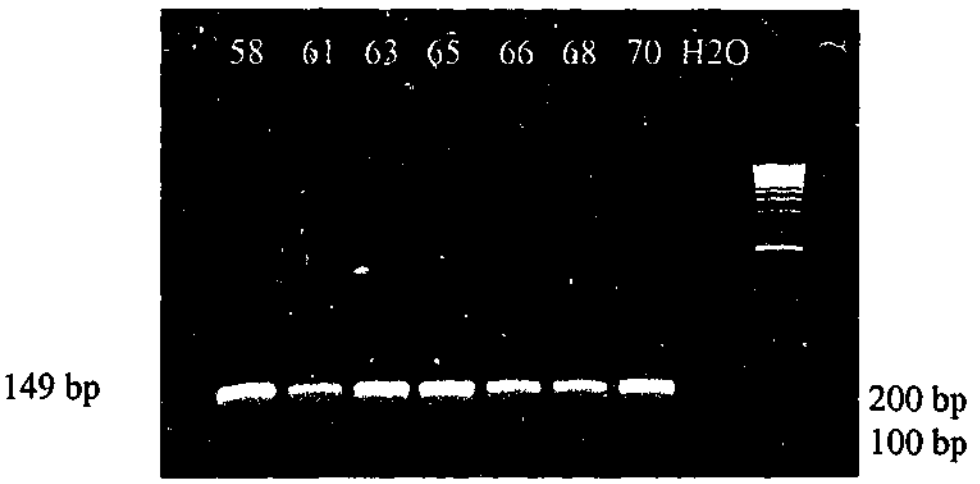

Figure 5-29 Effect of different annealing temperatures (58 – 68 °C) on P450c17 amplification. The P450c17 product was identified at 149 bp as expected. The optimum PCR temperature chosen was 65 °C.

5.3.8.2. P450c17 Optimal PCR Conditions

i. Magnesium Gradient – To determine the optimal magnesium concentration for the P450c17 real time PCR reaction, a serial magnesium gradient was performed and

run on an agarose gel to determine which concentration allowed the best amplification. See Figure 5-30 for the gel of the P450c17 magnesium gradient.

ii. Melting Temperature – Figure 5-31 shows the melting temperature of both the H_2O and standards.

iii. RT dilution – Figure 5-32 shows the RT dilution that amplifies at the same efficiency as the standard samples.

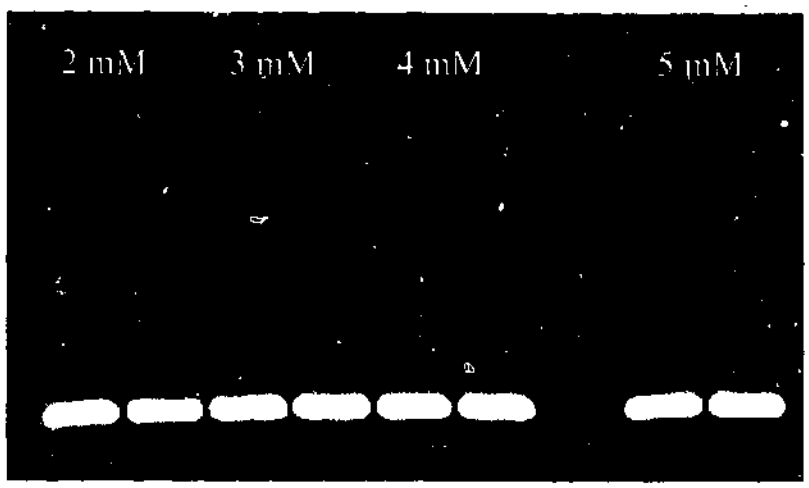

Figure 5-30 Effect of magnesium on the amplification of P450c17. Each magnesium concentration was performed in duplicate (2 lanes each on the gel). The optimum magnesium concentration chosen for P450c17 was 3 mM.

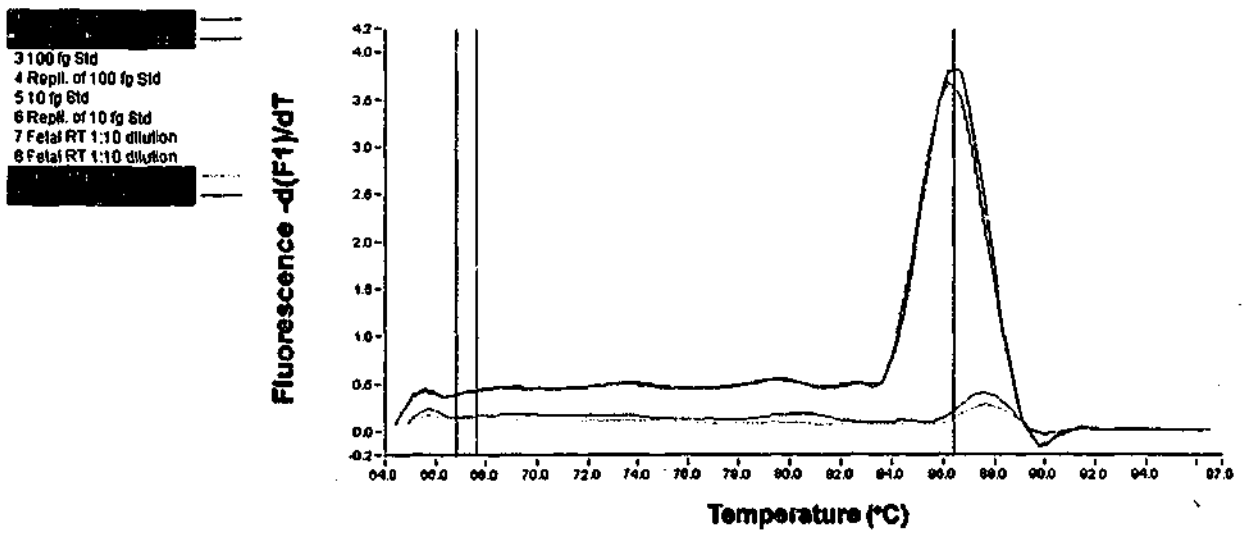

Figure 5-31 Melting curve analysis of the P450c17 amplicon. The blue vertical line indicates the temperature where the products from the tube containing the H_2O sample melts, representing primer dimer formation or contamination of samples. The red vertical line shows the melting temperature of the amplified product. From this, the temperature where fluorescence of subsequent runs was measured using this gene was determined to be 83 °C.

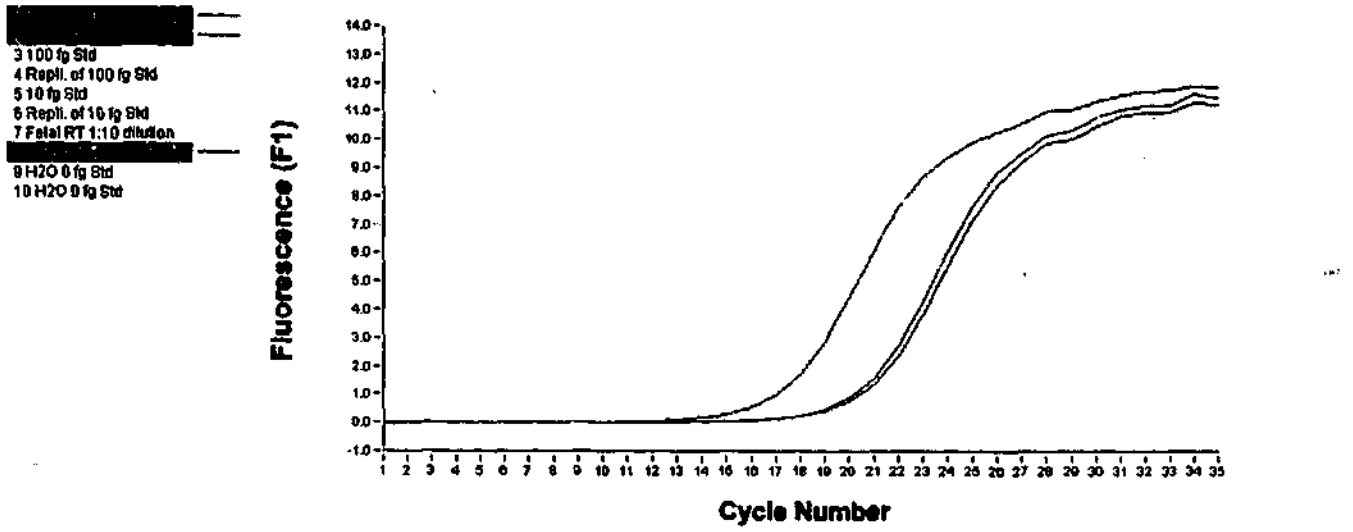

Figure 5-32 Effect of dilution of template DNA on the amplification of P450c17. A dilution is desired that amplifies in parallel to the template standard, indicating similar amplification efficiency between sample and std. The dilution that amplified at the same efficiency as the P450c17 standards was a 1:10 dilution.

5.3.9. Negative Controls

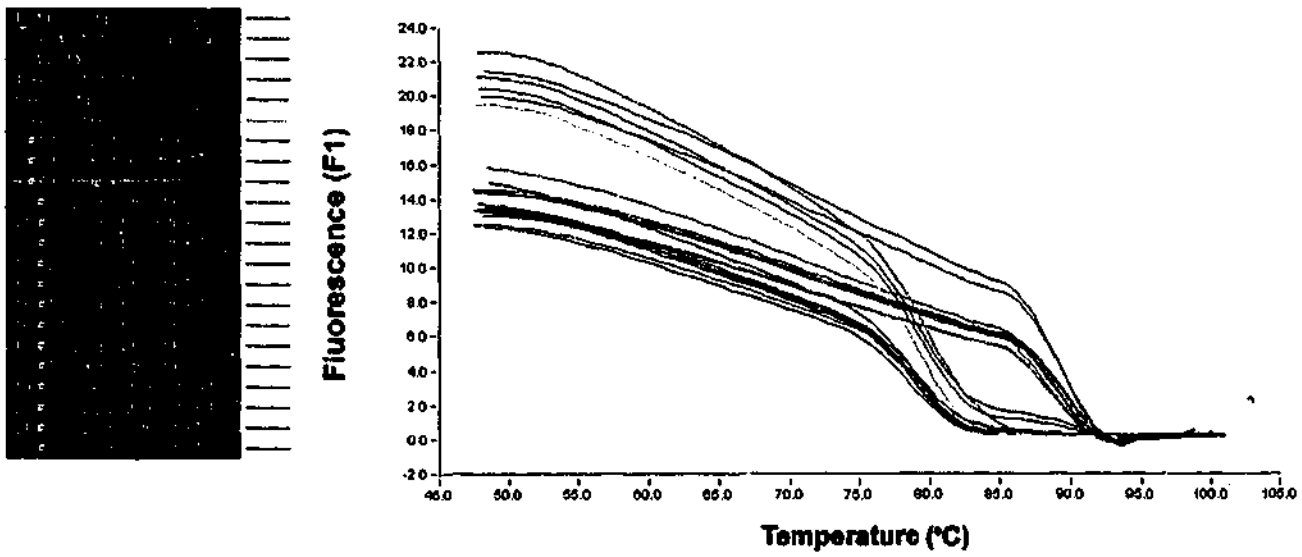

Figure 5-33 Melting curve of samples that were either reverse transcribed in the presence (red) or absence (black) of the Expand RT enzyme. The samples that did not contain the RT enzyme (black) did not amplify, and melted at the same time as the blank control (aqua) indicating no DNA contamination.

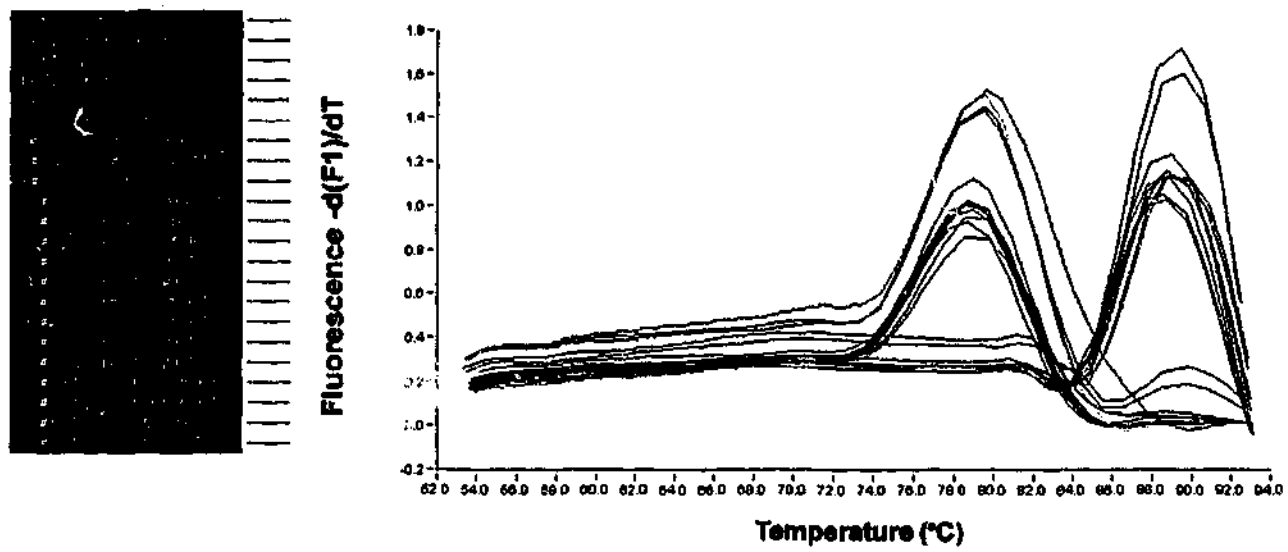

Figure 5-34 Melting graph showing 2 peaks. The samples that had been reverse transcribed in the absence of RT enzyme (black) melted at the same time as the blank H_2O controls (aqua), showing no amplification of the product. The samples that did contain RT (red) melted later, showing amplification of the desired product had occurred.

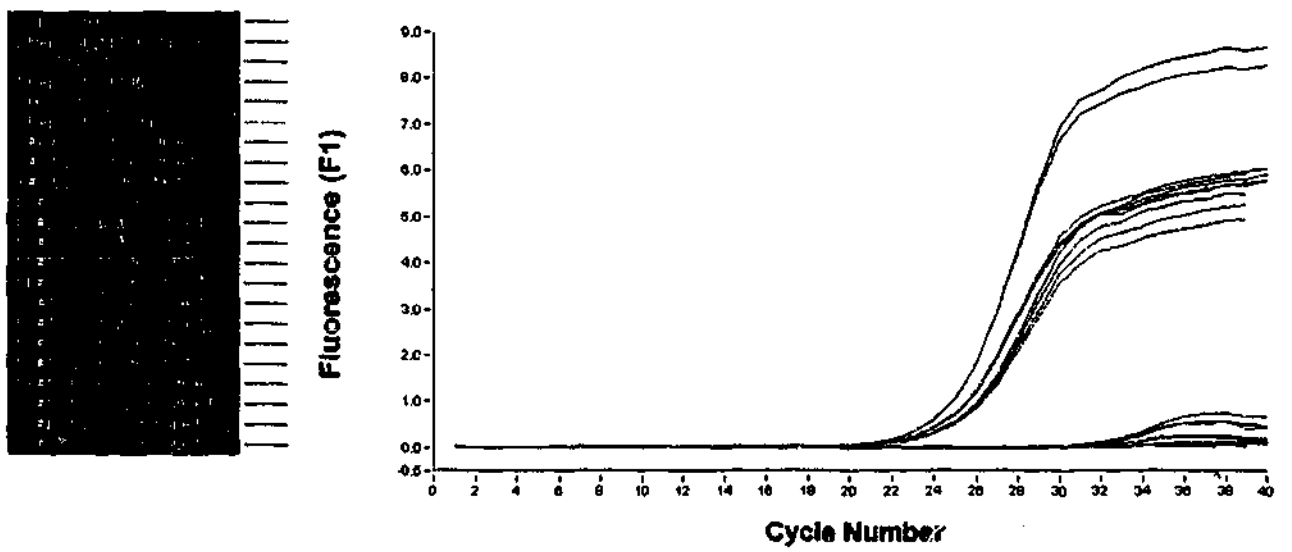

Figure 5-35 Amplification occurred in the standards (blue) and the samples that had undergone RT with the RT enzyme (red). Those samples that had not undergone RT with enzyme (black) did not amplify demonstrating no DNA contamination.

5.3.10. ACTH receptor real time final run

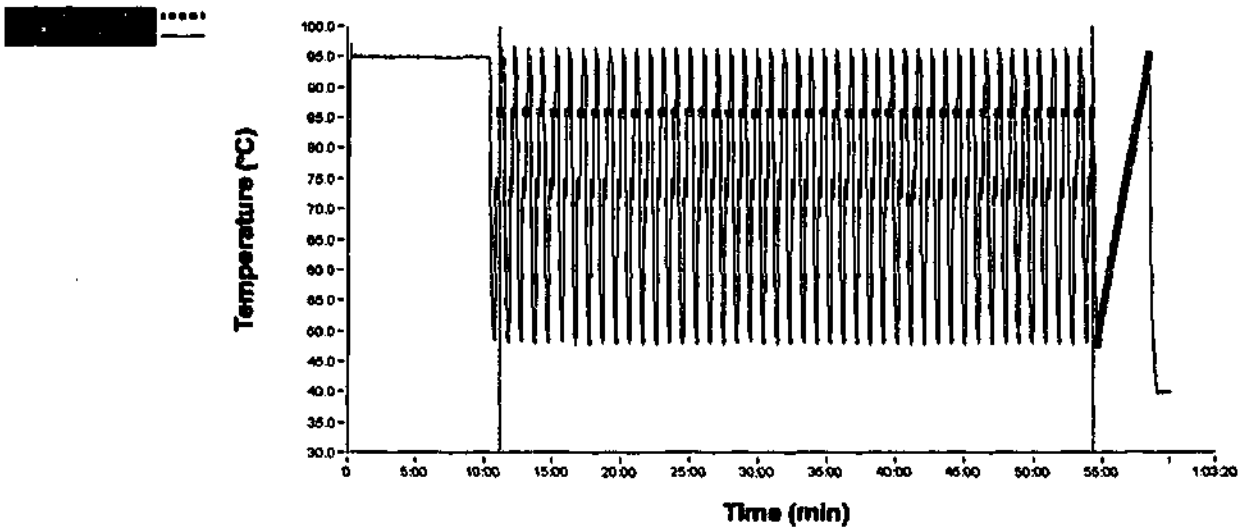

Figure 5-36 Example of a real time program that was used to determine the mRNA abundance for the ACTH receptor. The pink highlighted sections show where the fluorescence is trapped. The black line represents the temperature of the capillary tubes as they cycle through the chosen program.

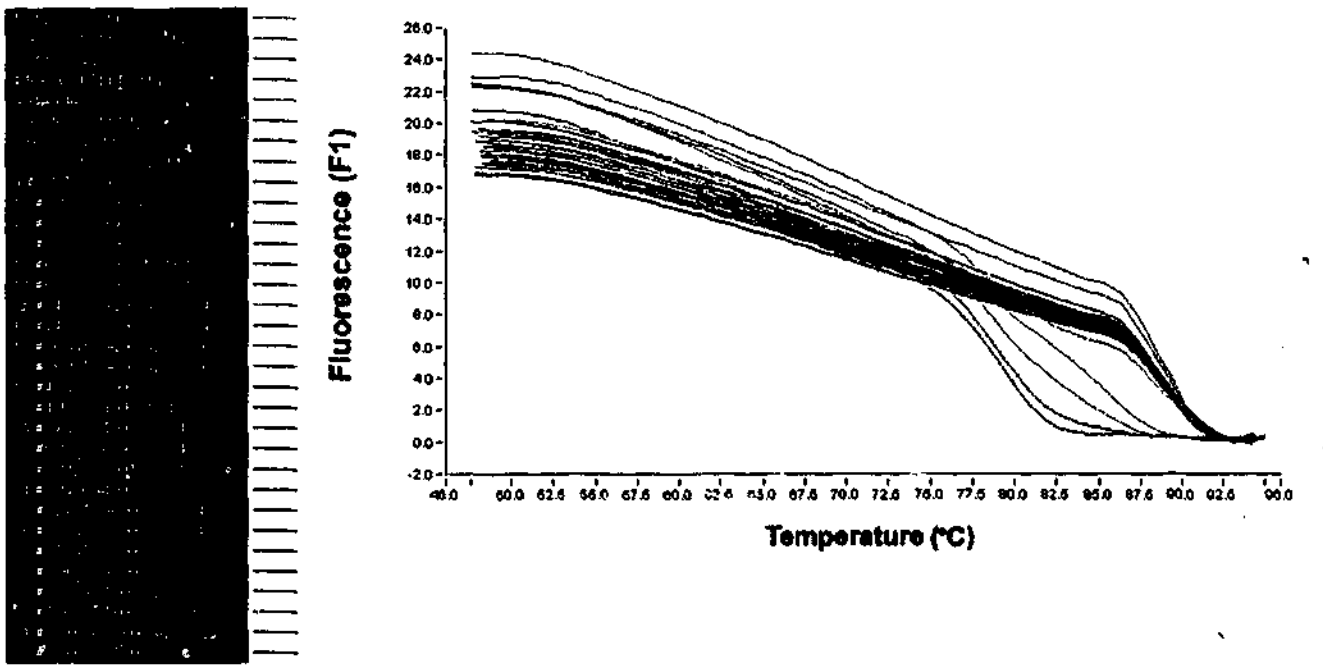

Figure 5-37 An example of the melting curve determined from one of the real time runs used to measure the mRNA abundance of the ACTH receptor. The amplified products from the unknown samples melt at approximately 87.5 °C. The two blank samples (aqua) melt at an earlier temperature indicating that they contain a product that is not the desired product, possibly primer dimer. All standards are presented in pink, INT/SAL +SAL are presented in red, INT/SAL + PGE_2 are in black and the HX/ACTH + SAL samples are in blue.

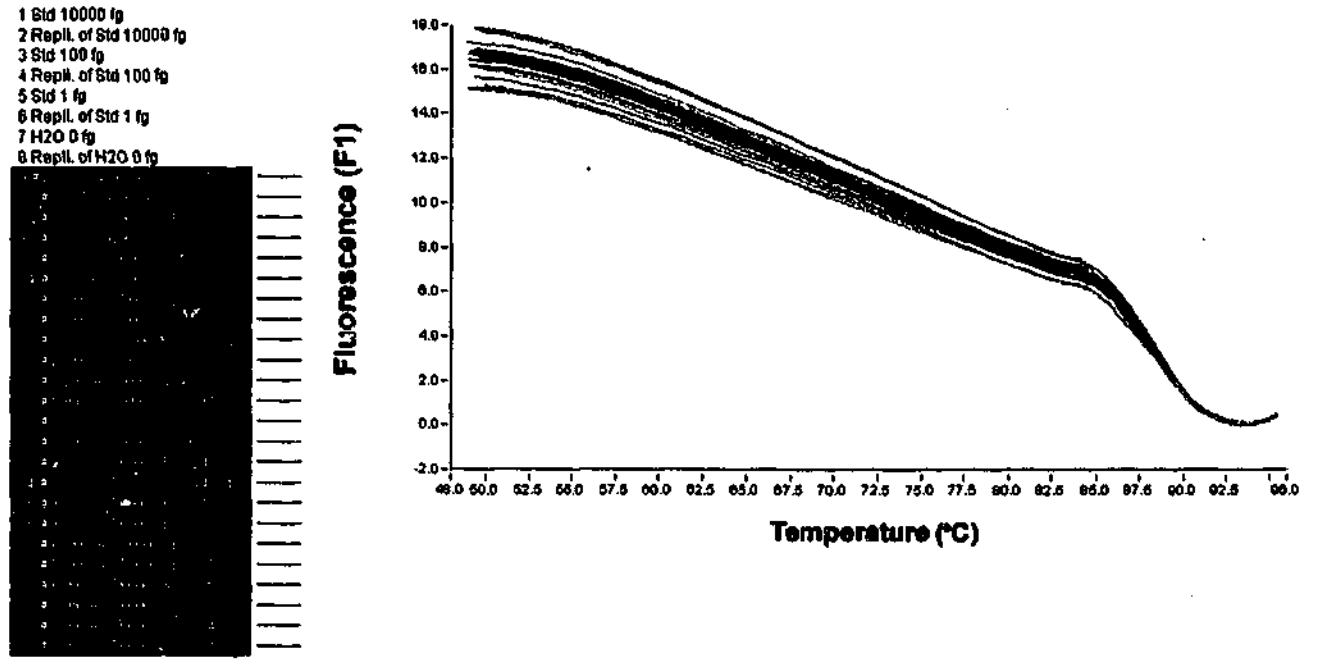

Figure 5-38 The melting curve determined from the other one of the real time runs used to measure the mRNA abundance of the ACTH receptor. The amplified products from all the unknown samples all melt at approximately 87.5 °C. INT/SAL +SAL are presented in red, HX/ACTH + SAL samples are in blue, and the HX/ACTH + PGE_2 samples are in green.

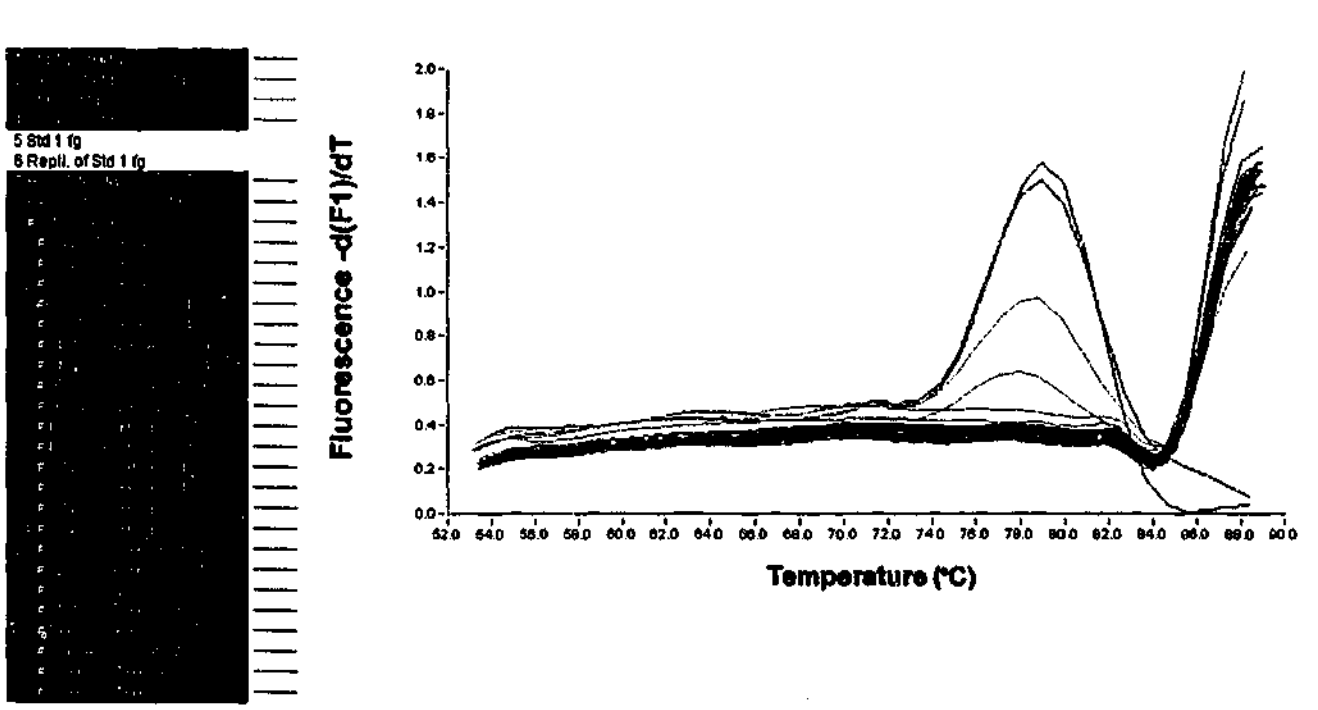

Figure 5-39 ACTH R melting graph in run 1, showing all unknown samples melting at the same temperature. The blank H_2O samples are in aqua and melt earlier, the standards are in pink, the INT/SAL + SAL samples are in red, INT/SAL + PGE_2 are in black and the HX/ACTH +SAL samples are in blue.

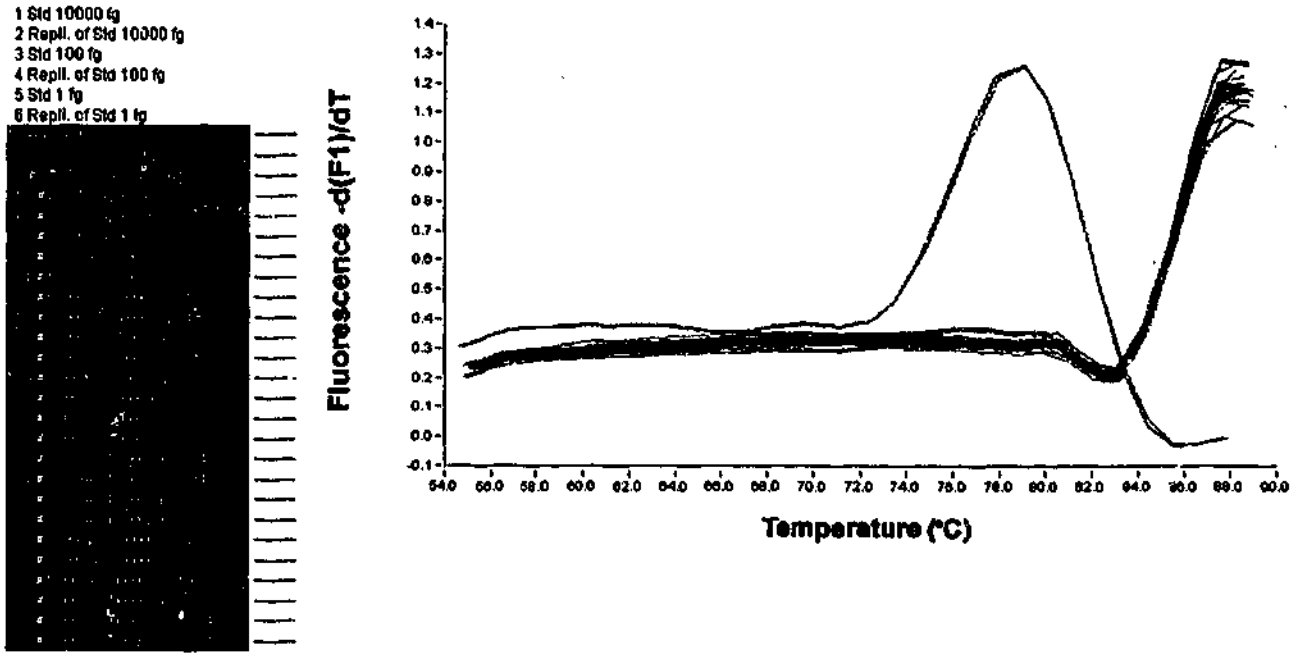

Figure 5-40 ACTH R melting graph in run 2, showing all unknown samples melting at the same temperature. The blank H_2O samples are in aqua and melt earlier, the INT/SAL + SAL samples are in red, the HX/ACTH +SAL samples are in blue and the HX/ACTH + PGE_2 samples are in green.

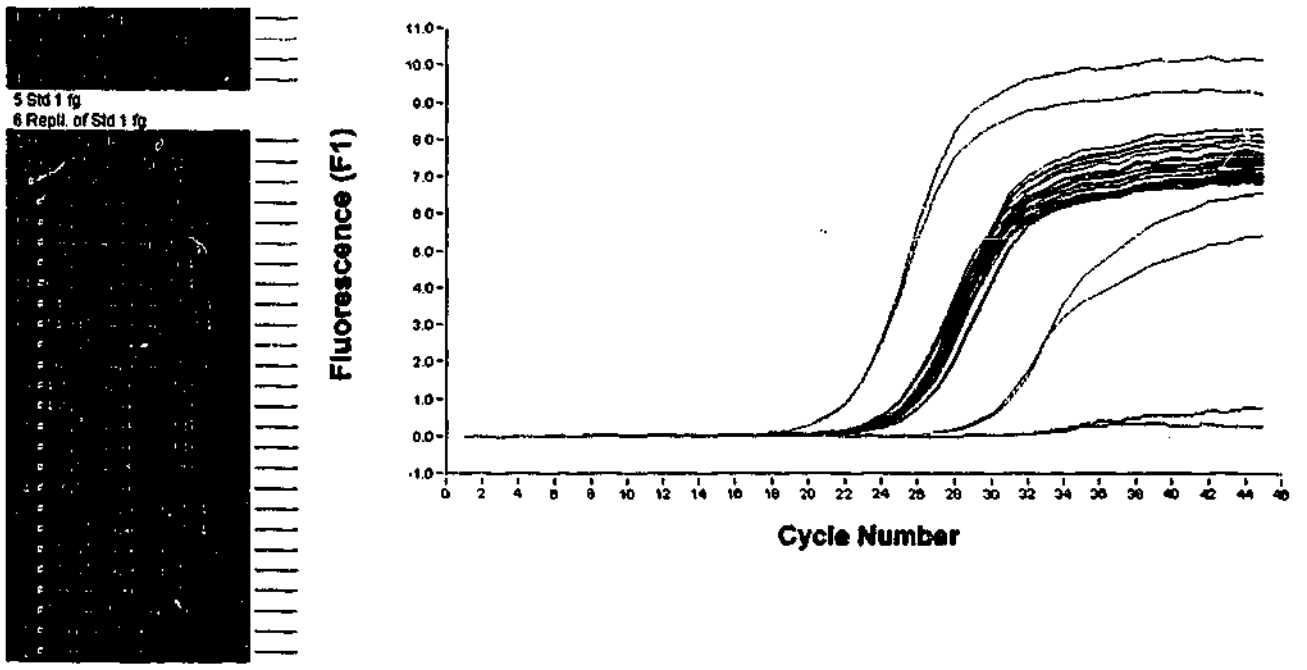

Figure 5-41 Real time amplification graph showing the amplification of the ACTH receptor mRNA in run 1. The standards are presented in pink, blank H_2O samples are presented in aqua, INT/SAL + SAL samples are in red, INT/SAL + PGE_2 samples are in black and the HX/ACTH + SAL samples are in blue.

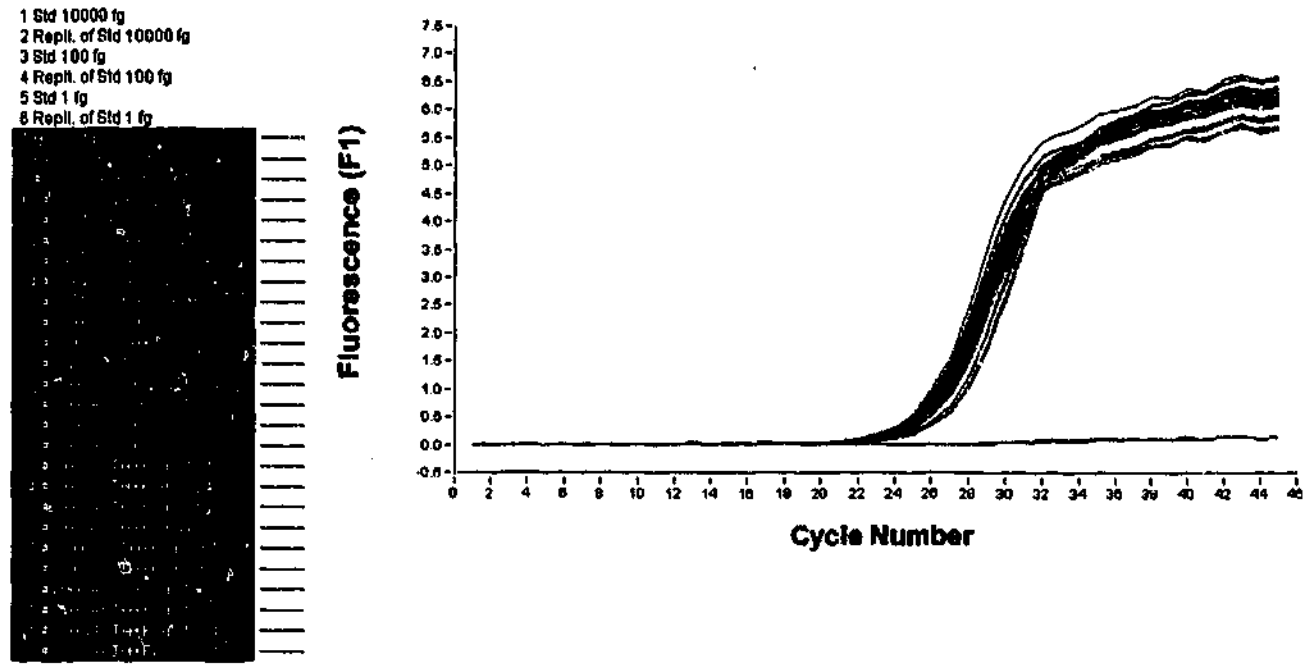

Figure 5-42 Real time amplification graph showing the amplification of the ACTH receptor mRNA in run 2. The blank H_2O samples are presented in aqua, INT/SAL + SAL samples are in red, the HX/ACTH + SAL samples are in blue and the HX/ACTH + PGE_2 samples are in green.

5.4. SUMMARY OF OPTIMISED CONDITIONS

In this chapter the optimal conditions for each of the genes of interest was determined. These conditions are summarised in the following tables. In Table 5-1 the optimised programs for each gene are summarised. In Table 5-2 the optimised conditions for each gene are summarised.

Gene	Denaturing Temp (°C)	Denaturing Time (secs)	Annealing Temp (°C)	Annealing Time (secs)	Extension Temp (°C)	Extension Time (secs)	Trap Fluro Temp (°C)	Melting Step Temps (°C)	No. cycles
EP1	95	10	59	12	72	10	86	59-99	45
EP2	95	10	60	6	72	10	84	60-99	55
EP3	95	10	51	5	72	10	88	51-99	55
EP4	95	10	55	6	72	10	85	55-99	50
ACTH R	95	10	49	6	72	10	85	49-99	45
P450scc	95	15	52	5	72	10	85	52-99	30
P450c17	95	15	65	5	72	10	83	65-99	35
3βHSD	95	10	57	6	72	10	84	57-99	30

Table 5-1 Summary of the final optimised programs for each of the genes. All programs started with 10 minutes at 95°C to activate the Taq enzyme and at the end of each program were followed by 1 minute at 40°C to cool the capillary tubes.

Gene	Product Size (bp)	Mg^{2+} Conc. (mM)	Optimum Annealing Temperature (°C)	Optimum RT Dilution	# PCR Cycles	Temp. to trap Fluorescence (°C)	Primer Dimer
EP1	214	3	59	1:30	45	86	Yes
EP2	108	1.5	60	1:20	55	84	Yes
EP3	264	2	51	1:20	55	88	
EP4	178	3	55	1:10	50	85	
ACTH R	157	3	49	1:20	45	85	
P450scc	127	3	52	1:20	30	85	
3βHSD	143	4	57	1:20	30	84	
P450c17	149	3	65	1:10	35	83	Yes

Table 5-2 Summary of the optimised conditions for real-time PCR for each of the genes of interest.

Chapter 6 QUANTITATION OF GENES ENCODING THE PGE_2 RECEPTORS, ACTH RECEPTOR & KEY STEROIDOGENIC ENZYMES IN THE OVINE FETAL ADRENAL BY REAL-TIME PCR

6.1. *INTRODUCTION*

In chapters three and four, the ability of PGE_2 to stimulate cortisol secretion directly from the ovine fetal adrenal has been demonstrated. In this chapter, the mechanisms underlying PGE_2-stimulated cortisol secretion are investigated.

PGE_2 can act through a number of receptors designated EP1 through to EP4. These receptors have only been partially cloned in the sheep so the following discussion refers to the human, rat and mouse EP receptors. The EP receptors act through different second messenger systems to mediate differential effects in diverse tissues. The EP receptors belong to the rhodopsin-type receptor family, which are G protein-coupled receptors that contain seven transmembrane spanning proteins (Narumiya *et al.*, 1999).

EP2 and EP4 are thought to be "relaxant" receptors as they act to relax smooth muscle, by signaling through G_s –adenylate cyclase mediated increases in cAMP (An *et al.*, 1993; Honda *et al.*, 1993; Bastien *et al.*, 1994; Coleman *et al.*, 1994; Regan *et al.*, 1994b; Katsuyama *et al.*, 1995; Breyer *et al.*, 1996). There is some evidence that the expression of EP2 can be induced, as administration of lipopolysaccharide to a mouse macrophage-like cell line increased EP2 mRNA abundance in a dose-dependant manner (Katsuyama *et al.*, 1998). EP2 is also inducible by gonadotrophins such as human chorionic gonadotrophin and pregnant mare's serum gonadotrophin in mouse uterine epithelial cells and because of

this the EP2 receptor has been implicated in the implantation process of mouse pregnancy (Katsuyama *et al.*, 1997; Lim & Dey, 1997).

EP1 differs from both EP2 and EP4 in that it is considered a "contractile" receptor that acts to contract smooth muscle. EP1 signals through a G_q mediated increase in phosphotidyl inositol turnover that elevates intracellular free Ca^{2+} (Funk *et al.*, 1993; Watabe *et al.*, 1993).

The EP3 receptor generally couples to G_i and is thus regarded as inhibitory (Narumiya *et al.*, 1999). There are a number of splice variants of the EP3 receptor, which differ by alternate splicing at the C-terminal tail and give rise to various isoforms of the EP3 receptor (Regan *et al.*, 1994a; Narumiya *et al.*, 1999). Namba *et al.* (1993) showed that the variation in the C-terminal end of the genes did not change ligand specificity of the receptor, but did affect which G-protein the receptor couples too and hence the second messenger cascade that is activated (Namba *et al.*, 1993). These isoforms have been named alphabetically, such as $EP3_A$ – $EP3_F$ in the human and $EP3_A$ – $EP3_D$ in the bovine model. The various isoforms have different binding capabilities but EP3 generally acts to inhibit adenylate cyclase induced cAMP formation although some variants appear to act via an increase in Ca^{2+} levels and other variants act to cause increases in cAMP (Narumiya *et al.*, 1999). In this chapter, the primers designed for the EP3 receptor bind to all four isoforms in the sheep.

6.2. EP RECEPTORS AND THE ADRENAL GLAND

In the adrenal, binding of PGE_2 has been reported. A number of reports suggest that binding is localised to the adrenal medulla, where PGE_2 plays an inhibitory role in the release of catecholamines, and in the zona glomerulosa of the adrenal cortex, where PGE_2 can stimulate the release of aldosterone (Karaplis & Powell, 1981a; Csukas *et al.*, 1998). Dazord *et al.* (1974) investigated the binding of radioactively labelled ^{3}H-PGE_2 to ovine and human subcellular adrenal preparations that had been decapsulated and the medulla removed. The authors reported specific binding sites for PGE_2 and that when PGE_2 bound these receptors there was an increase in adenylate cyclase activity which would be consistent with binding to EP2 or EP4 receptors. Saruta *et al.* (1972) also found an increase in cAMP when bovine adrenal slices were incubated in the presence of PGE_1. This

suggests that the predominant action of prostaglandins on the adrenal is through the adenylate cyclase-cAMP second messenger pathway of the EP2 and EP4 subtypes, the same pathway that the ACTH receptor acts through to stimulate cortisol secretion (Saruta & Kaplan, 1972). Subsequently, Karaplis and Powell (1981a) demonstrated PGE_2 binding sites in the fetal and adult adrenal medulla and reported that the concentration of PGE binding sites in the bovine adult adrenal medulla was 20 times the binding in the cortex. In that study the authors also attempted to separate the adrenal medulla and cortex from fetal calves and in contrast to the report of Dazord *et al.* (1974), found only low concentrations of PGE binding sites in the fetal adrenal cortex, with the binding sites in the medulla being approximately 5 times the concentration of the binding sites in the cortex (Karaplis & Powell, 1981a). More recently, Csukas *et al.* (1998) reported that PGE_2-induced aldosterone release in the adrenal zona glomerulosa is mediated by EP2 receptors, suggesting that PGE_2 receptors are present in the adrenal cortex. In the study presented in this chapter, no attempt was been made to separate the fetal adrenal and medulla, due to the size of the adrenals, difficulties in determining where the zones end (as the medulla has finger-like projections that extend into the cortex), and to ensure consistency between animals.

6.3. *Steroidogenic Enzymes in the Adrenal Cortex*

In contrast to prostaglandin receptors in the fetal adrenal cortex, the mRNA concentrations of a number of steroidogenic enzymes have been shown to increase in a fashion that corresponds to the increase in fetal cortisol concentrations in late gestation. These include the cholesterol side chain cleavage enzyme ($P450_{scc}$), 3β-hydroxysteroid dehydrogenase /Δ^4-Δ^5 isomerase (3βHSD), 17α-hydroxylase ($P450_{C17}$) and 21-hydroxylase ($P450_{C21}$) (Phillips *et al.*, 1996; Simmonds *et al.*, 2001). Changes in the expression of 11β-hydroxylase, the enzyme responsible for catalysing the final step of cortisol biosynthesis, have not been demonstrated in the late-gestation ovine fetal adrenal gland, suggesting that this enzyme does not play an acute regulatory role in the increase in cortisol synthesis (Myers *et al.*, 1992b). In addition, decreased cortisol concentrations were observed in PVN-lesioned fetuses that also have decreased mRNA expression of $P450_{scc}$ and $P450_{C17}$, but not 3βHSD nor $P450_{C21}$, this lesion also results in a failure of parturition in the ovine fetus (Myers *et al.*, 1993). Likewise, fetal HPD and HX, which results in low cortisol concentrations and failure of parturition, both also decrease the expression of

$P450_{scc}$ and $P450_{C17}$, as well as 3βHSD mRNA abundance (Phillips *et al.*, 1996; Ross *et al.*, 1997; Simmonds *et al.*, 2001). Furthermore, ACTH replacement into HX fetuses can restore the abundance of the mRNAs encoding these three steroidogenic enzymes (Simmonds *et al.*, 2001).

It has been suggested that the ACTH receptor is regulated positively by its own ligand, as previous reports show that treatment of adult adrenocortical cells with ACTH results in increases in the abundance of ACTH receptor mRNA (Picard-Hagen *et al.*, 1997). Durand *et al.* (1981b) reported that treatment of ovine fetuses for five days with ACTH enhanced the binding of ACTH to adrenal membranes when compared with controls. In contrast to these findings however, Carter *et al.* (2002) reported that there was no difference in the abundance of the ACTH R mRNA after a 24 hour infusion of ACTH to 126 d GA ovine fetuses as measured by ribonuclease protection assay. In addition, a study by Simmonds *et al.* (2001) also reported that ACTH infusion or HX had no effect on the abundance of the ACTH receptor mRNA in the ovine fetal adrenal.

6.4. *PROSTAGLANDINS AND STEROIDOGENESIS*

Prostaglandins have been implicated in steroidogenesis in all steroidogenic tissues. PGE_2 in particular, has been reported to be a positive regulator of 3βHSD, P450c17, P450scc and the ACTH receptor and it appears that much of the action of PGE_2 in adrenocortical steroidogenesis is activated by the cAMP second messenger system (Rolland & Chambaz, 1977; Rainey *et al.*, 1991a). Aldosterone synthesis can also be stimulated by PGE_2 (Campbell *et al.*, 1986).

PGE_2 has also been shown to have a role in steroidogenesis in the ovary, testis and placenta in a variety of species. In the rat ovary, PGE_2 stimulated an increase in ovarian cAMP accumulation and in rat corpora luteal and granulosa cells, progesterone synthesis was increased by PGE_2 (Schindler *et al.*, 1982; Horvath *et al.*, 1986; Fanjul *et al.*, 1992; Ahsan *et al.*, 1997). In goldfish testis and ovary, PGE_2 can stimulate testosterone production, the hormone produced in both sex organs (Wade & Van der Kraak, 1993; Mercure & Van Der Kraak, 1996), while in the bovine and porcine ovarian granulosa and corpora luteal cells and the sheep corpora luteal cells, PGE_2 has also been implicated in progesterone synthesis and release (McArdle, 1990; Wiesak *et al.*, 1992; Weems *et al.*,

1997; Kim *et al.*, 2001). Furthermore, in cultured porcine granulosa cells, Li *et al.* (1993) demonstrated that PGE_2 not only induces progesterone synthesis, but also induces the genes encoding the steroidogenic enzymes, 3βHSD and P450scc in a dose dependent manner. It is unknown whether PGE_2 can also induce steroidogenic enzyme mRNA expression in the adrenal cortex. Elvin and colleagues (2000) suggested that PGE_2-stimulated progesterone synthesis occurs through the EP2 receptor subtype. They administered butaprost, a specific EP2 agonist, to a mouse granulosa cell culture system and were able to stimulate progesterone synthesis to the same level as PGE_2 alone, which the authors suggested indicated that only the EP2 receptor subtype was involved in progesterone stimulation (Elvin *et al.*, 2000).

In chapters 3 and 4, I have demonstrated that exogenous PGE_2 is able to directly cause steroidogenesis in the ovine fetal adrenal, however it remains unclear if PGE_2, like ACTH, can influence the abundance of the mRNA encoding the steroidogenic enzymes. In this chapter, I will investigate if a 24 hour PGE_2 infusion into intact or HX fetuses receiving an ACTH replacement can influence the abundance of the mRNA encoding the PGE_2 receptor isoforms EP1, EP2, EP3 and EP4 as well as the ACTH receptor, $P450_{scc}$, 3β-HSD and $P450_{c17}$.

6.5. MATERIALS AND METHODS

6.5.1. Experimental procedures

The tissues used in this chapter were collected from the animals described in chapter 4. Briefly, there were four treatment groups INT/SAL + SAL (n = 5); INT/SAL + PGE_2 (n = 5); HX/ACTH + SAL (n = 5); and HX/ACTH + PGE_2 (n = 4).

The molecular methods employed in this chapter were described in chapter 5. Briefly, total RNA was extracted from the frozen adrenal glands and then quantified. RNA was reverse transcribed and then real time PCR was performed using the optimised conditions described in chapter 5 for each of the following: the PGE_2 EP receptor subtypes, the ACTH receptor and P450scc, P450c17 and 3βHSD. If any of the genes of interest had multiple isoforms, the primers were designed to a region common to all of the splice variants.

6.5.2. Real Time Analyses

Relative quantification, rather than absolute quantification of the mRNA of interest was used. Absolute quantification allows the absolute number of mRNA copies per capillary tube to be determined against a standard curve. It is useful if comparing data obtained on different days and using different reverse transcription (RT) reactions. In this study, the same RT reaction was used and each gene was measured at the same time, therefore relative quantitation was used. It is a method based on the expression ratio of the target gene between control and treatment groups. Trends in mRNA expression are better explained by relative quantitation (Pfaffl *et al.*, 2002). For each gene, the crossing points of eight quality control capillary tubes included in each run were compared and any differences between the runs were calculated and the other samples in the runs were normalised accordingly. The relative expression of the samples was determined by the following equation:

$$\text{Ratio} = (E_{target})^{\Delta CP_{target}(\text{mean control} - \text{mean sample})}$$

Where E is the real-time PCR efficiency of the gene. CP stands for the crossing point of the samples. The efficiency can be determined by the equation $E=10^{[-1/slope]}$ and the LightCycler determines the slope from known standard concentrations included in each reaction. As two runs were used to measure all unknowns, the mean of the two slopes were

used to calculate the efficiency for each gene. The mean crossing point for each treatment group was determined and the ratio was then calculated.

6.5.3. Statistical Analyses

Analysis of variance was used to determine if there were any differences in the ratio of the genes and to compare if there was an effect of operational status (INT or HC). It was also used to determine if there was an effect of the 24 hour PGE_2 treatment on any of the genes of interest. If the data was heterogeneous (EP2, EP3 and EP4) it was rendered homogenous by square root (EP4) or log transformation (EP2 and EP3).

6.6. RESULTS

6.6.1. EP1

Real-time analysis of the ratio of EP1 mRNA expression showed no effect of either operational status (INT/SAL or HX/ACTH; $P = 0.357$) or the saline or PGE_2 treatments ($P = 0.327$; See Figure 6-1).

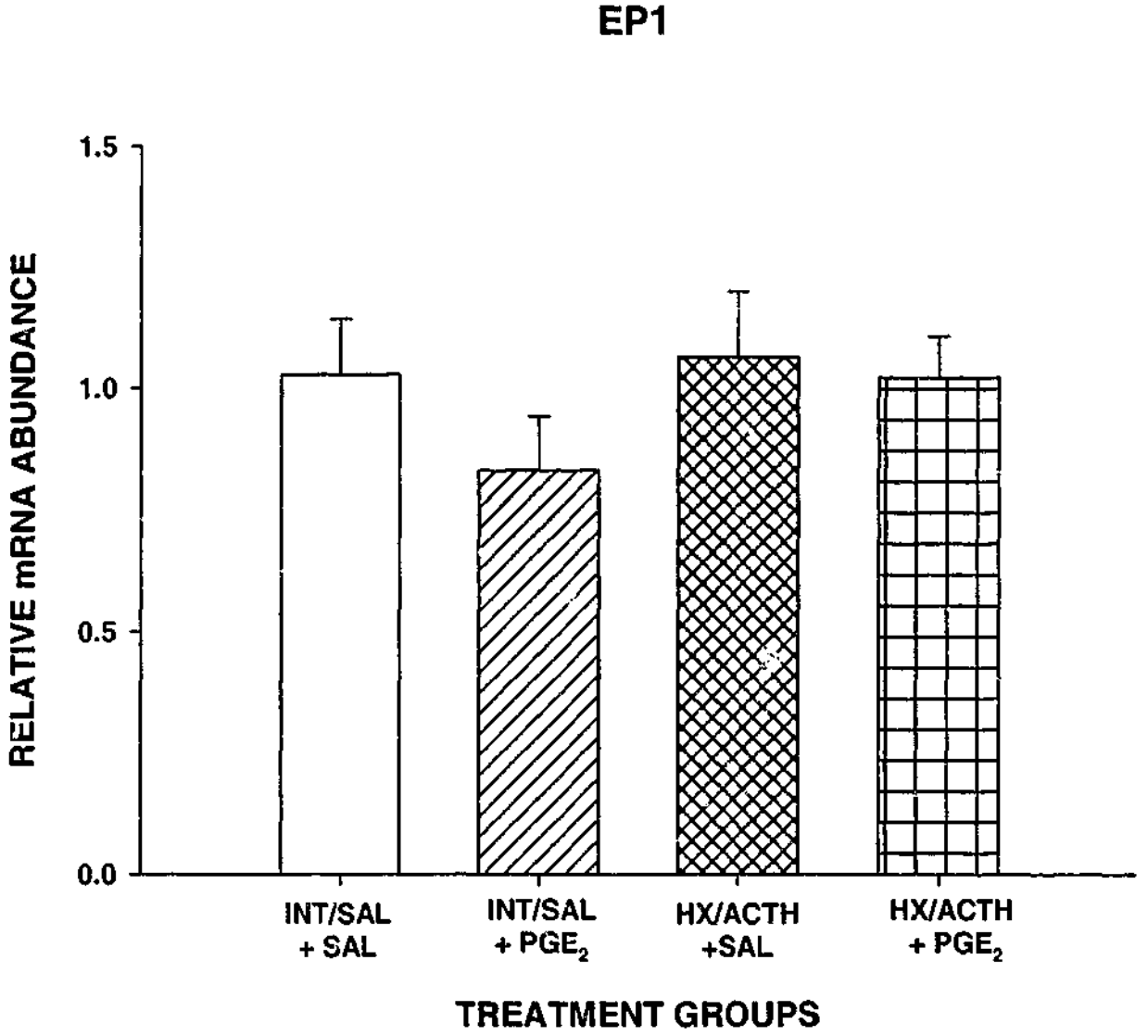

Figure 6-1 Relative expression of adrenal EP1 mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There were no differences in the expression of the mRNA encoding EP1 between any of the treatment groups.

6.6.2. EP2

There were no effects of either the operational status or PGE_2 treatment on the ratio of the expression of EP2 mRNA. The two HX/ACTH groups showed a non-significant trend towards higher expression of this mRNA than the two INT/SAL groups ($P = 0.06$). There

was no effect of PGE_2 treatment on the relative abundance of EP2 mRNA expression (P = 0.584; See Figure 6-2).

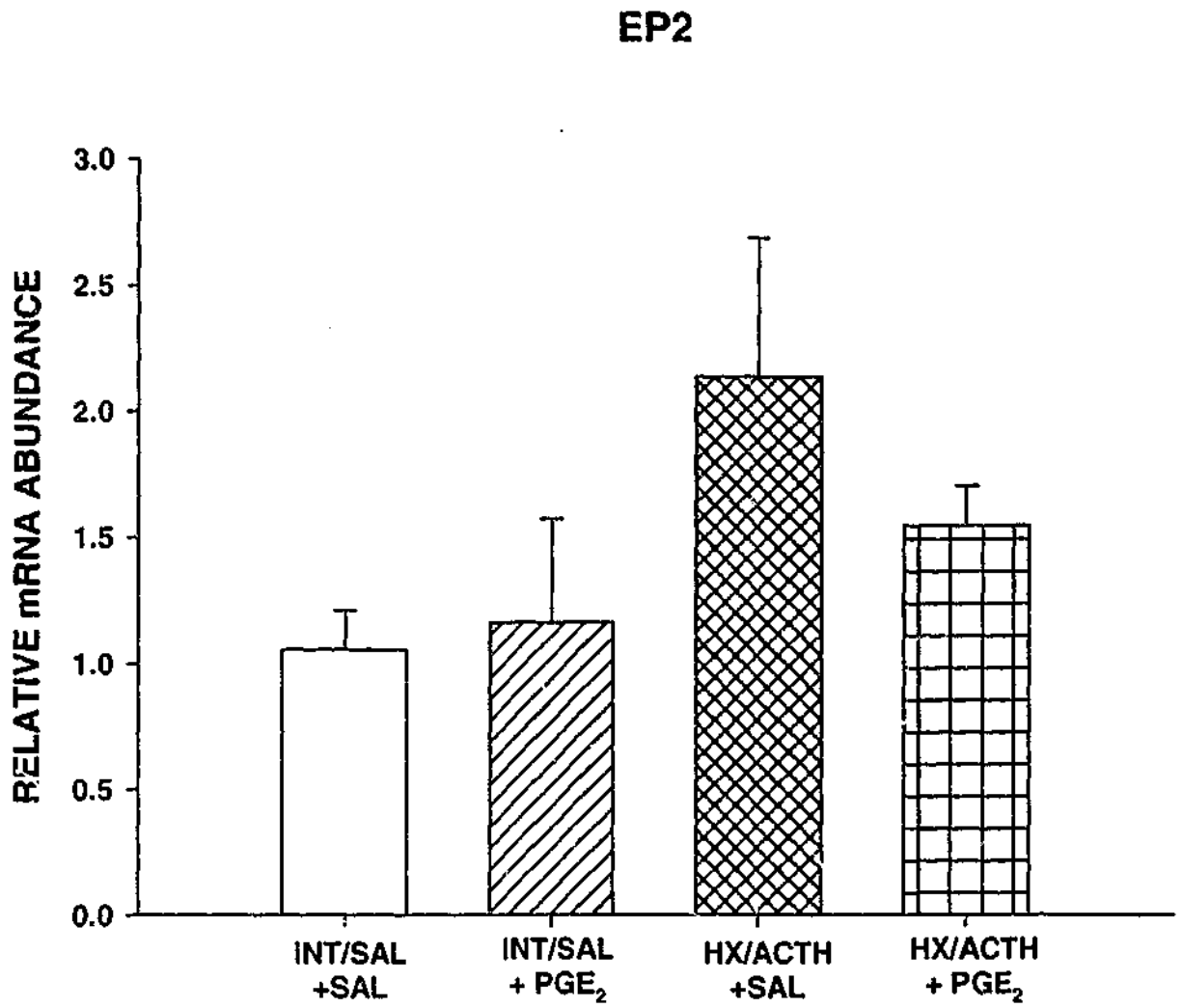

Figure 6-2 Relative expression of adrenal EP2 mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There were no differences in the expression of the mRNA encoding EP2 between any of the treatment groups. There was a non-significant trend for the HX/ACTH group to have higher EP2 mRNA expression than the INT/SAL group (P = 0.06).

6.6.3. EP3

There was a non-significant trend toward an increase in the abundance of the mRNA encoding EP3 with the PGE_2 treatment (P = 0.076). There was however, a significant effect of operation with the HX/ACTH group having an increased expression of EP3 with respect to that of the INT/SAL + SAL group (P = 0.027). The data showing the relative expression of EP3 abundance are presented in Figure 6-3.

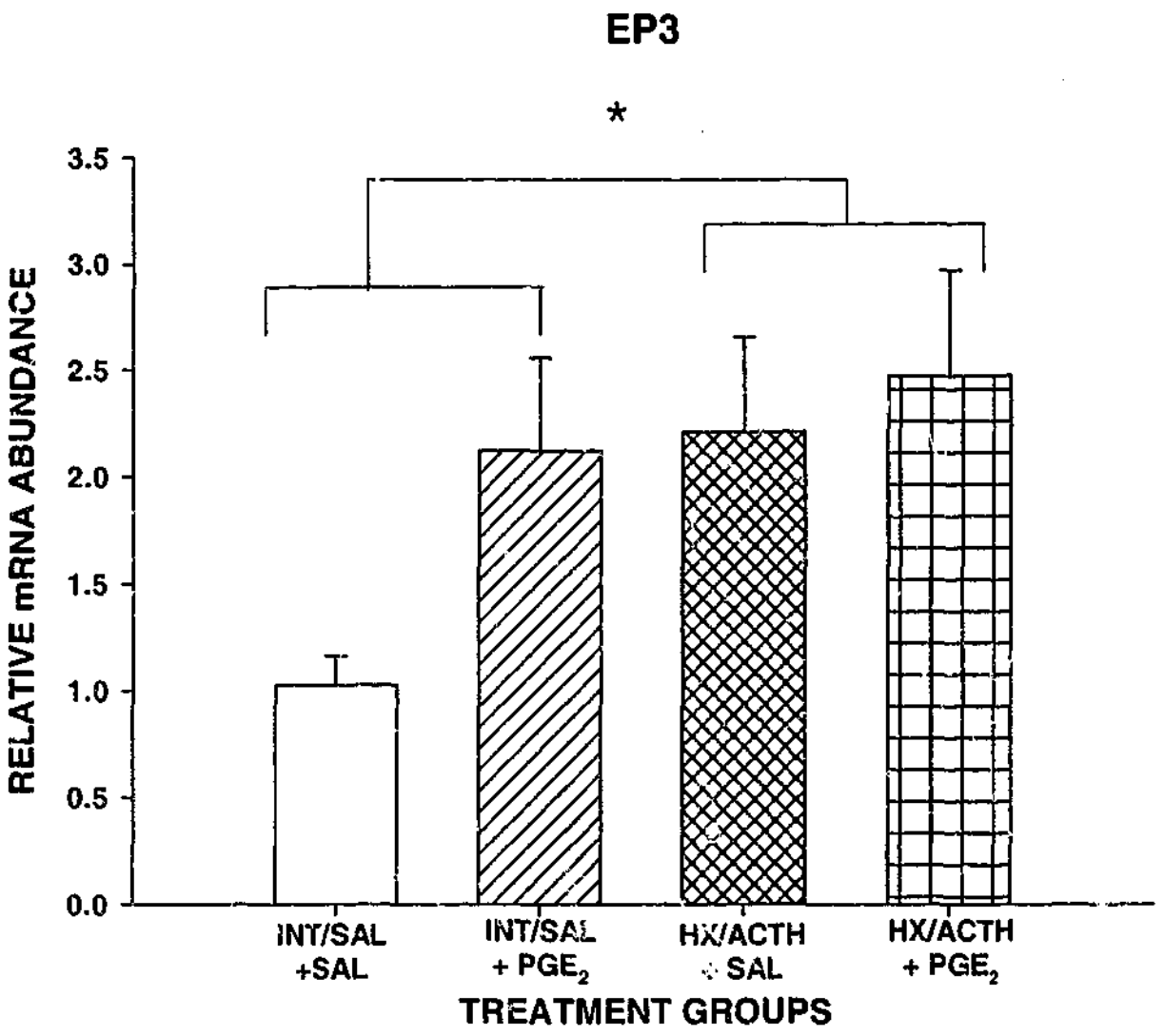

Figure 6-3 Relative expression of adrenal EP3 mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There was a non-significant trend for the PGE_2 infused groups to have higher expression of EP3 mRNA than the SAL infused animals (P = 0.076). There was a significant effect of operation with the two HX/ACTH groups having increased expression when compared to the INT/SAL groups (P = 0.027)

6.6.4. EP4

The mean relative expression of EP4 mRNA was not different between any of the four treatment groups. There was no effect of operational status (P = 0.304) or a 24 hour infusion of PGE_2 (P = 0.857) on the relative expression of EP4 mRNA in the ovine fetal adrenal gland (Figure 6-4).

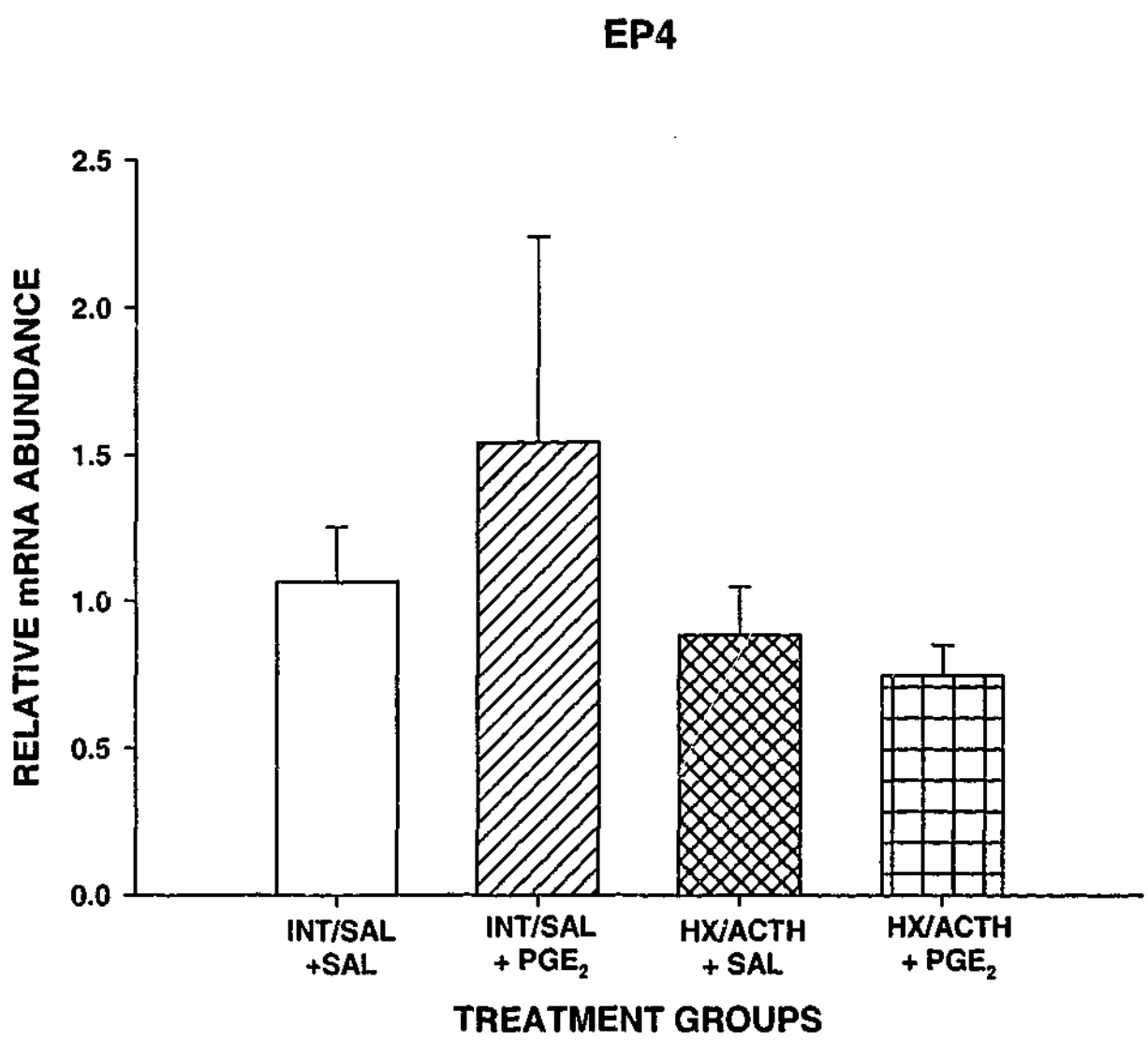

Figure 6-4 Relative expression of adrenal EP4 mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There were no differences in the expression of the mRNA encoding EP4 between any of the treatment groups.

6.6.5. ACTH Receptor

There was a highly significant decrease in the relative expression of the ACTH receptor mRNA in the two HX/ACTH groups when compared with the INT/SAL groups (P = 0.002). PGE_2 treatment had no effect on the expression of the ACTH receptor mRNA (P = 0.216; Figure 6-5).

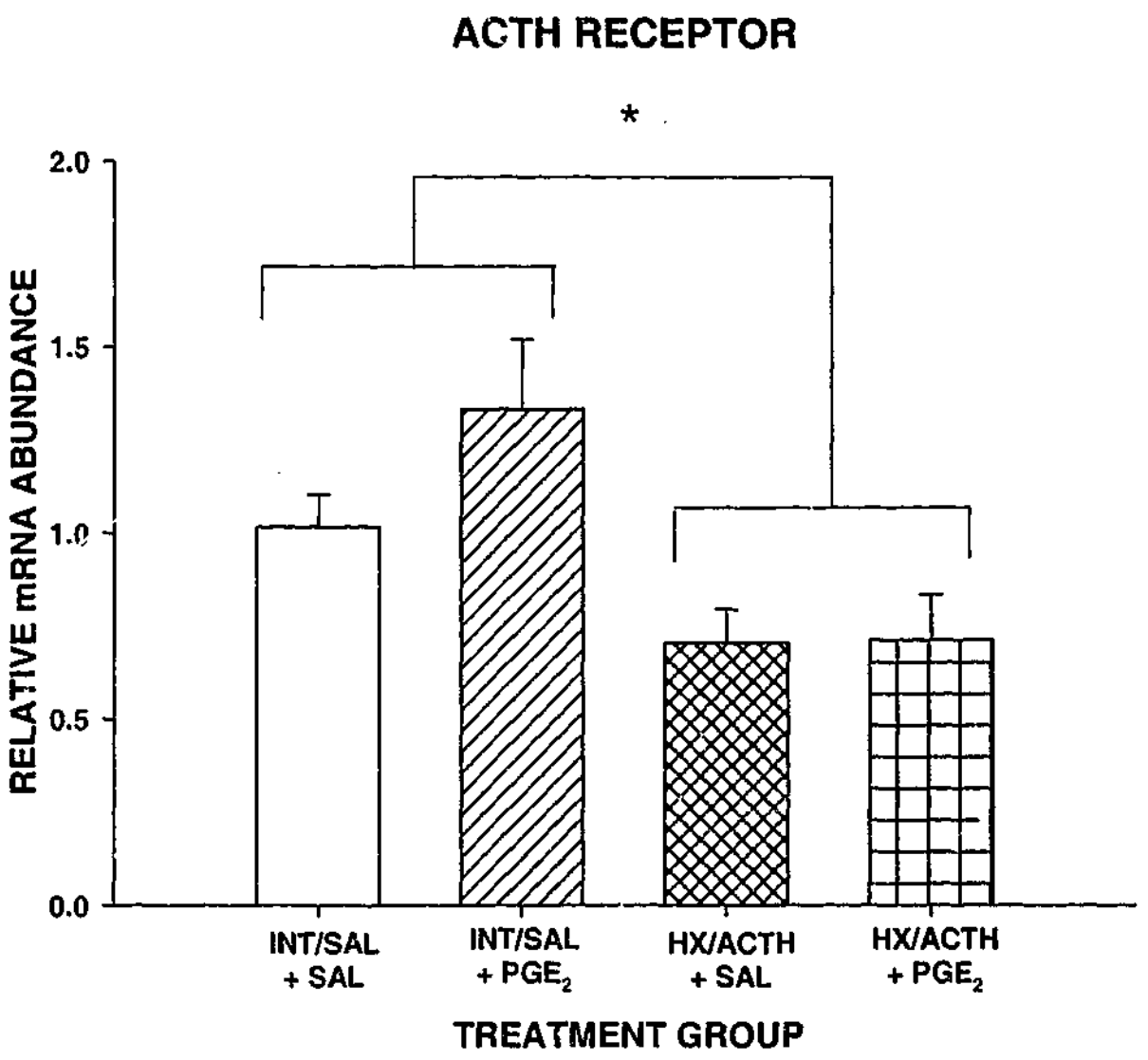

Figure 6-5 Relative expression of adrenal ACTH receptor mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There was a significant effect of operational status on the mRNA encoding the ACTH receptor as the two HX/ACTH groups had lower expression of this mRNA in comparison to the INT/SAL treatment groups (P = 0.002). There was no effect of PGE_2 treatment on the relative expression of the ACTH receptor mRNA.

6.6.6. P450scc

There was no effect of any of the treatments on the relative expression of P450scc mRNA. As shown in Figure 6-6, P450scc mRNA abundance was not affected by either operational status (P = 0.193) or by treatment with PGE_2 (P = 0.295).

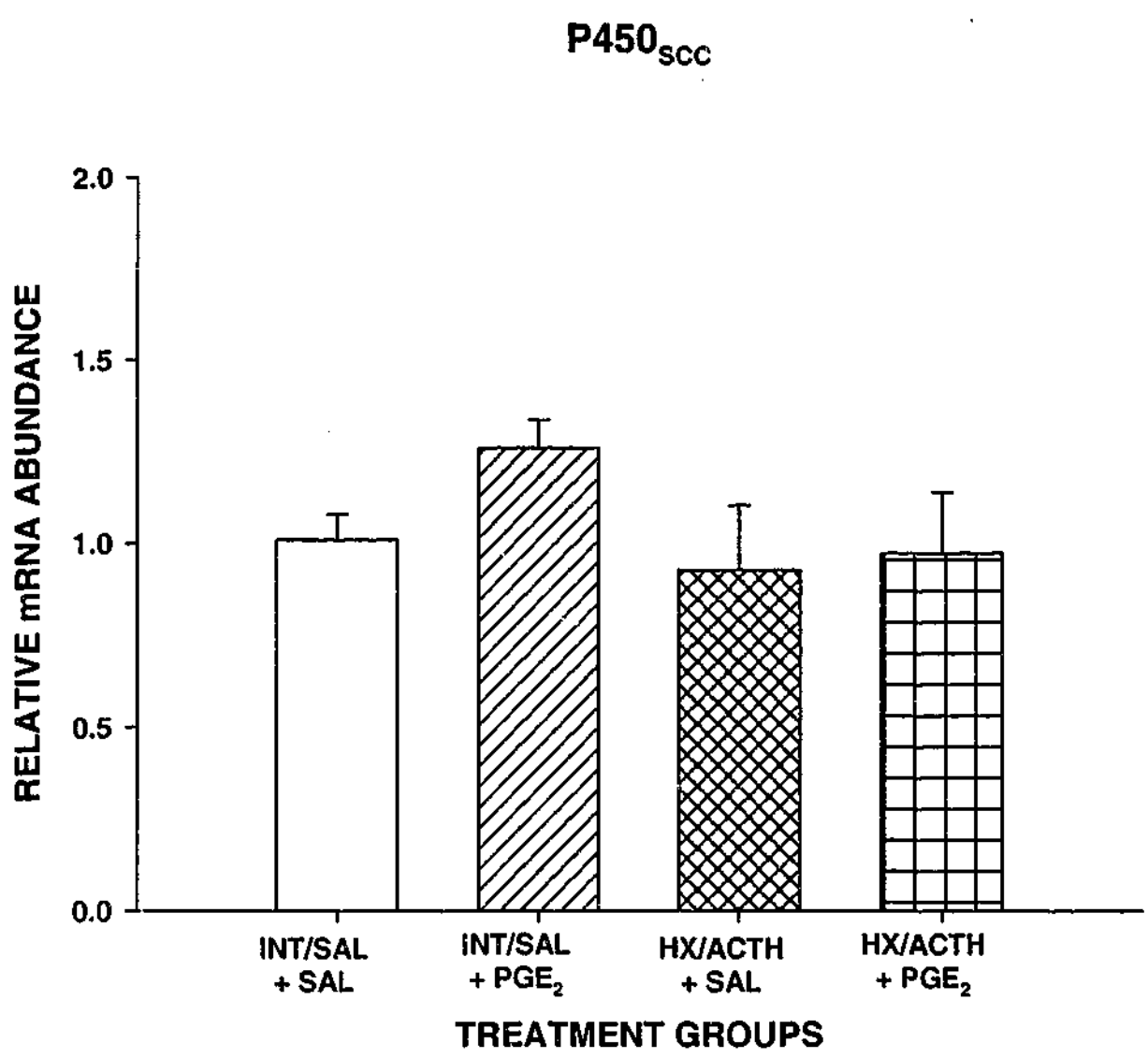

Figure 6-6 Relative expression of adrenal P450scc mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There were no differences in the expression of the mRNA encoding P450scc between any of the treatment groups.

6.6.7. 3βHSD

The relative expression of 3βHSD mRNA followed a similar profile to that of the ACTH receptor. There was a significant effect of operation with the two HX/ACTH treatment groups having lower mRNA expression than the INT/SAL treatment groups ($P = 0.011$). There was no effect of PGE_2 treatment on the relative abundance of 3βHSD mRNA between any of the treatment groups ($P = 0.650$; Figure 6-7).

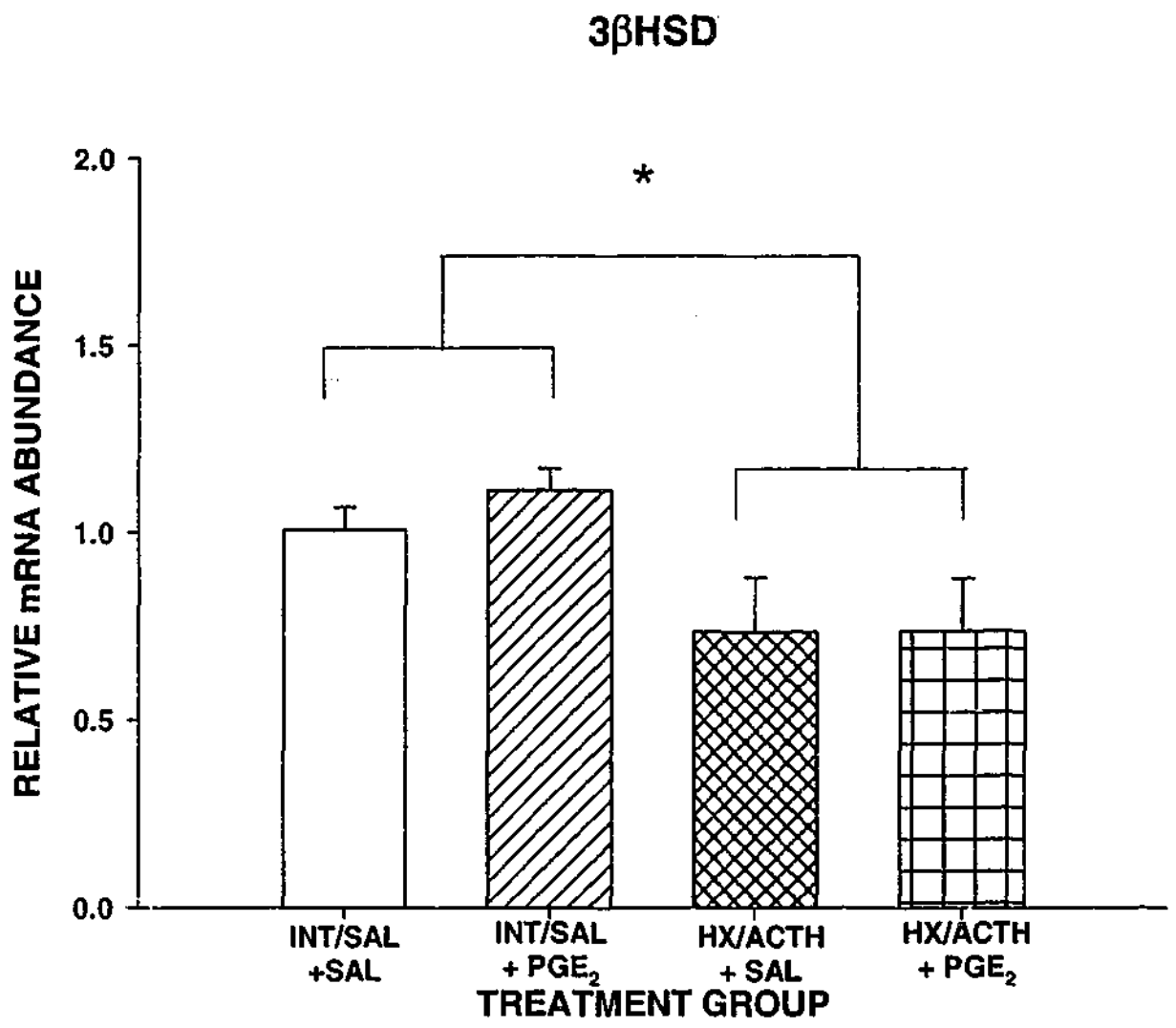

Figure 6-7 Relative expression of adrenal 3βHSD mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There was a significant effect of operation as the abundance of the mRNA encoding 3βHSD was lower in the HX/ACTH group when compared to the INT/SAL treatment groups (P = 0.011). There was no effect of PGE_2 treatment on the expression of 3βHSD mRNA between any of the treatment groups.

6.6.8. P450c17

There was no effect of either operational status (P = 0.653) or PGE_2 treatment (P = 0.932) on the relative expression of P450c17 mRNA. The data are presented in Figure 6-8.

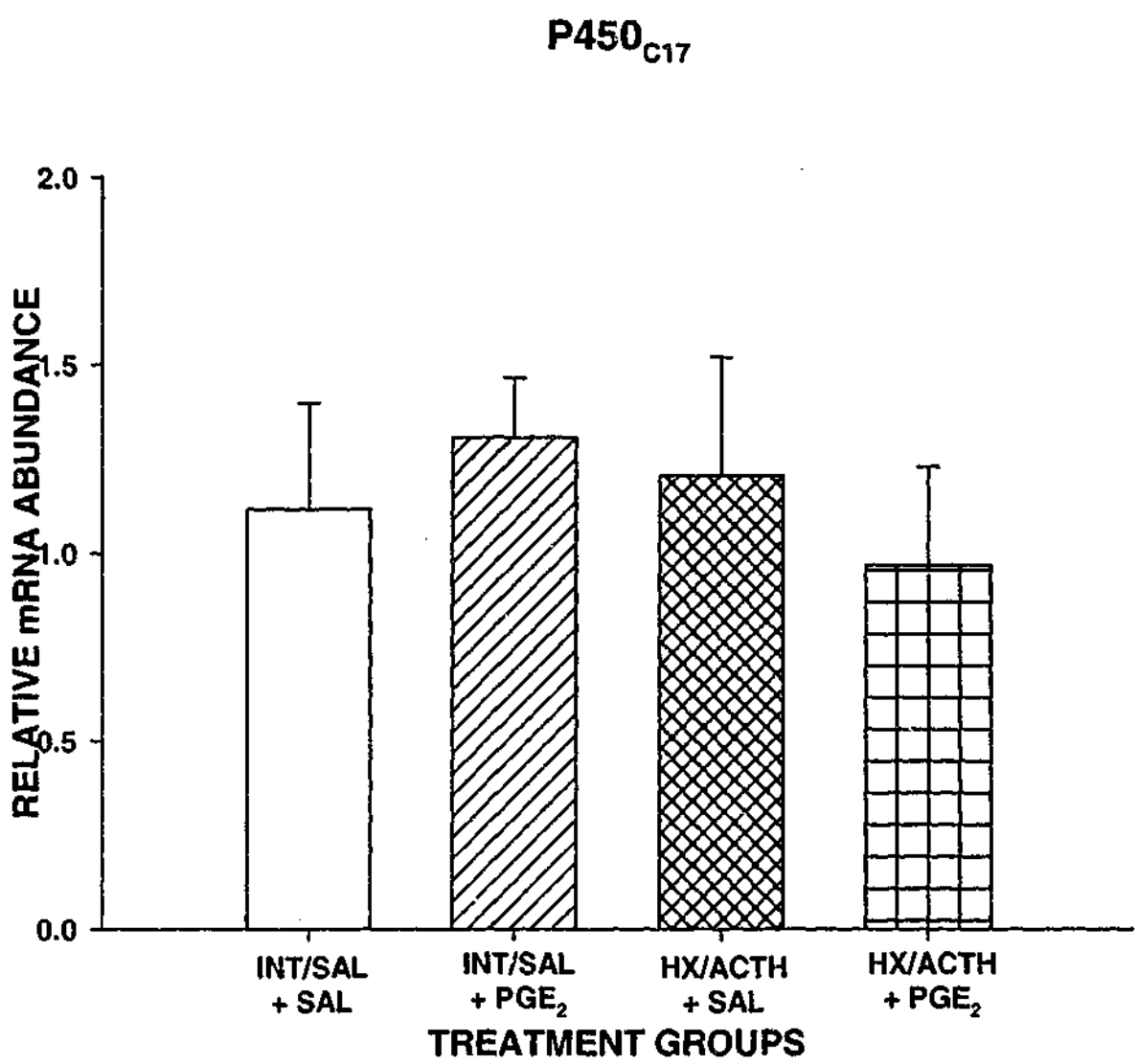

Figure 6-8 Relative expression of adrenal P450c17 mRNA in each treatment group. Results are expressed as mean ± s.e.m. The ratio of the mRNA abundance when compared with the INT/SAL + SAL control group (which is taken as 1) is shown. There were no differences in the expression of the mRNA encoding P450c17 between any of the treatment groups.

6.7. DISCUSSION

There was no effect of a 24 hour infusion of PGE_2 on the relative mRNA expression of any of the genes tested. The relative expression of the mRNA encoding the ACTH receptor and 3βHSD were both significantly decreased in the two HX/ACTH groups when compared to the INT/SAL groups. EP3 mRNA was significantly increased in the HX/ACTH groups in comparison to the INT/SAL groups. None of the other genes tested were affected by the hypophysectomy with ACTH replacement.

These results suggest that the replacement dose of ACTH administered to the HX/ACTH groups was sufficient to maintain the expression of the genes encoding P450scc and P450c17, but were not sufficient to sustain the relative abundance of 3βHSD and the ACTH receptor mRNA levels. This finding is quite interesting in light of previous studies that used either slot blot or northern blot analysis on ovine adrenal glands from HX or HPD fetuses and reported marked decreases in the abundance of the mRNA encoding P450scc, 3βHSD and P450c17 (Phillips *et al.*, 1996; Simmonds *et al.*, 2001). In the study by Simmonds *et al.* (2001), the expression of these three genes were restored to control levels in the HX group when a low dose infusion of ACTH was given, suggesting that these particular steroidogenic enzymes are closely regulated by the presence of ACTH. The ACTH replacement administered in that study was slightly higher than the dose administered in the present study, with a dose of 14.7 pmol/kg/hr versus 13.23 pmol/kg/hr being administered in the respective studies. It would appear from the present study that this lower dose is sufficient to restore P450scc and P450c17 mRNA levels but not 3βHSD mRNA abundance. This result suggests that ACTH tightly regulates 3βHSD and that this enzyme may be an important rate-limiting enzyme in the cortisol synthetic pathway in late gestation. Alternatively, the results could have been due to a type I statistical error, relating to a false positive. The real-time PCR technique used in the current study is far more sensitive to small changes than the previous northern blot method, which may also account for the differences observed.

In the present study and in direct contrast to the earlier study by Simmonds *et al.* (2001), the mRNA encoding the ACTH receptor was decreased in the HX/ACTH groups. In the previous study the mRNA encoding the ACTH receptor did not change with either HX alone or in combination with ACTH infusion. The differences between the two studies

could be accounted for by the different ACTH infusion that was administered, be due to type 1 statistical error or perhaps the differences may be a result of using a more sensitive experimental technique. Simmonds *et al.* (2001) did report that there was an absence of some of the transcripts that had been previously reported using the same probe for this mRNA, and that absence may have been due to a lack of sensitivity of the technique used in that study. In the present study, real-time PCR was employed, a technique that is far more sensitive to small changes in mRNA abundance. It is quite interesting that the ACTH receptor mRNA abundance was decreased in both the HX/ACTH groups, suggesting that this gene is regulated, at least in part, by some other pituitary dependent factor that is not ACTH. The results of this study do not preclude the possibility that a higher dose of ACTH may actually restore the levels of this mRNA back to control levels. Previous reports in human and rat adrenal tissue have shown that treatment with ACTH can increase the abundance of ACTH receptor mRNA (Morita *et al.*, 1995). Interestingly, the ACTH infusion restored plasma cortisol concentrations to normal levels suggesting that perhaps increased ACTH receptor number is not required for normal cortisol secretion. In this study we have only investigated the mRNA expression of these genes. Changes in mRNA expression may not be reflected in protein expression or protein activity.

Although there were no differences in the abundance of the four prostaglandin E_2 receptors between the treatment groups, the presence of each of the EP receptors was detected in the fetal adrenal gland. The present study confirms several reports that have previously shown prostaglandin E binding sites in the adrenal however the present study cannot elucidate the location of the EP receptors (Karaplis & Powell, 1981a, 1981b; Csukas *et al.*, 1998). Due to an absence of a clear line of demarcation between the medulla and cortex in the fetal adrenal gland, the separation of the two adrenal zones was not attempted in the present study to ensure a consistent result between individual animals. The presence of PGE binding sites has been examined in two studies by Karaplis & Powell (1981a, 1981b) and both have reported the presence of the EP receptors in the fetal adrenal gland. In these studies, most of the binding sites were present in the adrenal medulla, however there was a small proportion of binding sites located in the cortex of ovine, calf and human fetal adrenal glands (Karaplis & Powell, 1981a, 1981b). In addition, Csukas *et al.* (1998) reported that a selective EP2 antagonist in cultured bovine adult zona glomerulosa cells inhibited PGE_2 – mediated aldosterone release demonstrating that there are EP receptors in the adrenal gland outside the medulla.

Due to a large abundance of prostaglandin binding sites in other regions of the adrenal gland, any small changes in the abundance of the mRNA encoding the EP receptors in the cortex in response to either prostaglandin or HX/ACTH treatment may have been obscured. Previous studies have reported that in hypophysectomised fetuses there are no changes in the area of the adrenal occupied by cells that contained either adrenaline or noradrenaline, nor is there any change in the morphology of the fetal adrenal medulla when compared to intact fetuses (Coulter *et al.*, 1989).

Rainey *et al.* (1991a) investigated the effects of PGE_2 treatment on bovine adrenocortical cells and measured the abundance of P450c17 and 3βHSD mRNA and protein by northern blot and protein immunoblot analysis. They also measured the binding of ACTH to these adrenocortical cells after pre-treatment with PGE_2. They reported that chronic treatment with PGE_2 *in vitro* led to an increase in the expression of the mRNA and protein of both P450c17 and 3βHSD and that the response was dose dependent. Furthermore, they showed that ACTH receptor binding was also increased after PGE_2 treatment suggesting an up regulation in the number of receptors. Rainey *et al.* (1991a) treated the cells for 3 days with PGE_2, as opposed to 24 hours in the present study and used concentrations of PGE_2 ranging from 0.1 – 10 μM with the largest increases in mRNA and protein expression being observed at the highest dose, which may account for the differences observed in the two studies. The Rainey *et al.* (1991a) study could indicate that the infusion used in the present study may have needed to be longer to observe differences in the mRNA of the genes of interest. Li *et al.* (1993) reported that in cultured porcine granulosa cells, PGE_2 could increase the abundance of 3βHSD and P450scc mRNA approximately 2-fold after a 12 hour incubation, suggesting that a 24 hour infusion period should be adequate time for an increase in the abundance mRNA of the steroidogenic enzymes. It is unknown if any of the genes of interest increased transiently during the infusion period and had returned to control levels by the end of the 24 hour infusion. The dose administered to the culture medium by Li *et al.* (1993) was between 0.5 and 5 μg per well and while the local PGE_2 concentrations in the fetal adrenal were unknown during the infusion period, the total circulating plasma concentrations were approximately 9 μg (25 nM).

Elvin *et al.* (2000) infused growth differentiation factor-9 into a mouse ovarian granulosa cell culture system and observed an up-regulation of EP2 mRNA after 4 hours that

continued to increase over the 24 hours in culture. This suggests that the 24 hour infusion of PGE_2 used in the present study should have been adequate for the up-regulation of at least EP2 and perhaps all of the EP receptors. In addition, the infusion of the EP2 agonist, butaprost, over the same time frame as the growth differentiation factor-9, had no effect on the abundance of EP2 mRNA, suggesting that EP2 at least, is not regulated by its own ligand (Elvin *et al.*, 2000). Furthermore, the inclusion of a COX-2 inhibitor in the incubation also had no effect on the abundance of EP2 mRNA expression (Elvin *et al.*, 2000). The results of the present study suggest that none of the EP receptors in the ovine fetal adrenal are regulated by PGE_2.

It is possible that a 24 hour infusion of PGE_2 may not have allowed enough time for changes in the turnover of the mRNA encoding the genes of interest. It is unknown if a longer time frame or a higher dose of PGE_2 may have caused changes in the expression of these genes of interest. There was a non-significant trend for PGE_2 infusion to increase the abundance of EP2 and EP3. If more animals were performed these results may reach significance, as the expression between animals was variable.

In chapters three and four, PGE_2 was shown to increase cortisol secretion, although the receptor through which PGE_2 acts was uncertain. In this chapter we have examined the mRNA of the prostaglandin E receptors and several steroidogenic enzymes to elucidate if PGE_2 could regulate these genes. The INT/SAL + PGE_2 group had increased plasma concentrations of ACTH, cortisol and PGE_2 when compared with the INT/SAL + SAL group, yet there was no difference between the mRNA in this group and the INT/SAL + SAL group. The HX/ACTH + SAL group had similar plasma concentrations of ACTH, cortisol and PGE_2 to the INT/SAL + SAL group, while the HX/ACTH + PGE_2 group had elevated cortisol concentrations in comparison with the INT/SAL + SAL group. The two HX/ACTH groups both had decreased levels of the ACTH R and 3βHSD mRNA when compared to the INT/SAL groups and increased EP3 mRNA, suggesting that the ACTH replacement was sufficient to maintain the levels of P450scc and P450c17 but not 3βHSD and the ACTH R. The increase in EP3 mRNA in response to HX with ACTH replacement is a novel finding and may suggest some alteration in adenylate cyclase activity, as EP3 is known to act through a second messenger system to decrease AC activity (Narumiya *et al.*, 1999). As PGE_2 did not influence the mRNA of any of the genes of interest and the PGE_2

concentrations in the fetal plasma were supraphysiological, it may suggest that PGE_2 does not influence these genes except at higher pharmacological doses than those used in the present study. The mechanism of PGE_2-stimulated cortisol secretion still remains unclear.

Hypophysectomy removes all pituitary-derived hormones from the circulation, and in the present study only ACTH was replaced. As there was no change in the concentration of the EP receptors, P450scc and P450c17 with HX and ACTH replacement, a role for all the other pituitary derived hormones in the regulation of these genes can be excluded.

In this chapter, using real time PCR it has been demonstrated that the mRNAs encoding the four prostaglandin E receptor subtypes are present in the fetal adrenal gland, but apart from EP3, they are not influenced by either hypophysectomy with ACTH replacement, or by a 24 hour infusion of PGE_2. As has been previously shown, the mRNA encoding the ACTH receptor, P450scc, P450c17 and 3βHSD are present in the fetal adrenal gland, and we now show that they are not influenced by a 24 hour infusion of PGE_2. The abundance of P450scc and P450c17 are not altered when hypophysectomy occurs, if a replacement infusion of ACTH is administered, however, the abundance of the ACTH receptor and 3βHSD mRNA were not restored to control levels in the HX/ACTH groups.

Chapter 7 GENERAL DISCUSSION

The central aims of this thesis were to determine if PGE_2 could act directly at the late gestation ovine fetal adrenal gland to cause cortisol secretion *in vitro* (chapter 3), *in vivo* (chapter 4), to investigate whether the PGE_2 receptor subtypes were present in the adrenal and to examine if PGE_2 regulates the abundance of the mRNAs encoding these receptors, the ACTH receptor or key steroidogenic enzymes (chapter 6). I have investigated the interactions between the fetal adrenal gland and prostaglandin E_2 and have demonstrated that exogenous PGE_2 is able to act directly at the level of the adrenal gland to cause cortisol secretion (See Figure 7-1). The data from this thesis are consistent with the suggestion that PGE_2 does have a stimulatory action at the adrenal and may play a role at both the pituitary and adrenal cortex in the surge in cortisol preceding labour.

The suggestion that a factor other than ACTH contributes to the surge in cortisol in late gestation is not a new one, as many scientists have proposed the involvement of numerous factors including angiotensin II, CRH, HMW ACTH precursors, prolactin and PGE_2. This thesis has investigated the role of only one such factor that can stimulate cortisol secretion. ACTH plays an important, but permissive role in the cortisol surge as perturbation of secretion of ACTH by HX or HPD abolishes the cortisol surge and an increase in the secretion of ACTH causes a premature increase in cortisol (Liggins *et al.*, 1967; Liggins, 1968; Poore *et al.*, 1999). Since Poore *et al.* (1998a) demonstrated that HX and HPD fetuses infused with a chronic, low dose infusion of ACTH have a normal cortisol surge and timely labour, it can be argued that increases in ACTH are not a pre-requisite for the cortisol surge, but instead that ACTH plays only a priming or maintenance role to keep the adrenal gland active and functioning, while another factor drives the cortisol surge. PGE_2 has been shown to have many interactions with the HPA axis and has been shown to be a positive stimulator of both ACTH and cortisol secretion (Liggins *et al.*, 1982; Hollingworth *et al.*, 1995; Young *et al.*, 1996a; Young *et al.*, 1996b).

Using *in vitro* techniques, it was shown that PGE_2 could stimulate cortisol secretion from both the fetal and adult adrenal gland. Following these *in vitro* studies, it was established that PGE_2 could stimulate both ACTH and cortisol secretion in intact fetuses *in vivo*. In hypophysectomised fetuses that had received a constant, continuous, low-dose infusion of ACTH, the increase in cortisol secretion in response to PGE_2 occurred without an increase in ACTH suggesting that *in vivo*, exogenous PGE_2 can directly stimulate cortisol secretion from the fetal adrenal. Finally, using real-time kinetic PCR it was demonstrated that the four prostaglandin E receptor subtypes were present in the fetal adrenal gland but that increased cortisol secretion was not related to their expression or to that of key steroidogenic enzymes.

7.1. *In Vitro Studies*

The data presented in chapter three showed that the fetal adrenal responded to PGE_2, but not ACTH. This finding was unexpected but when the recovery of the agonists was examined 100% of the PGE_2 administered was recovered, while only 35% of the ACTH was recovered, suggesting that the fetal adrenal slices were stimulated by approximately 3.5×10^{-8} M. At this dose, Zambrano *et al.* (2001) also reported no response to ACTH in fetal adrenal cells. In contrast, the adult adrenal slices did respond to this dose, suggesting that the adult adrenal is more responsive than the fetal adrenal to ACTH. Further investigation is required to determine when the adrenal sensitivity to ACTH reaches adult levels. Madill and Bassett (1973) investigated the corticosteroid response to ACTH in late gestation, postnatal and adult ovine adrenal glands. They reported that adrenal tissue taken from fetuses at less than 137 d GA had a very small response to ACTH (< 2μg/100 mg tissue/3 hr), those at 145 d released 6.8 μg/100 mg tissue/3 hr and postnatal lambs less than 1 day old released 12.3 μg/100 mg tissue/3 hr, suggesting that the increase in adrenal sensitivity to adult levels may occur at birth (Madill & Bassett, 1973).

The *in vitro* study presented in chapter three used a primary explant culture, as Dazord *et al.* (1974) reported that co-incubation of adrenal cells with trypsin stopped PGE_2 binding. Cells were not dispersed in the present study and slices of adrenal glands were used instead, so this confounding problem was avoided. The trypsin-inhibition of PGE_2 binding may explain some of the inconsistency of results that has been seen in past studies when PGE_2 was administered to adrenal cells *in vitro*. (Durand *et al.*, 1981a; Chen & Nathanielsz, 1997)

It would be interesting to do a more comprehensive dose response curve to ACTH and PGE_2 to determine the range of sensitivity of the adrenal slices at a variety of ages, including postnatal lambs. The perifusion system used in chapter 3 would also be a good method to examine if other cortisol secretagogues could act at the fetal adrenal gland directly.

7.2. *IN VIVO STUDIES*

Chapter 4 demonstrated that a 24 hour exogenous infusion of a moderately supraphysiological dose PGE_2 into the fetus elicited a significant, sustained increase in cortisol concentrations. The increase occurred in the HX/ACTH fetuses without a measurable increase in ACTH concentrations. This is quite important as it confirms the finding *in vitro* that an increase in cortisol can occur without an increase in ACTH and offers an alternative stimulus for to the cortisol surge, as ACTH has not been shown to increase before the increase in cortisol in late gestation.

It would be interesting to extend the PGE_2 infusion until the animals went into labour, to determine if the infusion of PGE_2 would advance the timing of labour in either the intact or HX/ACTH fetuses. This proposed study could further elucidate the role of prostaglandin E_2 on the cortisol surge and the timing of labour. If the timing of the cortisol surge and labour was advanced in HX/ACTH/PGE_2 fetuses with respect to INT/SAL/SAL fetuses this would provide further evidence that ACTH plays only a permissive role in the timing of labour and that PGE_2 may help cause the increase in cortisol seen in late gestation.

The dose of PGE_2 administered in the *in vivo* studies presented in chapter 4 was supraphysiological. It is unclear if cortisol concentrations would still increase if a lower, physiological dose of PGE_2 was administered. It is possible that the increase in cortisol seen in chapter 4 was the result of supraphysiological concentrations of PGE_2 being administered and therefore the response was a pharmacological effect as opposed to a physiological one.

7.3. MOLECULAR STUDIES – EP RECEPTOR EXPRESSION

In chapter 6, the presence of the EP receptors in the fetal adrenal gland was demonstrated. In recent years, selective agonists for some of the EP receptors and a selective antagonist for EP1 has been developed and are commercially available through Cayman Chemical (EP1, EP2 agonists), Schering (EP1/3 agonist) or Wellcome (EP1 antagonist). When a full range of selective agonists and antagonists are available it may be possible to further delineate the mechanism through which PGE_2 acts to stimulate cortisol secretion. The EP receptors are not present in very high numbers in the adrenal, and have previously been reported to be present in greater numbers in the medulla (Karaplis & Powell, 1981a, 1981b). It is unclear from the studies presented in this thesis, if the PGE_2 receptors are present in the fetal adrenal cortex, or if the stimulation of cortisol secretion is via some indirect pathway through the medulla. This latter suggested mechanism is possible, however the adrenal slices in chapter 3 responded to PGE_2 within 10 to 20 minutes after administration of the agonist, so if the action of PGE_2 is mediated via the medulla, this indirect stimulation of the cortex occurs rapidly. In addition to sensitivity problems with techniques that do not amplify the signal, the confounding problem of the inability to carry out a uniform, total separation of the fetal adrenal cortex from the medulla makes localisation of the specific EP receptor subtypes somewhat difficult. In the future, it may be possible to localise and identify these sparsely populated receptors to the adrenal cortex, for example by *in situ* hybridisation.

There may have been changes in the various EP3 isoforms but as they were not separated in this study, further investigations will need to be conducted to examine their regulation and to determine if all of the isoforms are decreased by HX with ACTH replacement and if the decrease in this mRNA is greater in HX/SAL fetal adrenal glands.

It is unclear if a different dose, or a longer infusion period of PGE_2 was administered that a change in the mRNA abundance of either the EP receptors or key steroidogenic enzymes might have occurred. There is little information regarding the regulation of the EP receptors and this may be an avenue for further research. This chapter only investigated the mRNA levels of the expression of the EP receptors and key steroidogenic enzymes. It may be of interest to investigate protein level and enzyme activity of the EP receptors and key

steroidogenic enzymes, respectively to determine if any changes are observed with a PGE_2 infusion on those states.

The studies in this thesis have provided support for the proposal that PGE_2 may regulate fetal adrenal cortisol production but do not identify PGE_2 as the trigger for the cortisol surge. It is possible, that no factor alone is responsible and the surge results from the interaction of a number of hormonal and biochemical changes in the fetus and placenta. This thesis has examined the role of exogenous administration of PGE_2, which does not allow for conclusions to be made on the role endogenous PGE_2 may perform in late gestation. Further experimentation is required to elucidate the role of endogenous PGE_2 on the surge in cortisol.

In the past, infusions of inhibitors of PGE_2 synthesis (COX inhibitors) such as indomethacin have been administered, but because of the many actions of PGE_2, such as maintaining the patency of the ductus arteriosus and the role in fetal hemodynamics, such inhibitors have introduced confounding effects on fetal well being. More recently, selective COX-1 and COX-2 inhibitors have been developed which may allow long-term infusions, without the side effects seen with non-selective inhibitors. If a selective and specific COX-2 inhibitor can be infused for a number of weeks in the late gestation intact and hypophysectomised fetus with and without PGE_2 replacement, the role of PGE_2 in the cortisol surge may be determined.

PGE_2 may play a pivotal role in the initiation of parturition, or may be just one of a number of agents that aids in elevating cortisol concentrations in spite of negative feedback. Endogenous PGE_2 may act through stimulating ACTH release either directly from the fetal pituitary or indirectly via the hypothalamus, and as shown for the first time in this thesis, by stimulating cortisol secretion directly from the fetal adrenal.

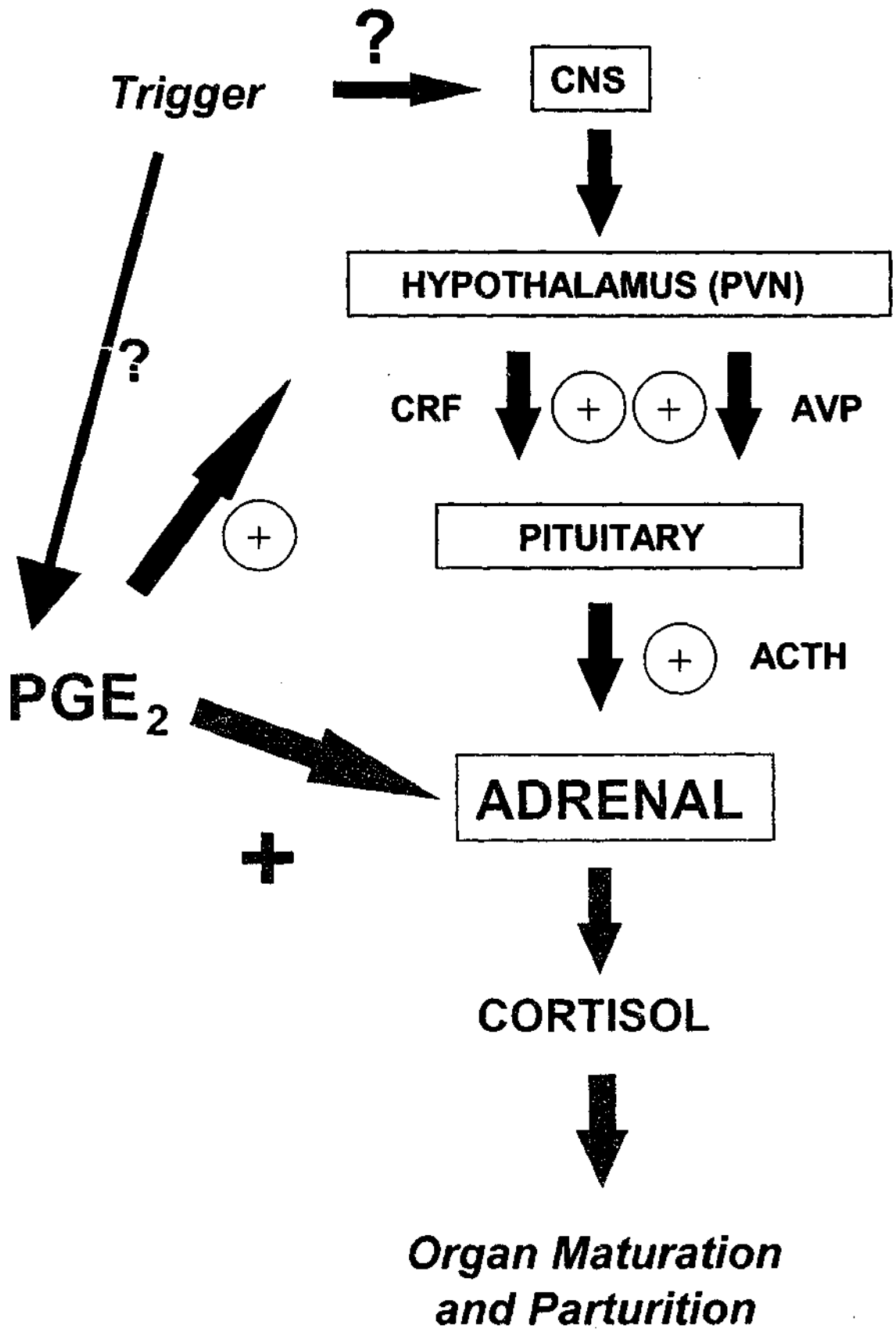

Figure 7.1 Schematic Diagram of the hypothalamo-pituitary-adrenal axis and the central finding of this thesis – PGE_2 can directly stimulate the ovine fetal adrenal gland to secrete cortisol.

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