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Studies on Adrenocortical Function in Cattle

A thesis

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by

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PREFACE

I dedicate the small drops of knowledge that these pages contain to the vast ocean. It is my hope that the future will see them become part of a tide which enables understanding for the benefit of all.

ABSTRACT

The hypothalamo-pituitary-adrenal (HPA) axis acts an integrator which recognises challenges to survival and initiates an appropriate neuroendocrine response with the intent of maintaining homeostasis. The end-point of the response is secretion of glucocorticoids by the adrenal cortex. The aim of these studies was to develop understanding of factors which may modify adrenocortical function in dairy cattle.

Cows, bulls and steers were treated with a range of doses (0.0125 to 0.4 mg/500 kg live-weight) of an adrenocorticotropic hormone (ACTH). Peak plasma cortisol concentrations (PCC) following ACTH were independent of dose except at the lowest dose; higher doses had a more prolonged effect. There was a linear relationship between the integrated adrenocortical response and $\log_{10}$ dose of ACTH. In post-pubertal bulls, mean peak PCC were less (68.4 ± 2.0, 72.5 ± 4.0 and 43.8 ± 2.4 ng/ml in cows, steers and bulls, respectively; $P<0.001$). Measurement of PCC in samples at 0, 50 and 120 min in relation to iv administration of 0.05 mg ACTH/500 kg live-weight was defined as an adrenal response test (ART) for assessment of adrenocortical function.

Treatment of cows with a range of doses (6.25 to 25 µg/500 kg live-weight) of synthetic bovine corticotropin-releasing hormone (bCRH) demonstrated that peak plasma ACTH concentrations (PAC) and PCC, as well as integrated pituitary but not adrenocortical responses were influenced by dose. There was a linear relationship between integrated pituitary responses and dose of bCRH, and between integrated adrenocortical responses and $\log_{10}$ PAC at 15 min after treatment. Injection of bCRH with measurement of PAC and PCC is suggested as a test of pituitary-adrenal function in cattle. Injection of bacterial lipopolysaccharide was investigated as a test for HPA axis integrity in cattle, but found to be unsuitable because results were highly variable.

There was a significant linear decline in results of ART during the third trimester of pregnancy. They also declined in bulls from 6 to 17 months of age and were low in bulls tested at 22 months and 5 years of age. Injection of dexamethasone suppressed results of ART for at least 17, 71 and 66 days in cows treated with sodium phosphate, trimethyl-acetate and 21-

isonicotinate formulations, respectively. Suppression lasted 9 and 16 days in bulls and steers, respectively, with dexamethasone sodium phosphate. Results of ART were suppressed until at least 3 weeks post-partum following dexamethasone-induced parturition. Plasma concentrations of progesterone, oestrone sulfate and prolactin followed a similar temporal pattern to those seen in naturally calving cows. Plasma α-lactalbumin concentrations were initially lower in more premature cows but were similar at parturition; plasma immunoglobulin G1 concentrations declined at a greater rate in more premature cows.

These studies have demonstrated that aspects of adrenocortical function in cattle are unique. There is marked sexual dimorphism in adrenocortical response to ACTH and responses may be further reduced with increasing maturity. The role of the adrenal cortex for the maintenance of metabolic homeostasis during stress may not be as critical in cattle as has been identified in other species; alternative mechanisms of response to stress may be more important.

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Verkerk, G. A., Macmillan, K. L., McLeay, L. M. (1994). Adrenal cortex response to adrenocorticotropic hormone in dairy cattle. *Domestic Animal Endocrinology*, 11: 115-123.

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List of Publications Arising from this Thesis

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Verkerk, G. A., Macmillan, K. L., McLeay, L. M., 1992. The effect of dexamethasone suppression on adrenocorticotropin response testing (ART) in the bovine. *Proceedings New Zealand Society of Endocrinology,* NZ26.

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McFadden, T. B., Hovey, R. C., Clark, B. A., Verkerk, G., 1994. Effect of induced premature calving on mammary development of dairy cows. *Journal of Dairy Science,* 77 (Suppl. 1): 226.

McFadden, T. B., Verkerk, G. A., Clark, B. A., Hovey, R. C., Macmillan, K. L., 1995. Effects of induced parturition on mammary function. *Proceedings of the New Zealand Society of Animal Production,* 55: 21-22.

List of Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase
aAUC	area under the plasma adrenocorticotropic hormone concentration.time curve
ACTH	adrenocorticotropic hormone
$ACTH_{1-24}$	adrenocorticotropic hormone consisting of the 24 N-terminal residues
$ACTH_{1-39}$	native adrenocorticotropic hormone
α-lac	alpha-lactalbumin
α-MSH	melanotropin stimulating hormone
ANOVA	analysis of variance
AP-1	activated protein-1
AR	androgen receptor
ART	adrenal response test
AUC	area under the curve
AVP	arginine vasopressin
bCRH	bovine corticotropin-releasing hormone
BNST	bed nucleus of the stria terminalis
BOH	β-hydroxy-butyrate
cAUC	area under the plasma cortisol concentration.time curve
CBG	corticosteroid binding globulin
CLIP	corticotropin-like intermediate lobe peptide
CNS	central nervous system
CRH	corticotropin-releasing hormone
cs	condition score

CV	coefficient of variation
ΔT	change in rectal temperature
DHEA	dehydro-epiandrosterone
DHEA-S	dehydro-epiandrosterone sulfate
DNA	deoxy-ribonucleic acid
DRC	Dairying Research Corporation
ELISA	enzyme-linked immunosorbent assay
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
GLM	general linear model
GnRH	gonadotrophin-releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotropin
HF	Holstein-Friesian
HPA	hypothalamo-pituitary-adrenal
hsp90	heat-shock protein 90
IGG1	immunoglobulin G1
im	intramuscular
IRMA	immuno-radiometric assay
iv	intravenous
J	Jersey
LH	luteinising hormone

LH-RH	luteinising hormone-releasing hormone
LIC	Livestock Improvement Corporation
LPS	lipopolysaccharide
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MW	molecular weight
[OS:P4]	oestrone sulfate:progesterone ratio
$P450_{C11}$	P450 cytochrome enzyme 11β-hydroxylase
$P450_{C17}$	P450 cytochrome enzyme 17α-hydroxylase
$P450_{C21}$	P450 cytochrome enzyme 21-hydroxylase
$P450_{scc}$	P450 cytochrome cholesterol side-chain cleavage enzyme
PAC	plasma adrenocorticotropic hormone concentration
PCC	plasma cortisol concentrations
PG	prostaglandin
PGFM	13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
POMC	pro-opiomelanocortin
PTC	plasma testosterone concentrations
PVN	paraventricular nuclei
RBC	red blood cells
RFM	retained foetal membranes
RIA	radio-immunoassay
RT°C	room temperature
sed	standard error of difference

sem	standard error of mean
std	standard deviation
Ttp	time to peak
VIP	vasoactive intestinal peptide
WBC	white blood cells

CHAPTER 1

INTRODUCTION

When Bartholomaeus Eustachius Sanctoseverinatus first recognised the adrenal gland as an organ in 1563, he could not have guessed that it would later shape medical philosophy because of its fundamental position in the stress response. The ability of an organism to cope with an immense variety of challenges or stressors, is essential for survival. The popular definition of stress relates to its use as a description of the state of the animal involved; yet after many decades of stress research, no acceptable stringent definition of the word has been achieved. The concept of stress requires an input by a stimulus, recognition by the organism that the stimulus represents a threat, and subsequent organisation of the stress response. The nature, duration and severity of stressors vary widely. Responses to these stressors have the overall intent of maintaining homeostasis, and may be behavioural, autocrine or neuroendocrine.

The hypothalamo-pituitary-adrenal (HPA) axis acts as an integrator for the recognition of challenge. The secretion of glucocorticoids by the adrenal cortex is the end-point of the neuroendocrine stress response. Glucocorticoids have a wide range of actions affecting many aspects of bodily function including metabolism, inflammation and immunity. Glucocorticoids control their own synthesis and release by completing negative feedback loops to higher levels of the HPA axis.

Apart from its role in the neuroendocrine stress response, the adrenal cortex of cattle has been investigated for its role in parturition and lactation and for its relationship to reproductive function. Recently the role of the HPA axis in relation to issues of animal welfare has been investigated. Increased understanding of the integrative role of the HPA axis in mediating many body functions has created a need to revisit the role of the axis in cattle. There is also concern that the practice of induction of premature parturition in dairy cows with synthetic glucocorticoids by many owners of New Zealand dairy herds may compromise their welfare.

AIMS OF THE RESEARCH

- To investigate some of the mechanisms which regulate function of the adrenal cortex in cattle;
- To investigate how physiological factors such as the presence of gonadal steroids, pregnancy and age can influence function of the adrenal cortex; and,
- To investigate changes in function of the adrenal cortex following dexamethasone administration, particularly in relation to induction of premature parturition.

THESIS OUTLINE

Chapters 2 and 3 review the literature - first to provide a general overview of function of the HPA axis and then to present current views specific to cattle. The assays used in the experimental chapters are detailed in Chapter 4. Experiments were conducted to provide a description of adrenocortical responses of cows, bulls and steers to a selected range of doses of adrenocorticotropic hormone (ACTH; Chapters 5 and 6). Chapter 5 also discusses the development and verification of an adrenal response test (ART) which was used subsequently. Later experiments assessed the responsiveness of the pituitary and of the whole HPA axis to challenges with corticotropin-releasing hormone or an endotoxin (Chapter 12).

Several physiological factors which may influence the response of the adrenal cortex to ACTH were investigated. The effect of time of day and repetitive dosing on adrenocortical response were examined as further verification of the ART (Chapter 7). This chapter also records the measurement of basal plasma cortisol concentrations at different months of the year. Changes in the results of ART during puberty and ageing in bulls (Chapter 8) and during pregnancy in cows (Chapter 9) are described.

The ability of the adrenal cortex to respond to ACTH following administration of the synthetic glucocorticoid dexamethasone was investigated. Chapter 10 describes adrenal suppression following administration of several pharmacological formulations of dexamethasone. Changes in adrenocortical responses to ACTH as well as changes in concentration of several circulating

steroids and proteins during induction of parturition with dexamethasone are presented in Chapter 11.

Chapter 13 provides a summary of the knowledge gained from the experiments carried out, and suggests where further research efforts might be concentrated to further our understanding of function of the HPA axis in cattle.

ANIMAL ETHICS COMMITTEE APPROVALS

All experiments described were approved by the Animal Ethics Committees of both AgResearch Ruakura and The University of Waikato, Hamilton, New Zealand.

CHAPTER 2

GENERAL REVIEW OF THE FUNCTION OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS

This chapter presents a general review of the function of the hypothalamo-pituitary-adrenal (HPA) axis. It is intended to cover the body of generally accepted knowledge across species. The literature relating specifically to HPA axis function in cattle is reviewed in the next chapter.

CHAPTER OUTLINE

2.1 Structure of the adrenal gland
- 2.1.1 Gross anatomy
- 2.1.2 Histology
  - 2.1.2.1 The adrenal cortex
  - 2.1.2.2 The adrenal medulla
- 2.1.3 Ontogeny

2.2 The nature of steroid hormones
- 2.2.1 Biochemistry of the steroid hormones
- 2.2.2 Steroidogenesis
- 2.2.3 Regulation of steroidogenesis
- 2.2.4 Steroid receptors
- 2.2.5 Metabolism and excretion

2.3 The function of adrenal steroid hormones
- 2.3.1 Mineralocorticoids
- 2.3.2 Adrenal androgens
- 2.3.3 Glucocorticoids
  - 2.3.3.1 Metabolic actions
  - 2.3.3.2 Actions on tissues
    - 2.3.3.2.1 Immature tissues
    - 2.3.3.2.2 Cells of the immune system
    - 2.3.3.2.3 Nervous tissue

2.3.3.3 Glucocorticoids and the foetus
2.3.4 Synthetic glucocorticoids
2.4 Modes of HPA axis activity
2.4.1 Basal activity
2.4.2 The stress response
2.5 Mechanisms of integrated HPA axis control
2.5.1 The pituitary
2.5.1.1 Adrenocorticotropic hormone (ACTH)
2.5.1.2 Endogenous opioids
2.5.2 The hypothalamus and ACTH secretagogues
2.5.2.1 Corticotropin-releasing hormone (CRH)
2.5.2.2 Arginine vasopressin (AVP)
2.5.2.3 Other neuropeptides and transmitter factors
2.5.2.4 Hypothalamic integration of HPA axis activity
2.5.3 The influence of centres in the central nervous system (CNS)
2.5.4 The role of steroid receptors in the CNS
2.5.5 Feedback control
2.5.5.1 A model for negative feedback control
2.5.5.2 Control in the lower axis
2.5.5.3 Control in the higher axis
2.6 The HPA axis and the adrenal medulla
2.7 The HPA axis and the gonadal axis
2.7.1 HPA axis regulation of the gonadal axis
2.7.2 Gender differences in HPA axis function
2.8 The HPA axis and ageing
2.9 Clinical methods for assessment of function of the HPA axis
2.10 Conclusion

2.1 STRUCTURE OF THE ADRENAL GLAND

2.1.1 Gross anatomy

The adrenal glands are situated retroperitoneally near the kidney surrounded by adipose tissue and the renal fasciae. The adrenal gland is composed of a central medulla which is distinct from the surrounding cortex (Labhart, 1986). These are traditionally regarded as separate entities. More than 90% of adrenal blood flow passes directly to the cortex. Cortical and medullary arteries have separate external origins and form distinct capillary beds. There is no portal system linking the cortex and medulla (Parker *et al.*, 1993).

Extrinsic innervation of the adrenal gland is by preganglionic sympathetic nerve fibres projecting via the splanchnic nerve. These fibres penetrate the capsule then branch. Most supply specific medullary cells but there is also some supply to cortical cells. A few form ganglia in the organ capsule, cortex and medulla from which postganglionic cells extend to blood vessel walls. No parasympathetic fibres are seen (Parker *et al.*, 1993).

2.1.2 Histology

2.1.2.1 The adrenal cortex

A capsule of irregular connective tissue surrounds the adrenal gland and extends as trabeculae into the cortex. A framework of reticular fibres extends between the trabeculae, the capsule and the blood vessels to support the secretory cells. An extensive arterial plexus branches into sinusoidal capillaries which surround cords of parenchymal cells. The sinusoids drain towards the centre of the gland where they merge with sinusoids of the medulla. The capillary endothelium is extremely attenuated and fenestrated so that only a thin diaphragm separates blood from the basal lamina. The cells have numerous microvilli and small infoldings to increase the surface area (Horst-Dieter and Dellman, 1971).

The adrenal cortex is in three concentric zones. The outermost layer is the *zona glomerulosa* which consists of small epithelioid cells, each containing a deeply staining nucleus. The size

and arrangement of the cells is dependent upon species. The cytoplasm is acidophilic with a few basophilic clumps. Small lipid droplets are common and numbers vary with the activity of the cell (Horst-Dieter and Dellman, 1971). Large numbers of polyribosomes and elongated mitochondria with lamellar cristae are distributed throughout the cytoplasmic matrix. The Golgi apparatus is well developed and closely associated with the nucleus (Nussdorfer, 1986).

The *zona fasciculata* is the widest zone. The cell columns of this zone are continuous with the *zona glomerulosa*. Cells are cuboidal or columnar and form long cords, one or two cells thick, arranged radially to the organ capsule. The intracellular structure indicates high metabolic activity and there are large numbers of lipid droplets in the cytoplasm. Agranular endoplasmic reticulum forms an extensive network with a few arrays of granular endoplasmic reticulum near the nuclei (Horst-Dieter and Dellman, 1971). Mitochondria are large and have numerous cristae. The Golgi apparatus is extensive with lysosomes and deposits of lipofuscin pigment often present (Nussdorfer, 1986).

The innermost zone is the *zona reticularis*. This continuation of the *zona fasciculata*, and is an irregular anastomosing network of polyhedral epithelioid cells which vary considerably in size and shape. 'Light' and 'dark' cells can be distinguished. The 'light' cells are similar to cells of the *zona fasciculata* but contain fewer lipid droplets. The 'dark' cells contain shrunken nuclei and large masses of lipofuscin pigment (Nussdorfer, 1986). Lipofuscin granules have been used as a marker of cellular ageing in the adrenal cortex of aged humans and rodents. The level of lipofuscin has been associated with the intracellular content of cholesteryl esters which have been shown to accumulate in an age-related manner (Cheng *et al.*, 1994).

The life history of an adrenocortical cell involves its progressive migration from the outer to the inner zones of the cortex. Cell division occurs under the capsule in the *zona glomerulosa* and possibly also in the outer *zona fasciculata*. The cells are pushed towards the *zona reticularis*, acquiring a increasing burden of lipofuscin as they go, possibly as a result of the production of free radicals during steroidogenesis. Apoptosis occurs in the *zona reticularis* (Nussdorfer, 1986). During this migratory course the cells are exposed to differing steroid gradients which may contribute both to the changes in steroid production and to their eventual degeneration (Hornsby, 1982).

2.1.2.2 The adrenal medulla

The adrenal medulla contains chromaffin cells which stain brown with chromium fixatives. These cells produce either adrenaline or noradrenaline, granules of which are seen in their cytoplasm and can be differentiated on the basis of their affinity for the stain azocarmine. Zonation of the medulla is evident in ruminants but not in other domestic mammals (Horst-Dieter and Dellman, 1971).

2.1.3 Ontogeny

The two components of the adrenal gland have differing embryonic origins. The cortex is of mesodermal origin, developing from coelomic mesothelium, while cells of the medulla are derived from neural ectoderm. Development of the human adrenal gland has been well described. In the 4 week old embryo, mesothelial cells situated between the root of the mesentery and the gonadal anlagen begin to proliferate. They invade the underlying mesenchymal tissue where they differentiate into compact epithelial masses - the primitive or foetal cortex (Labhart, 1986). During the 6th embryonic week, neuroectodermal elements from the sympathetic suprarenal paraganglia start to invade the cortex (Horst-Dieter and Dellman, 1971).

The adrenal gland of the sheep foetus can be recognised by day 28 of pregnancy but zonation is not apparent until after day 60, and no *zona reticularis* is evident during foetal life. Cells of the *zona fasciculata* remain immature until the last third of gestation. By day 130 the *zona fasciculata* is in two cell layers - an inner zone of "mature" cells (approximately 25% of cortical volume) and an outer "immature" zone. Weight of the foetal ovine adrenal gland increases exponentially during the last 15-20 days of gestation. This increase is associated with both hypertrophy of cells and increased deoxy-ribonucleic acid (DNA) synthesis (Challis and Brooks, 1989).

2.2 THE NATURE OF STEROID HORMONES

2.2.1 Biochemistry of steroid hormones

The steroids are structural derivatives of sterane, a cyclopentanoperhydro-phenanthrene nucleus consisting of three hexagonal carbon rings and one pentagonal ring. Steroids include pro-vitamin D, cholesterol, bile salts, and cardiac glycosides as well as hormones of the adrenal cortex, the gonads and the placenta. The steroid hormones of the adrenal cortex and the corpus luteum have 21 carbon atoms, and are derived from pregnane, a sterane with two angular methyl groups and an ethyl side chain. The male sex hormones are derived from androstane with 19 carbon atoms, and the female sex hormones from oestrane with 18 carbon atoms. The terms corticoid and corticosteroid refer to adrenocortical hormones with 21 carbon atoms which influence electrolyte and carbohydrate metabolism, as well as their metabolites with an intact side chain (Labhart, 1986).

Approximately 70 different steroids have been isolated from adrenal glands. The main secretory products of the human adrenal cortex are cortisol, corticosterone and androgens. Aldosterone, although physiologically important, is secreted in relatively small amounts. Human adrenals also secrete testosterone, but there is no direct evidence for secretion of oestrogen (Labhart, 1986).

2.2.2 Steroidogenesis

Cholesterol is the normal precursor of the adrenocortical hormones and can be fully synthesised in the adrenal cortex. Cholesterol esters are stored abundantly in the adrenal cortex but are only partly utilised in steroidogenesis. Cleavage of the cholesterol side-chain to yield pregnenalone is the initial step of the steroidogenic pathway (Fig. 2.1, Step 1). This process is catalysed by the P450 cytochrome cholesterol side-chain cleavage enzyme ($P450_{scc}$) located on the inner aspect of the inner mitochondrial membrane. Two other enzymes located in the mitochondrial matrix, adrenodoxin reductase, a flavoprotein, and adrenodoxin. an iron-sulfur protein, provide electrons for this reaction (Miller, 1988).

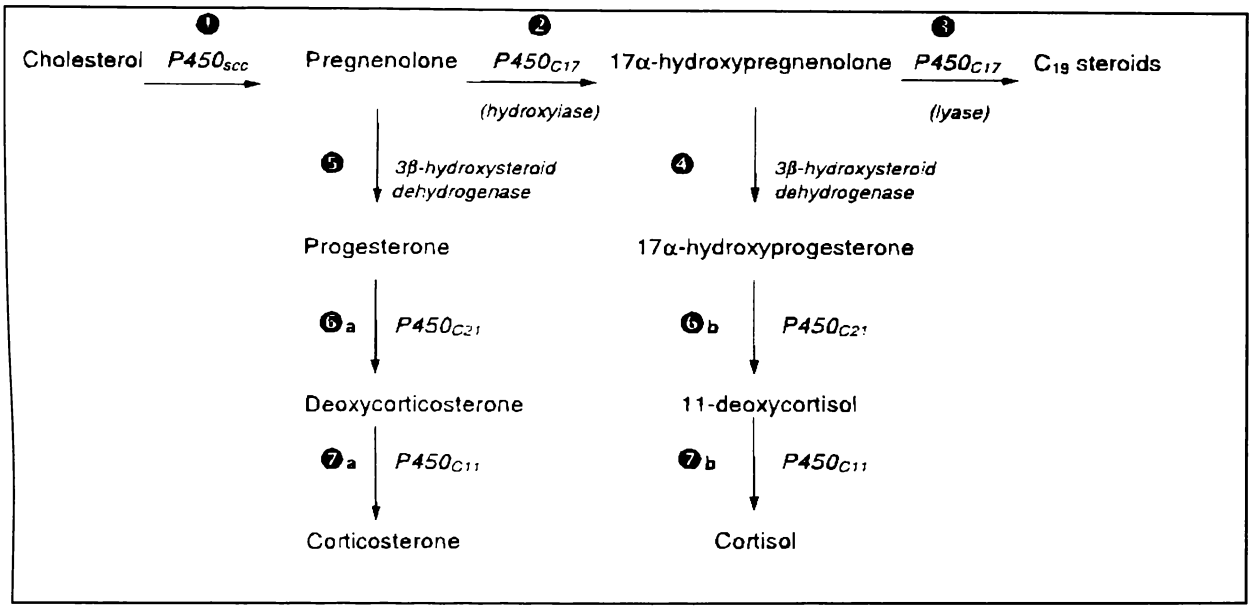


Figure 2.1: Steroidogenic pathways in the adrenal cortex. Numbers refer to steps as defined in text. Enzymes catalysing each step are represented in italics (from Miller, 1988).

The adrenal cortex exhibits functional zonation of further cholesterol processing. Aldosterone is only produced in the *zona glomerulosa*; glucocorticoids and adrenal androgens are exclusively synthesised in the two inner zones where processing is species specific. In the cow, human, sheep and pig, pregnenalone is converted to 17α-hydroxypregnenalone by the P450 cytochrome enzyme 17α-hydroxylase ($P450_{C17}$) located in microsomes (Fig. 2.1, Step 2). This enzyme also has 17,20-lyase activity which can further convert 17α-hydroxypregnenalone to the C_{19} steroids dehydro-epiandrosterone (DHEA) and androstenedione in those species (Fig. 2.1, Step 3). The balance of this dual activity is determined by the availability of electrons supplied by flavoprotein P-450 reductase, a protein present in relatively small amounts as compared to $P450_{C17}$. Increasing availability of electrons will favour lyase activity and yield C19 steroids in favour of 17α-hydroxypregnenalone (Miller, 1988).

Glucocorticoid production requires the hydroxylation of 17α-hydroxypregnenalone to 17α-hydroxyprogesterone by 3β-hydroxysteroid dehydrogenase (3βHSD; Fig. 2.1, Step 4). The adrenals of rats, rabbits, hamsters and guinea pigs do not contain $P450_{C17}$ but convert pregnenalone directly to progesterone, catalysed by 3βHSD (Fig. 2.1, Step 5). This conversion can occur in other species as well (Simpson and Waterman, 1988).

Hydroxylation of progesterone or 17α-hydroxyprogesterone occurs only in the inner zones and is catalysed by the microsomal cytochrome P450 enzyme 21-hydroxylase ($P450_{C21}$; Fig. 2.1, Steps 6a and 6b). This reaction produces deoxycorticosterone or 11-deoxycortisol. These leave the endoplasmic reticulum and re-enter the mitochondria where the P450 cytochrome enzyme 11β-hydroxylase ($P450_{C11}$) catalyses their conversion to corticosterone or cortisol respectively (Fig. 2.1, Steps 7a and 7b; Simpson and Waterman, 1988).

Steroidogenic cells in the gonads possess neither $P450_{C21}$ nor $P450_{C11}$ so that mineralocorticoids and glucocorticoids are not produced in the ovary or testis. The dual activity of $P450_{C17}$ to produce androgens is seen in testicular tissue (Simpson and Waterman, 1988). The intermediate product 17α-hydroxypregnenalone appears to be essential in the biosynthesis of androgenic steroids, as a direct conversion of cholesterol to the C_{19} steroids has never been demonstrated (Labhart, 1986).

2.2.3 Regulation of steroidogenesis

Adrenocorticotropic hormone (ACTH) is the principal regulator of steroid hormone production by cells of the adrenal cortex. It acts in the short-term to increase substrate availability (tropic action) and over a longer period to maintain levels of the steroidogenic enzymes and cholesterol (trophic action). Mobilisation of cholesterol provides for the rapid increase in cortisol secretion in response to ACTH. Binding of ACTH to specific cell-surface receptors activates adenylate cyclase to increase intracellular levels of cAMP. This leads to translocation of cholesterol to the mitochondria from lipid droplets in the cytoplasm. Intravenous injection of ACTH results in immediate depletion of adrenal cholesterol, but long-term treatment induces an increase in cholesterol content. ACTH mediates cholesterol uptake by regulating several other proteins. These include cell surface lipoprotein receptors, 3-hydroxy-3-methylglutaryl coenzyme A reductase which is a rate-limiting enzyme in cholesterol biosynthesis, and sterol carrier protein 2 which transports lipid stores to the mitochondria (Simpson and Waterman, 1988).

The trophic action of ACTH increases the rate of synthesis of adrenodoxin and the steroidogenic P450 cytochromes, particularly that of $P450_{C17}$ which could not be detected in cell

culture in the absence of ACTH. Cyclic AMP also mediates this action of ACTH. Both ACTH and cAMP increase the levels of messenger ribonucleic acid (mRNA) species encoding for steroidogenic enzyme precursors, indicating regulation of transcription. The proposed mechanism utilises a short-lived regulatory protein factor, tentatively named steroid hydroxylase inducing protein (Simpson and Waterman, 1988).

Factors other than ACTH have a role in regulating adrenal cortex activity. Some tissue-specific regulation occurs e.g. $P450_{C21}$ is expressed only in the adrenal cortex (Simpson and Waterman, 1988). $P450_{C11}$ exists in its reductive form for steroidogenesis in adrenal cortex tissue, but is more usually expressed in its oxidative form e.g. in hepatocytic microsomes (Monder and White, 1993). Calcium ions regulate corticosteroid production, probably as a component in the ACTH-activation pathway. They may also modulate adrenal androgen production. *In vitro* experiments with human adrenocortical cells demonstrated that Ca^{++} increased basal cortisol output but not that of androstenedione or DHEA. Following ACTH stimulation, Ca^{++} increased both cortisol and androstenedione output, but not DHEA (Fearon *et al.,* 1993).

Steroid levels themselves exert a controlling influence on enzyme activity in adrenocortical cells. Direct addition of secretory products to primary bovine adrenocortical cell cultures influenced both $P450_{C21}$ and $P450_{C11}$ activity. This action is likely also to occur *in vivo* and may provide a mechanism to establish zonation of steroid production. Androgen levels suppressed $P450_{C21}$ activity in particular, but not $P450_{C17}$ activity (Hornsby, 1982).

ACTH influences structural features of adrenocortical cells. Electron microscopy studies of adrenocortical cells have identified increases in the length of microvilli and in the amount of the cytoskeletal protein in cells of the *zona fasciculata* following ACTH stimulation. This may increase cell surface area and enhance release of steroids (Loesser *et al.*, 1994).

Sequestration of biosynthetic enzymes in cytoplasmic organelles provides spatial isolation for the steroidogenic pathway. Sequestration is seen in the testicular Leydig cell where luteinising hormone maintains cytoplasmic organelle structure for testosterone synthesis. The model of the Leydig cell where the drive of the trophic hormone controls cellular integrity could have

parallels in the adrenocortical cell. Spatial organisation of enzyme systems confers some important regulatory consequences:

- sequestration of enzymes in membranes facilitates the interaction of hydrophobic steroid molecules in the aqueous milieu;
- a membrane bound multi-enzyme sequence in a highly ordered state limits leakage of biosynthetic intermediates;
- separation of competing steroidogenic reactions in space limits the potential products; and,
- control points are established when a substrate is separated in space from the enzyme which catalyses its conversion (Ewing and Zirkin, 1983).

2.2.4 Steroid receptors

Steroid hormone receptors are members of an extended family of intracellular proteins which include receptors for thyroid hormone and vitamin A derivatives. Specific receptors for progesterone, androgens, mineralocorticoids and glucocorticoids belong to the subfamily of classical steroid hormone receptors. Receptors for hormones secreted by the thyroid gland form a second subclass, while the oestrogen receptor is classified alone (Williams and Franklyn, 1994).

Intracellular receptors have three functional domains:

a) a COOH-terminal ligand-binding sequence which is highly variable across the family, but with about 50% identity in the steroid receptor subfamily;

b) a DNA binding domain of 66 amino acids which form two folds known as the 'zinc fingers', with less than 40% identity across the family, and with 90% identity in the steroid receptor subfamily (only 4 to 6 of the 66 amino acids differ between the four members); and,

c) an NH_2-terminal sequence which is highly variable and which interacts with DNA once binding has occurred (Funder, 1993).

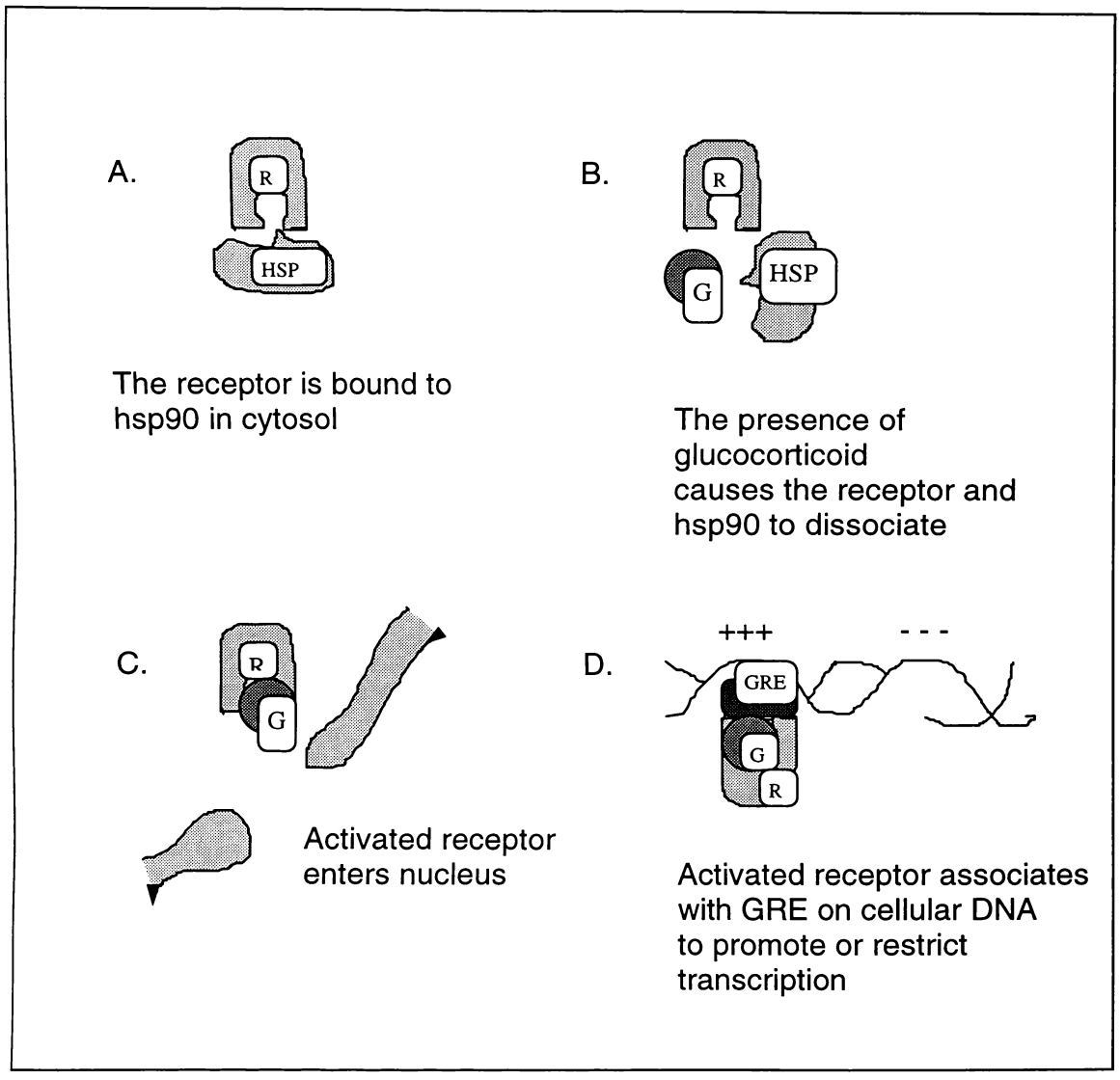


Figure 2.2: Schematic representation of the action of glucocorticoids via their specific intracellular receptors (from Arai and Chrousos, 1994).

Legend:	R	receptor
	HSP	heat-shock protein 90
	G	glucocorticoid
	GRE	glucocorticoid response element

The human glucocorticoid receptor (GR) is a 777-amino acid protein. The inactivated receptor is found in the cytoplasm as a complex with other proteins. The most important of these proteins is heat-shock protein 90 (hsp90; Fig. 2.2) which prevents unoccupied receptor l ocalising in the nucleus and may facilitate ligand binding. Binding of the ligand releases the receptor from hsp90 allowing access to the nucleus (Arai and Chrousos, 1994). There is a single class of GR which is expressed in almost every mammalian cell, with no evidence for

differing affinity in different tissues. The mineralocorticoid receptor (MR) also recognises glucocorticoids with high affinity (Barnes and Adcock, 1993).

Inside the nucleus, GRs exert their effects by either positive or negative regulation of gene transcription following interaction with specific DNA sequences in glucocorticoid-responsive genes known as glucocorticoid response elements (GREs), or by formation of intranuclear complexes with other transcription factors, to prevent their effect. Activated GRs may also control stability of specific mRNAs, alter levels of cGMP and Ca^{2+}, regulate phospholipase A_2 activity and influence cell membrane potentials (Arai and Chrousos, 1994).

Other activated steroid receptors apart from GRs can also bind to GREs reflecting the homogeneity of the DNA binding domain. Specificity of activity is obtained by further binding to composite response elements and particularly to binding sites for the transcription factor known as activated protein-1 (AP-1). This protein is a hetero-dimer of c-*fos* and c-*jun*, proto-oncogene products expressed in response to a variety of stimuli, particularly cytokines from activated white blood cells (WBC; Barnes and Adcock, 1993). The anti-growth and anti-inflammatory effects of glucocorticoids are due to formation of complexes which prevent AP-1 from exerting its effects on DNA response elements. While activated GRs can block AP-1 enhanced transcription, activated MRs cannot (Funder, 1993).

2.2.5 Metabolism and excretion

Glucocorticoids in plasma are bound to an α-globulin (a glycoprotein with molecular weight; MW; 52,000) known as transcortin or corticosteroid-binding globulin (CBG). CBG has a high affinity for cortisol but a limited binding capacity. When plasma cortisol concentrations (PCC) exceed the binding capacity of CBG, plasma albumin, which has low affinity and high capacity, acts as a transport protein. CBG levels are unaltered by ACTH so that stimulation of the adrenal cortex induces a comparatively greater increase in free than bound plasma cortisol. CBG protects cortisol from degradation by liver enzymes or filtration by the kidneys, and provides a buffer to prevent rapid changes in free cortisol levels. Oestrogens induce an increase in CBG levels in some species so that pregnancy or exogenous oestrogen therapy can elevate CBG levels. Oestrogens may also elevate total plasma glucocorticoid

concentrations, but the buffering action of CBG prevents the development of symptoms of glucocorticoid excess. Liver disease and dysproteinaemia can lead to a decrease in CBG levels and consequent lowering of total glucocorticoids in circulation. Cortisol metabolites, aldosterone and dexamethasone have very low or negligible CBG-binding affinity (Labhart, 1986).

The binding affinities and the points of saturation of CBG vary between species. In human plasma, 90% of plasma cortisol is bound to CBG up to a PCC of 200 ng/ml at which CBG is fully saturated. Above this level, free cortisol increases to 20-30% of total cortisol and the remainder is bound to albumin. Since cortisol levels vary between 60 and 250 ng/ml during the course of a day, binding of cortisol to CBG is an important buffer to maintain free PCC at constant levels (Labhart, 1986).

The liver has the most important role in the inactivation of the steroid hormones. In the hepatic microsomes, the redox balance of the cofactors NAD and NADP favour reduction of cortisol and corticosterone by $P450_{C11}$ to yield cortisone which is less metabolically active (Monder and White, 1993). Further reduction of the double bond between C_4 and C_5 is practically irreversible, and is probably the rate-limiting step for glucocorticoid breakdown. Thyroxine can alter the rate of steroid metabolism which is accelerated in hyperthyroidism. Subsequent 3-keto and 20-keto groups are reduced, and the various metabolites are excreted in urine. Free cortisol is filtered by the kidneys but most is reabsorbed by the tubules. In the human, less than 1% of cortisol is excreted unaltered in the urine (Labhart, 1986).

Clearance of plasma cortisol at physiological concentrations follows first order kinetics. Following intravenous injection of low doses of radioactive cortisol, the logarithm of the specific radioactivity decreases linearly per unit time after a short mixing period. With larger doses, clearance is according to a two pool model of distribution. The biologic half-life of plasma cortisol is prolonged by factors which increase levels of CBG, by liver disease, cachexia and old age, and is shortened in thyrotoxicosis and occasionally in obesity (Labhart, 1986).

2.3 THE FUNCTION OF ADRENAL STEROID HORMONES

The steroid hormones of the adrenal cortex exhibit some sharing of their functions e.g. cortisol is an important glucocorticoid, but has weak mineralocorticoid activity. Biological activity is always related to chemical structure, probably due to the steric nature of interactions with receptors and GREs. Effects on carbohydrate metabolism are generally paralleled by anti-inflammatory and ACTH-suppressive activity (Labhart, 1986).

2.3.1 Mineralocorticoids

The mineralocorticoids act predominantly on sodium and potassium balance. They have an α-ketol side chain at C_{17} but the specific structural basis of their activity is not understood. Aldosterone produced by the *zona glomerulosa* is the principle mineralocorticoid (Labhart, 1986).

2.3.2 Adrenal androgens

The role of adrenal androgens is uncertain since no apparent symptoms of withdrawal occur following adrenalectomy. The principal adrenal androgens are androstenedione and DHEA which is found both free and conjugated to sulfate. They are secreted in both male and female. DHEA is secreted in large quantities in the human and in some other species. Concentrations vary significantly with age. Plasma levels are high in new-born humans but decrease rapidly. Detectable levels reappear at puberty, are maximal between ages 20 and 40 and then decline with advancing age (Labhart, 1986). The function of DHEA has been described as "state dependent" meaning that expression of its activity is dependent upon the physiological state of the animal. DHEA may mediate lymphocyte responses by blocking corticosteroid action - it can restore immunoresponsiveness in aged mice (Regelson *et al.*, 1994).

2.3.3 Glucocorticoids

Glucocorticoids are pleiotropic hormones with many physiological functions. They exert effects on almost all tissues and organs, and tonic secretion is crucial for metabolic homeostasis.

Increased glucocorticoid secretion during stress assists adjustment of the organism by modifying energy expenditure, dampening the inflammatory-immune response and altering central nervous system (CNS) function (Arai and Chrousos, 1994).

Named for their predominant action on carbohydrate and protein metabolism, the glucocorticoids also have weak mineralocorticoid activity. They are structurally similar to mineralocorticoids but with a keto or hydroxyl group at C_{11}. Their activity is enhanced in those forms with an hydroxyl group at the $C_{17\alpha}$ position. The principal glucocorticoid is cortisol, except in rodents where corticosterone plays a predominant role. Cortisone is also secreted in small amounts, but is about 20% less active than cortisol (Labhart, 1986).

Overall the glucocorticoids are ergotropic, directing metabolism from a phase of growth and storage toward increased activity and energy consumption. Several controlling mechanisms may be available to an individual tissue. The interaction of activated receptors with components of cell nuclei confers variety of action. Specificity of activity in target tissues is achieved by multiple mechanisms including differing affinities of receptors for glucocorticoids and the presence of binding antagonists (Funder, 1993).

The enzyme $P450_{C11}$ which is widely distributed in tissues can modulate the activity of cortisol and corticosterone by influencing their oxidative state. In the 11-oxo form (cortisone and 11-dehydrocorticosterone) the glucocorticoids are less biologically active. The oxidative state is dependent upon the intracellular environment and provides a mechanism for variations in glucocorticoid activity between tissues. High levels of $P450_{C11}$ with oxidative capacity are found in placenta, and probably provide a barrier to the transfer of maternal glucocorticoids to the foetus. This is also important in aldosterone target tissues such as the kidney tubule where $P450_{C11}$ protects the MR against interference by glucocorticoids (Monder and White, 1993).

2.3.3.1 Metabolic actions

Glucocorticoids stimulate gluconeogenesis by the liver. This induces a negative protein balance due to mobilisation of amino acids from stored protein. Where pancreatic function is intact, there is moderate elevation of blood glucose and decreased insulin sensitivity. Blood

glucose is maintained without depleting liver glycogen reserves, although cortisol has a permissive effect on the glycogenolytic activity of adrenaline. The glucocorticoids have only weak mineralocorticoid activity but they do promote diuresis by increasing glomerular filtration rate and permitting water permeability of distal renal tubules. These effects may be due to modulation of vasopressin secretion. Glucocorticoids are also involved in maintenance of blood pressure in association with other hormones (Labhart, 1986).

2.3.3.2 Actions on tissues

2.3.3.2.1 Immature tissues

Glucocorticoids have a potent effect on immature tissues e.g. in immature connective tissue where exposure to glucocorticoids inhibits fibroblast growth and alters structure of collagen ibres. Activated GRs inhibit expression of the collagenase gene by binding to AP-1 which represses transcription (Barnes and Adcock, 1993).

2.3.3.2.2 Cells of the ilmmune system

Bi-directional communication exists between the HPA axis and the immune system allowing complex interactions. The cellular components of the immune system produce cytokines which act as chemical messengers in mediating the nature and persistence of the inflammatory response. Non-cognitive forms of stress such as the presence of endotoxins in circulation initiate cytokine release into circulation which in turn activates the hypothalamus (Blalock, 1989). Interleukins 1 and 6 and tumour necrosis factor α are particularly important in the activation of the HPA axis (Rook *et al.*, 1994).

Circulating cells of the immune system are influenced by glucocorticoids. Total leucocyte numbers are increased due to the retention of neutrophils in circulation. Activated GRs inhibit the expression of adhesion molecules by endothelial cells so that there is a reduction in diapedesis of neutrophils. There are fewer lymphocytes, with reduced cytoplasm, due to inhibition of mitosis. Eosinophils show an increased rate of apoptosis as the steroids inhibit

protective cytokines e.g. interleukin 5. This effect on eosinophils is more persistent than the effects on other leucocytes (Barnes and Adcock, 1993).

Although glucocorticoids influence circulating WBC numbers their effect on cellular immunity is more profound than on humoral immunity. Inflammatory change is inhibited which reduces granulation tissue, decreases capillary permeability, and reduces tissue infiltration of immune system components. Several cytokines are particularly sensitive to the inhibitory action of steroids since their transcription is directly controlled by the interaction of activated GRs with negative GREs e.g. the interleukin 6 gene has at least four negative GREs (Barnes and Adcock, 1993).

Activation of the HPA axis in animals suffering from inflammatory or toxic conditions has been associated with changes in immune tissues. Accelerated involution of the thymus in association with adrenocortical hypertrophy was reported in young lambs following exposure to sporedesmin. Plasma cortisol concentrations increased as the animals developed photodermatitis, rather than at the time that liver damage occurred, and sheep which did not show photosensitisation after sporedesmin dosing showed few adrenal-associated changes. This suggested that inflammation and pain associated with development of the skin lesions may have initiated the adrenocortical changes (Smith and Payne, 1991).

The HPA axis may have a role in maintaining immune efficiency and preventing diseases related to altered immunosurveillance. Corticostatins-defensins are cytokines released from activated neutrophils which may act in a paracrine manner to modulate glucocorticoid effects on natural killer cells and in an endocrine manner to alter adrenocortical function (Angeli *et al.*, 1994). Increased concentrations of glucocorticoids may alter cytokine production to influence the balance of T-helper lymphocytes types 1 and 2. These cells are proposed to exert mutual inhibition to balance cellular and humoral immune responses. Glucocorticoids may drive a shift towards type 2 T-helper lymphocytes which may favour reduction of humoral immunity. Such a mechanism has been suggested in the reactivation of latent viral infections during times of stress (Rook *et al.*, 1994).

2.3.3.2.3 Nervous tissue

In nervous tissue the glucocorticoids show both fast membrane-mediated effects and slower gene-mediated effects. Membrane-mediated effects occur through changes in ion flux and membrane potentials e.g. the binding of reduced steroid metabolites to γ-aminobutyric acid (GABA) receptors potentiates chloride conductance. These effects underlie the sedative and anti-convulsive effects of the glucocorticoids. Gene-mediated effects occur through altered transcription of neuropeptide precursors and transmitter-synthesising enzymes. Effects of this type are thought to be the basis for the influence of glucocorticoids on mating and singing behaviours in song-birds and on hibernation of rodents (de Kloet and Voorhuis, 1992).

2.3.3.3 Glucocorticoids and the foetus

Glucocorticoids have a maturational role in the foetus. They induce a wide range of enzymes upon which survival after birth is dependent - induction of surfactant and structural maturation to allow expansion of the lungs, glycogen accumulation and increased levels of antioxidants such as catalase and superoxide dismutase in the liver, catecholamine production by the foetal adrenal medulla, induction of β-adrenergic receptors in many cells, and altered thyroid hormone processing to increase levels of active thyroglobulin (Liggins, 1994).

Subject to species variation, changes in glucocorticoid metabolism in the foetus signal the time of birth to the dam. Feedback efficiency in the foetus alters, possibly due to changes in CBG, which leads to decreased free PCC. This may initiate feed-forward processes at the foetal pituitary and adrenal glands, and also in the placental and foetal membranes to further stimulate the axis. Resulting hormonal changes ready both the foetus and dam for the birth process (Challis and Brooks, 1989).

2.3.4 Synthetic glucocorticoids

The pharmacological development of synthetic glucocorticoids has aimed to increase desired therapeutic effects while diminishing undesirable side-effects. This has been achieved in several ways. Dehydrogenation of the A ring to form a double bond between C_1 and C_2 as in

prednisolone increases glucocorticoid activity and reduces mineralocorticoid activity, i.e., potency is increased. Fluorination at $C_{9\alpha}$ as in dexamethasone enhances both glucocorticoid and mineralocorticoid activity and prolongs metabolic half-life, i.e., duration of action is increased. Methylation or hydroxylation at $C_{16\alpha}$ as in dexamethasone and prednisolone respectively, decrease mineralocorticoid activity (Labhart, 1986).

Potency and duration of action of synthetic glucocorticoids must be clearly distinguished. Potency is influenced by chemical structure, and also formulation which determines availability e.g. a soluble formulation is more potent than a suspension. Duration of action is dependent upon route of administration, upon half-life of elimination which may also be influenced by chemical structure, and upon half-life of absorption which may be influenced by formulation. A suspension produces a more sustained effect than a solution (Baggot, 1977). The particle size and concentration in a suspension are critical determinants of absorption rate. Where particle size is large, the surface area is greater so the rate of uptake is increased. Where a suspension is more concentrated, the deposit formed within the tissues will be more compact, and absorption is delayed (MacDiarmid and Cooper, 1982).

Prednisone and prednisolone have negligible mineralocorticoid activity but their glucocorticoid activity is four to five fold greater than that of cortisol. Dexamethasone has the highest anti-inflammatory, hyperglycaemic and ACTH-suppressive activity per milligram of all corticosteroids used therapeutically, but no sodium-retaining activity. It is about 40 times more active than cortisol in suppressing ACTH secretion. It cannot be detected by most cortisol assays, and its metabolites in urine are quantitatively negligible (Labhart, 1986).

2.4 Modes of Hypothalamo-Pituitary-Adrenal Axis Activity

The HPA axis has two modes of activity. Basal activity follows a circadian rhythmicity which is co-ordinated and synchronised by daily activities and sleep-related events. Increased activity as a response to stressors provides a protective mechanism to assist the maintenance of homeostasis in unfavourable circumstances (de Kloet and Voorhuis, 1992).

Because the release of glucocorticoids in response to stressor challenge are frequently associated with negative effects on an individual's physiology, they are often considered to be harmful. In fact the opposite is true. The adrenal response to a stressor is essential for adjustments in biological function which allow the individual to cope with the stressor. Glucocorticoids are equally essential in the non-stressed animal to regulate normal biological functions such as metabolism, immunity, lactation and reproduction (Moberg, 1987).

2.4.1 Basal activity

Basal cortisol secretion occurs in bursts interspersed by periods of inactivity known as the ultradian rhythm. Appraisal of endogenous hormone secretion simultaneously with metabolic clearance has shown that 10-15% of daily cortisol production results from tonic glucocorticoid release with the remainder being pulsatile (Kerrigan *et al.*, 1993). In the human, between 7 and 13 phases of secretory activity occurred every 24 h, but the total time of these phases occupied only about one third of each day. The size of a burst is variable, even in the same individual. Aldosterone and DHEA are also secreted in bursts (Labhart, 1986).

Many species display a marked diurnal rhythm in the plasma concentration of glucocorticoids. Rhythms may be interrupted, or the phase of the rhythm may be shifted by prolonged increases in glucocorticoid levels (Persellin *et al.*, 1972). In the healthy human, PCC varies between 60 and 250 ng/ml during the course of a day with peak levels in early morning and a nadir around midnight. The subsequent rise of the diurnal rhythm is due to increased frequency and amplitude of secretory bursts, and increased responsiveness of the adrenal cortex to ACTH (Kerrigan *et al.*, 1993). The converse pattern is seen in nocturnally active species such as rats (Dallman *et al.*, 1987).

The diurnal rhythm of HPA axis activity is initiated in the CNS. Under free-running conditions the endogenous rhythm is 60-90 min longer than 24 h, but routines of daily activity influence periodicity, limiting it to a 24 h cycle (Sherman *et al.*, 1985). The circadian pacemaker is believed to reside in the area of the suprachiasmic nucleus with input altering activity throughout the HPA axis (Watts and Swanson, 1989).

Circadian and ultradian variations are well documented in sheep. Discrete peaks of activity were observed over 24 h. The adrenal glands of the sheep were estimated to be inactive for about 66% of the time (McNatty *et al.*, 1972). Frequency of secretory bursts were similar throughout the day but amplitude varied. PCC was greatest (≥100 ng/ml) at about midnight with lowest levels (≤3 ng/ml) in the afternoon (Fulkerson and Tang, 1979).

Diurnal patterns are easily disrupted by exogenous influences. Sheep adapting to a new environment exhibited diurnal rhythms with higher overall PCC during the period of adaptation (McNatty and Young, 1973). Patterns of PCC were synchronous during the first few waves of a sampling period in sheep then became asynchronous suggesting that the stress of extra handling at the beginning of the intensive sampling regime had set the pattern (Fulkerson, 1978).

2.4.2 The stress response

The HPA axis responds to exposure of the animal to various stressors which may be physical, psychological or non-cognitive in nature, with the end result being increased levels of plasma glucocorticoid. The stressor may act over acute, repeated acute, or chronic time spans. The response occurs in a manner graded to the severity of the stressor, but its range is limited and quickly reaches an effective maximum (Harbuz and Lightman, 1992).

The first attempt to explain the similarity of the end-point of the response of the HPA axis to stressors, i.e., elevated plasma glucocorticoid levels was the General Adaptation Syndrome. This hypothesis proposed that exposure to stressors of all forms led to a stereotypic response with specific alterations in the organism. The syndrome had three phases:

- the alarm reaction - characterised by shock ;
- adaptation - an increase in resistance to the stressor as the organism responded; and,
- exhaustion - if adaptation failed.

The adaptation process was further defined by the Pendulum Hypothesis where the individual's response would be self-correcting until equilibrium was achieved (Selye, 1946).

While Selye's hypothesis was supported by experimental data, it could not explain changes seen in response to chronic stressors or the sometimes apparently deleterious actions of glucocorticoids, particularly on the immune system. Based on the relationship between the pharmacological effects of the glucocorticoids and their physiological functions during stress a further hypothesis was proposed. Rather than protecting against the source of stress itself, stress-induced increases in glucocorticoid levels may prevent over-reaction which might in itself threaten homeostasis (Munck *et al.*, 1984).

2.5 Mechanisms of Integrated Hypothalamo-Pituitary-Adrenal Axis Control

2.5.1 The pituitary

2.5.1.1 Adrenocorticotropic hormone

Plasma glucocorticoid levels are controlled by plasma Adrenocorticotropic hormone levels. Synthesis and secretion of normal amounts of glucocorticoids and adrenal androgens occur only in the presence of ACTH. The adrenal cortex is unable to store its secretory products - an increase in PCC following stimulation by ACTH is due to increased production (Labhart, 1986). Increases in plasma corticosteroids are proportional to increases in plasma ACTH over the physiological range. At the lower end of the range, plasma corticosteroid levels are proportional to the logarithm of the plasma ACTH concentration up to a maximum corticosteroid secretory rate. Further increases in plasma ACTH concentrations prolong the duration of the maximum secretory rate such that the integrated response (area under the PCC.time curve) is proportional to the logarithm of plasma ACTH concentration (Keller-Wood *et al.*, 1983).

ACTH is a polypeptide of 39 amino acids with MW 4540. It is derived from the precursor protein pro-opiomelanocortin (POMC) which has a MW of 30,000. Melanotropin stimulating hormone (α-MSH) and β-endorphin. are also derived from POMC. The only POMC product with systemic activity is ACTH, while the other derivatives target the CNS, with various effects on behaviour and learning. Processing of POMC varies depending on the species, the tissue, and the developmental stage of the animal. In the adult rat, anterior pituitary POMC is

processed to ACTH and β-lipotropin. In the intermediate lobe of the rat pituitary, ACTH is further processed to yield α-MSH and corticotropin-like intermediate lobe peptide (CLIP), while β-lipotropin is converted to β-endorphin (Rosa *et al.*, 1980). Processing of POMC changes during foetal development. The anterior pituitary of the human foetus produces predominantly α-MSH and CLIP, but as term approaches ACTH production becomes predominant (Challis and Brooks, 1989).

Human, ovine and bovine ACTH differ in their amino acid sequences only between residues 25 and 33. A synthetic adrenocorticotropin analogue ($\beta_{1\text{-}24}$), which contains the first 24 amino acids of the NH_3-terminal sequence of complete corticotropin, is produced commercially for use in diagnostic tests. Its activity in humans is somewhat less than that of the $\beta_{1\text{-}39}$ corticotropin. Peptides with less than 16 amino acid residues have no corticotropic effect, but do exhibit MSH activity (Labhart, 1986).

The mechanism of transport of ACTH in the blood, and its metabolism and excretion have not been defined. Values reported for its biological half-life vary from less than 10 min to 2 h. ACTH is released from the pituitary in secretory bursts, with a temporal relationship between peaks of plasma ACTH concentration and the frequency of secretory bursts of cortisol. Basal plasma ACTH concentrations are considerably less those which give maximum stimulation of the adrenal cortex. In the human, basal plasma ACTH concentrations vary 4 fold from the nadir in the morning to the peak in the evening. Maximal stimulation of the adrenal cortex occurs at about 30 times the normal daily peak (Labhart, 1986).

2.5.1.2 Endogenous opioids

The opiate-like peptide β-endorphin is secreted by the anterior pituitary simultaneously with ACTH. This is not surprising since their common precursor is POMC. While β-endorphin does produce analgesic effects when injected peripherally, when administered centrally its effects are profound and may result in catatonia. Its role is proposed to be neurotropic or psychotropic, assisting the animal to adapt to painful stimuli (Guilleman *et al.*, 1977). The effect of opioids on the HPA axis varies between species. Opioids stimulate the release of corticotropin-releasing

hormone (CRH) in rats, mice and dogs, but inhibit secretion of ACTH in man. They appear to have little effect on ACTH secretion in adult sheep (Brooks and Challis, 1988; Nanda *et al.*, 1992).

2.5.2 The hypothalamus and ACTH secretagogues

A variety of stimuli can activate the HPA axis with the common end-point of an increase in circulating ACTH and increased secretion of glucocorticoids. Peripheral stimuli are integrated in the hypothalamus and elicit the release of several factors into the portal circulation to mediate the secretion of ACTH from the pituitary gland.

2.5.2.1 Corticotropin-releasing hormone

Corticotropin-releasing hormone (CRT) is a peptide of 41 amino acid residues, synthesised in the parvocellular cells of the medial division of the paraventricular nuclei (PVN) of the hypothalamus. Axons from these neurones terminate in the external zone of the median eminence where CRH is released into the hypothalamo-pituitary portal vein. In rodents, CRH is thought to be the only hypothalamic factor able to induce POMC gene expression (Harbuz and Lightman, 1992). The molecular structure of CRH is closely related to sauvagine (isolated from frog skin) and urotensin I (isolated from the urophysis of two fish species) both of which stimulate ACTH release *in vitro*. There is a close structural similarity between CRH of different species e.g. human and ovine CRH vary by only 7 residues (Vale *et al.*, 1983).

The increase in bioassayable CRH in the median eminence following stimulation is rapid and transient, lasting only 2-4 minutes. It is too rapid to reflect increased synthesis, so it must be due to activation of transport and mobilisation mechanisms (Vernikos-Danellis, 1965). Release of CRH is enhanced by serotonergic and inhibited by dopaminergic influences (Jacobson and Sapolsky, 1991). Noradrenaline and β-endorphin have inhibitory effects while acetylcholine has a stimulating effect on CRH secretion (Labhart, 1986). The action of CRH on the corticotrope is mediated by cAMP, levels of which increase within 60 sec after addition of CRH to pituitary cell cultures (Giguère *et al.*, 1982).

2.5.2.2 Arginine vasopressin

Arginine vasopressin (AVP) has a role in the control of ACTH secretion. Although AVP is primarily a product of the posterior pituitary gland, about 50% of the parvovellular cells of the PVN co-produce AVP with CRH (Harbuz and Lightman, 1992). The CRH-like activity of AVP varies with species. In the human and the rat, it is a weak ACTH secretagogue which potentiates CRH activity. In sheep, AVP is at least equipotent to CRH in the release of ACTH (Engler *et al.*, 1994). The action of AVP on the corticotrope is mediated by phospholipase C to generate an intracellular signal for an increase in levels of Ca^{2+} and protein kinase C. Activation of cAMP by CRH is dependent upon protein kinases and could be a mechanism whereby AVP potentiates the activity of CRH (Bilezikjian *et al.*, 1987).

2.5.2.3 Other neuropeptides and transmitter factors

Many neuropeptides and transmitter factors in neural tissue appear to have modulatory effects on HPA axis function. In the rat pro-enkephalin A has a role in ACTH release (Harbuz and Lightman, 1992). The neuropeptide galanin is co-localised with CRH and AVP in the PVN and was seen to increase plasma glucocorticoid levels in rats (Nowak *et al.*, 1994). Neurotensin is a neuropeptide found in the hypothalamus and pituitary gland. It has a net stimulatory effect on the rat HPA axis which can be attributed to enhancement of CRH release (Malendowicz and Nussdorfer, 1994). Neuropeptide-Y, which is widely distributed throughout the CNS, stimulated HPA axis activity when injected into the third ventricle of sheep, but had no effect on primary pituitary cell cultures. This suggested that it may exert a central effect (Brooks *et al.*, 1994). Other hormones for which an ACTH secretagogue role have been identified are oxytocin, adrenaline, noradrenaline and angiotensin II (Engler *et al.*, 1994).

Evidence for an inhibitory effect of the hypothalamus on pituitary release of ACTH has been seen in sheep following hypothalamo-pituitary disconnection. This technique preserved blood supply to the anterior pituitary. Although the response to stressors was abolished, mRNA encoding for POMC in the pituitary as well as basal plasma levels of ACTH and cortisol all increased. This led to the proposal that the hypothalamus secretes a corticotropin-release inhibitory factor (Engler *et al.*, 1994).

2.5.2.4 Hypothalamic integration of HPA axis activity

The hypothalamic response to a stimulus is influenced by the nature of the stressor and may result in the differential release of ACTH secretagogues. In the rat, increased levels of mRNA encoding for CRH were found after footshock, restraint and swimming, but not cold stress, which was associated with increased levels of pro-enkephalin A. Severe insulin-induced hypoglycaemia resulted in increased levels of CRH while mild insulin-induced hypoglycaemia increased AVP secretion. Experimentally-induced hypotension due to haemorrhage resulted in increases of both CRH and AVP, while hypotension in response to nitroprusside injection increased CRH alone. The mechanism of this differential activity is not known, but stimulation of different populations of hypophyseal cells to produce different ratios of CRH and AVP has been suggested (Harbuz and Lightman, 1992).

The HPA axis has an adaptive mechanism which operates after prolonged stress (either chronic or repeated acute stress) so that the response is attenuated. Circulating corticosteroid and ACTH levels return to the normal range despite continued exposure to the stressor. Habituation to prolonged stress is stressor specific - animals which have adapted to a chronic stimulus demonstrate either a normal or an exaggerated response to an alternative acute stressor (Harbuz and Lightman, 1992). Differential control during prolonged stress may be exerted by CRH and AVP. In a chronic stress model of adjuvant-induced arthritis in rats, circulating levels of corticosterone and levels of mRNA encoding for POMC in the pituitary remained elevated, but levels of mRNA encoding for CRH in the PVN fell. This was coincident with an increase in AVP release at the onset of joint inflammation and suggested that AVP was activating the HPA axis in the presence of permissive levels of CRH (Harbuz *et al.*, 1992).

2.5.3 The influence of centres in the central nervous system (CNS)

Axonal projections from the hippocampus to the PVN provided early evidence of limbic system involvement with HPA axis function. The hippocampus is structurally complex with many neuronal inter-connections which allow for cross-talk between cell fields. The lateral septum connects to the ventral hypothalamus and the bed nucleus of the stria terminalis (BNST). The fornix has projections extending to the septum, the cerebral cortex, the thalamic nuclei, and the anterior olfactory nucleus. This structure would allow integration of both excitatory and

inhibitory influences from interconnecting neurones with different activation thresholds (Jacobson and Sapolsky, 1991). The hippocampus may also have a direct influence on adrenal function via the splanchnic nerve (Edwards and Jones, 1993). Structures of the limbic system, especially the hippocampus, are highly susceptible to damage by insults such as ischaemia, hypoglycaemia and seizure, so that their integrity has implications for axis control (Labhart, 1986).

The hippocampus has been found to modulate the stress response and the circadian rhythm. It may exert its effects by mediating sensitivity to corticosteroid feedback inhibition. It dampens stress-induced HPA activity - rats with hippocampal lesions showed greater restraint-induced increases in plasma corticosteroids which were more sustained than in intact rats (Sapolsky *et al.*, 1984). Exposure of the hippocampus to dexamethasone reduces the corticosteroid response to stress. Following hippocampal and/or fornix lesions, increased doses of dexamethasone were required to achieve the same level of inhibition. The hippocampus may control plasma corticosteroid concentrations at the nadir of diurnal variation since higher concentrations were seen in lesioned animals (Jacobson and Sapolsky, 1991).

The BNST and the ventral hypothalamus probably provide important inputs to the hippocampus since they express GR. The BNST occupies a central position in neural pathways to the hypothalamus and may act as an independent corticosteroid feedback site (Jacobson and Sapolsky, 1991). Data from BNST-lesioned rats has suggested that the subnuclei of the BNST may have different roles in axis control. Subnuclei of the posterior BNST may have an integrative function to convert excitatory signals from the hippocampus into a negative signal to the PVN. Subnuclei of the anterior BNST receive little hippocampal input but exert positive regulation on tonic HPA axis function (Herman *et al.*, 1994).

2.5.4 The role of steroid receptors in the CNS

The actions of corticosteroids in the CNS are mediated by two types of receptors, classified according to their affinity for corticosterone and the synthetic corticosteroids. The Type I corticosterone receptor is similar to the classical MR, but with a relative binding affinity of:

$$\text{cortisol} \cong \text{corticosterone} \geq \text{aldosterone} >> \text{dexamethasone}.$$

Type I receptors are confined largely to the limbic system and some brain stem nucleii, and are absent in the PVN. Some nerve cells express aldosterone-selective MR as in peripheral tissue. The Type II receptor is a GR with a relative binding affinity of:

dexamethasone > cortisol > corticosterone >> aldosterone.

Type II receptors are widely distributed in the CNS particularly in regions associated with organisation of the stress response such as the PVN and hippocampus. The hippocampus has high levels of $P450_{C11}$ which converts glucocorticoids to less active metabolites. This may provide a mechanism whereby neurones can exert local control over receptor activation (Jacobson and Sapolsky, 1991).

Functional control appears to be exerted by the balance of activity of the two types of receptors. For example, in hippocampal CA_E neurones, corticosteroid control of ion regulation and transmitter responsiveness is differentially co-ordinated by co-localised receptors of both types. Type I receptors are involved in maintenance of excitability while Type II receptors suppress excitability (de Kloet, 1991).

The use of specific receptor antagonists has suggested that a number of behavioural patterns can be explained by the differential actions of the brain corticosteroid receptors. Information handling and the organisation of behaviour strategies have been associated with corticosterone effects on limbic Type I receptors, while fear and food motivated behaviour as well as information storage are mediated by steroid effects on Type II receptors. Aldosterone-selective Type I receptors mediate specific effects on salt hunger and functions associated with sodium homeostasis (de Kloet, 1991).

Corticosteroid concentrations exert a modulatory effect on receptor control mechanisms. High levels of glucocorticoids decrease numbers of hippocampal Type II receptors and increase Type I receptor capacity, while mineralocorticoids down-regulate both receptor types. Changes in the hippocampus associated with long-term glucocorticoid treatment or prolonged stress are similar to those seen in the ageing process. Individual susceptibility to stress and perhaps to stress-related brain diseases may be explained by idiosyncrasies of receptor balance. These findings place the Pendulum Hypothesis of the General Adaptation Syndrome in the

perspective of co-localised receptors mediating co-ordinated and differential effects to regulate cellular homeostasis and behavioural adaptation (de Kloet, 1991).

Hippocampal receptors may exert their effect following ligand binding by influencing cell membrane polarisation and Ca^{2+} influx to cells. Adrenalectomy reduced characteristics of cell membrane polarisation and the calcium-mediated action potential in CA_1 neurones. This reduction was restored by treatment with corticosterone. Stress and glucocorticoid treatment have been shown to increase these characteristics. Although the established roles of Type I and II receptors are clearly delineated in some situations, conflicting experimental data has been obtained in some circumstances. This has suggested that receptors exhibit a degree of plasticity which allows up and down-regulation of existing populations (Landfield and Eldridge, 1991).

2.5.5 Feedback Control

The negative feedback effect of the glucocorticoids upon the HPA axis is well established. Adrenalectomy results in hypersecretion of ACTH, and a corresponding increase in mRNA encoding for POMC in the anterior pituitary. Concentrations of CRH and AVP in the PVN both increase as do concentrations of the mRNAs encoding for CRH and AVP. Administration of exogenous corticosteroids reverse or prevent these changes (Harbuz and Lightman, 1992).

2.5.5.1 A Model for Negative Feedback Control

The model for control of PCC (Fig. 2.3) is of a closed-loop control system with negative feedback and a variable set-point. Control of the axis has been described as being one or several comparator units which compare aspects of plasma corticosteroid concentration with the intensity of the stimulus. The input signal is probably a combination of both concentration and timing of exposure to corticosteroids and may be a complex of both positive and negative signals. The comparator unit generates an output signal which determines the response of the axis. The unit may have a variable set-point which allows the level of response to be altered following different sets of input signals including circadian patterns or chronically elevated plasma corticosteroids. Several glucocorticoid feedback sites may exert their effects on the axis to provide a variable set-point (Keller-Wood and Dallman, 1984).

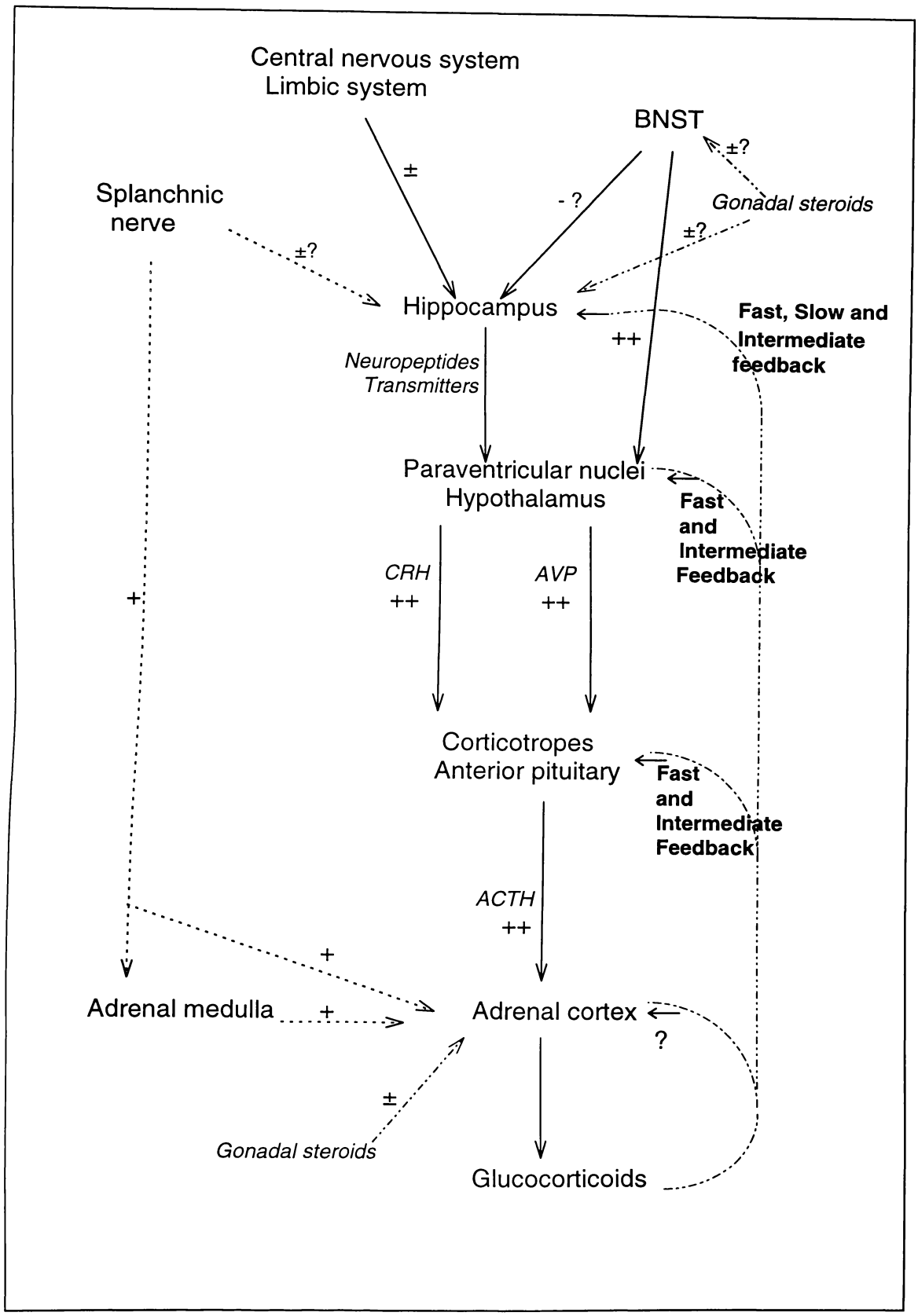


Figure 2.3: Schematic representation of feedback control mechanisms operating in the hypothalamo-pituitary-adrenal axis.

Three distinct time domains in which negative feedback is exerted can be identified. Fast feedback occurs within 10 minutes and does not involve protein synthesis. It may occur through changes at the level of the cell membrane and could include mechanisms such as the inhibition of CRH release from the median eminence. Change in membrane excitability following glucocorticoid exposure has been proposed as a mechanism. Fast feedback is desensitised by exposure to chronic stress (Keller-Wood and Dallman, 1984).

Intermediate feed-back mechanisms are expressed between 45 and 120 min after the signal. They involve gene-mediated effects and intracellular signal transduction pathways. Feedback in the intermediate time domain operates mainly at higher levels to influence CRH synthesis and release. Intermediate feedback appears to involve integrated control processes, since the level and duration of inhibition vary according to the type and intensity of the feedback signal (Keller-Wood and Dallman, 1984).

Slow feedback inhibits ACTH release and synthesis by the blockade of CRH and AVP gene expression in the PVN, and of POMC gene expression in the corticotropes (de Kloet, 1991). Only intermediate and fast feedback mechanisms would be expected to occur under physiological conditions. Slow feedback inhibition occurs only after prolonged periods of high plasma corticosteroids, i.e., in pathological conditions, or following pharmacological treatment particularly with potent synthetic glucocorticoids such as dexamethasone (Keller-Wood and Dallman, 1984).

2.5.5.2 Control in the lower axis

A direct inhibitory effect of cortisol on the adrenal cortex only occurs at extremely high levels, but prolonged inhibition of the HPA axis with loss of trophic support leads to atrophy of the adrenal cortex (Labhart, 1986).

ACTH secretion is subject to feedback inhibition both directly by control of pituitary activity and indirectly by control of ACTH secretagogues. The pituitary may be less sensitive to inhibition by increased endogenous glucocorticoid levels than the higher points in the axis, but it is more sensitive to inhibition by dexamethasone. Dexamethasone inhibited ACTH release from

perfused rat anterior pituitaries within 10-15 min of administration suggesting that it was exerting a fast feedback membrane stabilising effect (Keller-Wood and Dallman, 1984).

Glucocorticoids also exert feedback inhibition of ACTH secretion in the intermediate time domain by acting at a point distal to the production of cAMP following stimulation of the corticotrope by CRH (Giguère *et al.*, 1982). Glucocorticoids induce the production of lipocortin, an inhibitor of phospholipase A_2. This may provide a mechanism for intermediate feedback by its action on the cAMP-dependent protein kinase pathways responsible for ACTH production and release (Antoni, 1986). This is supported by the dexamethasone resistant nature of the action of AVP on the corticotrope. Treatment of dexamethasone-suppressed rat anterior pituitary cells with AVP allowed them to secrete ACTH at a level similar to that seen with CRH alone in untreated cells. This suggests that the glucocorticoid feedback mechanism is counteracted by protein kinase production induced by AVP (Bilezikjian *et al.*, 1987).

2.5.5.3 Control in the higher axis

The role of the hypothalamus in feedback control has been established by the observation of increases in PVN content of CRH and AVP following adrenalectomy and the reversal of these changes with glucocorticoid treatment. Feedback control is also exerted at the level of the hippocampus and it is likely that combinations of input at both of these levels determine the set-point of the system. Type I receptors in the hippocampus may mediate feedback at the nadir of diurnal variation which appears to be controlled by a corticosterone-preferring receptor (Dallman *et al.*, 1987). This is supported by the observation that type I antagonists increase corticosterone levels at the nadir (Ratka *et al.*, 1989). Normal plasma glucocorticoid levels probably saturate type I receptors but only 26% of receptors may be occupied at the circadian nadir (Cascio *et al.*, 1989). Type II receptors in the hippocampus may mediate stress-induced changes in glucocorticoid levels. While their occupancy rate is about 10% at the circadian nadir, this increases to about 75% at the circadian peak, following stress or after dexamethasone administration. Lower levels of receptor occupancy were required to inhibit AVP release than CRH release. Levels of CRH, AVP and oxytocin in adrenalectomised rats directly correlated with hippocampal type II occupancy, although levels of CRH were more accurately predicted by a combination of Types I and II receptor occupancy rate (Jacobson and Sapolsky, 1991).

Varying numbers and types of steroid receptors in control areas may contribute to differential feedback control. Use of selective agonists and antagonists for type I and II receptors has indicated that the balance of receptor types may set the sensitivity of the stress response system (de Kloet *et al.*, 1991). Individual differences in receptor number and plasticity may also influence the set-point (Jacobson and Sapolsky, 1991). This feedback control balance may have a regional bias. Dexamethasone pellets exerted control on CRH and AVP synthesis when the implantation site was directly over the PVN; corticosterone pellets exerted similar activity only when implanted over the dorsal hippocampus (Harbuz and Lightman, 1992).

2.6 The Hypothalamo-Pituitary-Adrenal Axis and the Adrenal Medulla

There is increasing evidence for the existence of medullary control over the adrenal cortex. Splanchnic nerve input can influence blood flow to the adrenal cortex influencing the presentation rate of ACTH to the adrenal cortex. Even so, experimental evidence suggests that the relationship may be more complex. Seven days after sympathetic denervation of the adrenal gland in calves, PCC following ACTH treatment were reduced compared to those in sham-operated calves. Stimulation of the splanchnic nerve of hypophysectomised calves had no effect on glucocorticoid secretion on its own, but resulted in a substantial increase in cortisol output during $ACTH_{1-24}$ infusion. The observed changes in cortisol output during splanchnic nerve stimulation were greater than can be accounted for by the increased ACTH presentation rate alone (Edwards and Jones, 1993). Hypophysectomised dogs showed increased production of cortisol following splanchnic nerve stimulation in a manner suggestive of a change in $P450_{c11}$ activity (Engeland and Gann, 1989).

Several neuropeptides found in the adrenal medulla may exert local effects on adrenocortical function. Stimulation of the splanchnic nerve increased output of a CRH-like peptide which was susceptible to feedback control by ACTH. Production of the peptide could also be stimulated by acetylcholine, suggesting that the release is stimulated by muscarinic receptors of the sympathetic system. Vasoactive intestinal peptide (VIP) is released from the adrenal medulla in response to splanchnic nerve stimulation in the conscious calf and may potentiate the response to ACTH. When infused into hypophysectomised calves, VIP produced a substantial increase in cortisol secretion in the presence of ACTH. When there was no ACTH, VIP mimicked the effect of splanchnic nerve stimulation to increase adrenal blood flow, but had no

effect on cortisol output. Since capillary beds supplying the cortex and medulla are largely separate, at least in the rat, the role of this peptide may be to regulate blood flow within the gland rather than being steroidogenic (Edwards and Jones, 1993).

Products of the adrenal medulla may also exert negative feedback control on the pituitary gland. Adrenomedullin, a peptide with has been isolated in rat and human tissues, was able to block ACTH secretion in pituitary cells *in vitro* at a point distal to cAMP accumulation (Samson *et al.*, 1995).

The role of the adrenal medulla in mediating the stress response in livestock requires further investigation. Catecholamines exert a direct steroidogenic effect on adrenocortical cells *in vitro*. This action is mediated by β_1 adrenoceptors whose activation promotes accumulation of mRNA for $P450_{scc}$ (Edwards and Jones, 1993). Products of the adrenal medulla may also mediate WBC responses to stress. Changes in WBC function following restraint-induced stress in sheep were greater than those seen than following infusion of cortisol at a rate to achieve similar PCC profiles (Minton, 1994).

2.7 THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS AND THE GONADAL AXIS

2.7.1 HPA axis regulation of the gonadal axis

The HPA axis plays a role in regulating the gonadal axis in all vertebrates. Adrenal regulation of reproductive function is seen across all classes of vertebrates which implies early evolutionary development of the regulatory mechanisms. Their persistence further implies their critical importance. The influence of hormones of the HPA axis on reproduction is highlighted by:

- disruption of normal reproductive function by the adrenal stress response as a proposed regulatory factor for the population size of wild animals;
- the influence of social hierarchies on the reproductive success of individuals within a social group; and,
- diminished reproductive function as observed in domestic animals maintained under intensive management conditions (Moberg, 1987).

The female may be more susceptible to the effects of acute stressors because the temporal relationship of the events that surround her reproductive efforts is critical; but stressors of a chronic nature may also jeopardise her reproductive success. The effects of stress are manifest both as an interruption of normal hormonal patterns and as disruption of behaviour associated with the expression of oestrus. These have been clearly demonstrated to reduce fertility in the female; but stress-induced changes, while interrupting reproductive function in the male, have not been investigated for their effects on fertility (Ehnert and Moberg, 1992).

Cortisol, ACTH and CRH may all have a role in the regulation of the gonadal axis. As with the exertion of negative feedback control of the glucocorticoids on the HPA axis, control may be effected at one or several of the hypothalamus, pituitary or gonad, depending on the species concerned (Armstrong, 1986; Moberg, 1987). The nature and duration of the stressor can influence the outcome, with chronic stressors being more deleterious to function of the gonadal axis (Rivier and Rivest, 1991). There is also evidence that follistatin, a gonadotrophin suppressing polypeptide, may be released as a result of HPA axis activation. An increase in circulating follistatin in ovariectomised ewes occurred following acute stressors such as jugular vein cannulation (Findlay *et al.*, 1992).

2.7.2 Gender differences in HPA axis function

Sex differences in the response of the HPA axis to stress, with female reactions being more robust than those of males, have been reported in a number of species including man. These differences are partly due to differences in circulating gonadal steroids, and probably originate both in the peripheral organs due to altered steroidogenesis and steroid metabolism as well as in higher levels of the axis (Handa *et al.*, 1994).

Several examples of local regulation of steroid metabolism by androgens exist. Gonadal steroids altered the manner in which corticosterone was metabolised in hepatocytes. Male and female rats demonstrate differences in the degradative pathways of glucocorticoids in the liver which could be mimicked by appropriate treatment of rat pups with testosterone or oestrogen soon after birth (Denef and de Moor, 1969). Gonadal steroids also alter steroidogenic pathways in the adrenal cortex. The enzyme 5α-reductase is normally found in testicular tissue

and converts testosterone to dihydrotestosterone. Activity of 5α-reductase in the adrenal is increased in gonadectomised rats which may lead to reduced secretion of corticosterone. Testosterone inhibited the activity of $P450_{scc}$ in rat adrenal tissue (Malendowicz, 1976). Androgens suppressed $P450_{C21}$ activity in cultured bovine adrenocortical cells resulting in reduced formation of glucocorticoids (Hornsby, 1982).

Human ACTH and cortisol secretory patterns show gender differences which are attributed to greater responsiveness, but not sensitivity of the adrenal gland to ACTH in females. Analysis of pulsatility over a 24 h period showed similar cortisol secretory patterns between men and women. ACTH secretion showed higher pulse frequency, greater mean peak amplitude and a higher overall mean in men (Horrocks *et al.*, 1990). The calculated half-lives of both ACTH and cortisol were similar in men and women (Roelfsma *et al.*, 1993).

Modulation of higher levels of the HPA axis by gonadal steroids have been demonstrated. Responsiveness to stress was altered by gonadectomy in rats. These changes were associated with alterations in levels of ACTH secretagogues, but anterior pituitary sensitivity to CRH did not appear to change. This suggested a site of action at the hypothalamus or higher (Handa *et al.*, 1994). Areas of the hippocampus such as the BNST contain significant numbers of androgen receptors (AR), suggesting that androgens may influence feedback mechanisms (Simerly *et al.*, 1990). Increased levels of CRH and AVP in the hypothalamus of male rats following gonadectomy were prevented by treatment with the non-aromatisable androgen dihydrotestosterone (Bingaman *et al.*, 1994). Attempts to co-localise AR and CRH in the hypothalamus have failed, and it appears that androgens exert their effect from higher in the CNS. In contrast, there is evidence that oestrogen may have a direct effect on the PVN as well as higher centres (Handa *et al.*, 1994). Chronic oestrogen treatment prolonged plasma ACTH and cortisol concentrations in rats exposed to stressors, suggesting that oestrogen was impairing intermediate negative feedback. Furthermore oestrogen treatment caused functional impairment of hippocampal GR (Burgess and Handa, 1992). Oestrogen may alter the plasticity of Type II receptors and their ability to self-regulate (Handa *et al.*, 1994). The sites and mechanisms of action of the gonadal steroids on the HPA axis require further elucidation.

2.8 THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS AND AGEING

Ageing is associated with a loss in resiliency of the HPA axis which is paralleled by changes seen following glucocorticoid exposure. This has led to a proposal known as the Glucocorticoid Cascade Hypothesis of Brain Ageing and Neurodegeneration. This postulates that once receptor degeneration in the hippocampus is initiated, the consequent inability to control glucocorticoid levels leads to positive feed forward and further degeneration (Sapolsky *et al.*, 1986). The hypothesis was modified following observations that ageing alters the balance of hippocampal receptors. It was proposed that receptor balance determines a negative feedback set-point which, when altered will lead to conditions of enhanced or reduced responsiveness to neurotransmitters and impaired adaptation to stress (de Kloet *et al.*, 1991).

Senescent hippocampal degeneration is a major contributor to HPA abnormalities seen in aged rats. They had higher unstressed corticosterone levels and slower recovery from stressor-induced corticosterone secretion (Sapolsky *et al.*, 1983). In young rats, persistent glucocorticoid exposure, due to either chronic stress or pharmacological treatment, resulted in neuronal degeneration in the hippocampus. Hippocampal lesions induced by trauma, as well as by glucocorticoid treatment, resulted in impaired ability to terminate the stress response (Jacobson and Sapolsky, 1991). Increased glucocorticoid levels may alter Ca^{2+} levels to change cell membrane conductance in neurones and also alter receptor plasticity to promote receptor imbalance (Landfield and Eldridge, 1991). Similar changes occurred in old dogs which showed a reduction in Type I receptor numbers (Rothuizen *et al.*, 1991). Up-regulation of mRNA encoding for both receptor types was seen following adrenalectomy in both young and aged rats, but the binding characteristics of the receptors varied between the two age groups. While corticosterone reversed the changes in mRNA encoding for both receptors in young rats, it was only effective in reversing the changes for Type I receptors in the older age group. This suggested that the age-associated decrease in receptors is functional (Morano *et al.*, 1994).

Structure-function relationships at lower levels of the axis may alter. Cells of the *zona fasciculata* in aged rats demonstrated an increased capacity to convert cholesterol to pregnenalone, but tended to accumulate this rather than utilising it for further steroidogenesis. This was associated with structural changes in mitochondria and cell membranes (Markowska *et al.*, 1994).

Parallels can be drawn between models of rat ageing and aspects of human ageing although the evidence for a loss of resiliency of the axis with age is not conclusive (Seeman and Robbins, 1994). Senescent loss of corticosteroid receptors and hippocampal degeneration have been observed in primates. Similar deficits in hippocampal function are associated with the degenerative changes of Alzheimer's disease (Jacobson and Sapolsky, 1991). Phase-shifts in the circadian rhythm were identified in ageing humans (Sherman *et al.*, 1985). Although clinical hypercortisolaemia is infrequent in aged humans, resting cortisol levels and post-dexamethasone cortisol levels may increase. Sensitivity to tropic drive through the axis is altered with ageing. The aged pituitary showed a dampened sensitivity to CRH, and the aged adrenal showed reduced sensitivity to ACTH. Although ACTH levels increased, correlated changes in glucocorticoids levels were more moderate (Markowska *et al.*, 1994). There is definitive evidence of increased prevalence of prolonged patterns of HPA response to challenge with increasing age as seen in rats. Groups of older humans demonstrate substantial heterogeneity. This has led to the proposal that a decline in HPA resilience in humans is related to a life-long cumulative exposure to glucocorticoids, and not simply to ageing itself (Seeman and Robbins, 1994).

The pineal gland may have a central regulatory role in the ageing process. Young-to-old and old-to-young pineal cross-plantation experiments in rats have demonstrated that the pineal gland can initiate or retard senescent change and may function as a regulatory "ageing clock" (Pierpaoli and Lesnikov, 1994). The potential role of HPA axis products other than glucocorticoids during senescent change requires elucidation. The $ACTH_{4-9}$ analogue known as ORG 2766 can selectively increase type I receptor density in the hippocampus and is used to improve concentration and attentiveness in ageing humans (de Kloet and Voorhuis, 1992). The age-dependent changes in plasma levels of the adrenal androgen DHEA seen in humans, and their relationship to senescent change, also require elucidation. It is uncertain whether the changes seen are due to age-related organ-failure, or to changes in feedback or regulatory mechanisms (Birkenhäger-Gillesse *et al.*, 1994).

2.9 Clinical Methods for Assessment of Function of the Hypothalamo-Pituitary-Adrenal Axis

Challenge of the adrenal cortex with ACTH is the most important test of adrenocortical function and adrenocortical reserve. Widely used in human medicine, adrenal response testing for the diagnosis of adrenocortical insufficiency has also been reported in dogs (Chen *et al.*, 1978) and cats (Smith and Feldman, 1987). Adrenal response testing has also been used to as a measure of adaptation to chronic stressors particularly in pigs (von Borell and Ladewig, 1992). Synthetic ACTH analogues e.g. $ACTH_{1-24}$ are usually used in clinical testing. ACTH is given by infusion, as an intravenous bolus, or by intramuscular injection. The levels of plasma and urinary cortisol and/or cortisol metabolites are assessed at various times in relation to the injection. Many modifications of the test exist, and critical evaluation of all the variations of this test is impossible. Traditionally, large doses of ACTH are used to assess adrenocortical reserve. The use of low doses of ACTH in the physiological range has allowed greater sensitivity in detection of alterations in adrenal cortex function (Crowley *et al.*, 1991). Hypersensitivity to ACTH can occur as a side-effect, particularly after repeated administration of ACTH. Diagnostic ACTH challenge is often performed under cover of oral dexamethasone which avoids side-effects, yet does not interfere with steroid analysis (Labhart, 1986).

Small doses of potent glucocorticoids such as dexamethasone will suppress ACTH secretion, yet contribute only minimally to urinary or plasma concentrations of 17-hydroxycorticoids. Response to dexamethasone can assist in the evaluation of the dependence of steroid production on ACTH, i.e., to differentiate steroid production from normal or hyperplastic adrenals from that of adrenal tumours, as well as assessment of the suppressibility of ACTH production, i.e., in pathological conditions of the pituitary. Various protocols exist which differ in the dose rate of dexamethasone and their subsequent interpretation (Fok *et al.*, 1991).

CRH challenge is used to assess the integrity of pituitary function and to differentiate between pituitary and hypothalamic insufficiency. CRH is given as an intravenous bolus injection and blood samples taken in relation to this injection for ACTH and cortisol assay (Schürmeyer *et al.*, 1985). When pituitary insufficiency is suspected but an ACTH test is normal, endogenous ACTH secretion may be stimulated with synthetic lysine-8-vasopressin, since the action of AVP is below the principal centres of fast negative feedback. Non-specific stimulation of the HPA

axis by insulin-induced hypoglycaemia, or fever provoked by pyrogen, are also useful tests of axis function as a rise in plasma corticoids is only elicited when the total HPA axis is intact (Schlaghecke *et al.*, 1992).

The adrenostatic drug metapyrone inhibits $P450_{C11}$ activity in the adrenal cortex to block synthesis of cortisol and corticosterone without impairing the production of their precursors. The consequent failure of negative feedback of cortisol on the hypothalamus results in maximal output of ACTH and allows an assessment of pituitary reserve of ACTH (Labhart, 1986).

2.10 Conclusion

The critical function of the HPA axis as an integrative and stabilising mediator for successful survival of the individual has been demonstrated. Its ability to receive both cognitive and non-cognitive input from a vast array of sources, and to translate these signals into an output which maintains homeostasis in a cohesive fashion underpins its complex function.

This chapter has provided only a broad overview of some aspects of HPA function. Differences identified between species add to the complexity of the system. Most knowledge to date has been gained from research on rodents and humans, and the extent to which these can be extrapolated to either ruminants or to farm livestock in general must be limited. Present interest in ensuring that farmed livestock enjoy appropriate freedom from management imposed stressors requires that we improve our understanding of the mechanisms surrounding the animal's perception of and response to them. This has led to the need to re-evaluate our knowledge of HPA function in farmed livestock.

CHAPTER 3

REVIEW OF THE FUNCTION OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN CATTLE

Much of our understanding of function of the hypothalamo-pituitary-adrenal (HPA) axis as described in Chapter 2 is based on research in non-ruminant species. Parallel investigations in ruminants in general, and cattle in particular, have not been carried out in many areas. Where information is available there are indications that cattle vary from other species in some aspects of function of the axis. This chapter reviews the literature relating to function of the HPA axis in cattle.

CHAPTER OUTLINE

3.1 Structure of the adrenal gland
- 3.1.1 Gross anatomy
- 3.1.2 Histology of the adrenal cortex

3.2 Hormones of the adrenal cortex
- 3.2.1 Steroidogenesis
- 3.2.2 Metabolism and excretion of glucocorticoids
- 3.2.3 Modes of glucocorticoid secretion
  - 3.2.3.1 Basal activity
    - 3.2.2.1.1 Rhythms of cortisol secretion
    - 3.2.2.1.2 Physiological influences on basal cortisol secretion
  - 3.2.3.2 Cortisol secretion in milk
  - 3.2.3.3 Cortisol secretion during stress
- 3.2.4 Other adrenal steroids

3.3 Mechanisms of HPA axis control
- 3.3.1 Adrenocorticotropic hormone (ACTH)
- 3.3.2 Hypothalamic secretagogues for ACTH
- 3.3.3 Endogenous opioids
- 3.3.4 The potential role of other factors in HPA axis control

3.4 Synthetic glucocorticoids
3.4.1 Plasma distribution and clearance
3.4.2 Effects of synthetic glucocorticoids
3.5 Glucocorticoids and bovine leucocytes
3.6 Pregnancy and parturition
3.6.1 The calf
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3.6.2.1 Spontaneous parturition
3.6.2.2 Induced parturition
3.7 Cortisol in lactogenesis and galactopoiesis
3.8 The inter-relationship of the adrenal and gonadal axes
3.8.1 HPA axis regulation of the gonadal axis
3.8.1.1 The cow
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3.8.2 Gonadal regulation of the HPA axis
3.8.2.1 The cow
3.8.2.2 The bull
3.9 Ageing
3.10 Changes in activity of the HPA axis during stress
3.10.1 Basal plasma cortisol concentrations
3.10.2 Responses to ACTH
3.11 Conclusion

3.1 STRUCTURE OF THE ADRENAL GLAND

3.1.1 Gross anatomy

The right adrenal gland of cattle is elongated and roughly V-shaped, and lies at the cranial pole of the right kidney. The ventral surface is grooved for the passage of the caudal vena cava. It weighs approximately 12 gm in adult cattle and measures about 8 cm in length and 2 cm in thickness. The left adrenal gland is slightly larger and more compact. It is located on the midline, 5-8 cm cranial to the left kidney. In adult cattle, the left gland weighs approximately 14.5 gm and measures about 8 cm in length, 6 cm in breadth and 3 cm thick (Venzke, 1975).

Each adrenal receives two arteries from either the aorta or the renal arteries. The right adrenal vein leaves the apex of the gland from its ventral surface and enters the caudal vena cava. The left adrenal vein leaves from the right face of the gland and enters the right renal vein. Lymph vessels leave with the veins and drain into renal lymph nodes (Venzke, 1975).

The extrinsic nerves arise from the last few thoracic spinal nerve segments and are nearly all sympathetic. They reach the adrenal through the adrenal plexus which is derived primarily from the coeliac plexus. Approximately 50-70% are myelinated preganglionic fibres which terminate on the medullary cells (Venzke, 1975).

3.1.2 Histology of the adrenal cortex

The *zona glomerulosa* is about 350 μm thick in mature cattle. The cells appear columnar (Venske, 1975) but irregular cell cords extend into the deeper layers of the adrenal cortex of the bull (Cupps *et al.,* 1954). The mature *zona fasciculata* is about 3 mm thick (Venske, 1975). Cells have a compressed appearance and contain abundant cytoplasm. The boundary between the *fasciculata* and the *reticularis* is very irregular (Cupps *et al.*, 1954 and 1956). The combined width of the *zona fasciculata* and *reticularis* was greater during the first year of life in bulls than in heifers (Macmillan, 1967).

The medullary cells are arranged with a zone of adrenaline-secreting cells at the outside and noradrenaline-secreting cells in the centre. Cords of cortical cells accompany nerves and arteries into the medulla and appear as islands in normal histological sections. Tumours of the medulla are more common than those of the cortex in cattle They are most often described as phaeochromocytomas and arise from chromaffin cells, but are usually benign (Appleby, 1978).

3.2 Hormones of the Adrenal Cortex

Cortisol and corticosterone are the principal products secreted by the bovine adrenal cortex. Their ratio may be dependent upon age. Corticosterone was not detected until about 10 days of age in calves. Breed differences in the cortisol:cortisone ratio have been reported in adult lactating cows. The ratio was 4:1 in Holsteins, 2.8:1 in Jerseys, and 1.5:1 in Guernseys

(Venkataseshu and Estergreen, 1969). This ratio was confirmed for Holstein cows (Wu *et al.*, 1990).

High performance liquid chromatography of adrenal cortex tissue extracts during the post-partum period demonstrated that cortisol was the major steroid present (3.3 μg/g tissue). Cortisone, corticosterone and 11-dehydrocorticosterone were present in lesser, but significant amounts. All other adrenal steroids were present at levels less than 0.8 μg/g tissue (Spicer *et al.*, 1989).

3.2.1 Steroidogenesis

Many *in vitro* investigations of adrenocortical steroidogenesis have used cells of bovine origin so the pathways are well understood. Bovine adrenocortical cells contain specific high-affinity, low-capacity receptors for adrenocorticotropic hormone (ACTH). Treatment of cells with ACTH reportedly caused an increase in ACTH binding, suggesting that ACTH had a positive tropic effect on target-cell responsiveness (Penhoat *et al.*, 1989). Activity of the P450 cytochrome enzyme 17α-hydroxylase ($P450_{C17}$) was specifically induced by ACTH in primary bovine adrenocortical cell cultures (Hornsby, 1982).

Bovine adrenocortical cells appear totally dependent upon the presence of exogenous precursors for cholesterol such as low density lipoproteins for the successful production of steroids. Adrenocortical steroids themselves can exert regulatory effects on steroidogenesis. The activity of the P450 cytochrome enzyme 21-hydroxylase ($P450_{C21}$) was reduced by exposure to pregnenalone, progesterone and its analogues, and to some C_{19} steroids (but not dihydro-epiandrosterone). In particular, the C_{19} steroid androstenedione inhibited $P450_{C21}$ activity *in vitro* at physiological concentrations. The C_{19} steroids may form a complex with $P450_{C21}$ which results in this loss of activity (Hornsby, 1982). Levels of cortisone were negatively correlated to levels of progesterone and 17α-progesterone. This suggested that cortisone might have an inhibitory action on steroidogenesis, possibly by inhibition of 3β-hydroxysteroid dehydrogenase activity (Spicer *et al.*, 1989).

3.2.2 Metabolism and excretion of glucocorticoids

The plasma half-life of cortisol in dairy cows was estimated by isotope dilution to be between 20 and 25 min (Christison and Johnson, 1972). In post-partum cows, plasma half-life for cortisol was estimated to be 30 min using a bolus injection method. Although basal plasma cortisol concentrations (PCC) were higher in suckled than non-suckled cows, clearance rates were similar. At 14 days post-partum the volume of distribution was 225 l, but by 28 days post-partum it had decreased. Since cow weights did not change, this decrease was considered to be due to a decrease in cortisol binding in tissues. Total body clearance was about 5 l/min (Dunlap *et al.,* 1981a).

The major protein which binds cortisol in serum of cattle is cortisol binding globulin (CBG) as in many other species. The proportion of free cortisol in serum of cows under basal conditions and following stimulation by ACTH was estimated to be about 6% of total cortisol (Shutt and Fell, 1985). Reported differences in CBG binding capacity between suckled and non-suckled cows were suggested to be related to effects of energy balance. Approximately 10% of total cortisol remained free in cows during the post-partum period with no difference detected in CBG binding capacity between well fed suckled and non-suckled cows. A similar change in ability of CBG to bind cortisol has been seen in sheep in negative energy balance (Faltys *et al.*, 1987). Progesterone was a competitive inhibitor of cortisol binding to CBG at 37°C, but notdexamethasone (Gorewit and Tucker, 1976).

3.2.3 Modes of glucocorticoid secretion

3.2.3.1 Basal activity

3.2.3.1.1 Rhythms of cortisol secretion

Early studies into diurnal rhythms of plasma glucocorticoids in cattle produced conflicting results. Sampling at low frequencies sometimes produced evidence of diurnal variations, including an early morning peak with lower levels in early evening (Wagner and Oxenreider, 1972; MacAdam and Eberhardt, 1972). Others failed to identify any rhythmicity (Paape *et al.*,

1972; Hudson *et al.*, 1975). When sampling occurred at high frequencies, there was good evidence that cortisol was secreted in a rhythmical fashion. Sampling of non-pregnant heifers at 10 min intervals demonstrated that ultradian rhythms occurred with a frequency of about 0.6 cycles/h. Pulse amplitudes were higher between midnight and mid-morning than in the afternoon (Fulkerson *et al.*, 1980). Bulls also showed spontaneous secretory episodes which varied in frequency and amplitude in a similar pattern to that seen in cows. Smallest peaks and lowest mean PCC occurred in early evening and activity increased at the onset of daylight (Thun *et al.,* 1981).

Circadian and ultradian rhythms in cattle were definitively characterised by Lefcourt and co-workers (1993). Potential influences which might obscure secretory patterns were reduced by stringent environmental controls, rigid management of the cows and automatic sampling techniques. Sampling was carried out at 15 min intervals over a 48 h period. The ultradian rhythm was strong and easily observed, suggesting that it may be intrinsic to mechanisms regulating peripheral glucocorticoid concentrations in cattle. The circadian rhythm was weak in amplitude compared to the ultradian rhythm. The variations in mean PCC during the diurnal cycle were relatively small - the difference in mean PCC between the peak (4.5 ng/ml) and the nadir (3.1 ng/ml) of the circadian rhythm was only one third of the mean peak PCC. Ultradian rhythms had amplitudes of up to 17 ng/ml, and a periodicity of 120 min which varied among cows and within individuals over time (Lefcourt *et al.*, 1993).

Secretory patterns in ACTH secretion have not been defined. When cows were sampled at 20 min intervals for 6 h starting at 1000 h, no pulsatile patterns of either cortisol or ACTH concentration in plasma were identified (Sartin and Kemppainen, 1988). Plasma ACTH concentrations in samples taken from cows at 5 min intervals for 190 min varied from 24-50 pg/ml and no secretory pattern was identified; but sampling was done only 60 min after the catheters had been introduced, and animals escaped during the sampling period. As a consequence of these stressors, the HPA axis may have been refractory. Furthermore, there appeared to be no consideration of time of sampling in relation to circadian rhythms (Hegstad *et al.,* 1992).

3.2.3.1.2 Physiological influences on basal cortisol secretion

Regular behaviours such as feeding can alter patterns of cortisol secretion. Differences in PCC were identified when pre-ruminant calves were fed milk that either curdled or failed to curdle in the abomasum, or were fasted for 24 h. There was a sharp post-prandial decrease in fed calves which was more intense following the morning meal than the evening meal. Mean PCC after feeding was lower in the calves fed uncurdled milk than in the other two groups. Fasted calves showed no change in PCC whereas the other groups showed a post-prandial decrease (Gardy-Godillot *et al.*, 1989).

Variations in cortisol secretion were documented in bulls which had been relocated after puberty to places which were geographically separated in longitude. Their basal PCC were assessed by sampling at 20 min intervals over a 24 h period on three occasions which were 6, 12 and 18 months after relocation. There were differences due to location - mean PCC was lower at locations which were closer to the equator and hotter (Berardinelli *et al.*, 1992).

Duration of light influenced basal plasma glucocorticoid concentrations in a group of prepubertal bulls held at constant temperature and humidity. Animals were sampled at the midpoint of the "lights on" period which corresponded to the same "clock-time" for each animal. Plasma glucocorticoids decreased when light exposure increased from 8 to 16 h, and *vice versa*. Changes in light intensity and wavelength had no effect on glucocorticoid levels (Leining *et al.*, 1980).

Season of the year did not influence basal PCC although it did alter luteinising hormone (LH) secretion in ovariectomised cows (Critser *et al.*, 1983). Patterns of PCC were not assessed when plasma LH concentrations in steers and bulls were measured. Although steers showed an influence of season on mean plasma LH concentrations, those in bulls showed no such effect. Pulse amplitude of LH was greatest at the summer solstice which corresponded with the time of highest mean plasma testosterone concentrations (Stumpf *et al.*, 1993).

Genotype may influence adrenocortical activity. Mean basal serum cortisol concentrations measured in suckled cows at 17 and 34 days post-partum were lower in Brahman than in

Angus animals (Byerley *et al.*, 1992). In contrast there was no detectable influence of breed on PCC when bulls were relocated following puberty (Berardinelli *et al.*, 1992).

Lactational state and level of production can also influence basal PCC. Mean PCC were higher in pregnant lactating cows than in pregnant non-lactating cows (MacAdam and Eberhart, 1972; Wagner and Oxenreider, 1972). Suckling or machine milking both resulted in a brief increase in plasma glucocorticoids (Wagner and Oxenreider, 1972; Hall *et al.*, 1991). The ultradian rhythm had lower amplitude at 30 days than at 90 days post-partum. Level of production influenced amplitude at 90 days post-partum. Cows in the top quartile of the herd for milk volume had a lower pulse amplitude than cows in the bottom quartile (Sartin and Kemppainen, 1988).

3.2.3.2 Cortisol secretion in milk

Cortisol passes freely across the mammary barrier to be secreted in milk. This transfer is probably passive, with levels in milk being proportional to levels in the serum during the period prior to milk sampling. Glucocorticoid concentrations were similar in skim and whole milk suggesting that the cortisol did not associate with the milk fat (Gwazdauskas *et al.*, 1977). Cortisol in milk from cows in established lactation is about 6% of total cortisol, similar to the proportion of free cortisol in serum (Shutt and Fell, 1985). Changes in milk cortisol concentrations closely reflected the changes in PCC following injection of cows with ACTH or trucking. Milk cortisol concentrations increased and milk volumes decreased, but milk cortisol concentrations were better indicators of PCC than calculated total cortisol outputs. There was marked individual variability in milk cortisol concentrations within treatment groups as for basal PCC (Bremel and Gangwer, 1978).

Milk cortisol concentrations appear to be in dynamic equilibrium with blood cortisol, and are not altered by dilution from continued milk synthesis. Although increases in cortisol secretion were followed by increases in milk cortisol concentrations, levels declined again rapidly when increments of cortisol in blood were not sustained (Fox *et al.*, 1981).

3.2.3.3 Cortisol secretion during stress

Diurnal and ultradian patterns of cortisol secretion in cattle are easily disrupted by exogenous influences. Patterns of PCC were synchronous at the beginning of a sampling period, but then became asynchronous, suggesting that the initial stress of the sampling regime had set the pattern (Fulkerson *et al.,* 1980).

The stress of restraint and handling is sufficient to cause an increase in PCC in cattle. Commonly practised veterinary procedures, including venipuncture and rectal palpation of the reproductive tract, increased PCC three to six-fold above basal levels. Levels were raised for up to 80 min following the manipulations (Alam and Dobson, 1986).

Genotype may influence PCC during exposure to stressors. Calves of *Bos indicus* genotype had higher basal levels of PCC following restraint, weaning and transport than calves of *Bos taurus* genotype (Zavy *et al.*, 1990). When *Bos indicus* and *Bos taurus x Bos indicus* calves were exposed at birth to cold temperatures the crossbred calves had lower PCC and blood glucose levels and were better able to maintain their body temperatures (Godfrey *et al.*, 1991).

Cattle are sensitive to heat stress. Cows exposed to 35°C for 4 h showed an increase in PCC which peaked after about 2 h. When continuously exposed to the same temperature for 7-10 weeks there was a depression in PCC (Christison and Johnson, 1972). Thermal stress caused an increase in mean plasma corticoid levels in cows which had no shade compared to cows which did have shade. Shaded cows had lower rectal temperatures, greater forage intake and less water intake (Roman-Ponce *et al.*, 1981a).

Prolonged stress may have differential effects on PCC. Basal PCC of calves were high during the days following weaning and restraint. Plasma cortisol concentrations were further increased on the day following trucking but fell to low levels one week later. This may have been due to desensitisation of the HPA axis by negative feedback (Zavy *et al.*, 1990).

Dietary influences on basal PCC are not clear. Increases in PCC occurred during acute fasting in both bulls and steers (Ward *et al.*, 1992). By contrast, inadequate nutrition over a prolonged

period may not produce changes. There was no difference in basal PCC during 15 min sampling for 8 h in the post-partum period of suckled beef cows where animals were on diets at two different energy levels, even though one group maintained their weight while the other group lost about 0.6 kg/day (Hall *et al.*, 1991).

Disease can alter basal PCC and the degree of change may be related to prognosis. Calves which had greater increases in plasma glucocorticoid levels during acute viral enteritis were more likely to die (Lopez *et al.*, 1975). During bouts of acute bacterial enteritis, scouring calves had increased basal plasma corticoid levels. Calves in the group which developed diarrhoea were those which had tended to have higher plasma glucocorticoid levels during the first four days of life (Hudson *et al.*, 1976a).

Psychological stressors can activate the HPA axis. Unfamiliar environments and disturbance by surprising events may elicit a fear response which is characterised by an increase in PCC. Psychological reactions are complex and also involve the sympathetic nervous system e.g. to alter heart and respiration rates (Boissy and Bouissou, 1994).

3.2.4 Other adrenal steroids

Apart from glucocorticoids, progesterone is the only steroid secreted from the bovine adrenal cortex in significant quantities, and then it is only secreted in minimal amounts. Secretion increases during periods of high plasma ACTH concentration, probably as overflow from excess production of cortisol precursor (Dunlap *et al.*, 1980). Detectable levels of androstenedione were found in plasma from cycling cows and did not vary during the oestrous cycle as did levels of testosterone; but treatment with betamethasone did not alter plasma androgen concentrations in these cows suggesting that they were not of adrenal origin (Kanchev *et al.*, 1976).

Androstenedione and testosterone are present in moderate quantities in the plasma of pubertal and post-pubertal bulls, but are principally of testicular origin (Bedair and Thibier, 1979). Androstenedione may have a role in maturation of the HPA axis at the onset of pubertal change in bull calves. Plasma concentrations increased, but the increase was greater in bulls than in steers indicating that the principal source was testicular (Amann *et al.*, 1986).

3.3 Mechanisms of Hypothalamo-Pituitary-Adrenal Axis Control

3.3.1 Adrenocorticotropic hormone

Sensitivity to (ACTH) can be considered to be the minimum dose required to elicit a consistent measurable response in PCC. The response to low doses of ACTH is considered to represent steroidogenesis by the pool of available secretory enzymes rather than by the induction of additional enzymes. Following administration of low doses of ACTH, response peaks were characteristically rapid, with PCC returning to pretreatment levels by 90 min (Dickstein *et al.*, 1991; Crowley *et al.*, 1991). The adrenal cortex of dairy cows is highly sensitive to ACTH. A measurable increase in PCC which lasted for one hour resulted from the intravenous injection of only 1 IU of ACTH in adult dairy cows (Paape *et al.*, 1977). Even so, this is 10 times the ACTH dose rate of 0.0014 ng/kg$^{0.75}$ reported to be the minimal dose required to elicit a measurable response in sheep (Fulkerson and Jamieson, 1982). By contrast the equivalent dose for young adult men was 3.5 ng/kg$^{0.75}$ (Crowley *et al.*, 1991).

The adrenal cortex responds in a graduated manner to increasing doses of ACTH. When a range of doses of ACTH (0.03 to 0.4 mg) were administered to cows by intramuscular (im) injection, peak PCC and the integrated response (area under the PCC.time curve) increased linearly with dose of ACTH up to 0.12 mg. Above this, the integrated response continued to increase linearly, but the rate of increase of peak PCC was logarithmic. The mean peak PCC achieved following 0.4 mg ACTH was not different from that achieved following 0.12 mg (Alam *et al.*, 1986).

Responsiveness of the adrenal cortex is influenced by previous exposure to ACTH. Daily dosing of cows with 100 IU or 200 IU of ACTH for 3 days resulted in increases in PCC during the 8 h following each injection. At the lower dose, the integrated response increased from day 1 to day 2 but decreased from day 2 to day 3. At the higher dose, the response was similar between days 1 and 2, but decreased from day 2 to day 3. This suggested that prolonged exposure to high levels of ACTH may make the gland refractory, and that this effect may be dose dependent (Gwazdauskas *et al.*, 1980). When cows were given two injections of 40 IU ACTH at 2 h apart, adrenocortical responses were greater after the second injection than after

the first. This suggested that a moderate dose of ACTH could increase adrenal responsiveness (Fox *et al.,* 1981).

The adrenocortical response to exogenous ACTH can exceed that seen physiologically in response to stressors. Plasma cortisol concentrations were similar following an im injection of 100 IU of ACTH and intramammary infusion of *Escherichia coli* endotoxin, but were greater following administration of 200 IU of ACTH (Paape *et al.*, 1977). Adrenocortical responses to exogenous ACTH are often more prolonged than responses to stressors such as jugular catheterisation (Alam and Dobson, 1986).

Production of adrenal steroids other than cortisol may be stimulated by ACTH. This response may be dependent upon both the dose of ACTH and the physiological status of the animal. Plasma progesterone levels in post-partum cows increased following intravenous (iv) injection of 200 IU ACTH. The increase was negatively correlated to time post-partum, and was greater in non-suckled than in suckled cows (Dunlap *et al.*, 1980). Plasma cortisol concentrations increased but there was no change in plasma concentrations of progesterone or testosterone following treatment with a depot form of ACTH (Dobson and Kanchev, 1977). Treatment of cows with 0.1 or 0.2 mg ACTH increased plasma progesterone but not testosterone concentrations (Alam *et al.*, 1986). Plasma corticosterone concentrations in dairy cows were not altered by treatment with ACTH. This suggested that ACTH was preferentially stimulating the 17α-hydroxylating enzyme system to decrease substrate available for production of corticosterone (Venkataseshu and Estergreen, 1969).

Administration of ACTH can alter levels of other metabolites in circulation. Injection of 6 IU ACTH altered plasma levels of amino acids; concentrations of phenylalanine increased while those of asparagine and glycine decreased. Plasma glucose and valerate concentrations increased, but urea concentrations were not altered (Ndibualonji *et al.*, 1994).

Age and the physiological state of the animal may be associated with variations in adrenocortical response to ACTH. During the first week of life, both peak and duration of response to ACTH increased (Steffen, 1989). Age, stage of lactation and environmental temperature all had significant effects on adrenocortical responses to ACTH in a group of

lactating cows. Age was negatively correlated to response, but the effect of age was much less than stage of lactation or environmental temperature. Adrenocortical responses decreased when environmental temperatures increased above 21°C (Shayanfar *et al.*, 1975). When response to ACTH was compared in post-partum beef cows, it was greater in non-suckled than in suckled cows, and increased with time post-partum. Maximum responses were reached at 28-29 days post-partum in the non-suckled cows, and at 56-57 days post-partum in the suckled cows. Increased negative feedback to the HPA axis following suckling-induced cortisol release may have decreased responsiveness to ACTH, or metabolic clearance of cortisol may have been greater in the lactating cows (Dunlap *et al.*, 1981b).

Responses to ACTH may vary during pathological conditions. A syndrome of adrenocortical insufficiency was reported in lactating cows with periparturient udder oedema (van der Kolk *et al.*, 1991b). Cows with cystic ovarian disease showed lower basal plasma glucocorticoid concentrations and a reduced adrenocortical response to ACTH. This may have resulted from suppression of adrenal function by prolonged elevations of plasma oestrogen. The cows demonstrated normal increases in plasma progesterone concentrations following stimulation with ACTH, which suggested that the alteration in function was occurring at the level of steroidogenesis (Trocaniz, 1973).

3.3.2 Hypothalamic secretagogues for ACTH

The pituitary secretion of ACTH in cattle is mainly regulated by corticotropic-releasing hormone (CRH) and arginine vasopressin (AVP). The bovine pituitary can respond to either peptide given alone, but the combination of the two augments the secretion of ACTH. A bolus treatment of 10 IU of AVP increased plasma glucocorticoid concentrations for 20-25 min (Wagner and Oxenreider, 1972). Ovine CRH administered by iv injection produced a rapid elevation in plasma ACTH concentrations (PAC) which persisted for 1 h (Welsh *et al.*, 1985). When exogenous bovine CRH (0.3 μg/kg live-weight) and AVP (0.1 μg/kg live-weight) were administered to cows by iv injection either alone or in combination, tightly coupled increases in PAC and PCC were seen at 1 and 10 min following treatment (Carroll *et al.*, 1993). Following iv administration to heifers of an equivalent dose of bovine CRH in combination with a 10-fold greater dose of AVP, peak PAC lasted about 40 min, and PCC were still elevated at the end of

the 2 h sampling period. The response to the combination was more than three-fold the response to treatment with CRH alone, while CRH alone resulted in greater PAC than did AVP alone. Increases in PCC were similar following treatment with either CRH alone or for a combination of CRH and AVP, but were twice as great as those seen following AVP alone (Jacob and Minton, 1993).

3.3.3 Endogenous opioids

The HPA axis of dairy cows may be under suppressive opioidergic control. The synchronous release of both endogenous opioids and ACTH from the pituitary may modulate negative feedback mechanisms (Guillemin *et al.*, 1977). Treatment of anoestrus beef cows with the opioid antagonist naloxone produced variable changes in serum cortisol concentrations but with a tendency for increased levels (Whisnant *et al.*, 1986). When lactating dairy cows were treated with a higher dose of naloxone, the response was more consistent. There was an immediate increase in PCC, reaching a peak within 30 min of treatment. In contrast, morphine suppressed basal cortisol secretion (Nanda *et al.*, 1992).

3.3.4 The potential role of other factors in HPA axis control

Endogenous prostaglandins may have a role in control of function of the HPA axis. Evidence that the bovine pituitary is sensitive to endogenous secretion of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) was seen in bulls. Plasma concentrations of PGFM showed a pulsatile pattern when measured at 2 min intervals, and the frequency of PGFM peaks increased prior to the LH surge (Wallace *et al.*, 1985).

Prostaglandins administered to cattle initiate secretion of cortisol. Infusion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) into the carotid artery at a rate of 2 μg/min increased plasma LH, testosterone and glucocorticoid concentrations in bulls, but infusion into the jugular vein did not. Higher doses by both routes resulted in increases in these hormones. This suggested that the site of action of $PGF_{2\alpha}$ in stimulating release of adenohypophyseal hormones was in the head (Haynes *et al.*, 1977). Prostaglandin E_2 also stimulated increases in PCC following im injection of bulls. Peak PCC were positively correlated to doses of prostaglandin (Collier *et al.*, 1979). Similarly im

injection of ovariectomised cows with $PGF_{2\alpha}$ resulted in an increase of PCC (Watson and Hinrichs, 1989).

Prostaglandins may be involved in the activation of the HPA axis by cytokines. About 30 min after iv injection of cows with bacterial endotoxin, serum prolactin and cortisol concentrations increased and remained elevated for up to 7 h. Serum PGFM increased immediately and remained elevated for 4 h (Jackson *et al.*, 1990). Time delay in the reaction supports the hypothesis of an occurrence of a cascade of endocrine events in cytokine activation of the HPA axis. Endotoxin may trigger release of interleukin 1 and tumour necrosis factor α from peripheral monocytes and liver Kuppfer cells. These may in turn stimulate prostaglandin release and activation of the HPA axis (Lohuis *et al.*, 1988). Similar disturbances (but no prolactin response) were seen following intramammary infusion of endotoxins but maximum effects occurred at about one-fifth of the dose, and the time-lag to the increase in PCC was greater. This suggested that there is release of active compounds from the mammary gland rather than diffusion of the endotoxin into the circulation. Variations in the nature of cytokines released may account for these differences of action (Jackson *et al.*, 1990).

3.4 SYNTHETIC GLUCOCORTICOIDS

3.4.1 Plasma distribution and clearance

The formulations of the synthetic glucocorticoids used in cattle confer a wide variety of potency and duration of action. Prednisolone has a shorter plasma half-life and greater body clearance rate than dexamethasone which is similar to betamethasone. In general the plasma half-lives of the synthetic glucocorticoids are longer in cattle than in horses, dogs and humans. This suggests that cattle may have a low hepatic capacity to metabolise synthetic corticosteroids (Toutain *et al.*, 1985). Prednisolone can bind to transcortin with a high affinity but low capacity. It also binds to albumin by a non-saturable mechanism. This produces differences in distribution and clearance in many species, but may not be important in cattle because transcortin levels are low and binding sites are always saturated over the range of measurable prednisolone concentrations (Alvinerie *et al.*, 1988).

When solutions of dexamethasone alcohol or dexamethasone 21-isonicotinate were injected iv into cattle, the kinetics of distribution were similar, and fitted a two compartment open model. Plasma half-life of elimination had a mean of 5.5 h but there was individual variation with a range from 3.3 to 6 h (n = 4). Volume of distribution was about 1.2 l/kg and body clearance rate was 0.145 l/h/kg. After im injection of equivalent doses the half-life of absorption was 1.3 h for both solutions with maximal plasma concentration being achieved after about 4 h (Toutain *et al.*, 1982).

Following the im injection of a suspension of dexamethasone 21-isonicotinate, dexamethasone concentrations in plasma were undetectable. To assess the effects of this treatment, basal PCC and PCC following ACTH challenge were measured in treated cows at various intervals. Plasma cortisol concentrations were suppressed in samples taken under basal conditions until 45 days after injection of dexamethasone. Adrenocortical responses to ACTH did not return to pre-injection levels until 52 days after treatment (Toutain *et al.*, 1982).

When cows were injected with tritiated dexamethasone trimethyl-acetate, radioactivity in plasma was detectable at a low level for up to 30 days (McGowan *et al.,* 1975). Following injection of tritiated dexamethasone sodium phosphate, plasma concentrations rose sharply to peak in 20 min at levels of 24-70 ng/ml but were undetectable after 72 h. Following im injection of 20 mg dexamethasone trimethyl acetate, plasma concentrations achieved a broad peak of about 1 ng/ml from days 2 to 6 post-injection, and were undetectable by day 14 post-injection. Concentrations of dexamethasone in milk followed a similar pattern to plasma concentrations but at about 40% of that level (Fairclough *et al.*, 1981).

Patterns of prednisolone absorption and excretion in cows were similar to dexamethasone. An im injection of prednisolone succinate resulted in rapid absorption with plasma concentrations reaching a peak by 8 min and being undetectable beyond 14 h. PCC were suppressed from 1 h until 2 days post-injection. Prednisolone acetate had a half-life for absorption of 2 days following im injection. Mean plasma concentrations showed a broad peak from 2 to 10 h, then decreased slowly. The time at which prednisolone became undetectable varied between individuals from 1 to 6 days. PCC were suppressed from 2 h post-injection, remained undetectable for 3 weeks, and regained normal values only after 6 weeks (Toutain *et al.*, 1985).

3.4.2 Effects of synthetic glucocorticoids

There is extensive literature regarding the effect of treatment with synthetic glucocorticoids on milk yield. Dexamethasone can be detected in milk within 15 min of iv injection, and has a powerful suppressive effect on milk yield (van der Kolk, 1990). Dexamethasone treatment was associated with diminished glucose uptake and utilisation by the mammary gland (Hartmann and Kronfeld, 1973). Receptors with a high affinity for dexamethasone are found in mammary tissue. Dexamethasone may compete with cortisol for binding to receptors in mammalian epithelial cells. Dexamethasone is more potent than cortisol in altering glucose metabolism in mammary tissue so that its overall effect is suppressive rather than permissive as for cortisol (van der Kolk, 1990).

Synthetic glucocorticoids interrupt reproductive function by actions at different levels of the gonadal axis. Cows treated with betamethasone had an extended luteal phase and lower circulating levels of testosterone (Kanchev *et al.*, 1976).

Basal and induced gonadotrophin secretion were altered in bulls treated with dexamethasone (Thibier and Rolland, 1976; Chantaraprateep and Thibier, 1978). Receptor binding of GnRH was not altered by glucocorticoid treatment suggesting that the effect was exerted on LH synthesis or release (Padmanabhan *et al.*, 1983). Failure of LH support of the testis may also alter spermatogenesis. Mature bulls treated with a dexamethasone preparation showed marked histological changes in the testis (Renaville *et al.*, 1993). Dexamethasone blocked 17α-hydroxylase activity in bovine testicular cells without altering 17,20-lyase activity. This reduced progesterone production and decreased gonadotrophin-stimulated production of androstenedione and 17α-hydroxyprogesterone (Welsh *et al.*, 1982).

Synthetic glucocorticoids are commonly used to maintain blood glucose concentrations during ketotic episodes in dairy cows, but their use can lead to impaired glucose utilisation. The gluconeogenic effect of synthetic glucocorticoids may be by alteration of phosphoenol-pyruvate carboxykinase activity. This liver enzyme helps control the rate of gluconeogenesis. Flumethasone and dexamethasone were most effective in maintaining blood glucose levels (Braun *et al.*, 1970). Plasma glucose levels were significantly elevated for 8 days following

injection of 20 mg of betamethasone in suspension. Gluconeogenesis as a result of glucocorticoid treatment occurred at the expense of protein balance, and may have been detrimental when long-acting preparations were used (MacDiarmid and Cooper, 1983).

3.5 Glucocorticoids and Bovine Leucocytes

Synthetic glucocorticoids produce a similar effect on circulating (WBC) in cattle as they do in other species. Treatment with 9α-fluoroprednisolone acetate increased total WBC in a dose dependent manner, primarily due to neutrophilia. Lymphocytes in blood and somatic cells in milk were not altered (Convey *et al.*, 1971). Following injection with 20 mg betamethasone in a suspension, neutrophil numbers increased, eosinophil numbers decreased, and lymphocyte numbers remained the same. The change in eosinophil numbers was still detectable 9 days after treatment (MacDiarmid and Cooper, 1982).

The distribution of WBC was also altered by increased endogenous PCC following stress or administration of ACTH. Treatment with ACTH produced an increase in total WBC which paralleled the increase in PCC but was more prolonged (Paape *et al.*, 1977). Sequential administration of ACTH resulted in neutrophilia, lymphopenia and eosinopenia in a dose dependent manner (Gwazdauskas *et al.*, 1980).

Leucocyte function tests might be a better measure of the effect of glucocorticoids than WBC counts. Repeated doses of dexamethasone given to calves only slightly increased WBC counts, but responses of T-lymphocytes to phytohaemagglutinin and concanavalin A were suppressed (Pruett *et al.*, 1987). The chemiluminescent response of phagocytes was not affected at a similar dose rate of dexamethasone (Phillips *et al.*, 1987). Increased endogenous glucocorticoid concentrations following prolonged treatment of steers with ACTH altered WBC function in a different manner to dexamethasone. The rate ingestion of bacteria, oxidative metabolic activity and antibody-dependent cytotoxicity of phagocytes were reduced by dexamethasone but not by endogenous glucocorticoids (Roth *et al.*, 1982). Endogenous glucocorticoids enhanced random migration but decreased the activity of the myeloperoxidase-catalysed bactericidal mechanisms of the neutrophils, and depressed T-lymphocyte blastogenesis in response to phytohaemagglutinin and concanavalin A (Roth *et al.*, 1982).

3.6 PREGNANCY AND PARTURITION

Maintenance of pregnancy and the initiation of parturition in cattle is dependent upon the normal function of the HPA axis (Jöchle, 1973).

3.6.1 The calf

Normal parturition is induced by developmental changes in the foetal HPA axis. Prolonged pregnancy can occur when the foetal HPA axis is non-functional as in the inherited conditions of adenohypophyseal hypoplasia in Guernsey cattle and a familial adrenal defect seen in Holstein cattle (Jöchle, 1973). When changes in glucocorticoids were measured in cannulated foetuses approaching full-term, PCC increased from about 14 days before parturition. The increase followed an exponential pattern, and peaked on the day of parturition at a value which was about six times that seen 14 days earlier (McGowan *et al.*, 1975; Fairclough *et al.*, 1975a). Plasma ACTH concentrations in bovine foetuses increased in the 8th and 9th months of pregnancy (Takeishi *et al.*, 1989).

Administration of a single large dose of ACTH, directly to foetal calves in the 8th month of gestation, induced parturition in 6 days. The adrenal glands of induced calves were three times heavier than adrenal glands from control calves which had been injected with saline and delivered by caesarean section 2 weeks later (Welch *et al.*, 1973a).

Increasing levels of glucocorticoid in foetal circulation following activation of the adrenal glands alter placental steroid metabolism. In sheep placenta, there is increased activity of 17α-hydroxylase, 17,20-lyase and aromatase. Placental progesterone is converted, via androstenedione as an intermediate, to oestrogenic compounds. The altered balance between progesterone and the C_{18} compounds initiates maturation of the placenta and assists in preparation of the foetus and dam for parturition. Similar changes in steroid concentrations are seen in the cow; but their origin may not be totally the same. Bovine placentae may also synthesise some oestrogens from pregnenalone following exposure to glucocorticoids as is seen in goat placenta (Flint *et al.*, 1979).

The origin of the increases in activity of the foetal HPA axis which initiate parturition is not clear. Isotope studies in sheep suggest that most foetal cortisol before 120 days gestation is derived from the maternal compartment. Changes thereafter are dependent upon the presence of the foetal pituitary. Foetal plasma ACTH and cortisol concentrations increase progressively during the last 35 days of pregnancy. Increases are associated with increases in responsiveness of the pituitary to hypothalamic secretagogues and of the adrenal cortex to ACTH. The concurrent increase in ACTH and cortisol suggests alterations in the set-point of negative feedback, or inputs which are not subject to negative feedback controls. A range of high molecular weight peptides derived from pro-opiomelanocortin (POMC) have been identified in foetal plasma. These are probably released from the intermediate pituitary lobe of the foetus, and may have adrenocorticotrophic activity (Challis and Brooks, 1989). Endogenous opioids were able to stimulate the HPA axis in foetal sheep from day 125 of gestation, but naloxone did not influence tonic release of ACTH and cortisol (Brooks and Challis, 1988).

Increases in CRH and AVP content of the foetal hypothalamus also occur in late gestation. It is uncertain whether these changes reflect increased production or retention due to development of negative feed-back loops. Increased foetal adrenal weight and basal ACTH and cortisol concentrations followed administration of CRH to foetal sheep, but pre-term delivery was induced inconsistently (Challis and Brooks, 1989). Activity of CRH may be enhanced by AVP, but administration of AVP antagonist to foetal lambs in the last 10 days of gestation did not alter basal ACTH or cortisol levels (Apostolakis *et al.*, 1991).

The placenta may be a source of substances which exert trophic actions on the HPA axis. Significant amounts of CRH, ACTH and POMC-derived peptides are present in human placenta, with glucocorticoids able to increase CRH and prostaglandin output. This has led to the proposal that a positive feed-forward cascade involving the foetal HPA axis is activated at term in primate pregnancy (Waddell, 1993). There is immunocytological evidence of CRH and ACTH in sheep placenta, but there appears to be little change in content as a function of gestational age (Challis and Brooks, 1989).

Events occurring around parturition produce large variations in PCC in the calf. New-born calves had greater PCC than their dams within 5 min of birth, but levels dropped to about one

third by 12 h and were similar to adult cattle by 12 days of age (Eberhart and Patt, 1971). There was no relationship between PCC of the calf and PCC of the dam either during or the day following parturition (Hudson *et al.*, 1976b).

3.6.2 The dam

3.6.2.1 Spontaneous parturition

Changes in basal function of the HPA axis of cattle during pregnancy may differ from those of other species. Basal PCC fell during the 4th month of pregnancy after which levels remained low until the 9th month (Eissa and El-Belely, 1990). Variations in adrenocortical responses to ACTH were reported during lactation but could not be attributed to stage of pregnancy (Shayanfar *et al.*, 1975).

Basal PCC in the parturient cow increased acutely at calving. As parturition approached, maternal plasma corticosteroid levels remained low despite the increase in foetal corticosteroid levels (Fairclough *et al.*, 1975a). Increases in PCC were evident from about 2 days before calving, peaked at calving and returned to low levels by 2 days after calving (Brush, 1958; Arije *et al.*, 1974; Hudson *et al.*, 1976b).

Maternal plasma concentrations of other steroid hormones also vary in relation to parturition. Serum progesterone levels fell gradually from about the 6th day prepartum, then declined suddenly in association with luteolysis to be undetectable during the final 2 days of pregnancy. The ovary is the major source of circulating progesterone in the cow in late pregnancy, although ovariectomy in the last trimester does not jeopardise pregnancy (Evans and Wagner, 1976). Serum oestradiol-17β, oestrone and oestrone sulfate concentrations increased from day 12 prepartum until parturition, then fell rapidly to be negligible by day 6 post-partum (Arije *et al.*, 1974; Humphrey *et al.*, 1983, Guilbault *et al.*, 1985).

Plasma prolactin concentrations increased in the last 15 days of pregnancy to peak just prior to parturition. These changes are coincident with the prepartum changes in plasma oestrogen and progesterone concentrations (Eley *et al.*, 1981a).

Detectable levels of PGFM were seen in maternal plasma from about 60 days prepartum. Plasma PGFM concentrations from the utero-ovarian vein increased during the 48-72 h before parturition, and rose sharply during labour. The changes in PGFM and progesterone concentrations were negatively correlated from 5 to 60 days prepartum. The sharp increase in plasma PGFM concentrations may have initiated luteolysis as it corresponded in time with the rapid decline in plasma progesterone concentrations (Edqvist *et al.*, 1978). Peripheral serum concentrations of PGFM peaked from 1 to 3 days postpartum, and remained elevated for a further 10 days post-partum (Fairclough *et al.*, 1975b; Eley *et al.*, 1981a). The source of PGFM is probably both maternal caruncular tissue and foetal cotyledon tissue (Peter and Bosu, 1987); but the possibility of the mammary gland as a source of both oestrogens and prostaglandins cannot be discounted. Since progesterone can block release of prostaglandins, and since oestrogens can induce parturition, it was proposed that the oestrogen:progesterone ratio may be an important determinant in the provocation of prostaglandin synthesis (Eley *et al.*, 1981b).

Activation of uterine $PGF_{2\alpha}$ production may be dependent upon adequate levels of circulating cortisol. Inhibition of 3β-hydroxysteroid dehydrogenase in adrenalectomised and hypophysectomised sheep foetuses at term delayed delivery compared to intact foetuses. It was proposed that the low circulating levels of cortisol prior to delivery failed to activate adequate placental steroidogenesis which led to insufficient uterine $PGF_{2\alpha}$ production (Silver and Fowden, 1991).

During the last trimester of pregnancy the myometrium displays a regular electromyographic activity with long periods of quiescence. There is a temporary loss of uterine tone immediately before initiation of parturition which is replaced by a pattern characterised by short bursts of activity and short periods of rest as the first stage of labour begins. The period of quiescence was synchronous with the abrupt changes in plasma PGFM and progesterone concentrations (Janszen *et al.*, 1990). It corresponded in time with prostaglandin-induced luteolysis, but the period of quiescence still occurred when cows were supplemented with a progestagen during luteolysis. This suggested that a factor released during luteolysis may exert an inhibitory effect on myometrial activity at this time (Janszen *et al.*, 1993).

Increases in plasma AVP and oxytocin also occur during the birth event, but not until the second stage of parturition when vaginal distension occurs (Landgraf *et al.*, 1983).

3.6.2.2 Induced parturition

The increase in glucocorticoid output by the foetal adrenal gland which initiates parturition can be mimicked during the third trimester of pregnancy by exogenous glucocorticoids (Jöchle, 1973). This has led to the use of artificial induction of parturition as a management tool in the seasonal dairy industry in Australia and New Zealand to reduce numbers of late calving cows. Various combinations of synthetic glucocorticoids and/or prostaglandin treatments have been tested. Preparations of either dexamethasone or betamethasone have been most effective in initiating parturition (Welch, 1971; Jöchle, 1973; Welch *et al.*, 1973b, 1977 and 1979; Day, 1979; MacDiarmid, 1979). The technique causes some undesirable side-effects including increased calf mortality (Welch and Kaltenbach, 1977) and increased incidence of retained foetal membranes, metabolic disease and septiceamia (Morton and Butler, 1995). Nevertheless many farmers consider that the apparent benefits are greater than the disadvantages and the technique is widely used. In a survey of 1982 calving season data from 35 dairy farms in the Waikato region, induction was used in 31 herds, with 11.3% of cows which were 3 years and older being induced (Macmillan *et al.*, 1990). A larger survey of 200 Taranaki dairy farmers in 1991 showed 76% of respondents induced some cows in their herds, with a total of 8.1% of all cows in surveyed herds being induced (Bunny, 1992).

Physiological processes during induced parturition follow a similar pattern to those seen in natural parturition. Changes in plasma concentrations of progesterone, oestrone and PGFM, following injection of 20 mg dexamethasone trimethyl-acetate on days 30 and 29 prepartum, closely resembled those seen prior to natural parturition, except that they occurred over a 5-7 day period instead of a 20-30 day period (Evans and Wagner, 1976; Edqvist *et al.*, 1981). In some cows, plasma oestrogen concentrations increased but progesterone concentrations failed to decrease completely. These cows exhibited a delay in parturition which suggested that the ratio of progesterone:oestrogen was an important factor for successful initiation of parturition (Evans and Wagner, 1976). Maximal oxytocin concentrations at parturition were not different from those of naturally calving cows (Landgraf *et al.*, 1983). Induced parturition did not affect the ability of calves to respond to ACTH (Steffen, 1989).

3.7 CORTISOL IN LACTOGENESIS AND GALACTOPOIESIS

Glucocorticoids have a role in the physiological control of mammary growth, lactogenesis and lactation of cows. With prolactin and growth hormone, glucocorticoids can induce the two-stage mechanism of lactogenesis. Cytological and enzymatic differentiation of alveolar cells prior to parturition marks the first stage. In the alveolar cell, cortisol induced differentiation of rough endoplasmic reticulum and Golgi apparatus. Prolactin stimulates the development of casein and α-lactalbumin synthesis by mammary tissue, but glucocorticoids stabilise casein messenger ribonucleic acid (mRNA). The second stage of lactogenesis is the initiation of milk secretion which begins about 4 days before parturition and extends into the early post-partum period. Glucocorticoids are very potent in initiating secretion of copious quantities of milk from well-developed mammary glands. Progesterone plays an inhibitory role during lactogenesis possibly by competitive binding to glucocorticoid receptors in mammary tissue (Tucker, 1981; Mepham, 1987).

At low plasma concentrations glucocorticoids may have a permissive role in galactopoiesis of established lactation. Milking and suckling induce the release of glucocorticoids along with prolactin and oxytocin into blood. At low levels glucocorticoid binding in mammary tissue is correlated with glucose uptake. The galactopoietic effect of glucocorticoids at low doses may be due to maintenance of levels of enzymes for glucose degradation in the alveolar cell (Tucker, 1981; Mepham, 1987).

High levels of endogenous glucocorticoids have a detrimental effect on milk production. Injection of a long-acting preparation of ACTH depressed milk yield but increased fat and protein content. Fat characteristics also changed and carotene content was lowered (Campbell *et al.*, 1963). Repeated injections of ACTH to produce maximal adrenal stimulation resulted in a decline in milk yield after the first 8 h of treatment. Milk protein yields decreased, and fat percentage of milk increased, but fat yield was unaffected. Somatic cell counts were unchanged, but sodium increased and potassium decreased (Varner and Johnson, 1983).

3.8 THE INTER-RELATIONSHIP OF THE ADRENAL AND GONADAL AXES

There is a close relationship between the adrenal and gonadal endocrine axes in cattle. Most research has concentrated on the effect of glucocorticoids on gonadotrophic and gonadal hormones, but some evidence suggests that modulation in the opposite direction can also occur.

3.8.1 HPA axis regulation of the gonadal axis

3.8.1.1 The cow

Activation of the HPA axis suppresses function of the female gonadal axis in a complex manner involving products of both pituitary and adrenal cortex. The influence of the HPA axis on LH secretion may differ for basal and gonadotrophin-releasing hormone (GnRH) stimulated release. Exogenous cortisol suppressed LH secretion in response to LH-releasing hormone (LH-RH), but had no effect on basal LH secretion (Matteri and Moberg, 1982). Higher basal PCC in suckled cows were correlated to low mean plasma LH concentrations, implicating cortisol as a cause of suckling anoestrus (Dunlap *et al.*, 1981c); but LH levels increased despite high PCC upon removal of calves (Whisnant *et al.*, 1985; Faltys *et al.*, 1987). Non-shaded cows in a tropical environment had higher PCC and lower fertility than shaded cows, but LH levels were also higher (Roman-Ponce *et al.*, 1981a).

Adrenal progesterone was implicated as a product of the HPA axis which might suppress LH secretion. Basal secretion of LH was reduced following treatment with ACTH but not with cortisone in intact cows; but was not altered by either treatment in adrenalectomised cows. The GnRH-stimulated release of LH was also reduced following treatment with ACTH in intact cows but not in adrenalectomised cows. Infusion of cortisol in adrenalectomised cows only partly restored these effects of ACTH (Li and Wagner, 1983). Even though small doses of ACTH produced significant increases in serum progesterone concentration, the amounts were not considered sufficient to exert negative feedback suppression of LH secretion (Watson and Manor, 1984).

Increases in endogenous products of the HPA axis particularly interfere with the events surrounding ovulation. Restraint and electric shock of heifers increased PCC for about 30 min. When applied twice daily to heifers in the follicular phase of the oestrous cycle, the pre-ovulatory LH surge was blocked. Basal LH concentrations were not altered (Stoebel and Moberg, 1982). High endogenous PCC, induced by transport, reduced pituitary responses to injection of GnRH (Dobson, 1987). The LH surge induced by injection of oestradiol was impaired following transport during the early post-partum period, but not when cows had resumed cycling. This suggested that the gonadal axis might be more vulnerable to the influence of stressors during the early post-partum period (Nanda *et al.*, 1990).

The gonadotroph may be directly influenced by ACTH levels. Suppression of the LH response to LH-RH was more profound in cows which received ACTH than in cows which received cortisol alone. Priming of the gonadotroph with LH-RH before ACTH treatment could overcome the blockade of gonadotrophin response (Matteri and Moberg, 1982). Based on *in vitro* studies, the effect of ACTH on gonadotrophin secretion may be attributed to different portions of the ACTH molecule or even other fragments of the POMC molecule (Moberg, 1987).

Corticosteroids can alter secretion of progesterone and oestradiol-17β from granulosa cells directly, but the practical significance of these changes is uncertain. Cortisol treatment of granulosa cells *in vitro* inhibited oestrogen production but enhanced progesterone production by blocking FSH-induced aromatase activity (Da Rosa and Wagner, 1981). The total deoxy-ribonucleic acid (DNA) content of cortisol treated granulosa cells did not change even though the number of LH receptors was reduced in a dose dependent manner (Kawate *et al.*, 1993).

Products of the HPA axis were shown to alter sexual behaviours in sheep. Expression of behavioural oestrus following oestrogen treatment of progesterone-primed ovariectomised ewes was blocked or delayed following transportation. No equivalent experiments have been done in cattle (Ehnert and Moberg, 1991).

3.8.1.2 The bull

The HPA axis can modulate the activity of the male gonadal axis. An inverse relationship was demonstrated between PCC and both basal and LH-induced testosterone secretion (Welsh *et al.*, 1979). Increases in endogenous plasma cortisol and progesterone concentrations seen during handling and electro-ejaculation were associated with suppression of both LH and testosterone secretion (Welsh and Johnson, 1981a and 1981b). Similar changes were seen following injection of ACTH (Johnson *et al.*, 1982). High PCC following ACTH administration did not influence LH release following GnRH, but FSH levels were reduced (Barnes *et al.*, 1983). Corticosteroids may influence specific mating behaviours of bulls. Sexual activity increased endogenous PCC but not plasma testosterone concentrations. When PCC was increased due to administration of ACTH, levels of plasma testosterone were suppressed, and mating behaviours towards an oestrous female were decreased (Borg *et al.*, 1991). These results suggest that the influence of the HPA axis on the gonadal axis may operate through several mechanisms.

3.8.2 Gonadal regulation of the HPA axis

3.8.2.1 The cow

Oestrogen treatments alter HPA axis function in rodent species and in humans, but such a direct physiological effect has not been recorded in cattle. Even so, there is evidence that oestrogens may influence adrenal function in cattle: adrenal weights were greater following treatment of heifers and steers with stilboestrol implants. Oestrogens can increase plasma levels of CBG in many species to delay metabolism of glucocorticoids. The significance of this in cattle with only low CBG levels is uncertain (Malendowicz, 1994). Cystic ovarian degeneration associated with increased levels of circulating oestrogens have been shown to alter adrenal secretion. Cows with cystic ovarian disease had lower basal concentrations of plasma glucocorticoids and reduced adrenocortical responses to ACTH (Trocaniz, 1973). Enlargement of the adrenal gland may also be seen in cystic ovarian disease leading to the suggestion that stress is an aetiological factor in the condition (Lopez-Diaz and Bosu, 1992).

Patterns of adrenocortical response to ACTH during the oestrous cycle have not been defined. Basal PCC varied throughout the oestrous cycle but with no clear pattern. There was a tendency for higher levels on the day of oestrus, but the sampling technique involved disturbance of the animals and no firm conclusions could be drawn. Any increases in PCC associated with day of oestrus may be due to increased activity in relation to behavioural changes. The oestradiol surge at oestrus is brief, and unlikely to influence PCC in a prolonged or detectable manner (Echternkamp and Hansel, 1973).

3.8.2.2 The bull

Products of the testis appear to modulate activity of the HPA axis in bulls. Bovine testicular hormones comprise a combination of testosterone, dehydro-epiandrosterone, androstenedione, 5-androstenediol and progesterone (Ganjam and Amann, 1976). Basal PCC were higher in steers than in bulls (Barnes *et al.*, 1983). Absolute values for both free and total PCC were higher in steers than bulls, but the proportion of free cortisol was higher in bulls. This was attributed to differences in binding to plasma albumin. The relative affinities of steroid binding sites on albumin for testosterone and cortisol were 2:1. Displacement of cortisol by testosterone could result in a higher proportion of free plasma cortisol in bulls. This may increase negative feedback and lead to a reduction in trophic drive to the adrenal cortex (Ward *et al.,* 1992).

Some differences between bulls and steers in their response to exogenous ACTH have been demonstrated. Peak PCC following iv treatment with 0.3 mg ACTH/500 kg live-weight occurred earlier in steers than in bulls, but reached similar levels (Barnes *et al.*, 1983). Adrenocortical responses to ACTH increased following castration along with an increase in plasma LH concentration. These changes were only partially inhibited by testosterone supplementation (Bass *et al.*, 1982). Testosterone feedback mechanisms are complex and may not be exerted only at the level of the pituitary. Repeated injections of testosterone following castration of prepubertal bulls failed to suppress increases in basal plasma LH concentrations and increased responsiveness of the pituitary to GnRH (McCarthy and Swanson, 1976).

Further evidence of the ability of testosterone to suppress adrenocortical response to ACTH was seen in heifers treated with testosterone proprionate. Treatment for 100 days reduced

both behavioural and cortisol responses to fear-eliciting events and also reduced adrenocortical responses to ACTH treatment. It was suggested that the site of the latter action could be by direct influence on steroidogenesis at the adrenal gland, by alteration of pituitary secretion of endogenous opioids, or by modulation of neuroendocrine systems in higher brain centres (Boissy and Bouissou, 1994).

Other androgenic steroids may have modulatory effects on adrenal steroidogenesis. The C_{19} steroid androstenedione can block the activity of $P450_{C21}$ in bovine adrenocortical cells. This would reduce the availability of substrate for production of cortisol (Hornsby, 1982).

3.9 Ageing

Age has been identified as a factor which may influence adrenocortical function in cattle. Basal PCC were negatively correlated to age in bulls studied after geographical relocation (Berardinelli *et al.*, 1992). Responses to ACTH in lactating cows were also negatively correlated to age, but the influence of age was much less than those of environmental temperature or stage of lactation (Shayanfar *et al.*, 1975).

Responses to ACTH, but not basal PCC, were related to age in a group of bulls aged from 3 months to 15 years. Following infusion of ACTH for 2 h, the bulls aged from 2 to 5 years showed increases in plasma glucocorticoid levels between 65% and 185% of basal levels; but the bulls from 8 to 15 years showed increases between 2% and 120% of basal levels. This suggested that the adrenal cortex became less responsive to circulating ACTH with age (Riegle and Nellor, 1967).

3.10 Changes in Activity of the Hypothalamo-Pituitary-Adrenal Axis during Stress

3.10.1 Basal plasma cortisol concentrations

Changes in basal PCC, often in conjunction with observations of behavioural activity, have been used to determine the harmfulness of stimuli which arise due to management and animal husbandry. For example, the behaviour and adrenocortical responses to three methods of

castration of bull calves at three ages were compared. The greatest change in PCC was seen in 6 week old calves castrated surgically, while the shortest responses occurred with the Burdizzo method. The timing of the expression of acute behavioural changes correlated well with the time changes in PCC, but the relationship between behaviour and pain intensity could not be clearly interpreted (Robertson *et al.*, 1994).

One week following transport stress, calves showed a sudden decrease in basal PCC which may have been associated with a desensitisation of the HPA axis by delayed feedback inhibition (Zavy *et al.*, 1990).

3.10.2 Responses to ACTH

Changes in adrenocortical responses to ACTH have been used to assess the effects of management imposed stressors (Friend *et al.*, 1977). Adrenocortical responses may increase or decrease following prolonged activation. The stimulatory action of ACTH on cells of the adrenal cortex leads to the accumulation of steroidogenic enzymes and membrane receptors for cholesterol uptake (Miller, 1988). ACTH can positively regulate its own receptors on adrenocortical cells (Gallo-Payet, 1987). Chronic activation may lead to depressed adrenocortical responses, i.e., "adrenal fatigue" due to activation of negative feedback mechanisms (Roman-Ponce *et al.*, 1981b). Reported changes in adrenocortical responses to ACTH following periods of stress portray a complex mechanism of HPA axis control. The extent to which the various levels of the axis are involved is unclear, and may be dependent on the nature and duration of the stressor.

When adrenocortical responses to ACTH were used to assess the effect of high housing density for 3 days, the integrated responses to ACTH became progressively greater with time. This increase was suggested to represent adaptation to the stressor (Friend *et al.*, 1977). Adrenocortical responses to ACTH were less in non-shaded cows than in shaded cows during high ambient temperatures (Roman-Ponce *et al.*, 1981a). Assessment of adrenocortical function over two lactations in cows on different diets suggested that diet could alter a cow's ability to withstand stressful periods. Interactions between diet and year were complex and the conclusions were unclear (Gwazdauskas *et al.*, 1986). Responses to ACTH in calves were

more prolonged following weaning and transportation than following initial handling at weaning (Zavy *et al.*, 1990).

Cows with Downer Cow Syndrome had higher basal and ACTH-stimulated glucocorticoid levels than their counterparts which had experienced uncomplicated parturition. The prognosis was poorest in those animals in which response was greatest (Nakao and Grunert, 1990). Pituitary and adrenocortical response to CRH and ACTH, respectively, during the course of infection with *Trypanosoma congolense* demonstrated a decrease in pituitary responsiveness during the acute phase. Basal ACTH secretion was also reduced in infected animals, but adrenocortical responses were increased when considered on the basis of cortisol released per unit of ACTH. The changes in pituitary function may have been a direct effect of parasitic invasion of pituitary tissue, or secondary to immune system activation (Abebe *et al.*, 1993).

3.11 Conclusion

Investigations of function of the HPA axis of cattle have concentrated mainly on its relationship to reproduction and production. Many aspects of the physiological control of the HPA axis in cattle remain unclear. Our understanding of the role of the HPA axis in maintenance of homeostasis during exposure to environmental or management imposed stressors is poor. The influence of products of the HPA axis on the immune system and their relationship to the sympatho-adrenal medullary axis remain to be elucidated.

CHAPTER 4

ASSAYS

This chapter describes the assays used in the experiments reported in this thesis.

CHAPTER OUTLINE

4.1 Introduction
4.2 Cortisol assay
4.2.1 Principles of the fluorescence polarisation immunoassay
4.2.2 Manufacturer's specifications
4.2.3 Assay validation
4.2.4 Discussion
4.3 Adrenocorticotropic hormone assay
4.3.1 Principles of the assay
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4.4 Progesterone assays
4.4.1 Plasma progesterone
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4.6 Oestrone sulfate assay
4.7 Prolactin assay
4.8 Alpha-lactalbumin assay
4.9 Immunoglobulin G1 assay

4.1 INTRODUCTION

This chapter describes the various assays used during the experiments of this thesis. Assays were either of commercial origin or carried out by other laboratories which had developed the appropriate methodology. The author analysed approximately half of the plasma samples for

cortisol and all the samples for adrenocorticotropic hormone (ACTH). The assays for plasma cortisol and ACTH both required validation prior to their use with bovine plasma. These validation procedures were carried out by the author.

4.2 CORTISOL ASSAY

4.2.1 Principles of the fluorescence polarisation immunoassay

The TDx System by Abbott, (North Chicago, Illinois) is an automated system which utilises fluorescence polarisation immunoassay technology. It can perform a variety of laboratory tests including assays for therapeutic drugs, hormones and clinical chemistries, either as individual units or in batches.

The assay process includes an extraction step to release cortisol from plasma binding proteins. The second step of the assay uses competitive binding immunoassay methodology. Tracer-labelled antigen (cortisol-fluorescein) competes with sample antigen for binding sites on antibody molecules. The cortisol antiserum is of combined mouse monoclonal and goat origin. When the reaction reaches steady state, samples with low cortisol concentration will have a high proportion of bound tracer and *vice versa*.

The level of bound tracer is measured by its reaction to polarised light. The tracer is excited by polarised light of a single wavelength (blue: 485 nm). As it returns to steady state the tracer emits a light of different wavelength (green: 525-550 nm). The rotational properties of the molecules in solution determine the degree of polarisation of the emitted light. If the tracer is bound to a very large antibody molecule and cannot rotate freely, the emitted light will be in the same plane as the excitation light and polarisation is retained. If the tracer is free to rotate because it is not bound to antibody, polarisation is lost. Samples with high proportions of bound tracer will have high proportions of polarisation in the emitted light (Abbott, TDx Manual, 1990).

The assay was considered suitable for use to measure plasma cortisol concentrations (PCC) in samples of bovine origin both because an extraction step was included, i.e., it measured total PCC; and, because the cortisol molecule is identical across species. The ability to perform

assays on small batches of samples added to its suitability because it allowed close monitoring of hormone levels. This was an advantage with some of the experimental procedures.

4.2.2 Manufacturer's specifications

Calibration of the instrument involved establishing a standard curve from six standards which ranged from 0 to 600 ng/ml. Control samples were low (30-50 ng/ml), medium (133-167 ng/ml) and high (356-444 ng/ml). Since PCC of cattle are low compared to the range over which the assay is calibrated, only the low and medium controls were used for quality control procedures.

The assay specifications showed that interference from elevated plasma protein (up to 10 g/dl), high lipid content (up to 500 mg/dl), cholesterol, haemolysis (up to 0.5 g/dl haemoglobin) or heparin was less than 5%, but elevated bilirubin (greater than 10 mg/dl) could result in greater than 10% interference (Abbott, TDx Manual, 1990).

The precision of the assay in human serum was reported as coefficients of variation (CVs) of 8.4%, 4.1% and 2.7% within an assay run, and 7.2%, 1.0% and 2.7% between assay runs, for the standards of 40, 150 and 400 ng/ml, respectively. The lowest measurable PCC distinguishable from 0 with 95% confidence was 4.5 ng/ml. Recovery of added cortisol in human serum was 104% at 25 ng/ml, 100% at 100 ng/ml and 102% at 250 ng/ml (Abbott, TDx Manual, 1990).

Reported cross-reactivities in human serum (Abbott, TDx Manual, 1990) were:

11-deoxycortisol	4.7%
corticosterone	4.5%
cortisone	1.1%
17-OH-progesterone	0.5%
progesterone	0.1%
testosterone	0.1%
prednisolone	23.8%
dexamethasone	1.1%

Correlations between PCC for human samples measured by both radio-immunoassay (Diagnostic Products: Coat-A-Count®) and TDx Cortisol were 0.960. Correlations between PCC for samples measured by both high pressure liquid chromatography and TDx Cortisol were 0.983.

4.2.3 Assay validation

To perform an assay run, samples are loaded into disposable cups on a carousel which is placed in the machine. Each carousel can hold up to 20 cups for a single assay run. Each assay run included the low (30-50 ng/ml) and medium (133-167 ng/ml) controls supplied by the manufacturer, two unknown samples in duplicate (at positions 1 and 17, and, 10 and 18), and 14 other unknown samples. This allowed calculation of "within run" (intra-assay) CVs from the results of the analyses of the duplicated unknown samples, and "between run" (inter-assay) CVs from the results of the analyses of the control samples.

The assay was validated for use with bovine plasma samples. Selected samples (n = 45) covering a range of PCC from 0.5 to 75 ng/ml were assayed in triplicate on 2 days. CVs were calculated following these assays. Table 4.1 gives the CVs from the validation procedures and summarises the mean CVs obtained for each experiment reported.

Cross-reactivity of steroids in bovine plasma were tested by mixing the steroid with bovine plasma. The bovine plasma was pooled from samples which had been assayed previously and shown to have PCC <0.5 ng/ml. Steroids were added at three different concentrations and each mixture assayed in duplicate. For this validation, cross-reactivity was calculated as:

(measured concentration) / (actual concentration) %

Cross-reactivities were:

progesterone	0.4%
testosterone	0.2%
dexamethasone	2.3%

4.2.4 Discussion

The manufacturer's specifications reported that, for its use in analysis of clinical medical samples, the lowest discernible PCC within a 95% confidence interval was 4.5 ng/ml. The machine was able to discern lower PCC and reported down to 0.5 ng/ml (Abbott, TDx Manual, 1990). Thus 0.5 ng/ml was considered as the sensitivity of the assay in the experiments described in this thesis.

The TDx Cortisol assay was least accurate at the lower range of PCC from 0.5 to 10.0 ng/ml (Table 4.1) so that basal PCC measurements reported must be regarded as having low accuracy. This was not considered to be a problem as most experiments required measurements in samples with more than 10 ng/ml, especially following adrenocortical stimulation. The advantages of being able to assay samples in small batches during experiments which required frequent monitoring of changes in adrenocortical response, and the low labour input to assays (16 unknown samples per 20 minutes) due to the automated system outweighed this problem. Further difficulties were occasionally experienced with accuracy so that variation in CVs in the range of PCC above 10 ng/ml occurred from time to time (Table 4.1) due to mechanical problems with machine and light maintenance. Use of a spike suppresser on the power supply reduced some of these problems. At the beginning of 1994, Abbott extended the range of the calibration standards. This improved the standard curve and resulted in smaller CVs.

Experiment	Intra-assay CVs			Inter-assay CVs	
	0.5-10 ng/ml	10.1-30 ng/ml	30+ ng/ml	40 ng/ml	150 ng/ml
Validation I	31.1%	16.5%	6.4%	-	-
Validation II	32.7%	15.2%	7.8%	-	-
5.1, 5.2, 5.3	21.8%	15.0%	6.9%	11.2%	4.8%
6.1	36.1%	11.8%	6.1%	7.6%	4.0%
7.1, 7.2	44.0%	19.4%	8.4%	10.3%	7.3%
8.1	25.8%	13.8%	4.6%	8.3%	3.8%
8.2	28.3%	14.8%	7.1%	15.4%	6.6%
8.3	10.0%	8.7%	3.2%	7.9%	4.0%
9.1	40.6%	13.6%	5.6%	9.5%	4.4%
10.1	42.8%	19.6%	8.6%	11.5%	7.8%
10.2	38.0%	30.6%	11.1%	13.5%	5.5%
10.3	67.6%	7.4%	3.0%	8.5%	4.5%
10.4	19.4%	11.3%	3.4%	4.9%	3.1%
11.1	44.3%	29.3%	6.0%	8.4%	4.5%
11.2	26.7%	12.3%	3.4%	10.1%	4.2%

Table 4.1: Mean coefficients of variation (CVs) of plasma cortisol assays for validation experiments and for each animal experiment. Validation CVs were calculated from measurements in triplicate of plasma cortisol concentrations in 45 samples on two seperate occasions. Intra-assay CVs were calculated from measurements in duplicate of 2 unknown samples in each assay run. Inter-assay CVs were calculated from measurements of control samples for each assay run in which samples from the experiment were analysed.

4.3 Adrenocorticotropic hormone assay

4.3.1 Principles of the assay

Determination of plasma adrenocorticotropic hormone (ACTH) concentrations were by a commercial non-extracted "two-site" immuno-radiometric assay (ACTH-Fast IRMA [^{125}I], Eurodiagnostica B.V., Apeldoorn, The Netherlands). This assay is a specific and sensitive measure of $ACTH_{1-39}$, since it utilises an antibody raised in sheep which specifically labels the 2-16 N-terminal sequence, and an antibody raised in rabbits which labels the 34-39 C-terminal sequence of the $ACTH_{1-39}$ molecule. This technique effectively reduces cross-reactivity with other pro-opiomelanocortin derivatives and it has no cross-reaction with $ACTH_{1-24}$ or with corticotropin-like intermediate lode peptide (CLIP), even at concentrations which are 1000 times higher than the highest $ACTH_{1-39}$ standard (Hodgkinson *et al.*, 1984).

4.3.2 Assay validation

Assay characteristics were validated with bovine plasma. Samples with high (420 pg/ml) and medium (320 pg/ml) ACTH concentrations which had been serially diluted with the 0 pg/ml standard supplied in the commercial kit to examine for parallelism with the standard curve. Recovery was assessed by combining samples of high and medium/low ACTH concentrations in 1:1 and 1:3 proportions. These mixtures were assayed in duplicate and the recoveries were calculated. Precision of the assay was determined by assaying two samples ten times each.

The serially diluted samples demonstrated parallelism with the standard curve. The precision of the assay was 6.0% and 12.0% for samples with mean ACTH concentrations of 176 and 61 pg/ml, respectively. Recovery of ACTH was 102%.

The sensitivity of the assay was not revalidated, but was taken to be 8 pg/ml in accordance with that reported by its developers (Hodgkinson *et al.*, 1984).

4.4 Progesterone assays

4.4.1 Plasma progesterone

Plasma progesterone concentration was measured in duplicate using a commercial solid phase ^{125}I radio-immunoassay (RIA; Count-a-Coat®, Diagnostic Products Corp., Los Angeles, California). Validation of this assay in bovine samples was reported by McDougall (1994). The antiserum was highly specific for progesterone, having a cross-reactivity of <2% with naturally occurring steroids and therapeutic drugs (McDougall, 1994).

Assay precision was reported as intra-assay CVs of 13.0% and 10.9% for samples with mean concentrations of 3.9 and 1.7 ng/ml of progesterone, respectively; and inter-assay CVs of 14.7% and 16.6% for samples with mean concentrations of 3.9 and 1.7 ng/ml of progesterone, respectively. The sensitivity of the assay was <0.2 ng/ml (McDougall, 1994).

4.4.2 Milk progesterone

Milk progesterone concentration was also determined in duplicate using the Count-a-Coat® (Diagnostic Products Corp., Los Angeles, California) commercial solid phase ^{125}I RIA. This assay had been previously validated for use with fresh bovine milk. Precision was reported as intra-assay CVs of 5.3% and 6.0% for samples with mean concentrations of 3.7 and 1.7 ng/ml of progesterone, respectively; and inter-assay CVs of 15.2% and 9.0% for samples with mean concentrations of 3.7 and 1.7 ng/ml of progesterone, respectively. The sensitivity was 0.08 ng/ml (McDougall, 1994).

4.5 Testosterone assay

Plasma testosterone concentrations were measured in duplicate using a commercial solid-phase ^{125}I RIA (Count-a-Coat® Total Testosterone, Diagnostic Products Corp., Los Angeles, California). The antibody had a cross-reactivity of 20% with the testosterone precursor 4-oestren-17-ol-3-one and 3.4% with 5α-dihydrotestosterone. Cross-reactivities with other naturally occurring steroids were <2% (Diagnostic Products Corp., Count-a-Coat® manual,

1994). This assay was validated for use in cattle samples (Berardinelli *et al.*, 1992). The sensitivity was 0.7 ng/ml for bull serum, and precision was reported as intra-assay and inter-assay CVs of <12% for serum pools containing 9.4 and 76.0 ng/ml testosterone.

4.6 Oestrone sulfate assay

Plasma oestrone sulfate was determined in duplicate using an RIA which had been developed at AgResearch Wallaceville, New Zealand for measurement of oestrone sulfate in milk. This used a monoclonal antibody which had a cross-reactivity of 31% with oestrone and <1% with other naturally occurring steroids. When used for plasma analyses, the standards for the standard curve were prepared in ovariectomised cow plasma and the volume of the sample to be assayed was reduced to 5 or 25 µl. This assay could discern plasma oestrone sulfate concentrations up to 35 pg/ml. Precision of the assay was 7% for intra-assay CVs and 12.0% for inter-assay CVs. The assay sensitivity was 0.5 ng/ml (Henderson *et al.*, 1992).

4.7 Prolactin assay

Plasma prolactin concentration was assayed using a double antibody assay which has been previously described (Barnes *et al.*, 1985). The assay was established at AgResearch Ruakura, New Zealand, according to the described method except that the second antibody dilution was at 1/20. The assay was validated following its development. Sequentially diluted samples demonstrated parallelism with the standard curve, and recovery of prolactin was 92%. Precision of the assay was 3.8% for intra-assay CVs and 6.2% for inter-assay CVs. The sensitivity of the assay was 0.2 ng/ml (T.B. McFadden, unpublished).

4.7 Alpha-lactalbumin assay

The alpha-lactalbumin (α-lac) assay was developed at AgResearch Ruakura, New Zealand. It was an RIA which utilised a rabbit antibody, and had low cross-reactivity for other peptides occurring in plasma. Parallelism of serially diluted samples containing high α-lac concentrations with the standard curve was demonstrated and recovery was 106%. Precision

of the assay was 9.5% for intra-assay CVs and 13.2% for inter-assay CVs. The sensitivity of the assay was 0.2 ng/ml (Prosser *et al.*, 1992).

4.8 Immunoglobulin G1 assay

Plasma immunoglobulin G1 (IGG1) concentrations were measured by a non-competitive "single-site" enzyme-linked immunosorbent assay (ELISA) based on a published technique (Hadsell *et al..*, 1993). The assay had been established at AgResearch, Ruakura, New Zealand. The primary antiserum was a monoclonal mouse anti-bovine IGG1, with a sheep anti-mouse IGG1 conjugated to horse-radish peroxidase being the secondary antibody. Plasma samples were diluted 1/50,000. Parallelism was confirmed in randomly selected samples, and recoveries of IGG1 were 110%. Cross-reactivity to immunoglobulin G2 and to bovine serum albumin were not detected. Precision of the assay was 9.9% for intra-assay CVs and 12.4% for inter-assay CVs. Sensitivity of the assay was not determined but was <1 ng/ml (T.B. McFadden, unpublished).

CHAPTER 5

ADRENOCORTICAL RESPONSES TO ADRENOCORTICOTROPIC HORMONE IN COWS

This chapter describes the adrenocortical responses of cows to adrenocorticotropic hormone (ACTH). An earlier version of the chapter was published in *Domestic Animal Endocrinology* (1994) 11: 115-123 and is in Appendix 1.

CHAPTER OUTLINE

5.1 Abstract

5.2 Introduction

5.3 Materials and Methods

- 5.3.1 Experiment 5.1 - Responses to ACTH in cows
- 5.3.2 Experiment 5.2 - Low dose ACTH treatments
- 5.3.3 Experiment 5.3 - Field Test Verification
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5.1 ABSTRACT

Adult Holstein-Friesian cows were treated by intravenous (iv) injection with a range of doses (0.0125 mg to 0.4 mg per animal) of synthetic adrenocorticotropic hormone ($ACTH_{1-24}$) and plasma cortisol concentrations (PCC) were measured. At 50 min after administration of ACTH, PCC were similar for all doses except 0.0125 mg. Increasing dose led to a prolongation of elevated PCC. There were linear relationships between adrenocortical responses and log_{10} dose of ACTH. Individual cows had a significant effect on the *y*-axis intercept of these relationships but not on the slope. A dose rate of 0.05 mg ACTH iv was identified as a suitable dose rate for use in clinical assessment of adrenal cortex function, with 0, 50 and 120 min following ACTH being critical sampling times to identify the response.

5.2 INTRODUCTION

Assessment of plasma cortisol levels following the administration of ACTH is a recognised method for evaluating adrenocortical function in human and veterinary clinical medicine. Peak cortisol, the increment of peak above basal cortisol level, and the integrated cortisol response over time following ACTH treatment are used as measures of adrenocortical responsiveness (Dickstein *et al.,* 1991; Crowley *et al..*, 1991).

Research into the responses of cattle to ACTH has most commonly employed a standard dose of 0.25 mg (Friend *et al.*, 1977; Gwasdauskas *et al.,* 1986) Reported responses are prolonged (6-8 h) when compared to responses to stressors such as jugular catheterisation (less than 1 h: Alam and Dobson, 1986). Recent evidence from human medicine suggests that when minimal doses of ACTH are used, the response reflects the true physiological capacity of the adrenal cortex so that more accurate inferences of function might be made (Dickstein *et al.,* 1991; Crowley *et al.*, 1991). Most reports of ART in cattle have used the intramuscular route for administration of ACTH. This might result in slower and more variable absorption and contribute to greater variation in the cortisol response curve as compared to intravenous (iv) administration.

The aim of the experiments described here was to determine the adrenocortical responses of dairy cows to a range of low dose rates of ACTH given by the iv route. This information could then be

used to determine a suitable dose rate and sampling schedule for a field test of adrenocortical function.

5.3 MATERIALS AND METHODS

5.3.1 Experiment 5.1 - Responses to ACTH in cows

Five parous Holstein-Friesian (HF) cows aged 3 to 6 years, with a mean weight of 471 kg (range: 377 to 568 kg), were first trained to accept the experimental handling procedures. These cows were not lactating at the time of the experiment. Routine haematology and screening for liver function showed that the cows were clinically healthy during the experiment. The cows' oestrous cycles were synchronised with progesterone-releasing CIDR™ devices (Eazi-Breed CIDR-B, InterAg Pty. Ltd., Hamilton, NZ) and prostaglandin $F_{2\alpha}$ (Lutalyse, Upjohn) so that each animal was in the luteal phase of its cycle (day 5-17) on any treatment day (Macmillan and Peterson, 1993). An indwelling catheter was inserted into a jugular vein of each cow at least 24 h before treatment. The catheters were used for blood sample collection and for bolus injection of treatments. On each day of sampling, cows were restrained indoors in a head bail and were fed hay *ad lib.* Water was offered regularly.

Sampling always began at 0900 h. Samples were taken at -60, -30, 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 180, 240, 300 and 360 min, with administration of the treatment following the 0 min sampling. Each treatment, *viz.,* normal saline (2 ml), 0.05, 0.1, 0.2 or 0.4 mg $ACTH_{1-24}$ (Synacthen, CIBA-Geigy, Switzerland; 1 mg equivalent to 800 IU) was administered twice to each cow. ACTH was diluted in normal saline to a total volume of 2 ml. Treatments were assigned randomly in a replicated five by five Latin square design. The interval between consecutive treatments was at least 72 h. Blood samples were collected in lithium heparin tubes in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 2 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. One sample collected from each cow on each treatment day was assayed for progesterone to confirm dioestrus. Cows were considered to be dioestrous when plasma progesterone concentrations were <2 ng/ml (McDougall, 1994). Details of the assays are reported in Chapter 4.

5.3.2 Experiment 5.2 - Low dose ACTH treatments

Initial results from Expt. 5.1 indicated the need to test lower ACTH doses. The five cows from Expt. 5.1 were treated with either saline, 0.0125 mg, 0.025 mg or 0.05 mg ACTH in a Latin square design with replication of the two lower dose treatments. Blood samples were taken at -30, -15, 0, 15, 30, 45, 60, 90, 120, 150, and 180 min with treatment following the 0 min sample. All other aspects of the experimental procedure were identical to Expt. 5.1.

5.3.3 Experiment 5.3 - Field Test Verification

A group of 20 lactating cows comprising 10 sets of monozygous twins, aged 3 to 6 years and either HF (n = 16), Jersey (J; n = 2) or J x HF (n = 2) were used for this experiment. Cows were in the first trimester of pregnancy and had a mean weight of 406 kg (range: 333 to 476 kg). Cows in twin pairs were allocated to a treatment of either normal saline or 0.05 mg ACTH in 2 ml saline. Treatments were reversed one week later in a cross-over design. Treatments began at 0900 h each day. All samples were collected and treatments administered by coccygeal venipuncture. Samples were processed as described previously. Cows were sampled immediately before ACTH administration, then again at 50 and 120 min after treatment.

5.3.4 Statistical analyses

Results are reported as a mean $\pm$ sem. Since all cows had returned to pretreatmentlevels of PCC by 240 min post-treatment in Expts. 5.1 and 5.2, this was used as the end-point for statistical analysis. Plasma cortisol concentrations for individual cows following saline and following the dose of 0.05 mg ACTH in Expts. 5.1 and 5.2 were compared by paired Student's *t*-Test. Since there were no significant differences between the two experiments, data were combined for further analysis.

Peak PCC and time to peak (Ttp) were estimated for each cow at each dose and dose occasion, using a computer-aided curve-fit of a fourth order polynomial curve (Kaleidagraph, Synergy Software, Reading, Pennsylvania). Since PCC tended to fall during the three pretreatment samples, 0 min PCC was used as baseline PCC. The integrated responses (areas under the

PCC.time curve) for each cow at each dose and dose occasion were calculated by integration using full data (AUC240) and data corrected for 0 min PCC (dAUC240). Visual inspection of the PCC.time curves (Fig. 5.1) identified 50 min and 120 min as critical points following ACTH administration for determining the shape of the curve. These values (50 min PCC and 120 min PCC, respectively) and their increments above 0 min PCC (d50 min PCC and d120 min PCC) were also used as response variables.

The response variables of 50 min PCC, 120 min PCC, AUC240, and their respective increments above 0 min PCC, as well as peak PCC and Ttp were subjected to analyses of variance for effects associated with dose, dose occasion and cow, and for cow-dose interactions. Data for all cows on all dose occasions were subjected to regression analyses (Minitab version 8.2, Minitab Statistical Software, State College, Pennsylvania) to determine prediction equations of best fit. These analyses examined sequentially for linear and quadratic relationships to $\log_{10}$ dose and individual cow effects. The relationships of 0 min PCC to peak PCC, AUC240 and dAUC240 across all dose rates were examined by regression analyses.

For data from Expt. 5.3, integrated responses were calculated to 120 min following administration of ACTH (AUC120 and dAUC120). Results were examined by analyses of variance for effects associated with cow, live-weight and breed. Among twin pair variation was compared with total variation by calculation of intra-class correlation coefficients (r_I = the proportion of the total variance of an observation that is associated with the twin set to which it belongs; Ostle, 1963).

5.4 RESULTS

5.4.1 Experiments 5.1 and 5.2 - Responses to ACTH in cows

Plasma progesterone concentrations were >2 ng/ml at all treatment occasions.

Saline control:

The overall mean PCC for all samples from control cows given saline was 12.2 $\pm$ 0.9 ng/ml. Mean PCC for saline treated animals at each sampling time did not differ significantly from 0 min PCC except at the 10 min sample (19.8 $\pm$ 0.9 ng/ml; $P<0.02$; Fig. 5.1).

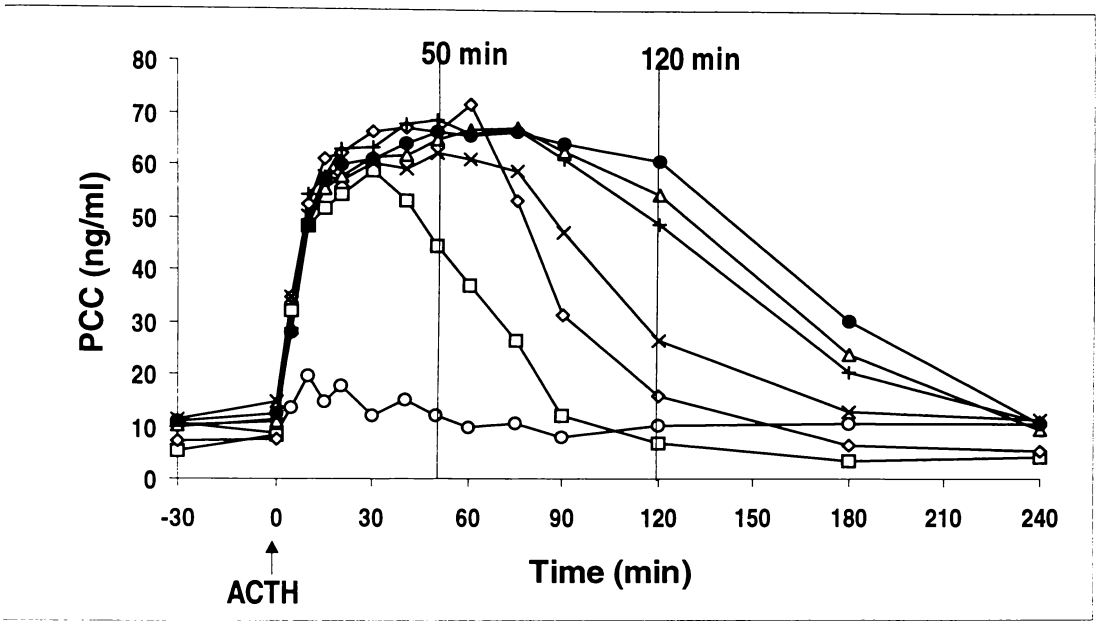


Figure 5.1: Mean plasma cortisol concentrations (PCC; ng/ml) following administration of a graded range of doses of ACTH (mg/500 kg) to cows.

Legend:
- O - saline
- □ - 0.0125 mg ACTH
- ◆ - 0.025 mg ACTH
- x - 0.05 mg ACTH
- + - 0.1 mg ACTH
- Δ - 0.2 mg ACTH
- ● - 0.4 mg ACTH

n = 10 for each value based on duplicated treatments in each of 5 cows.

Peak PCC and Ttp:

The PCC.time curves for each dose of ACTH are shown in Fig. 5.1. Each dose produced a significant increase ($P<0.01$) in PCC from 5 min after treatment with ACTH until 90 min post-treatment for the dose of 0.0125 mg ACTH, to 120 min post-treatment for the doses of 0.025 and 0.05 mg ACTH, and to 180 min post-treatment for the doses of 0.1, 0.2 and 0.4 mg ACTH.

Peak PCC (Table 5.1) for the doses of 0.0125 and 0.05 mg ACTH were less than for the other doses ($P<0.05$). There were no significant differences in peak height between 0.025 mg, and the three higher doses. Time to peak (Table 5.1) was less for the dose of 0.0125 mg than for 0.025 mg ($P<0.05$), and less for both of the lower doses than for the higher doses ($P<0.001$).

Dose of ACTH	Peak PCC*	Time to peak*
0.0125 mg (10 IU)	62.1 (3.1)	31.4 (0.9)
0.025 mg (20 IU)	73.0 (3.5)	35.7 (1.3)
0.05 mg (40 IU)	62.3 (2.8)	50.5 (1.6)
0.1 mg (80 IU)	71.8 (2.5)	56.9 (1.6)
0.2 mg (160 IU)	69.7 (3.2)	62.5 (1.3)
0.4 mg (320 IU)	71.2 (2.9)	65.0 (1.7)

Table 5.1: Peak plasma cortisol concentrations (PCC; ng/ml) and times to peak (Ttp; min) following administration of a graded range of doses of ACTH (mg/500 kg) to cows [mean (sem)].

* represents significant differences within columns between doses of ACTH ($P<0.05$).
n = 10 for each value based on duplicated treatments in each of 5 cows.

PCC at 50 and 120 min (Table 5.2):

Plasma cortisol concentrations at 50 min were lower ($P<0.001$) with the lowest ACTH dose, but then did not vary with the other five doses. Similarly d50 min PCC for 0.0125 mg and 0.05 mg were lower ($P<0.05$) than the other doses which did not vary. Differences ($P<0.05$) in 50 min PCC for individual cows were noted at the 0.025 mg, 0.1 and 0.2 mg doses, but only at 0.1 mg for d50 min PCC. No cow-dose interaction was statistically significant.

With each of the three lowest doses, 120 min PCC varied ($P<0.05$) and were less ($P<0.05$) than for the higher doses which did not differ amongst themselves. The response variables of d120 min PCC were similar at the three lower doses, which were less ($P<0.01$) than the three higher doses which were also similar. There were no significant differences in 120 min PCC between individual cows at any dose and no significant cow-dose interactions were detected.

Dose of ACTH	50 min PCC*	d50 min PCC*	120 min PCC*	d120 min PCC*
saline	12.1 (1.1)	0.3 (0.9)	10.3 (1.3)	-1.5 (1.7)
0.0125 mg (10 IU)	44.5 (3.0)	40.1 (3.9)	7.9 (1.0)	3.5 (2.1)
0.025 mg (20 IU)	66.6 (4.0)	57.9 (3.3)	16.0 (1.6)	7.3 (2.6)
0.05 mg (40 IU)	62.1 (3.0)	47.1 (2.5)	26.4 (2.3)	11.4 (1.9)
0.1 mg (80 IU)	68.6 (2.3)	57.4 (1.7)	48.5 (4.9)	37.3 (4.4)
0.2 mg (160 IU)	64.8 (2.2)	53.8 (1.8)	54.4 (2.7)	43.4 (2.5)
0.4 mg (320 IU)	66.6 (3.7)	54.2 (4.0)	60.8 (3.1)	48.5 (3.7)

Table 5.2: Absolute and incremental plasma cortisol concentrations (PCC; ng/ml) at 50 and 120 min following administration of a graded range of doses of ACTH (mg/500 kg) to cows [mean (sem)].

There were significant differences ($P<0.01$) between saline and all doses of ACTH for 50 min PCC and d120 min PCC. Saline and the dose of 0.0125 mg ACTH were similar for 120 min PCC and d120 min PCC.
* represents significant differences within columns between doses of ACTH (not saline; $P<0.05$).
n = 10 for each value based on duplicated treatments in each of 5 cows.

Integrated response variables (Table 5.3):

With the 0.0125 mg dose, AUC240 was less ($P<0.05$) than with the 0.025 mg dose, which was less ($P<0.01$) than the 0.05 mg dose; all three lower doses were less ($P<0.01$) than the three higher doses which did not vary. Similarly with the 0.0125 mg dose, dAUC240 was less ($P<0.02$) than the 0.025 mg dose. Doses of 0.025 mg and 0.05 mg gave similar responses which were less ($P<0.001$) than the higher doses. There were no significant differences between individual cows at any dose level and no cow-dose interaction was detected.

Three response variables (dAUC240, 120 min PCC and d120 min PCC) were found to be linearly related to $\log_{10}$ dose ACTH ($r = 0.69$, 0.82 and 0.76, respectively). The response variables of AUC240, 50 min PCC and d50 min PCC showed an additional quadratic relationship ($r = 0.83$, 0.64 and 0.36, respectively). Fig. 5.2 shows the dose response curve based on $\log_{10}$ dose vs AUC240 ($r = 0.83$).

Dose of ACTH	AUC240*	dAUC240*
saline	2640 (216)	724 (231)
0.0125 mg (10 IU)	3873 (442)	2656 (215)
0.025 mg (20 IU)	4672 (316)	3495 (267)
0.05 mg (40 IU)	8107 (454)	4731 (254)
0.1 mg (80 IU)	10260 (475)	7711 (488)
0.2 mg (160 IU)	10566 (322)	7781 (199)
0.4 mg (320 IU)	11452 (534)	8204 (414)

Table 5.3: Absolute and incremental areas under the plasma cortisol concentration.time curve over 240 min (AUC240) following administration of a graded range of doses of ACTH (mg/500 kg) to cows [mean (sem)].

There were significant differences ($P<0.01$) between saline and all doses of ACTH for AUC240 and dAUC240.
* represents significant differences within columns between doses of ACTH (not saline; $P<0.05$).
n = 10 for each value based on duplicated treatments in each of 5 cows.

The value of the *y*-axis intercept differed ($P<0.02$) for individual cows for AUC240 (n = 2), for 50 min PCC and for d50 min PCC (n = 3). There was no individual cow effect on the slope of any regression relationship, i.e., dose response curves for individual cows tended to be parallel.

5.4.2 Experiment 5.3 - Field test verification

Administration of ACTH produced an increase ($P<0.001$) in all parameters when compared to saline administration (Table 5.4). Response to ACTH was not influenced by either breed or live-weight. The response parameters for Expt. 5.3 were not different from those calculated using the data for the same sampling points, i.e., 0, 50 and 120 min for the 0.05 mg dose in Expt. 5.1. Using data from Expt. 5.1, a comparison was made of dAUC120 calculated by using these three sampling points and the full data over 120 min. This had a significant correlation coefficient ($r = 0.97$).

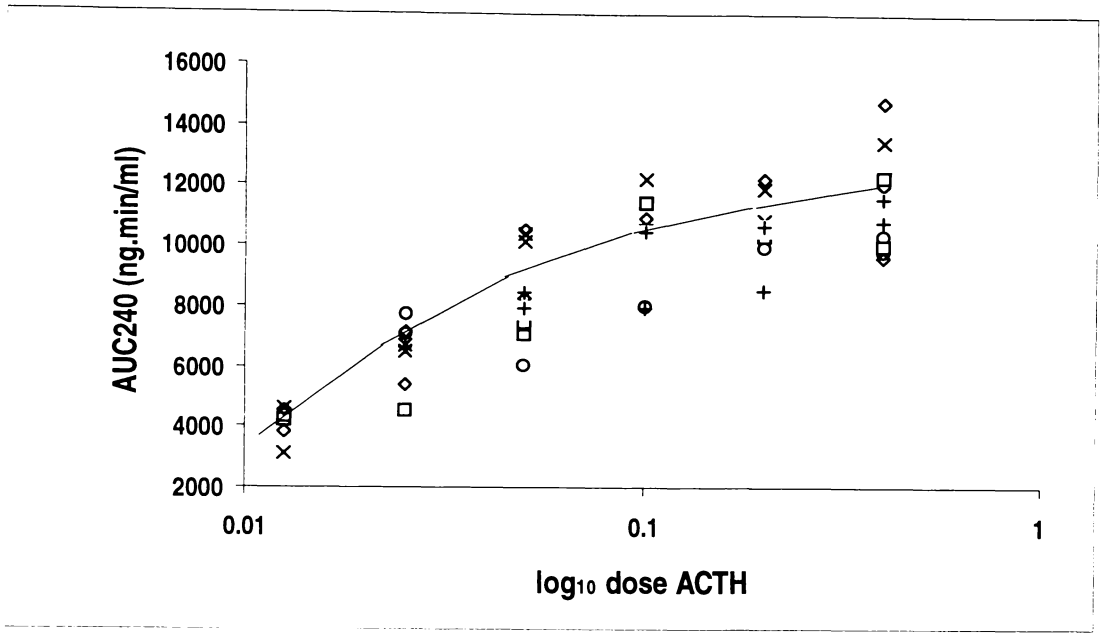


Figure 5.2: Areas under the plasma cortisol concentration.time curve over 240 min (AUC240; ng.min/ml) following administration of a graded range of doses of ACTH (mg/500 kg) to cows.

Different symbols represent values for individual cows. Cows which had significant individual effects on the regression relationship are represented by ◊ ($P < 0.02$) and x ($P < 0.01$).

$AUC240 = 13817 + 4678 \log_{10} dose - 2925(\log_{10} dose - mean \log_{10} dose)^2 + cow\ effect$

$(r = 0.83; P_{(regression)} < 0.01)$

Response variable	Saline	ACTH
0 min PCC	6.0 (1.1)	7.1 (1.3)
50 min PCC	8.4 (2.5)	59.0 (2.7)
d50 min PCC	2.4 (2.8)	51.8 (2.5)
120 min PCC	5.9 (1.6)	24.5 (2.8)
d120 min PCC	-0.1 (2.1)	17.4 (2.9)
AUC120	858 (179)	4576 (231)
dAUC120	141 (218)	3723 (208)

Table 5.4: Absolute and incremental plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min, and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH and saline to cows [Expt. 5.3; mean (sem)].

No differences in the responses were detected between the lactating cows in the first trimester of pregnancy, and the non-pregnant, non-lactating cows. The combined data from Expts. 5.1 and 5.3 showed the integrated plasma cortisol response to 0.05 mg ACTH to have a mean value of 3548 ± 104 ng.min/ml, with a 99% confidence interval of 1900 to 5300 ng.min/ml.

The only response variable in which twin pairs showed a similarity in response was dAUC120 ($P<0.05$) which had an associated intra-class correlation coefficient of $r_I = 0.51$. By comparison, r_I for live-weight and milk fat% in these twin pairs were 0.95 and 0.98, respectively.

5.5 Discussion

The preceding experiments have shown that healthy dairy cows respond to synthetic $ACTH_{1-24}$ administered iv in a dose dependent manner. Peak response was independent of dose but higher doses had a more prolonged effect. These results are consistent with observations in other species that the integrated adrenocortical response to ACTH is proportional to dose (Keller-Wood *et al.*, 1983).

Previous reports of responses to ACTH in dairy cows have utilised doses of 0.25 mg, long-acting preparations, and/or intramuscular administration. This makes direct comparison of results difficult. Alam *et al.* (1986) used a range of doses (0.03 to 0.4 mg), administered intramuscularly to cows in the follicular stage of the oestrous cycle. The cortisol response curve tended to be more prolonged with a peak 2 h after ACTH administration at all dose rates; peak response was up to 36.4 ng/ml for 0.4 mg ACTH. This is less than we obtained.

Van der Kolk *et al.* (1991a) administered 6 IU of synthetic $ACTH_{1-24}$ (Cortrosyn, Organon, Oss, The Netherlands; 0.06 mg equivalent to 6 IU) intravenously to lactating cows. The mean PCC one hour later was 148 ± 34.2 ng/ml and cortisol levels remained elevated for 5 h. This greatly exceeded the responses found in this study: a dose of 0.125 mg (equivalent to 10 IU) produced a peak PCC of 62 ± 3.1 ng/ml at 31 ± 1 min, PCC of 37.1 ± 4.4 ng/ml at 60 min after administration, and PCC had returned to basal levels after 90 min. It was also a more variable response. The cows described by van der Kolk *et al.* (1991a) were in early to mid-lactation (67 ± 14 days compared to >100 days in Expt. 5.3) and the age range was greater (25 to 128 months compared to 42 to 78

months in Expt. 5.3). These authors suggested that the high PCC which they measured could be due to a more sensitive assay, or the alternative ACTH preparation. A further explanation might be that management conditions experienced by the cows altered adrenal responsiveness.

5.5.1 Sensitivity of the adrenal cortex to ACTH

Sensitivity to ACTH can be considered as the minimum dose required to elicit a consistent measurable response in PCC. The results of Expt. 5.1 and 5.2 confirm that the adrenal cortex of dairy cows is highly sensitive to ACTH. The dose of 0.0125 mg used in the experiments reported here is approximately equivalent to 0.14 ng/kg$^{0.75}$. This produced a measurable response in all cows with a peak PCC being achieved at 30 min (Table 5.1). This is a similar response to that seen in a group of healthy young men at a dose rate of 3.5 ng/kg$^{0.75}$ which was reported to be the minimum dose required to evoke a response. Even so, the smallest dose used here was 10-fold greater than the minimum dose observed to evoke a response in cows (Paape *et al.*, 1977) and 100 fold greater than the minimum dose identified for sheep (Fulkerson and Jamieson, 1982). Even smaller doses than those used here may be capable of eliciting responses.

5.5.2 Influence of basal PCC on response to ACTH

Ultradian and circadian rhythms might influence both basal cortisol levels (Fulkerson and Jamieson, 1982) and responsiveness to ACTH (Fulkerson *et al.*, 1980). These influences were minimised by using the same starting time each day. Stress experienced during the sampling procedure may also influence PCC (Alam *et al.*, 1986). The transient rise seen in PCC for saline-treated cows probably represents a response to handling during the period of increased sampling frequency. Using animals accustomed to the experimental procedures kept these influences to a minimum.

5.5.3 Consistency of response by individuals

The classification of cows into high or low responding groups required repeated sampling at several dose rates to achieve statistical validity. As a consequence, high and low responding cows could not be identified in Expt. 5.3. The finding that the dose response curves for individual cows

were parallel implies that there was a consistent ability across all cows for the response to increase as ACTH dose rate increased. Classification of animals as high or low responders may reflect differences between individuals in the *y*-axis intercept of the dose response curves, i.e., in their overall level of response. This might vary due to factors such as relative adrenal size, or relative numbers of ACTH receptors in the adrenal cortex.

The identification of sub-populations which differ in ACTH response is in agreement with work in pigs (von Borell and Ladewig, 1992) where response was shown to be consistent in individuals over time, and in rats where response could be correlated to behavioural traits (Dallman *et al.*, 1987). The failure here to demonstrate strong similarities within twin pairs suggests that the consistency in response may not reflect genetic similarities. Rather, environmental and management experiences may modulate an individual's adrenocortical responses to ACTH, and overshadow genetic influences.

5.5.4 Development of the field test

It has been demonstrated in humans that a minimal dose of ACTH is more sensitive in detecting adrenocortical dysfunction than one in the pharmacological range (Dickstein *et al.*, 1991; Crowley *et al.,* 1991). The plasma cortisol responses to 0.0125 mg and 0.025 mg ACTH were short-lived and might be difficult to detect where groups of cows are being tested in a field situation, i.e., timing of sampling would be more critical. For this reason, 0.05 mg ACTH was selected as the dose for use in field test situations. Critical sampling times were 50 and 120 min following ACTH administration to allow the integrated response to be calculated. Selection of this protocol was confirmed by comparison of the data from the selected points with the full data. When the protocol was tested in Expt. 5.3, it produced response parameters which were not different from those defined in Expt. 5.1. This demonstrates that the technique can be used to assess adrenocortical response to ACTH in untrained animals in the field.

This newly developed ACTH response test has potential uses in furthering our understanding of adrenocortical function, and changes which might occur physiologically or where function is modified by drugs such as glucocorticoids. In such situations, the use of pretreatment measurements will allow an animal to act as its own control, reducing the influence of individual variation.

5.6 CONCLUSIONS

Adrenocortical responses in dairy cows to a range of doses (0.0125 mg to 0.4 mg) of synthetic $ACTH_{1-24}$ have been defined. The integrated response was proportional to the dose, but the response measured as 50 min PCC was independent of dose in the range of 0.025 to 0.04 mg ACTH. Although the responses of individual cows at each dose were consistent, variations between individuals were demonstrated when responses across the range of doses were considered. A suitable dose rate and sampling schedule for a test of adrenocortical function which can be readily applied in a field situation was defined and verified.

CHAPTER 6

ADRENOCORTICAL RESPONSES TO ADRENOCORTICOTROPIC HORMONE IN BULLS AND STEERS

This chapter describes the adrenocortical responses of bulls and steers to adrenocorticotropic hormone (ACTH).

CHAPTER OUTLINE

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- 6.3.2 Statistical analyses

6.4 Results

6.5 Discussion

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- 6.5.2 Basal plasma cortisol concentrations
- 6.5.3 Comparison with cows
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- 6.5.6 Modulation of the hypothalamus and pituitary

6.6 Conclusions

6.1 ABSTRACT

Plasma cortisol concentrations (PCC) were measured in Holstein-Friesian bulls and steers at 11 months of age following treatment with a range of doses of a synthetic $ACTH_{1-24}$ (from 0.0125 mg to 0.2 mg/500 kg for steers and from 0.05 to 0.2 mg/500 kg for bulls). The means of peak PCC at the three highest dose rates were not different within groups, but did differ between groups (72.5 ± 4.0 ng/ml in steers compared to 43.8 ± 2.4 ng/ml in bulls; $P<0.001$). Responses were significantly less

in bulls for all of the examined response variables at the common doses, except for the means of PCC following saline treatment (5.4 $\pm$ 0.4 ng/ml for both bulls and steers) and the means of time to peak PCC (56 $\pm$ 2 min and 57 $\pm$ 4 min for steers and bulls, respectively). Both bulls and steers showed elevations of PCC for similar periods of time (180 to 240 min). The results suggest that testosterone or some other testicular hormone may modulate function of the hypothalamo-pituitary-adrenal axis in cattle.

6.2 INTRODUCTION

Activity of the hypothalamo-pituitary-adrenal (HPA) axis is modified by activity of the hypothalamo-pituitary-gonadal axis. Evidence for sexual dimorphism in HPA axis function was discovered first in rodents (Kitay, 1961), but its extent differs among species (Malendowicz, 1994). Differences are not limited to the adrenal cortex, but are evident through all levels of the HPA axis (Bingaman *et al.*, 1994).

Interrelationships between the adrenal and gonadal endocrine axes have been documented in cattle, but most research has concentrated on the modulation of pituitary and/or gonadal hormones by glucocorticoids (Moberg, 1987). Basal plasma cortisol concentrations (PCC) were lower in bulls than in steers, but responses to a pharmacological dose of ACTH were similar (Barnes *et al.*, 1983). Total and free cortisol levels differed between bulls and steers (Ward *et al.*, 1992). Adrenocortical responses of bulls to a synthetic ACTH increased by 9 days after castration (Bass *et al.*, 1982).

The adrenocortical responses of cows to a range of low doses of ACTH were consistent with variations between individuals being demonstrated when responses across a range of doses were considered (Chapter 5). The aim of the experiment described here was to determine adrenocortical responses of young bulls and steers to a similar range of doses of ACTH given by the intravenous route. This should provide comparable data to that of Chapter 5 to determine whether there is sexual dimorphism in HPA axis function in cattle, and whether testicular hormones have a modulatory effect on adrenocortical responses to ACTH.

6.3 MATERIALS AND METHODS

6.3.1 Animals and sampling regime

Eight male Holstein-Friesian animals were selected as calves and accustomed to regular handling and restraint. They were born in July 1992 following artificial insemination in October 1991, of cows in a single herd. Three different sires were represented in the group. Four animals were surgically castrated in November 1992, when they were 3 months old. The other four remained intact. Their average growth rate from weaning onto a ryegrass/clover pasture diet until one year of age, was 0.7 kg/day.

The first period of intensive sampling was in June/July 1993, when animals were 11 months of age and had mean live-weights of 278 $\pm$ 4 kg for steers (range: 263 to 286 kg), and 293 $\pm$ 9 kg for bulls (range: 274 to 311 kg). Routine haematology and screening for liver function indicated that the animals were clinically healthy during this period. An indwelling catheter was inserted into a jugular vein of each animal at least 24 h before treatment. The catheters were used for blood sample collection and for bolus injection of treatments. On each day of sampling, the animals were restrained indoors in a head-bail and were fed fresh cut pasture (ryegrass and clover) or pasture silage *ad lib.* Water was offered regularly.

Sampling always began at 0830 h. Samples were taken at -30, 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 210 and 240 min, with administration of a treatment immediately following sampling at 0 min. Treatments were assigned randomly in a replicated Latin Square design. Each treatment, *viz.,* 0, 0.03, 0.06 or 0.12 mg $ACTH_{1\text{-}24}$ (Synacthen, CIBA-Geigy, Switzerland; 1 mg equivalent to 800 IU) was administered in a total of 2 ml saline. The interval between consecutive treatments was at least 48 h. Samples could not be collected due to catheter failure on two occasions, once with a dose of 0.06 mg for a steer, and the other with a saline dose for a bull.

A second sampling period for the steers, to test two lower doses of 0.01 and 0.02 mg ACTH, was completed in December (17 months old) when they had a mean live-weight of 483 $\pm$ 12 kg (range: 459 to 506 kg). The steers also received saline and a dose of 0.04 ACTH mg to link the data to

that derived during the first sampling period. Treatments were assigned randomly with replication of the two low doses of ACTH. Samples could not be collected due to catheter failure on one occasion with a dose of 0.01 mg. The bulls were not available to be resampled at this time.

Each blood sample was placed in a vacutainer containing lithium heparin, held in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 2 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. Details of the assays are reported in Chapter 4.

6.3.2 Statistical analyses

Each result is reported as a mean ± sem. Response variables of 0, 50 and 120 min PCC, AUC240 and their respective increments, as well as peak PCC and time to peak (Ttp) were derived as in Chapter 5. Response variables for the steers given saline and ACTH at the dose of 0.03 mg were compared between the two sampling periods using a paired Student's *t*-Test. As there were no significant differences between these two data sets, the data for the lower doses were included with those of the first sampling period for further analyses.

To facilitate comparison of the doses, these were standardised to a dose rate per 500 kg live-weight, i.e., the range of doses was from 0.0125 mg/500 kg live-weight up to 0.2 mg/500 kg live-weight. Mean PCC at each sampling point was compared to that at 0 min both within and between bulls and steers using a Student's *t*-Test. The response variables of 50 and 120 min PCC, AUC240, and their respective increments as well as peak PCC, and time to peak (Ttp) were examined by analyses of variance (ANOVA) using a General Linear Model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania). The model examined for effects associated with dose, sexual status, individual and dose occasion (i.e., 1st or 2nd treatment with a particular dose).

Data for all animals on all dose occasions were subjected to regression analyses using Minitab (version 8.2) to determine prediction equations of best fit, examining for linear and quadratic relationships as well as individual animal effects. These analyses first used data from the three higher dose rates for bull *vs.* steer comparison and within bull group comparisons, then from all five dose rates for analyses within steer group.

Values for exponential decay constants for the decline phase of each PCC.time curve were calculated using Genstat (version 5.3.1). These values were examined by ANOVA using a GLM for effects of dose and sexual status both within and between groups.

6.4 RESULTS

Mean PCC.time curves following each dose of ACTH or saline are shown in Figs. 6.1 and 6.2. Tables 6.1 and 6.2 give mean values for the response variables at each dose of ACTH or saline. The incremental response variables of dAUC240, d50 min and d120 min PCC were highly correlated to the variables from which they were derived (r = 0.98, 0.99 and 0.99, respectively) and have not been reported further.

Dose of ACTH	0 min PCC	50 min PCC	120 min PCC**	AUC240**	Peak PCC	Ttp*
saline	2.7 (0.9)	6.9 (1.6)	2.7 (1.4)	1390 (250)	-	-
0.0125 mg (10 IU)	2.2 (1.1)	58.8 (3.1)	6.0 (1.1)	4490 (320)	58.9 (3.5)	38.3 (0.4)
0.025 mg (20 IU)	2.4 (1.1)	64.8 (2.6)	11.2 (1.2)	5770 (360)	64.6 (2.4)	42.6 (1.1)
0.05 mg (40 IU)	3.7 (1.0)	68.8 (4.0)	29.7 (1.7)	8240 (450)	71.1 (3.4)	48.6 (1.1)
0.1 mg (80 IU)	4.3 (1.5)	69.9 (4.8)	51.9 (3.0)	9950 (510)	76.8 (4.4)	57.4 (2.1)
0.2 mg (160 IU)	2.3 (0.5)	71.1 (3.9)	56.9 (4.3)	10630 (700)	79.1 (4.3)	60.5 (2.2)

Table 6.1: Plasma cortisol concentrations (PCC; ng/ml), areas under the PCC.time curve over 240 min (AUC240; ng.min/ml) and times to peak (Ttp; min) following administration of a graded range of doses of ACTH (mg/500 kg) to steers [mean (sem)].

There were significant differences ($P<0.001$) between saline and all doses of ACTH for 50 and 120 min PCC and for AUC240.

** represents significant differences within columns between doses of ACTH (not saline; $P<0.001$).

n = 8 for each value based on duplicated treatments in each of 4 steers except for doses of 0.0125 and 0.1 (n = 7).

Saline control:

The overall mean PCC for all samples from control animals was 5.4 ± 0.4 ng/ml for both bulls and steers. There was no difference in response to saline between bulls and steers. Mean PCC for saline treated animals did not vary from the overall mean on any sampling occasion.

Dose of ACTH	0 min PCC	50 min PCC	120 min PCC**	AUC240	Peak PCC	Ttp
saline	5.1 (0.9)	5.1 (1.6)	6.3 (1.7)	1410 (140)	-	-
0.05 mg (40 IU)	4.8 (1.9)	43.2 (2.7)	18.1 (2.2)	4920 (280)	43.6 (1.9)	50.3 (2.0)
0.1 mg (80 IU)	3.3 (0.9)	39.1 (2.8)	31.8 (3.8)	5900 (570)	42.9 (3.5)	55.1 (7.6)
0.2 mg (160 IU)	3.3 (1.1)	38.4 (2.0)	35.1 (3.7)	6450 (600)	44.7 (2.8)	67.3 (4.5)

Table 6.2: Plasma cortisol concentrations (PCC; ng/ml), areas under the PCC.time curve over 240 min (AUC240; ng.min/ml) and times to peak (Ttp; min) following administration of a graded range of doses of ACTH (mg/500 kg) to bulls [mean (sem)].

There were significant differences ($P<0.001$) between saline and all doses of ACTH for 50 and 120 min PCC and for AUC240.
** represents significant differences within columns between doses of ACTH (not saline; $P<0.01$).
n = 8 for each value based on duplicated treatments in each of 4 bulls except for saline (n = 7).

ACTH treatments:

All ACTH treatments resulted in an elevation of PCC ($P<0.001$) compared to saline treatments (Tables 6.1 and 6.2) for at least 120 min following treatment (Figs. 6.1 and 6.2). There were significant elevations of PCC in bulls above the 0 min value from 5 min until 180 min post-treatment at the ACTH dose rate of 0.05 mg/500 kg, and until 240 min for the two higher dose rates. The equivalent elevations in steers also occurred within 5 min, and persisted until 120 min post-treatment for the lowest dose of 0.0125 mg/500 kg live-weight, through 180 min (0.025 mg/500 kg), 210 min (0.05 and 0.1 mg/500 kg) and 240 min post-treatment (0.2 mg/500 kg).

When PCC for the equivalent time periods after ACTH administration were compared between groups, the values for steers were higher ($P<0.05$) than for bulls from 5 min to 150 min post-

treatment at the dose rates of 0.05 and 0.1 mg/500 kg, and to 120 min post-treatment at 0.2 mg/500 kg.

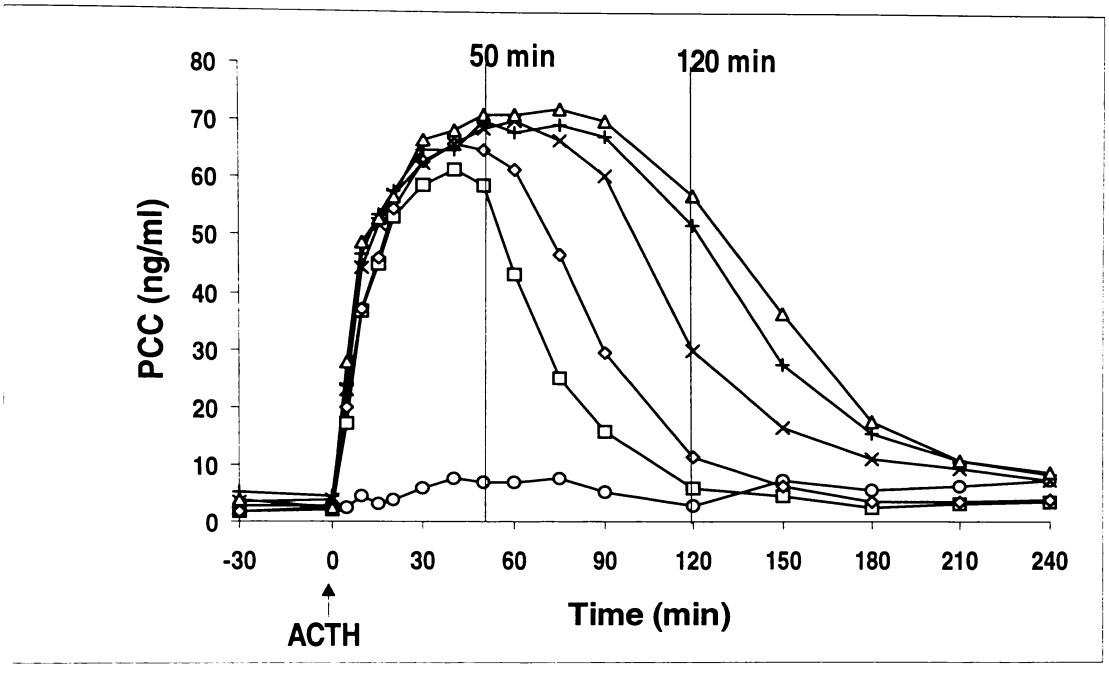


Figure 6.1: Mean plasma cortisol concentrations (PCC; ng/ml) following administration of a graded range of doses of ACTH to steers.

Legend:

- O - saline	- □ - 0.0125 mg ACTH
- ◆ - 0.025 mg ACTH	- x - 0.05 mg ACTH
- + - 0.1 mg ACTH	- Δ - 0.2 mg ACTH

n = 8 for each value based on duplicated treatments in each of 4 steers except for doses of 0.0125 and 0.1 (n = 7).

Effect of dose occasion (ANOVA):

Mean 0 min PCC was lower at the second dose occasion than the first for steers (4.0 ± 0.7 ng/ml and 1.8 ± 0.4 ng/ml for 1st and 2nd dose occasions, respectively; $P<0.01$) but not for bulls. There was no effect of dose occasion on any other response variable.

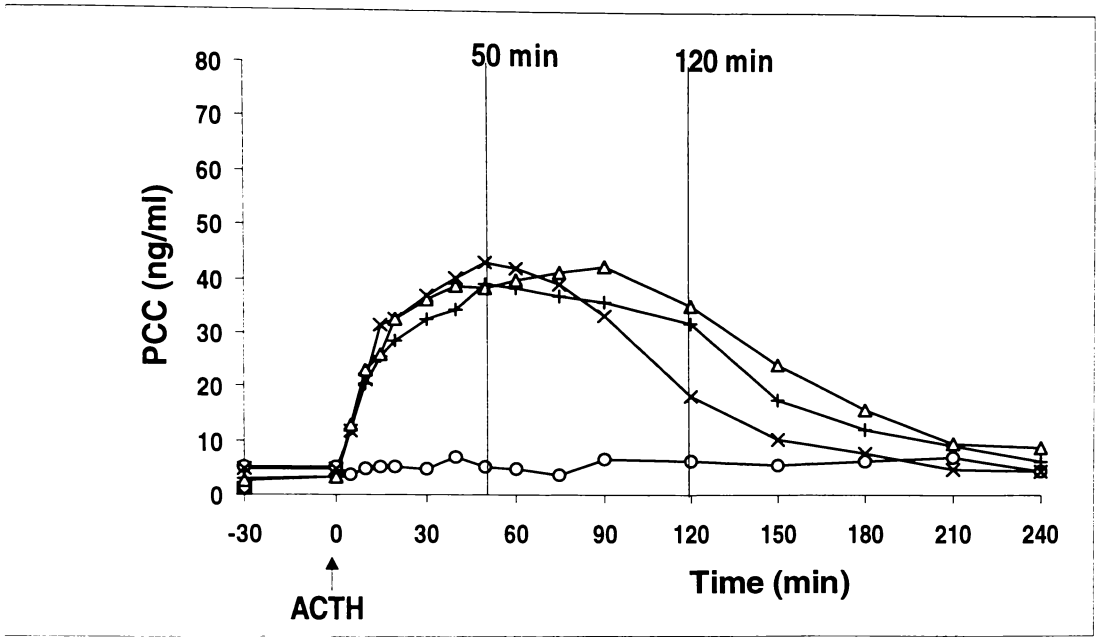


Figure 6.2: Mean plasma cortisol concentrations (PCC; ng/ml) following administration of a graded range of doses of ACTH to bulls.

Legend: - O - saline - x - 0.05 mg ACTH
- + - 0.1 mg ACTH - Δ - 0.2 mg ACTH

n = 8 for each value based on duplicated treatments in each of 4 bulls except for saline (n = 7).

Effect of individual animals (ANOVA):

Significant differences were detected among individual steers, but only with the two lower dose rates of ACTH. One steer had lower values ($P<0.04$) for 50 min PCC than its three companions following the two lowest dose rates, e.g., 50 min PCC with a dose rate of 0.025 mg/500 kg was 53.8 ± 1.4 ng/ml *vs.* 66.8 ± 1.4, 67.8 ± 1.4 and 70.9 ± 1.4 ng/ml. Because 50 min PCC was strongly correlated with peak PCC (r = 0.96) and AUC240 (r = 0.85), differences among the steers were also significant for these variables.

Comparison of bulls and steers (ANOVA):

There were significant differences between bulls and steers in all variables except 0 min PCC (3.4 ± 1.0 *vs.* 3.8 ± 1.3 ng/ml) and Ttp (56 ± 2 *vs.* 58 ± 5 min). Dose rate influenced only 120 min PCC in the bulls but also AUC240 and Ttp in the steers (Tables 6.1 and 6.2).

Regression analyses:

Significant linear relationships were found between $\log_{10}$ dose and some of the variables for both bulls and steers (Tables 6.3 and 6.4, Fig. 6.3). There were no significant quadratic or exponential components to any relationship. Two individual bulls had significant effects on the *y*-axis intercept of the relationship between $\log_{10}$ dose and 120 min PCC ($P<0.01$; $r = 0.58$ with individuals included *vs.* $P<0.005$; $r = 0.45$ without). One steer had a similar effect on the relationships between $\log_{10}$ dose and both 50 min PCC and peak PCC; but only when the analyses included data from all dose rates of ACTH, e.g., for the relationship between $\log_{10}$ dose and peak PCC, $P<0.001$ and $r = 0.55$ with the steer included *vs.* $P<0.001$ and $r = 0.38$ without. Individual animal effects did not alter the slope of any regression relationship.

	Significance	50 min PCC	120 min PCC*	AUC240*	Peak PCC	Ttp
Bulls (3 doses)	P	ns	0.002	0.039	ns	0.029
	r		0.37	0.18		0.20
Steers (3 doses)	P	ns	<0.001	0.006	ns	<0.001
	r		0.61	0.31		0.50
Steers (5 doses)	P	0.016	<0.001	<0.001	<0.001	<0.001
	r	0.15	0.86	0.75	0.38	0.80

Table 6.3: Statistical significances (P) and r values for prediction of responses to ACTH from ($\log_{10}$ dose) in bulls (based on 3 doses of ACTH) and steers (based on 3 and 5 doses of ACTH).

Plasma cortisol clearance:

The time taken for PCC to reduce by half during the decline phase of the PCC.time curve was not different, either between bulls and steers, or among doses (42.2 ± 0.5 *vs.* 43.5 ± 0.4 min for steers and bulls, respectively).

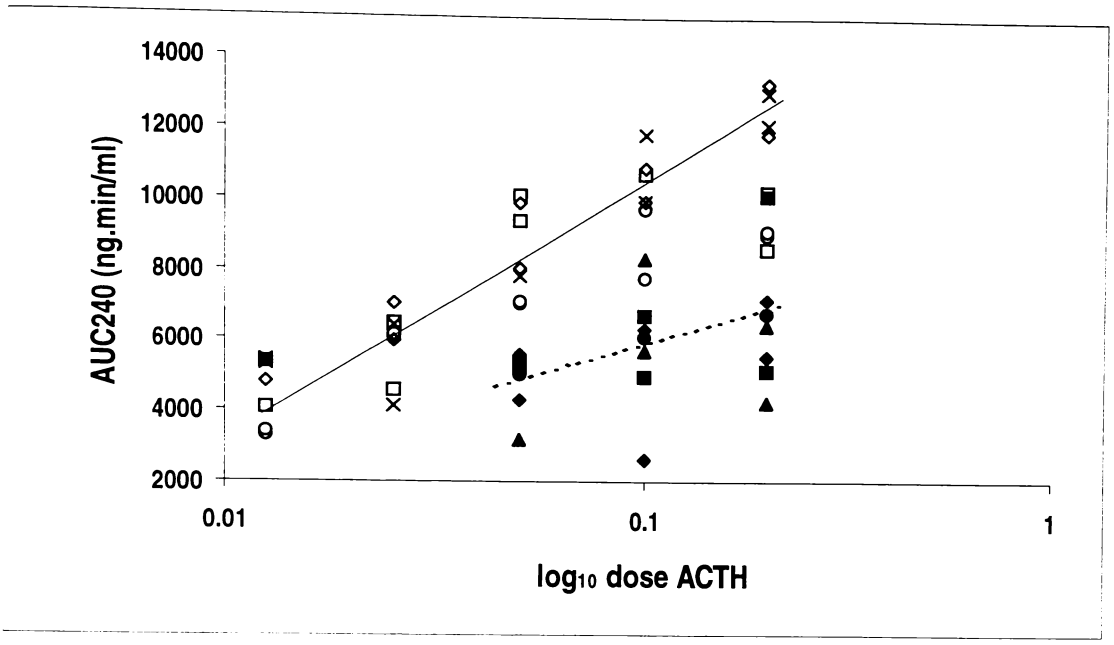


Figure 6.3: Areas under the plasma cortisol concentration.time curve over 240 min (AUC240; ng.min/ml) following administration of a graded range of doses of ACTH to bulls and steers.
Open symbols represent values for individual steers. Closed symbols represent values for individual bulls. No individual animal had significant effects on the regression relationship. Prediction equations are given in Table 6.4.
Legend: _____ steers
_ _ _ _ _ _ _ bulls

	Constant	Slope	n	r	P
Steers (5 doses)	14886 (714)	5444 (525)	38	74.9%	<0.001
Bulls (3 doses)	8303 (1196)	2546 (1162)	24	17.9%	<0.01

Table 6.4: Regression equations (response variable = constant + slope*$\log_{10}$ dose ACTH) for prediction of absolute areas under the plasma cortisol concentration.time curves over 240 min (AUC240) following administration of ACTH (mg/500 kg) from ($\log_{10}$ dose ACTH) in steers and bulls.

Standard errors for the coefficients are given in parentheses.

6.5 Discussion

6.5.1 Comparison of bulls and steers

This experiment has demonstrated that there are significant differences in adrenocortical responses to a range of low doses of ACTH between 11 month old bulls and steers of the same

age which had been orchiectomised before puberty. Although 0 min PCC were similar steers had 50 min and peak PCC which were 1.5 times the comparable averages in bulls at each of the three common dose rates of ACTH (Tables 6.1 and 6.2; $P<0.001$). Even though dose rate had differing effects on the response variables, the duration that PCC was significantly elevated from 0 min levels was similar for both steers and bulls. Furthermore, direct comparison of the data from the steers and the bulls showed that PCC were not different by 180 min post-treatment for dose rates of 0.05 and 0.1 mg/500 kg, and by 150 min for the dose rate of 0.2 mg/500 kg.

Measurement of the decay constant for PCC showed no difference between bulls and steers. This indicates that plasma half-life of cortisol is similar for bulls and steers. The plasma half-life for cortisol in post-partum cows was reported to be about 30 min, with no differences observed between suckled and non-suckled animals, or time post-partum (Dunlap *et al.,* 1981a). No equivalent calculations have been made for bulls.

Since PCC were elevated for a similar duration both in bulls and steers, despite much higher peaks in the steers, this suggests that the secretory response to the same dose of ACTH was more persistent in the bull although maximum rate of output was less. This difference could arise either by longer plasma half-life of $ACTH_{1-24}$ or by more persistent enzyme activity following adrenocortical stimulation in bulls. There is no published evidence for differences in plasma half-life for $ACTH_{1-24}$ due to sexual status (Malendowicz, 1994). Plasma half-life of $ACTH_{1-24}$ in humans is reported to be about 8 min (CIBA-Geigy, Switzerland), but there is no equivalent data for cattle. Further work is required to determine the clearance rates of plasma cortisol and ACTH in bulls and steers.

6.5.2 Basal plasma cortisol concentrations

Overall mean PCC during saline treatments were the same for bulls and steers (5.4 ± 0.4 ng/ml for both groups). Steers showed a lower 0 min PCC at the second dose occasion than at the first which suggests that some habituation developed during the course of the experiment. The absence of an effect of saline treatment indicates that the animals were not stressed by the experimental procedures. One should be cautious in making inferences about basal PCC when sample collection involves contact between animal and handler, even where animals are well

adjusted to the procedures. The low precision of the assay in the range of 0.5 to 10 ng/ml (CV = 36.1%; Table 4.1) prevents further conclusions regarding basal plasma cortisol levels in this experiment.

6.5.3 Comparison with cows

The data for the steers are similar to those obtained using similar experimental procedures with cows (Chapter 5). The 50 min PCC were very similar at each of the dose rates of 0.025, 0.05, 0.1 and 0.2 mg/500 kg, but were lower in cows at the lowest rate of 0.0125 mg/500 kg. This suggests that this dose rate may have been approaching a sensitivity threshold in the cows, but not in the steers. This could have been influenced by age, gender, season of the year or physiological state.

The prediction equation relating $\log_{10}$ dose and AUC240 for steers (Table 6.4, Fig. 6.3) is similar to that for cows. Both differ from that for bulls in the *y*-axis intercepts and the slopes. When individual animal differences were identified, the effect was on the intercept with the *y*-axis, not the slope. Fewer individual animal effects were seen in the steer and bull groups than in the cows. This may be due either to selection of a more homogenous group of animals than were selected in the study with cows, or to the use of fewer measurements per animal (12 per cow *cf.* 10 per steer and 6 per bull).

6.5.4 Modulation of cortisol secretion by other steroid hormones

Sexual dimorphism in adrenocortical function observed in rats led to the proposal that differences in oestrogen levels influenced the secretion of cortisol from the adrenal cortex (Critchlow *et al.,* 1963). Differences in ACTH secretion between men and women have also been attributed to the influence of oestrogens, with results suggesting that the human female adrenal cortex is more responsive to ACTH (Horrocks *et al.*, 1990; Roelfsema *et al.,* 1993).

The differences between steers and bulls suggest that the adrenocortical secretory process of cattle may be modulated by a hormone of testicular origin. This is further supported by the similarity of the responses of the steers to those reported in cows. The principle testicular hormones of bulls include testosterone, dehydro-epiandrosterone, androstenedione, 5-androstenediol and progesterone (Ganjam and Amann, 1976).

Several reports have been presented in the literature which would also support such a conclusion. Adrenocortical responses increased following castration of post-pubertal bulls. Supplementation with testosterone only partially inhibited increases in adrenocortical responses to ACTH measured as PCC, and in plasma luteinising hormone concentrations following castration; but the rate of testosterone supplementation may have been insufficient to provide full replacement (Bass *et al.,* 1982). Testosterone treatment of heifers for 100 days reduced adrenocortical responses to ACTH challenge, as well as behavioural responses to fear-eliciting events and subsequent increases in PCC (Boissy and Bouissou, 1994).

Androgens influence both adrenal steroid content and concentration, as well as steroid output *in vitro* (Malendowicz, 1994), but most research relates to rodents, where castration reduces corticosterone output. Only in the guinea pig is the converse true, as orchiectomised males have a higher mean basal cortisol concentration than intact males. Adrenocortical responses to ACTH have not been reported in guinea pigs *in vivo*, but there is *in vitro* evidence for a direct inhibitory effect of androgens on adrenocortical function. Bélanger *et al.* (1992) found that dihydrotestosterone administration inhibited both cortisol and corticosterone production by guinea pig adrenocortical cells.

6.5.5 Direct modulation of steroidogenesis

Androgenic steroids may directly influence the activity of steroidogenic enzymes in the adrenal cortex. Androstenedione inhibited the activity of the P450 cytochrome enzyme 21-hydroxylase ($P450_{C21}$) in adrenocortical cell cultures derived from post-pubertal bulls. This occurred in a manner which suggested that the inhibition was not by simple binding. The concentrations of androstenedione in adrenal venous blood is within the range of concentrations which inhibited $P450_{C21}$ activity. Adrenocortical cells may be exposed to increasing concentrations of androstenedione as they are pushed towards the centre of the adrenal gland by cell division (Hornsby, 1982). Where androstenedione of testicular origin is also present in blood, and where adrenocortical thickness is greater, i.e., the male, this inhibitory activity may be enhanced.

Androgens may also influence the direction of the steroidogenesis by modulating the P450 cytochrome enzyme 17α-hydroxylase ($P450_{c17}$) which has dual hydroxylase and lyase activity (Fig. 2.1). *In vitro* studies have shown that increasing the availability of electrons, mediated by flavoprotein P450 reductase, will favour lyase activity to yield dehydro-epiandrosterone and androstenedione (Miller, 1988). Conditions in the adrenal cortex of bulls may favour lyase activity which would reduce levels of substrate for production of glucocorticoid.

6.5.6 Modulation of the hypothalamus and pituitary

It is unlikely that the differences observed between bulls and steers were due only to direct modulation of the adrenal cortex. There may be differences in trophic drive from higher levels of the HPA axis either by altered negative feedback, or by altered input to the hypothalamus from the central nervous system.

The percentage of free cortisol was higher in bulls than in steers. Albumin has twice the affinity for testosterone as for cortisol so that competition for binding sites on blood proteins will increase levels of free plasma cortisol in bulls. This in turn could increase negative feedback to higher points of the HPA axis, and reduce trophic drive to the adrenal cortex (Ward *et al.*, 1992).

Areas of the hippocampus such as the bed nucleus of the stria terminalis (BNST) contain high levels of androgen receptors. These areas have been demonstrated in rodents to exert an inhibitory effect on hypothalamic secretion of corticotropin-releasing hormone. This could reduce trophic drive through the HPA axis, and so result in a reduction in ability to produce corticosteroids (Bingaman *et al.*, 1994).

6.6 Conclusions

There are marked differences in the adrenocortical responses of bulls and steers to low doses of ACTH, evident as differences in peak PCC following ACTH administration, as well as other altered characteristics of the PCC.time curve and dose response regression relationships. Testosterone, or some other testicular androgen, may modulate HPA axis function in cattle to a significant extent.

CHAPTER 7

CIRCADIAN PATTERNS OF ADRENOCORTICAL FUNCTION IN THE COW

This chapter presents experiments which investigated some physiological factors which might alter the adrenocortical response to adrenocorticotropic hormone (ACTH) in cows. The first experiment investigated circadian and seasonal influences on basal plasma cortisol concentration (PCC). The second experiment investigated the effect of time of dosing and repetitive dosing with ACTH on the pattern of response of this hormone.

CHAPTER OUTLINE

7.1 Abstract

7.2 Introduction

7.3 Experiment 7.1 - Circadian variations in basal activity

- 7.3.1 Aims
- 7.3.2 Materials and Methods
- 7.3.3 Results
- 7.3.4 Discussion

7.4 Experiment 7.2 - Circadian patterns of adrenocortical responsiveness and the effects of repetitive challenge with adrenocorticotropic hormone

- 7.4.1 Aims
- 7.4.2 Materials and Methods
- 7.4.3 Results
  - 7.4.3.1 Effects of time of dosing on adrenocortical responsiveness
  - 7.4.3.2 Effects of repetitive challenge on adrenocortical responsiveness
- 7.4.4 Discussion
  - 7.4.4.1 Effects of time of dosing on adrenocortical responsiveness
  - 7.4.4.2 Effects of repetitive challenge on adrenocortical responsiveness

7.5 Conclusions

7.1 ABSTRACT

Expt. 7.1: Plasma cortisol concentrations (PCC) were measured in samples from non-lactating dairy cows taken every 2h for 24 h. This sampling regime was repeated in eight consecutive calendar months. No diurnal pattern of PCC was detected but differences between individuals and between calendar months were observed. Variations between months may have been due to environmental factors causing restlessness in the cows, e.g., flies.

Expt. 7.2: Non-lactating dairy cows were treated with either 0.05 or 0.2 mg of a synthetic adrenocorticotropic hormone ($ACTH_{1-24}$) at either 12 h intervals for 7 occasions, or intermittently. Cows showed greater adrenocortical responses to ACTH in the evening, but only at the higher dose. Repetitive dosing at 12 h intervals increased responses to ACTH, particularly with the higher dose (50 min PCC increased from 61.0 $\pm$ 1.0 to 72.7 $\pm$ 1.4 ng/ml). These results demonstrate that the bovine adrenal cortex may increase its steroidogenic capacity with repeated stimulation by ACTH. Experiments which utilise challenge of the adrenal cortex with ACTH to monitor adrenocortical function should ensure that the choice of the time of day of the challenge or the use of repetitive challenges do not bias results.

7.2 INTRODUCTION

Basal PCC exhibit a dynamic profile of pulsatile cortisol secretion. This ultradian rhythm is superimposed over a diurnal pattern. Definitive identification of circadian and ultradian rhythms in cattle was achieved when animals were studied in controlled environmental conditions, and with intensive sampling frequency (Lefcourt *et al.*, 1993). Many exogenous and endogenous factors may influence adrenocortical secretory rhythms in cattle including reproductive status (Sartin and Kemppainen, 1988) and routine daily activities such as feeding (Gardy-Godillot *et al.*, 1989). Diurnal patterns in the responsiveness of the adrenal gland to ACTH have been observed in rats and in humans (Dallman *et al.*, 1987; Dickstein *et al.*, 1991).

Responsiveness of the adrenal cortex may be influenced by previous exposure to ACTH. Repeated doses of 40 IU led to increased adrenal responsiveness, while repetitive doses of

100 and 200 IU led to the development of a refractory state (Gwasdauskas *et al.*, 1980; Fox *et al.*, 1981).

The aims of the following experiments were to investigate whether circadian variations might alter basal PCC or adrenocortical responsiveness to ACTH in cows. A further aim was to investigate whether repetitive challenge by ACTH might influence interpretation of results of tests of adrenocortical function. This aim was particularly relevant in the context of this thesis, because repetitive adrenocortical challenges were used in some experiments to monitor changes in function of the adrenal cortex.

7.3 EXPERIMENT 7.1 - CIRCADIAN VARIATIONS IN BASAL ACTIVITY

7.3.1 Aims

The aims of Expt. 7.1 were:

- to determine basal PCC levels in non-lactating New Zealand cows under pastoral grazing conditions;
- to identify the occurrence of any diurnal patterns in basal PCC; and,
- to identify seasonal patterns in basal PCC.

7.3.2 Materials and Methods

Animals and sampling regime:

Five parous Holstein-Friesian cows, aged 3 to 6 years, were accustomed to frequent handling and to the sample collection routine. These cows were not lactating at the time of the experiment. They were fed ryegrass/clover pasture and were weighed every 2 weeks. Monthly haematology and screening for liver function indicated that the animals were clinically healthy.

The cows' oestrous cycles were synchronised with progesterone-releasing CIDR™ devices (Eazi-Breed CIDR-B, InterAg Pty. Ltd., Hamilton, NZ) and prostaglandin $F_{2\alpha}$ (Lutalyse, Upjohn) so that each animal was in the luteal phase of its cycle (day 5-17) during sampling periods (Macmillan and Peterson, 1993).

At least 24 h prior to the start of sample collection, a catheter was placed into a jugular vein of each cow to facilitate sampling. A 10 ml blood sample was taken from each cow at 2 hourly intervals for 24 h. This was done monthly from October 1991 to May 1992. For the sampling periods in October to December 1991, the cows were restrained in head bails in an open barn. They were fed hay *ad lib.* and offered water every 4 h when in the barn. Thereafter, from January to May 1992 cows were grazed in a paddock and moved quietly to a nearby yard for blood collection. This change occurred because accidental catheter removal was a problem when the cows were restrained for long periods in the head bails, and because the cows were irritated by increased numbers of flies in the summer months.

The facility at which sampling took place was situated at 37° 47' South and 175° 19' East of Greenwich. Minimum and maximum daily temperatures, and the time of sunrise and sunset were noted at each sampling period.

Each blood sample was placed in a vacutainer containing lithium heparin, held in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 4 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. The first sample on each day was also assayed for progesterone to confirm dioestrus. Cows were considered to be dioestrous when plasma progesterone concentrations were >2 ng/ml (McDougall, 1994). Details of the assays are reported in Chapter 4.

The experiment was terminated in May 1992. This was because PCC were at the limit of sensitivity of the assay. In addition, it was apparent that environmental influences (e.g. flies) were sufficiently large to mask variations due to physiological factors.

Statistical analyses:

Results are given as mean ± sem or mean ± sed as appropriate. The means and standard deviations (std) of PCC from all samples for each cow during each month were calculated. This approach was taken to overcome statistical difficulties associated with repetitive measures. The means and std values were examined by analyses of variance (ANOVA) using a General Linear Model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania). The model examined for effects associated with month and individual.

To allow for changes in day-length and changes between New Zealand Day-light Saving Time and New Zealand Standard Time, the times of sampling were classified as either "daylight" (sample taken after at least 2 h of daylight) or "dark" (sample taken after at least 2 h of dark). Means of PCC within this classification were calculated for each cow at each month. The mean differences between "daylight" and "dark" were then calculated and examined by ANOVA using a GLM for effects associated with month and individual.

7.3.3 Results

Progesterone concentrations were >2 ng/ml at all sampling times tested. Due to catheter failure, samples were not collected from Cow 6752 in October, Cow 7714 in April or Cow 8741 in March, while some samples were not collected from Cow 6752 in December, Cow 8720 in December or Cow 8741 in March. Data were discarded for Cow 8720 in October since PCC were greater than 20 ng/ml during the hours of darkness, indicating her failure to accept the sampling routine.

Minimum and maximum daily temperatures, the times of sunrise and sunset, and mean cow live-weights for each sampling period are given in Table 7.1. Mean PCC for each month calculated across all cows and for each cow calculated across all months are given in Tables 7.2 and 7.3, respectively. Overall mean PCC was 5.0 ± 0.3 (range: 0.5 to 22.4 ng/ml).

There was a significant effect of individual ($P<0.03$) and month ($P<0.001$) on PCC. Analyses of the std of mean PCC values showed no difference in either the within cow variation or the within month variation. Analyses of the mean PCC differences during "daylight" and "dark" periods showed a significant effect of month ($P<0.05$), but not of individual cow. Mean differences in PCC between "daylight" and "dark" periods for each month are given in Table 7.4.

Date	Max temp	Min temp	Sunrise	Sunset	Cow weight
22 Oct 91	19.1	7.4	0625	1944	425.2 (18.0)
19 Nov 91	17.5	10.1	0600	2013	459.4 (20.6)
10 Dec 91	20.0	11.7	0555	2032	485.4 (20.5)
20 Jan 92	20.7	9.4	0624	2040	496.8 (20.7)
12 Feb 92	24.9	12.1	0649	2022	503.6 (23.2)
11 Mar 92	20.8	15.6	0718	1945	476.0 (21.0)
15 Apr 92	21.5	3.0	0647	1756	472.0 (21.4)
14 May 92	14.7	-2.6	0714	1723	494.2 (18.2)

Table 7.1: Maximum and minimum air temperatures (°C), times of sunrise and sunset (h), and cow live-weights [kg; mean (sem)] for the sampling periods of Expt. 7.1.

Month	n	PCC*	Std
October	3	5.5 (1.3)	3.8 (0.8)
November	5	2.6 (0.4)	3.4 (0.4)
December	5	13.2 (0.9)	2.8 (0.5)
January	5	5.8 (0.9)	4.1 (0.4)
February	5	7.4 (1.1)	4.6 (0.5)
March	4	1.3 (0.5)	1.9 (1.2)
April	4	2.2 (0.4)	3.4 (0.8)
May	5	2.9 (0.8)	3.6 (0.8)

Table 7.2: Plasma cortisol concentrations (PCC; ng/ml) and means of standard deviations (std) for assessment of between cow variability [mean (sem)] and number of cows included in the observations for each month (Expt. 7.1).

* represents significant differences within column between months ($P<0.001$).

Cow	n	PCC*	Std
6726	8	4.6 (1.4)	3.1 (0.6)
6752	7	3.7 (1.5)	2.6 (0.5)
7714	7	6.9 (1.7)	4.3 (0.5)
8720	7	5.7 (1.9)	3.9 (0.5)
8741	6	6.0 (1.4)	3.4 (0.6)

Table 7.3: Plasma cortisol concentrations (PCC; ng/ml) and means of standard variations (std) for assessment of between month variability [mean (sem)]and number of months included in the observations for each cow (Expt. 7.1).

* represents significant differences within column between cows (P<0.03).

Month	n	PCC_{Lgt}-PCC_{Dk}*
October	3	0.0 (1.2)
November	4	0.1 (0.3)
December	5	1.0 (0.4)
January	5	3.8 (0.8)
February	5	-0.6 (0.8)
March	4	0.2 (0.5)
April	4	2.4 (0.8)
May	5	-0.8 (0.8)

Table 7.4: Differences between plasma cortisol concentrations [PCC_{Lgt}-PCC_{Dk}; ng/ml; mean (sed)] in samples classified as "light" or "dark" according to the time of their collection from cows, and number of cows observed in each month (Expt. 7.1).

* represents significant differences within column between months (P<0.03).

A positive difference indicates greater mean PCC during "daylight" than "dark".

7.3.4 Discussion

At the time that this experiment was planned, studies on diurnal rhythms of PCC in cattle had produced conflicting results. Some evidence of diurnal patterns had been identified, including an early morning peak with lower levels in early evening (MacAdam and Eberhardt, 1972), and the disappearance of ultradian rhythms in the afternoon (Fulkerson *et al.*, 1980). In 1993, definitive evidence of secretory rhythms of cortisol secretion in cattle was published. Influences which might have obscured secretory patterns were reduced by stringent environmental controls, rigid management of the cows and automatic sampling at 15 min intervals over a 48 h period. A strong ultradian rhythm was observed with amplitudes up to 17 ng/ml, and a periodicity of 120 min. The circadian rhythm was weak in amplitude compared to the ultradian rhythm. The peak of the circadian rhythm occurred in early morning and was 4.5 ng/ml. The nadir of 3.1 ng/ml occurred in the evening (Lefcourt *et al.*, 1993). Basal PCC measured in this experiment were in a similar range to those reported by Lefcourt *et al.*, (1993).

Although mean PCC (Tables 7.2 and 7.3) showed variations by month and by individual there was no discernible pattern. The std of the means were similar, indicating that the degree of variation was similar between months and between individuals. No secretory rhythms could be discerned from the data. The precision of the assay in the range of 0.5 to 10 ng/ml (CV = 44.0%) was too great to allow detection of subtle variations. The sampling interval of 2 h precluded estimation of the true mean PCC where the ultradian rhythm was running at an oscillation frequency in the same order of time.

The design of experiments to examine basal cortisol secretory patterns must include frequent sampling over a prolonged period. Time of day is also important. When cows were sampled at 20 min intervals over 6 h starting in mid-morning (1000 h), no pulsatile patterns of either cortisol or ACTH concentration in plasma were identified (Sartin and Kemppainen, 1988). This sampling period may have coincided with a decline phase prior to the nadir of the diurnal cycle of cortisol secretion.

The differences in PCC between months may be due to environmental factors. Environmental temperatures were higher from December to April (Table 7.1). Cows were reported to be

sensitive to environmental temperatures and demonstrated increased PCC in response to acute thermal stress (Christison and Johnson, 1972; Roman-Ponce *et al.*, 1981a). The increases in environmental temperature experienced by the cows in this experiment may not have constituted thermal stress, as the daily temperature maximum exceeded the reported thermo-neutral point for cattle of 21.1°C only in February and April (Christison and Johnson, 1972).

Since the diurnal rhythm identified in cows has a peak in early morning and a nadir in early evening, one might expect no difference in mean PCC between the "daylight" and "dark" hours, i.e., the periods would represent the decline and the incline phases, respectively. The finding that there were differences which tended to be positive (Table 7.4) suggests that basal PCC were higher during the daylight hours. Environmental factors acting during daylight hours, e.g., flies, or the increased activity of farm-staff and/or other animals in the vicinity of the experimental subjects may have contributed to this difference.

7.4 Experiment 7.2 - Circadian Patterns of Adrenocortical Responsiveness and the Effects of Repetitive ACTH Challenge

7.4.1 Aims

The aims of this experiment were:

- to determine whether there were differences in adrenocortical responses to ACTH administered either in the early morning or early evening; and,
- to determine whether repetitive dosing with ACTH over several days altered adrenocortical responses to ACTH.

7.4.2 Materials and Methods

Animals and treatment regime:

The five cows from Expt. 7.1 were used following sampling in January 1992. They were randomly assigned to receive either saline (n = 1) or a synthetic $ACTH_{1-24}$ (Synacthen, CIBA-Geigy, Switzerland) at doses of 0.05 mg (40 IU; n = 2) or 0.2 mg (160 IU; n = 2). The 0.05 mg

dose was selected because it had been selected as the field test dose and would be applied in later experiments for this thesis. The 0.2 mg dose was selected because it represented a supra-physiological stimulus (Chapter 5). Those receiving ACTH were further assigned within dose group to either "intermittent" or "repetitive" treatment (Fig. 7.1). For the "intermittent" treatments, ACTH was administered at 0600 h and 1800 h on the first and third days only. For the "repetitive" treatments, ACTH was administered at 0600 h and 1800 h on consecutive days for a total of seven dose occasions. There was a 48 h rest period at the conclusion of the treatment schedule. The schedule was then repetitive in a crossover design within dose group. The saline treated cow served as a control. It received saline without ACTH and was sampled at the same frequency as cows in the "repetitive" treatment group.

Day:	1		2		3		4
	0600	1800	0600	1800	0600	1800	0600
	am	pm	am	pm	am	pm	am
Treatments:	1	2	3	4	5	6	7
"Intermittent"	↑	↑			↑	↑	
"Repetitive"	↑	↑	↑	↑	↑	↑	↑

Figure 7.1: Treatment schedules for Expt. 7.2.

Cows already had pre-placed jugular catheters following the sampling for Expt. 7.1 in January 1992. A 10 ml blood sample was taken immediately before the administration of ACTH (in saline to a total volume of 2 ml) or 2 ml saline. Further blood samples were taken at 50 min and at 120 min after ACTH administration (adrenal response test; ART; Chapter 5). Each blood sample was placed in a vacutainer containing lithium heparin, held in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 2 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. The first sample on every third day was assayed for progesterone to confirm dioestrus (McDougall, 1994). Details of the assays are reported in Chapter 4.

Statistical analyses:

Results are given as mean ± sem or mean ± sed as appropriate. Areas under the PCC.time curve for each animal at each treatment were calculated by integration over 120 min following ACTH administration, using full data or data corrected for 0 min PCC (AUC120 and dAUC120 respectively). Increments of PCC over 0 min PCC were calculated at 50 min and 120 min (d50 min and d120 min PCC respectively). These values, along with 0 min, 50 min and 120 min PCC were used as the response variables (Chapter 5).

a) Effects of time of dosing on adrenocortical responsiveness:

The means of the response variables for each cow at am and pm treatments were calculated (am treatment, n = 6 for each cow; pm treatment, n = 5 for each cow, Fig. 7.1). These means were compared by paired Student's *t*-Test within each dose rate. Mean differences between am and pm treatments were calculated for each cow and were analysed by ANOVA using a GLM to examine for effects associated with dose.

b) Effects of repetitive challenge on adrenocortical responsiveness:

The data were classified as "repetitive" if the cow had received two doses of ACTH in the previous 24 h (i.e., the 3rd to 7th doses in the "repetitive" treatment schedule). The rest of the data were classified as "intermittent" (i.e., 1st and 2nd doses in the "repetitive" treatment schedule and all doses in the "intermittent" schedule). The means of the response variables for each cow were calculated for each classification ("repetitive": n = 5 for each cow; "intermittent": n = 6 for each cow). These means were compared by paired Student's *t*-Test within each dose rate. Mean differences between "repetitive" and "intermittent" treatments were calculated for each cow and were further analysed by ANOVA using a GLM to examine for effects associated with dose. Response variables for which there was a significant effect of dose were subjected to linear regression analyses to determine orthogonal contrasts.

7.4.3 Results

7.4.3.1 Effects of time of dosing on adrenocortical responsiveness

Saline treatment had no significant effect on PCC on any occasion in which it was administered to the control cow. At the dose of 0.05 mg ACTH, there were no statistically

significant differences between means of response variables at am or pm administration. For four of the six response variables examined at the dose of 0.05 mg ACTH, am responses were greater than pm responses, e.g., for 120 min PCC the mean difference was 13.3 ± 1.2 ng/ml between am and pm responses. For the dose of 0.2 mg ACTH, mean 120 min PCC was less at the am treatment than at the pm treatment (60.2 ± 0.3 ng/ ml *vs.* 64.0 ± 0.5 ng/ ml, respectively; $P<0.03$). For all the response variables examined at the dose of 0.2 mg ACTH, the differences were negative, i.e. the response was greater at the pm dose than the am dose.

The mean differences between am and pm treatments showed significant effects of dose for the response variables of 120 min PCC, d120 min PCC and dAUC120. Responses to 0.05 mg ACTH were greater at the am than at the pm treatment. This was reversed for 0.2 mg ACTH, where responses were less at the am than at the pm treatment (120 min PCC: 13.3 ± 1.2 for low dose *vs.* -3.7 ± 0.1 for high dose, $P<0.01$; d120 min PCC: 10.9 ± 1.9 for low dose *vs.* -9.9 ± 2.3 for high dose, $P<0.03$; dAUC120: 209 ± 37 for low dose *vs.* -894 ± 160 for high dose, $P<0.03$).

7.4.3.2 Effects of repetitive challenge on adrenocortical responsiveness

Table 7.5 gives the mean value of each response variable for "intermittent" and "repetitive" treatments at each of the two doses of ACTH. There were significant differences between "intermittent" and "repetitive" treatments for the response variables of 120 min PCC and d120 min at the lower dose and for all response variables except 0 min PCC at the higher dose.

Dose had a significant effect on the values for 120 min PCC and d120 min PCC ($P<0.01$ and $P<0.05$, respectively, Fig. 7.2). Regression analyses indicated that this difference was due to a difference between the *y*-axis intercepts but not between the slopes, e.g., for 120 min PCC, slopes differ ($P<0.01$):

With 0.05 mg ACTH: 120 min PCC = 33.9 (4.2) + 12.9 (6.5) "repetitive"
P(regression) <0.1 r = 0.21

With 0.2 mg ACTH: 120min PCC = 53.6 (3.5) + 18.7 (5.2) "repetitive"
P(regression) <0.01 r = 0.45

Response variable	0.05 mg ACTH			0.2 mg ACTH		
	Intermittent	Repetitive	P	Intermittent	Repetitive	P
0 min PCC	6.3 (1.1)	1.8 (0.2)	ns	7.4 (1.7)	4.5 (0.8)	ns
50 min PCC	63.4 (7.0)	70.8 (11.4)	ns	61.0 (1.0)	72.7 (1.4)	0.02
120 min PCC	33.9 (6.0)	47.7 (6.2)	0.01	53.6 (0.5)	72.3 (0.3)	<0.01
d50 min PCC	56.9 (7.7)	69.0 (11.6)	ns	53.6 (2.7)	68.2 (2.2)	0.02
d120 min PCC	27.4 (6.7)	45.9 (6.4)	<0.01	46.2 (1.2)	67.8 (0.6)	0.02
AUC120	5154 (610)	5957 (895)	ns	5720 (40)	7003 (52)	0.02
dAUC120	4377 (697)	5751 (929)	ns	4833 (202)	6462 (151)	0.02

Table 7.5: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min, and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg or 0.2 mg ACTH to cows [mean (sem)] according to "intermittent" and "repetitive" treatment regimes, and the statistical significance (P) of the differences between these treatment regimes at each dose as determined by paired Student's t-Test (Expt. 7.2).

n = 2 cows for each dose rate based on means of 6 "intermittent" treatments of 5 "repetitive" treatments per cow.

7.4.4 Discussion

7.4.4.1 Effects of time of dosing on adrenocortical responsiveness

The results suggest that, at the dose rates tested, adrenocortical responses were not affected by the time of administration. The only response variable which was influenced by time of administration was 120 min PCC ($P<0.03$), and only at the dose of 0.2 mg. It was higher at the pm treatment than at the am treatment. At a dose of 0.2 mg ACTH the response must be considered supra-physiological (Chapter 5). Since higher 120 min PCC represent a more prolonged response, this could indicate that the steroidogenic processes of the adrenocortical cells were stimulated more at the pm dose. This would be unexpected because the nadir of diurnal variation should represent a time of maximum dampening (Dallman *et al.*, 1987).

Alternatively this result may be a Type I statistical error. The failure to detect differences in the other response variables would support this alternative.

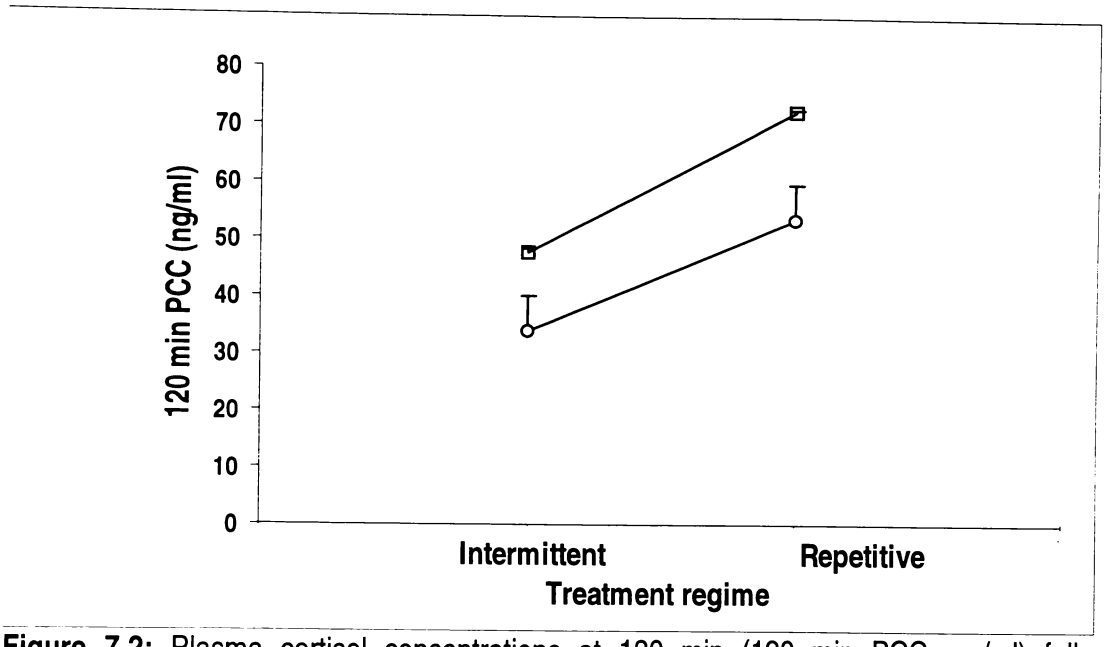


Figure 7.2: Plasma cortisol concentrations at 120 min (120 min PCC; ng/ml) following administration of 0.05 or 0.2 mg ACTH to cows under "intermittent" and "repetitive" treatment regimes (Expt. 7.2).

Legend: - O - 0.05 mg ACTH - □ - 0.2 mg ACTH
Refer to text for regression analyses (section 7.4.3.2). There was a significant difference ($P<0.01$) between *y*-axis intercepts but not between slopes. Error bars represent sem.

Dose rate had a significant effect on the mean differences between am and pm treatments for three of the response variables (120 min PCC, d120 min PCC and AUC120). At the low dose of ACTH, the am responses were greater than the pm values, as would be expected if the nadir of adrenocortical responsiveness occurred in early evening (Lefcourt *et al.,* 1993). Yet, at the higher dose, the pm responses were greater than the am responses. This suggests that the supra-physiological dose of 0.2 mg might overpower any natural tendency of the gland to be quiescent in the diurnal cycle.

7.4.4.2 Effects of repetitive challenge on adrenocortical responsiveness

Repetitive dosing, particularly at the dose of 0.2 mg, increased adrenocortical responses to ACTH. Dose rate influenced the degree of increase for the response variables of 120 min PCC

and d120 min PCC (Table 7.5, Fig. 7.2). These results are similar to reported observations where repeated injections of 40 IU or 100 IU ACTH given to cows over several days increased corticosteroid output by the adrenal cortex. At a dose of 200 IU, cows became refractory to further stimulation (Fox *et al.,* 1981; Gwasdauskas *et al.,* 1980). Doses of ACTH employed in the experiment reported here were equivalent to 40 IU and 160 IU. The similarity of the slope of the regression lines at these two doses (Fig. 7.2) indicates that the cows did not become refractory in their response to the higher dose rate.

The differences in 120 min PCC between the two treatments suggests that the effect of repetitive treatment was to prolong the secretory response of the adrenal cortex. This further suggests that the steroidogenic processes within the adrenocortical cells were stimulated to a greater degree. This could occur by accumulation of steroidogenic enzymes and membrane receptors for cholesterol uptake (Miller, 1988) or by the action of ACTH to positively regulate of its own receptors in adrenocortical cells (Gallo-Payet, 1987). Prolonged elevation of PCC following adrenocortical stimulation may be due to failure of negative feedback mechanisms. Inability of the hypothalamo-pituitary adrenal axis to terminate the response to stress following a challenge is seen in ageing and chronic stress and may be due to incompetence of hippocampal negative feedback circuits (Jacobson and Sapolsky, 1991). In the model employed here, the stimulus was applied directly to the adrenal cortex over a short-term period of several days. In this instance, the delay in termination of the response may have been due to functional hypertrophy from increased exposure to ACTH rather than to failure of feedback mechanisms.

This experiment has shown that care must be taken in the design of experiments where adrenocortical responses to ACTH are measured on a repetitive basis. Too frequent administration of ACTH may bias results. The dose of 0.05 mg ACTH may be more appropriate for repetitive dosing for routine ART.

7.5 CONCLUSIONS

The experiments reported here have shown that:

- basal PCC levels in cows managed under NZ pastoral grazing routines were similar to those reported in cows overseas under intensive management routines;
- no diurnal patterns in basal PCC were detected in cows managed under pastoral grazing routines with sampling frequency of 2 h;
- environmental factors produced significant variations in basal PCC of cows;
- differences in the adrenocortical responses of cows to ACTH administered in the early morning or early evening were undetectable at a dose of 0.05 mg and only small at a dose of 0.2 mg; and,
- repeated administration of ACTH to cows for several days altered adrenocortical function.

CHAPTER 8

ADRENOCORTICAL RESPONSES TO ADRENOCORTICOTROPIC HORMONE DURING PUBERTY AND MATURATION IN DAIRY BULLS

This chapter describes three experiments which examined adrenocortical responses to adrenocorticotropic hormone (ACTH) in dairy bulls of differing ages. Expt. 8.1 was a longitudinal study of bulls and steers which measured adrenocortical responses to a synthetic ACTH at various intervals from 3 to 17 months of age. The other experiments measured adrenocortical responses to a synthetic $ACTH_{1-24}$ in bulls aged 22 months and 5 years.

CHAPTER OUTLINE

8.1 Abstract
8.2 Introduction
8.3 Experiment 8.1 - Adrenocortical responses from 3 to 17 months of age
8.3.1 Materials and Methods
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8.4.1 Materials and Methods
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8.5 Experiment 8.3 - Adrenocortical responses at 5 years of age
8.5.1 Materials and Methods
8.5.2 Results
8.6 Discussion
8.7 Conclusions

8.1 ABSTRACT

Adrenocortical responses to a synthetic adrenocorticotropic hormone ($ACTH_{1-24}$) were measured in bulls and steers of differing ages. Responses were measured as plasma cortisol

concentrations (PCC) at 0, 50 and 120 min in relation to administration of ACTH, and as areas under the PCC.time curve over 120 min (AUC120).

Expt. 8.1: Holstein-Friesian (HF) bulls (n = 7) and steers (n = 4) were treated with a synthetic $ACTH_{1-24}$ at a dose rate of 0.01 mg/100 kg live-weight at intervals from 3 to 17 months of age. There was an increase in responses from 3 to 6 months of age. Thereafter, responses in steers were similar on three further occasions up to 15 months of age. There was an increase in response from 15 to 17 months of age. Responses of bulls declined after 6 months of age ($P<0.05$), with 50 min PCC being 47.2 $\pm$ 3.4, 37.5 $\pm$ 3.4 and 29.6 $\pm$ 2.5 ng/ml for tests at 6, 8 and 9 months, respectively; equivalent AUC120 were 4247 $\pm$ 2669, 3052 $\pm$ 277 and 2286 $\pm$ 312 ng.min/ml at 6, 8 and 9 months, respectively.

Expt. 8.2: Adrenocortical responses to 0.05 mg $ACTH_{1-24}$ were measured in HF bulls (n = 10) aged 22 months (mean live-weight 515 kg). Mean 50 min PCC was 26.9 $\pm$ 1.7 ng/ml, and AUC120 was 2504 $\pm$ 185 ng.min/ml. Responses of control animals (n = 3) treated with saline were significantly different (2.4 $\pm$ 1.9 ng/ml and 301 $\pm$ 155 ng.min/ml, respectively; $P<0.001$).

Expt. 8.3: A group of HF bulls (n = 23) aged approximately 5 years (mean live-weight 1032 kg) were treated with 0.01 mg $ACTH_{1-24}$. Mean 50 min PCC was 17.4 $\pm$ 0.9 ng/ml and AUC120 was 1725 $\pm$ 105 ng.min/ml. These responses were greater ($P<0.001$) than those of control animals (n = 6) treated with saline (5.4 $\pm$ 1.4 ng/ml and 806 $\pm$ 185 ng.min/ml, respectively).

The results of these experiments confirmed that adrenocortical responses to exogenous ACTH were reduced in bulls after puberty. Further reductions in the ability of the adrenal cortex to respond to ACTH may occur as bulls age. Although the onset of changes were associated with puberty and were negated by castration at three months of age, they may not be modulated by testosterone alone.

8.2 INTRODUCTION

Ageing has been associated with a loss of resiliency in the ability of the hypothalamo-pituitary-adrenal (HPA) axis to respond to stimulation. Evidence from rodent studies suggests that ageing is associated with a loss in sensitivity to negative feedback signals which result in increased basal secretion of both ACTH and corticosterone, and prolonged activation following stimulation. Studies in humans have failed to demonstrate changes in adrenal responses to exogenous ACTH, but have not fully examined recovery following stimulation (Seeman and Robbins, 1994).

Marked differences between the adrenocortical responses to ACTH of steers and bulls at 11 months of age were described in Chapter 6. Changes in adrenocortical responsiveness are seen in male rodents at the onset of puberty, but presence of circulating androgens results in an increase in mean basal corticosterone concentrations. Only in the guinea pig have increases in mean basal corticosterone concentrations following castration been reported (Malendowicz, 1994). Changes in adrenocortical function during ageing in bulls have been reported. Data suggested that the adrenal cortex was less responsive to ACTH as cattle grew older (Riegle and Nellor, 1967). Basal PCC were shown to be negatively correlated to age in both cows and bulls (Shayanfar *et al.*, 1975; Berardinelli *et al.*, 1992).

The experiments described here examined responses to ACTH in a group of bulls and steers which were challenged with a synthetic ACTH at intervals from 3 to 17 months of age. Also described are the results of adrenal response tests (ART) in two groups of bulls aged 22 months and 5 years, respectively.

The aims of these experiments were:

- to measure adrenocortical responses to ACTH during puberty and maturation in dairy bulls; and,
- to measure adrenocortical responses to ACTH in mature dairy bulls at two different ages.

8.3 EXPERIMENT 8.1 - ADRENOCORTICAL RESPONSES FROM 3 TO 17 MONTHS OF AGE

8.3.1 Materials and Methods

Animals:

The eight male Holstein-Friesian (HF) animals described for the experiments in Chapter 6, as well as three others which were derived from the same herd were used for this study. The three additional animals were managed with the other bulls. Rearing and management conditions have been described previously in Chapter 6.

The first ART was performed 2 weeks following weaning but before orchiectomy of the four steers (3 months of age). Further ART were completed at 6, 8, 9, 15 and 17 months of age. The period of intensive sampling for the animals described in Chapter 6 occurred at 11 months of age. One bull (Bull 211) was euthanased at 13 months of age, having suffered injuries from fighting after his return to the group at the end of the intensive sampling period. The intact bulls were used as herd sires between the ART at 15 and 17 months of age.

Adrenal response tests:

Each ART was made according to the field test developed previously (Chapter 5), except that the dose of $ACTH_{1-24}$ was calculated in proportion to live-weight to be approximately 0.01 mg/100 kg live-weight. Blood sampling and ACTH injections were by jugular venipuncture for the first four ART (<1 yr old) and by coccygeal venipuncture for the last two tests. Immediately after a 10 ml blood sample was taken using a vacutainer containing lithium heparin (0 min), the $ACTH_{1-24}$ (Synacthen, CIBA-Geigy, Switzerland) was administered in saline to a total volume of 2 ml. Further blood samples were taken at 50 min and 120 min after ACTH administration. Each blood sample for cortisol analysis was held in iced water until centrifuged (2500 rpm for 20 min at RT°C) within 3 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. An aliquot of plasma from the first blood sample of each ART occasion was stored until assayed for testosterone. Details of the assays are reported in Chapter 4.

Statistical analyses:

Results are reported as mean ± sem. Increments of plasma cortisol concentration (PCC) over 0 min PCC were calculated at 50 min and 120 min (d50 min and d120 min PCC respectively). Areas under the PCC.time curve for each animal at each ART were calculated by integration over 120 min following ACTH administration, using full data or data corrected for 0 min PCC (AUC120 and dAUC120 respectively). These values, along with 0 min, 50 min and 120 min PCC were used as the response variables (Chapter 5).

The response variables were examined by analyses of variance (ANOVA) using a General Linear Model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania). All the data were combined to examine for effects associated with age at ART and sexual status. The data for bulls and steers were further examined separately for effects associated with age at ART and individual animals. The analyses of bull data included 0 min PCC and plasma testosterone concentration as covariates, since both these variables were shown to be associated with age at ART ($P<0.01$). The differences between consecutive tests were further examined by paired Student's *t*-Test for bull and steer data separately.

The data for the bulls and the steers following castration were examined by regression analyses using Minitab (version 8.2). Regression models were fitted to the data to examine relationships between response variables, for effects associated with age at ART and individual animals. Analyses for bulls included examinations for effects associated with plasma testosterone concentrations (PTC) and 0 min PCC. A regression model to examine the relationship between PTC and age at ART was also fitted.

8.3.2 Results

Live-weights and doses of ACTH used at each ART are given in Table 8.1. Values for response variables of bulls and steers are given in Table 8.2. Plasma testosterone concentrations at each test for bulls are given in Table 8.2a. There was no testosterone detected in any sample from the steers.

Age	Live-weight		Dose ACTH
	Bulls	Steers	
3	135 (3)		0.013
6	190 (7)	198 (6)	0.02
8	216 (7)	227 (10)	0.02
9	257 (8)	245 (3)	0.025
15	462 (14)	406 (5)	0.04
17	498 (12)	483 (12)	0.05

Table 8.1: Ages (months) and live-weights (kg) of bulls and steers [mean (sem)] and doses of ACTH (mg) used at each adrenal response test (Expt. 8.1).

ANOVA:

There was no variation of 0 min PCC with age at ART, either when the data were combined or when the steers were considered alone; but age at ART had a significant effect on 0 min PCC in the bulls ($P<0.01$). There was a poor correlation between the incremental response variables dAUC120, d50 min and d120 min PCC and their parent variables (for bulls: $r = 0.54$, 0.68 and 0.81, respectively; for steers: $r = 0.09$, 0.67 and 0.43, respectively). Data for all response variables have been included in Table 8.2. Despite the low levels of correlation between absolute response variables and their respective increments, the ANOVA identified similarities in the effects associated with the factors examined. The text will only report cases where the incremental response variables did not behave in the same manner.

Sexual status had a significant effect on 50 min PCC ($P<0.001$) and AUC120 ($P<0.04$), but not on 120 min PCC. For the steers, age at ART had a significant effect on 50 min PCC ($P<0.05$), 120 min PCC, AUC120 and dAUC120 ($P<0.02$), but not d50 min PCC or d120 min PCC. Age had a significant effect on all response variables for the bulls ($P<0.001$; except for AUC120 for which $P<0.02$). There was a significant association between PTC and 50 min PCC ($P<0.05$), and between 0 min PCC and both 50 min PCC and AUC120 ($P<0.02$). Figure 8.1 displays mean data for 50 min PCC and PTC for each sexual status group.

a) Bulls

Age	0 min PCC	50 min PCC	120 min PCC	d50 min PCC	d120 min PCC	AUC120	dAUC120	PTC
3	15.0 (2.8)	29.7 (2.3)	8.8 (1.5)	14.7 (3.4)	-6.2 (2.5)	2469 (198)	665 (279)	2.0 (0.6)
6	15.5 (2.7)	47.2 (3.4)	29.4 (2.5)	31.7 (2.2)	14.0 (3.7)	4247 (269)	2392 (184)	2.1 (0.8)
8	6.9 (1.2)	37.5 (3.4)	17.9 (2.4)	30.6 (2.4)	11.0 (2.2)	3052 (277)	2222 (211)	1.5 (0.7)
9	0.5 (0)	29.6 (2.5)	14.1 (5.0)	29.1 (2.5)	13.6 (5.0)	2286 (312)	2226 (312)	3.6 (0.9)
15	13.3 (2.8)	29.0 (3.1)	4.8 (1.9)	15.6 (3.2)	-8.6 (2.6)	2239 (265)	639 (255)	9.5 (1.5)
17	3.8 (1.9)	30.0 (3.3)	11.4 (2.7)	26.2 (3.1)	7.6 (1.9)	2292 (314)	1834 (248)	11.2 (2.3)

b) Steers

Age	0 min PCC	50 min PCC	120 min PCC	d50 min PCC	d120 min PCC	AUC120	dAUC120
6	11.0 (3.8)	52.0 (2.2)	17.4 (2.7)	41.1 (2.1)	6.4 (4.3)	4002 (232)	2688 (233)
8	10.7 (7.1)	49.2 (2.0)	11.0 (1.8)	38.5 (6.2)	0.4 (8.0)	3600 (251)	2322 (643)
9	12.5 (4.1)	46.5 (2.6)	14.6 (4.1)	34.0 (5.5)	2.1 (5.9)	3613 (208)	2113 (486)
15	4.5 (1.7)	51.1 (4.4)	9.1 (1.1)	46.6 (3.0)	4.6 (2.0)	3492 (295)	2952 (131)
17	18.5 (3.5)	67.3 (7.7)	22.1 (5.2)	48.9 (4.8)	3.6 (2.3)	5273 (724)	3056 (362)

Table 8.2: Absolute and incremental plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min, and absolute and incremental integrated responses over 120 min (AUC120; ng.min/ml) following administration of ACTH at a dose rate of 0.05 mg/500 kg live-weight, and plasma testosterone concentrations (PTC; ng/ml) at 0 min [mean (sem)] at each age tested (months) in bulls (a) and steers (b; Expt. 8.1).

n = 11 for the test at 3 months of age, 7 for the tests at 6 to 9 months of age, and 6 for the test at 15 and 17 months of age for the bulls; n = 4 for all tests for the steers.
Refer to text for significant differences within columns between ages.

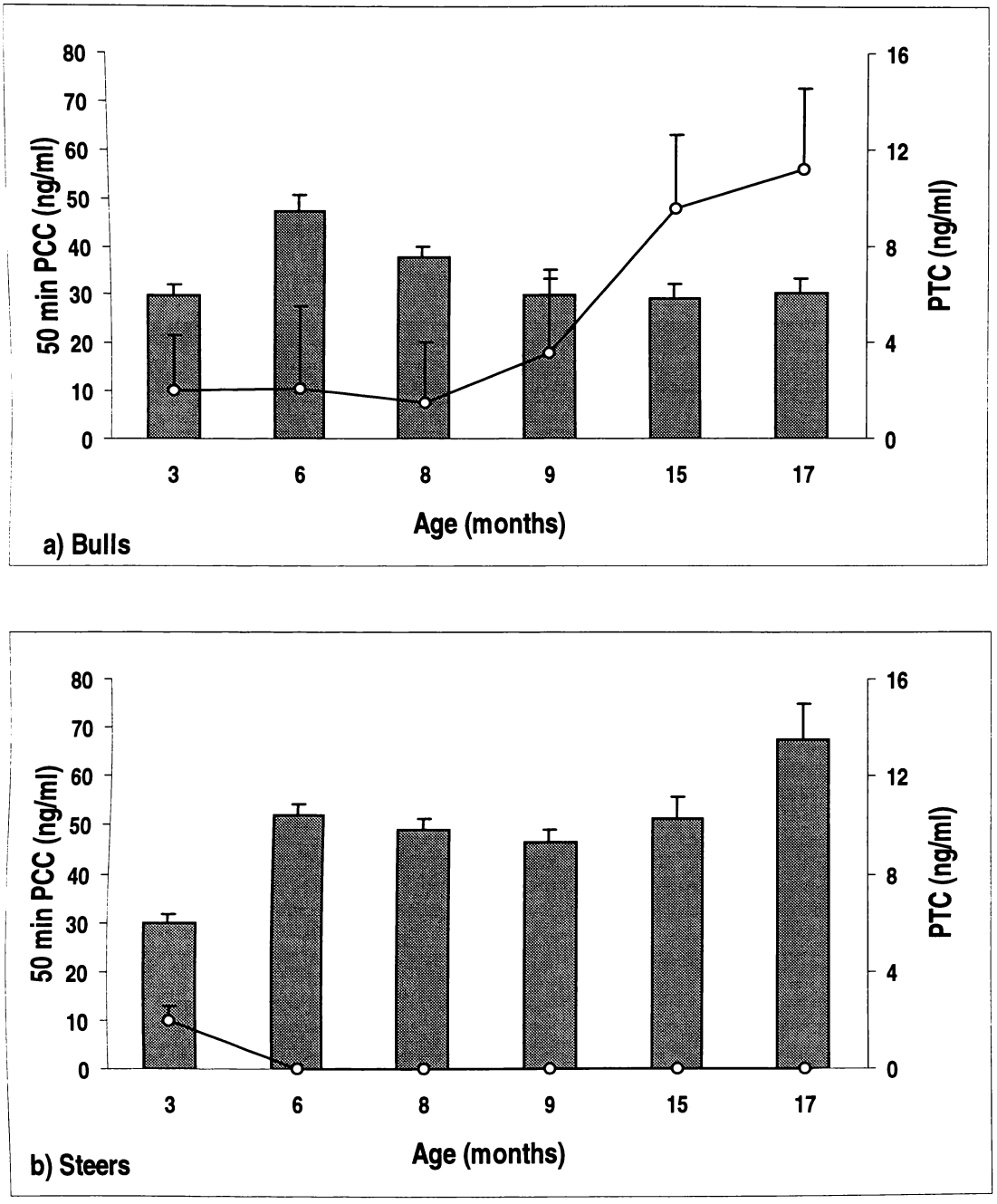


Figure 8.1: Plasma cortisol concentrations (PCC; ng/ml) at 50 min following administration of ACTH at a dose rate of 0.05 mg/500 kg live-weight and plasma testosterone concentrations (PTC; ng/ml) at 0 min at each age at which adrenal response testing was carried out in bulls (a) and steers (b; Expt. 8.1).

Legend: Columns represent 50 min PCC
Lines represent PTC
Error bars represent sem.

Paired Student's *t*-Tests identified significant increases between values of the response variables 50 min and 120 min PCC and AUC120 at 3 months and 6 months for both bulls ($P<0.01$) and steers ($P<0.03$; Table 8.2; Fig. 8.1). A significant decline ($P<0.05$) in 50 min PCC, 120 min PCC and AUC120, but not in the incremental variables, occurred between 6 and 8 months in the bulls (Table 8.2a, Fig. 8.1a). These same response variables increased in the steers between the tests at 15 and 17 months ($P<0.01$; Table 8.2b; Fig. 8.1b).

Regression analyses:

The only significant regression relationship identified in the data from the steers related to d50 min PCC, but was not strong ($r = 0.45$, $P<0.05$). Age was not a statistically significant factor in this relationship, only individual animal ($P<0.03$), with two steers having lower d120 min PCC than the others.

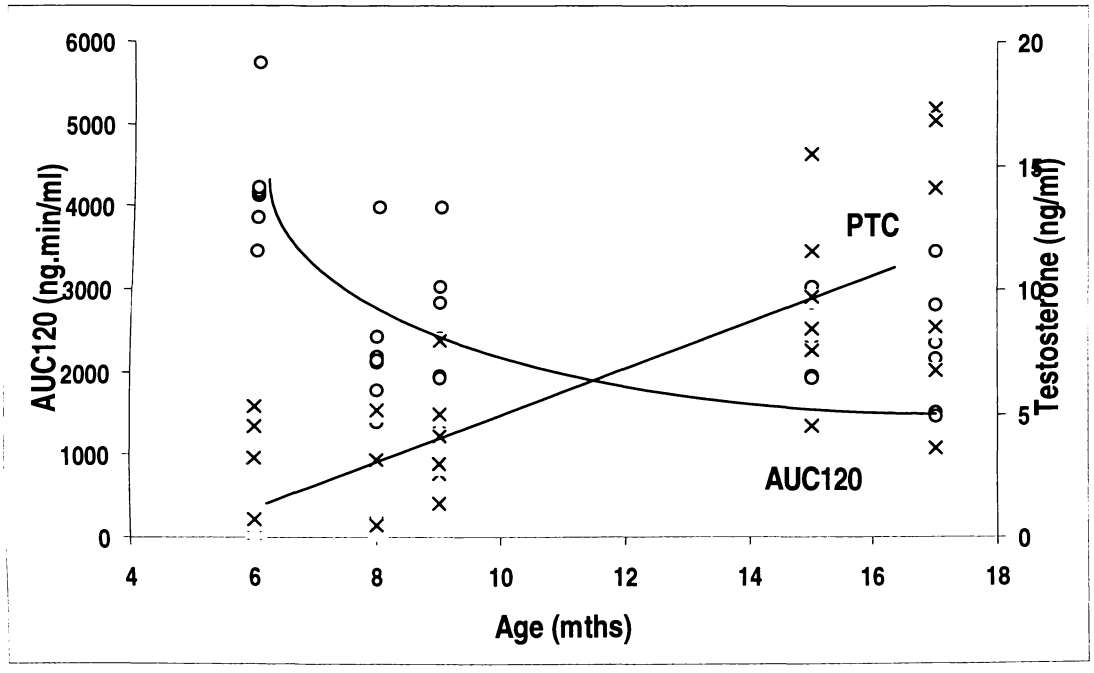


Figure 8.2: Areas under the plasma cortisol concentration.time curve over 120 min (AUC120) following administration of ACTH at a dose rate of 0.05 mg/500 kg live-weight and plasma testosterone concentrations (PTC) in bulls at selected ages from 6 to 17 months (Expt. 8.1).

Legend: - O - AUC120
- x - PTC

Refer to text for prediction equation for AUC120.

Prediction equation for PTC:

PTC	=	- 2.6	+ 0.7 age
	se:	± 1.2	± 0.1
	P:	<0.04	<0.001

r = 0.59 P(regression) <0.001

Significant relationships (P<0.001) were identified in the bull data between the response variables, age and 0 min PCC, but not PTC. Responses to ACTH demonstrated a curvilinear relationship with age (months). The inverse of ($\log_e$ age) was a significant (P<0.001) predictor of both 50 min PCC and AUC120. There was a significant positive effect (P<0.01) of 0 min PCC on all response variables except 120 min PCC, i.e., higher 0 min PCC was associated with a higher overall response. Individual animals also influenced these relationships, in particular Bull 211 which showed higher values (P<0.01) for all response variables, and Bull 214 which showed lower values (P<0.02) for 50 min PCC. Fig. 8.2 displays the relationships between AUC120, PTC and age (months) at ART in bulls. The best-fit regression relationship for AUC120 in the bulls was described by:

$$AUC120 = -14847 + 457(age) + 28739(1/\log_e age) + 41.4(0\ min\ PCC) + individual$$

	r = 0.91	P<0.001
For age:	se ± 76;	P<0.001
For ($1/\log_e$ age):	se ± 3778;	P<0.001
For 0 min PCC:	se ± 9.3;	P<0.001

8.4 Experiment 8.2 - Adrenocortical Responses at 22 months of Age

8.4.1 Materials and Methods

Animals:

Thirteen HF bulls aged 22 months (mean live-weight 515 kg, range 462 to 578 kg) which were accustomed to regular yarding for weighing and blood sampling by coccygeal venipuncture were used for this study. These animals had been born to cows in the DRC herds in August 1990, and reared to achieve high growth rates as control animals in an experiment to compare carcass composition with other breeds.

Adrenal Response Tests:

Adrenal response tests were carried out as described for Expt. 8.1 but using a dose of 0.05 mg synthetic $ACTH_{1-24}$ in 2 ml saline. Three of the animals were selected randomly to receive 2 ml saline without ACTH.

Statistical analyses:

Results are reported as mean ± sem. Response variables were calculated as described for Expt. 8.1. Data were examined by ANOVA using a GLM with Minitab (version 8.2). Data were examined for effects of treatment, with PTC as a covariate.

8.4.2 Results

The mean values for response variables following either saline or ACTH, and mean PTC are reported in Table 8.3. The incremental response variables of dAUC120, d50 min and d120 min PCC were highly correlated to the variables from which they were derived (r = 0.82, 0.89 and 0.91, respectively) and are not reported further.

	ACTH	Saline
0 min PCC	7.7 (2.0)	4.4 (2.0)
50 min PCC	26.9 (1.7)	2.4 (1.9)
120 min PCC	20.0 (3.4)	1.3 (0.4)
AUC120	2504 (185)	301 (155)
PTC	8.3 (1.4)	9.0 (3.5)

Table 8.3: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min, and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH (n = 10) or saline (n = 3), and plasma testosterone concentrations at 0 min [mean (sem)] in bulls aged 22 months (Expt. 8.2).

There were no differences between treatment groups for either 0 min PCC or PTC, and the latter was not significantly associated with any response variable. There were significant differences between treatment groups for 50 min PCC and AUC120 ($P<0.001$) and for 120 min PCC ($P<0.02$).

8.5 EXPERIMENT 8.3 - ADRENOCORTICAL RESPONSES AT 5 YEARS OF AGE

8.5.1 Materials and Methods

Animals:

Twenty-nine HF bulls aged 5 years (live-weight 1032 kg, range: 923 to 1157 kg) were made available by the Livestock Improvement Corporation (LIC). These bulls were from the 1987 (n = 23) and 1989 (n = 6) intakes of the LIC Sire-proving Scheme. They had been farmed on an LIC property in small groups of 6 to 10 animals for the preceding 3 years following collection of semen for sire-proving purposes.

Adrenal Response Tests:

Adrenal response tests were carried out as for Expt. 8.2, except that the dose of synthetic $ACTH_{1\text{-}24}$ was 0.1 mg in 2 ml saline. The 23 animals from the 1987 intake were tested in 1992. Six of these bulls were selected randomly to receive 2 ml saline without ACTH. The 6 animals in the 1989 intake were tested in 1994. Because of difficulties in moving the animals between sampling occasions, they were held unrestrained in compartments of a stock-race for the entire 120 min of the ART. This was a restraint procedure which they had experienced several times previously. During this time they could hear but not see the other bulls.

Statistical analyses:

Results are reported as mean $\pm$ sem. Response variables were calculated as for Expt. 8.1. Data were examined by ANOVA using a GLM with Minitab (version 8.2). Data were examined for effects associated with treatment and intake group, with PTC as a covariate.

8.5.2 Results

The mean values for response variables following either saline or ACTH, and mean PTC are reported in Table 8.4. The incremental response variables of dAUC120, d50 min and d120 min PCC were not highly correlated to the variables from which they were derived (r = 0.38, 0.34 and 0.38, respectively). There were no differences between the two intake groups for any response variable or for PTC. There were no differences between treatment groups for either 0

min PCC, 120 and d120 min PCC or PTC, and the latter was not significantly associated with any response variable. There were differences between treatment groups for 50 min PCC and AUC120 ($P<0.001$), dAUC120 ($P<0.005$) and d50 min PCC ($P<0.04$).

	ACTH	Saline
0 min PCC	7.1 (1.2)	6.7 (2.6)
50 min PCC	17.4 (0.9)	5.4 (1.4)
120 min PCC	14.4 (1.5)	8.9 (3.1)
d50 min PCC	10.2 (1.3)	3.5 (3.4)
d120 min PCC	7.3 (1.4)	5.2 (4.3)
AUC120	1725 (105)	806 (185)
dAUC120	871 (98)	2 (221)
Testosterone	14.2 (1.4)	15.6 (1.7)

Table 8.4: Absolute and incremental plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min, and absolute and incremental integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.1 mg ACTH (n = 23) or saline (n = 6), and plasma testosterone concentrations at 0 min [mean (sem)] in bulls aged 5 years (Expt. 8.3).

8.6 Discussion

The results of the three experiments suggest that adrenocortical responses to ACTH are reduced in bulls after puberty and that this reduction may become progressively greater with increasing age.

Many of the cattle used in these experiments were relatively unaccustomed to regular handling compared to the animals used elsewhere in this thesis for studies of adrenocortical function. Some resisted the experimental procedures and this probably accounts for the variability of 0 min PCC. As a consequence the incremental response variables of d50 min, d120 min PCC and dAUC120 were not highly correlated to the variables from which they were derived, e.g., the values for r between absolute and incremental AUC120 in Expt. 8.1 were 0.54 and 0.09 for bulls and steers, respectively. When 0 min PCC were included as covariates

in the ANOVA, they were shown to be positively associated with 50 min PCC and AUC120. This is different to the data in cows (Chapter 5) where there was no relationship between 0 min PCC and peak PCC. This association probably accounts for age being associated with changes in adrenocortical responses in the steers. From 6 to 15 months of age, responses were similar in the steers; but responses measured as 120 min PCC and AUC120 increased by the ART at 17 months of age ($P<0.04$). The regression analyses of the steer data failed to demonstrate a significant effect of age on response to ACTH.

Adrenocortical responses of all animals increased significantly between 3 and 6 months; AUC120 increased from 2469 $\pm$ 198 to 4247 $\pm$ 269 and 4002 $\pm$ 232 ng.min/ml for bulls and steers, respectively. After 6 months of age, the responses of the bulls and steers were significantly different ($P<0.001$). Subsequent ART of the bulls demonstrated a graded reduction in response between 6, 8 and 9 months, after which the responses plateaued until the final test at 17 months. PTC was significantly associated with these changes as a covariate ($P<0.05$). Considered alone, PTC demonstrated a significant linear relationship ($P<0.001$) to age, but was not a significant predictor of adrenocortical response to ACTH in the bulls, which showed an inverse curvilinear relationship to age (Fig. 8.2a).

Although the adrenocortical responses of the 22 month and 5 year old bulls cannot be directly compared to those of the bulls in the longitudinal study, response variables were also low and appeared to decline further as age increased (AUC120 being 2292 $\pm$ 314, 2504 $\pm$ 185 and 1725 $\pm$ 195 ng.min/ml for bulls aged 17 months, 22 months, and 5 years, respectively). These changes are similar to a previous report where PCC approximately doubled following a 2 h infusion of ACTH in 2 and 3 year old bulls, but only increased by about 50% in older bulls (Riegle and Nellor, 1967).

Plasma testosterone concentrations appeared to be greater in the older bulls (11.2 $\pm$ 2.3, 9.0 $\pm$ 3.5 and 15.6 $\pm$ 1.7 ng/ml for bulls aged 17 months, 22 months and 5 years, respectively). Measurement of PTC by a single point sample does not accurately describe an individual's mean basal PTC. Testosterone is released in a pulsatile manner associated with luteinising hormone (LH) secretion, with peaks up to 20 ng/ml occurring about once every 8 h (Kiser *et al.*, 1976). A negative relationship between basal cortisol and testosterone concentrations has

been demonstrated (Welsh *et al.*, 1981). Stress will reduce episodic secretion of LH and delay secretion of testosterone following an LH pulse, possibly by modulation of pituitary function by cortisol (Welsh and Johnson, 1981a). Mean basal PTC increases in the bull calf from 12 weeks of age in association with the onset of spermatogenesis. Circulating androstenedione levels also increase at about this time, but then decline while PTC continues to increase, reaching adult plasma concentrations of about 4-6 ng/ml at about 24 weeks (Amann, 1983).

The initiation of the processes leading to altered adrenocortical responsiveness appear to be associated with pubertal change, but whether these changes are due to increased PTC alone or involve other factors as well is uncertain. The higher levels of response observed in the steers from 6 months of age imply that some factor of testicular origin is responsible for initiating the changes in adrenocortical response. The physiological basis whereby circulating testosterone may alter adrenocortical function has been discussed in Chapter 6.

Although the data presented suggest that PTC increased as the bulls matured after puberty, in fact mean basal PTC probably remained relatively constant. Bulls exhibited mean PTC between 2.4 and 6.2 ng/ml from 22 months to 34 months of age with variation due to season but not to increasing age (Stumpf *et al.,* 1993). The PTC reported were derived from single point sampling and may be higher than true mean basal PTC. There was a statistical association between age at ART and PTC, but PTC alone was not a significant predictor of response in the data for Expt. 8.1. This suggests that changes in PTC may not be directly responsible for the apparent continued reductions in adrenocortical responsiveness to ACTH even though inspection of the data might suggest otherwise. Other androgens of testicular origin such as androstenedione may also contribute to the observed changes. Although testosterone is a likely factor in the initiation of changes, other changes may also occur in the adrenal cortex at a structural level, and may contribute to the continuing decline in response with increasing age.

8.7 CONCLUSIONS

Adrenocortical responses to exogenous ACTH were reduced in bulls after puberty, and the results suggest that further reductions may occur as bulls age. Although the onset of these changes were associated with puberty and required the presence of a testis, they may not have been modulated by testosterone alone.

CHAPTER 9

ADRENOCORTICAL RESPONSES TO ADRENOCORTICOTROPIC HORMONE DURING THE THIRD TRIMESTER OF PREGNANCY

This chapter describes a longitudinal study of adrenocortical responses to adrenocorticotropic hormone (ACTH) in dairy cows during the third trimester of pregnancy.

CHAPTER OUTLINE

9.1 Abstract
9.2 Introduction
9.3 Materials and Methods - Experiment 9.1 - Adrenocortical responses during the third trimester of pregnancy
9.4 Results
9.5 Discussion
9.6 Conclusions

9.1 ABSTRACT

Changes in adrenocortical responses to 0.05 mg of a synthetic $ACTH_{1-24}$ were observed during the third trimester of pregnancy in ten multiparous Holstein Friesian dairy cows. Responses were measured as plasma cortisol concentrations (PCC) at 0, 50 and 120 min in relation to administration of ACTH, and as areas under the PCC.time curve over 120 min. Responses were significantly less ($P<0.05$) at four tests in the 2 months prior to calving than during mid-pregnancy or at 3 weeks after parturition. Mean PCC at 50 min showed a significant linear decline with advancing pregnancy and responses of individual cows differed consistently.

9.2 INTRODUCTION

Maintenance of pregnancy and the initiation of parturition in cattle is dependent upon the normal function of the hypothalamo-pituitary-adrenal (HPA) axis. Normal parturition is initiated

by developmental changes in the foetal HPA axis which lead to an increase in glucocorticoid output by the foetal adrenal gland. This increase is mimicked when exogenous glucocorticoids are used to induce parturition in pregnant cattle during the third trimester of pregnancy (Jöchle, 1973). Increasing levels of glucocorticoids in circulation alter the steroidogenic activity of the placenta leading to a change in the ratio of progesterone and C_{18} compounds. These changes mature the placenta as well as preparing the foetus and dam for parturition (Flint *et al.*, 1979).

Adaptation of the maternal HPA axis during pregnancy might be crucial to the successful outcome of pregnancy since an over-responsive maternal axis in late pregnancy could trigger premature parturition. Such an adaptation might occur by alterations in axis feedback mechanisms during pregnancy. Some indications that adrenocortical responses to ACTH might change during pregnancy were observed during preliminary experiments to examine the effect of glucocorticoid induction treatments on HPA axis function. The purpose of this experiment was to describe adrenocortical responses to a synthetic ACTH by multiparous dairy cows during the third trimester of pregnancy.

9.3 Materials and Methods

Animals:

Ten pregnant Holstein-Friesian cows, aged 3 to 7 years, and having experienced at least one lactation, were selected from the herd at Dairying Research Corporation No 2 Dairy. Selection criteria were that each cow had a history of good health, its production was within the upper half of the herd, and its due calving date was between 20th July and 30th August 1993. The final criteria for selection were that results of routine haematology and liver function tests were in the normal range of veterinary reference values; and, that results of an adrenal response test (ART) at mid-pregnancy fell within the range of expected values for lactating cows in mid-pregnancy (Chapter 5).

Cows had been bred in a synchronised oestrus programme (Macmillan and Peterson, 1993) during the previous breeding season. This allowed selection of two groups, each of five cows (Calving Groups 1 and 2) which would calve 3 weeks apart. This grouping was used so that in the event that conditions in the spring of 1993 became stressful for the animals, (e.g., from

inclement weather conditions or lack of pasture growth), these potential effects on adrenocortical function might be identified statistically.

Cows were weighed and their body condition scored on a scale of 1 to 10 by experienced operators every 2 weeks as well as on the day after parturition (condition score; cs; Macdonald and Macmillan, 1993). During the 8 weeks prior to calving, while cows were not lactating, they were fed pasture supplemented with silage to a level which either maintained cs or increased it by no more than 0.5 cs per month. After calving, cows were fed pasture supplemented by silage and maize according to their production requirements.

General health monitoring:

On three occasions (27/5/93 - following the end of the 1992/93 lactation; 15/7/93 - pre-calving; and 12/8/93 - post-calving) blood samples from each cow were submitted to a clinical pathology laboratory (Animal Health Laboratory, Ruakura, NZ) for routine haematology and liver function tests, as well as estimations of serum magnesium, calcium, phosphorus, and β-hydroxybutyric acid.

Adrenal response tests:

Each ART was made according to the field test developed previously (Chapter 5). Blood sampling and ACTH injections were by coccygeal venipuncture. Immediately after a 10 ml blood sample was taken using a vacutainer containing lithium heparin (0 min), a dose of 0.05 mg $ACTH_{1-24}$ (Synacthen, CIBA-Geigy, Switzerland) was administered in saline to a total volume of 2 ml. Further blood samples were taken at 50 min and at 120 min after ACTH administration. Each blood sample was held in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 3 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. Details of the assays are reported in Chapter 4.

Each cow was tested on either 9 (Calving Group 1) or 10 (Calving Group 2) occasions. The first test was on 24/2/93 (during cow selection: mid-pregnancy), then again on 7/4/94 and 20/5/93, which were three weeks either side of the end of lactation. Thereafter cows were tested at two weekly intervals until 26/8/93. Calving Group 2 had a final test on 23/9/93.

Statistical analyses:

Results are reported as mean ± sem. Increments of plasma cortisol concentrations (PCC) over 0 min PCC were calculated at 50 min and 120 min (d50 min and d120 min PCC, respectively). Areas under the PCC.time curve for each animal at each ART were calculated by integration over 120 min following ACTH administration, using full data or data corrected for 0 min PCC (AUC120 and dAUC120, respectively). These values along with 0 min, 50 min and 120 min PCC were used as the response variables (Chapter 5).

The response variables were examined by analyses of variance (ANOVA) using a General Linear Model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania). The data were assigned to test occasions (n = 8) by determining when the test was carried out in relation to week of calving (week 0) for each cow. This assignment led to the exclusion of data from tests 3 and 4 for Calving Group 1 since there were no equivalent test times for Group 2. The model examined for effects associated with test occasion, calving group and individual cow within calving group.

All data up to week 0 were subjected to regression analyses using Minitab (version 8.2). Since no differences between groups had been identified by ANOVA, data from both groups were pooled. The time of test was identified in relation to week prepartum.

9.4 RESULTS

Animals:

Calving occurred in two groups with mean calving dates of 24/7/93 and 17/8/93. Gestation length was 283.8 ± 1.5 days. Details of age, calving date, sire of calf, sex of calf, and calf weight at birth for individual cows are given in Table 9.1. Weights and condition scores at intervals in relation to calving are given in Table 9.2.

Cow			Calf		
ID	Age	Calving date	Breed of sire	Sex	Birth-weight
0760	3	14/8/93	HF	M	33.5
6737	7	20/7/93	HF	F	38.5
7131	6	15/8/93	HF	F	33.0
8724	5	27/8/93	J	F	32.5
8730	5	24/7/93	J	F	38.5
8754	5	25/7/93	HF	F	35.5
8755	5	25/7/93	HF	M	41.5
8760	5	28/7/93	HF	M	36.0
8763	5	16/8/93	HF	F	31.5
9751	4	4/8/93	HF	F	32.0

Table 9.1: Details of cows and calves in Expt. 9.1.

Legend: Ages of cows: years; birth-weights of calves: kg.
Breeds of sire: Holstein-Friesian (HF) and Jersey (J).

Time	Condition score	Live-weight
Two weeks prepartum	5.8 (0.3)	565 (20)
One day post-partum	5.4 (0.3)	514 (20)
One month post-partum	4.9 (0.4)	475 (18)

Table 9.2: Condition scores and live-weights [kg; mean(sem)] of cows at selected intervals in relation to parturition.
Condition score units are on a scale of 1 to 10 (Macdonald and Macmillan, 1993).

General health monitoring:

All results for the health tests following the end of the 1992/93 lactation (27/5/93) were within the normal reference range for the laboratory except for four cows which had serum magnesium below normal levels. At the pre-calving health test (15/7/93), three cows had red blood cell (RBC) counts which were slightly below the normal reference range [two at 4.80 x 10^{12} RBC/l (Cows 7131 and 8754) and one at 4.90 x 10^{12} RBC/l (Cow 9751) *cf.* normal reference range of 5.00 to 7.00 x 10^{12} RBC/l]. At the post-calving health test (12/8/93) two cows still had RBC counts lower than the normal reference range [3.40 x 10^{12} RBC/l (Cow 8754) and 4.80 x 10^{12} RBC/l (Cow 7131)]. At this test, occasional *Theileria* spp. were observed in the RBC from these two cows, andfrom two further cows (Cows 8763 and 9751) for which haematological parameters were within the normal reference range. All other metabolic and liver function tests results were in the normal range.

ANOVA:

There was no variation of 0 min PCC due to test occasion, calving group or individual cows. Test occasion had a significant effect ($P<0.05$) on the response variables of 50 min PCC, d50 min PCC, AUC120 and dAUC120 (Table 9.3; Figs. 9.1 and 9.2). Individual cows differed ($P<0.05$) for all response variables except dAUC120 for Calving Group 1.

Regression analyses:

There was a significant ($P<0.001$) relationship between week prepartum and both 50 min PCC and AUC120. Three cows (Cows 6737, 8754 and 9751) had significant effects on this relationship which was described as:

50 min PCC = 38.4 -	0.86(week) -	6.73(Cow 6737) +	10.0(Cow 8754) +	6.25(Cow 9751)	
(± se)	(± 1.7)	(± 0.14)	(± 3.2)	(± 3.2)	(± 2.8)
		r = 0.48	P(regression) <0.001		

There was also a significant relationship between AUC120 and week prepartum ($P<0.001$) but it was not as strong as that for 50 min PCC ($r = 0.33$ *cf.* 0.48). Only Cows 6737 and 9751 had a significant effect on this relationship.

Since two of the cows identified as having individual cow effects on the regression relationship also had low RBC counts (Cows 8754 and 9751), *post hoc* ANOVA, with RBC numbers as a covariate, were carried out. There were no significant associations between RBC numbers and any of the response variables.

Week	0min PCC	50 min PCC*	120 min PCC	AUC120*	d50 min PCC*	d120 min PCC	dAUC120*
-22	6.8 (1.2)	58.7 (2.7)	16.8 (2.4)	3461 (270)	51.8 (3.4)	10.0 (2.8)	2642 (384)
-16	3.3 (1.3)	57.2 (1.6)	22.2 (3.2)	3891 (133)	53.8 (1.5)	18.9 (3.1)	3492 (232)
-7	5.2 (1.1)	42.0 (2.3)	22.5 (3.0)	2813 (213)	36.8 (2.2)	17.3 (2.6)	2191 (254)
-5	2.9 (0.7)	44.0 (2.8)	23.1 (2.5)	3178 (201)	41.1 (2.8)	20.3 (2.5)	2832 (236)
-3	4.2 (1.4)	39.3 (2.8)	15.4 (1.7)	2497 (220)	35.1 (3.0)	11.2 (2.0)	1991 (334)
-1	6.7 (1.3)	43.5 (2.0)	20.4 (2.1)	2686 (212)	36.8 (2.3)	13.7 (2.8)	1879 (334)
1	6.4 (1.4)	49.8 (6.3)	23.0 (2.9)	3277 (416)	43.5 (5.8)	16.7 (3.2)	2515(425)
3	5.3 (1.0)	55.7 (4.8)	22.1 (3.5)	3618 (439)	50.5 (5.4)	16.9 (4.3)	2986 (535)

Table 9.3: Absolute and incremental plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min following administration of ACTH, and absolute and incremental integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to pregnant cows [mean(sem)] for each week tested in relation to parturition (week 0).

* represents significant differences within columns between week of ART ($P<0.005$ for 50 min PCC and d50 min PCC; $P<0.01$ for AUC120; $P<0.05$ for dAUC120).

9.5 Discussion

The results indicate that there is a decrease in adrenocortical responses to ACTH during the third trimester of pregnancy in cows. Variations in adrenocortical responses to ACTH have been reported during lactation but may have been confounded by stress due to high environmental temperatures. No changes could be attributed to stage of pregnancy - cows were at the 140th day of gestation at the end of sampling, approximately the stage of the first observations described here (Shayanfar *et al.*, 1975). The values of the response variables are

less than those reported in non-pregnant, non-lactating cows (Chapter 5). A reduction in responses appears to coincide with the end of lactation (Fig. 9.1). The time of this reduction in relation to stage of pregnancy cannot be identified because the schedule of ART was infrequent early in the experimental period.

Chronic or intermittent acute stressors have been observed to alter adrenocortical responses to ACTH (Friend *et al.*, 1977). Feed shortage or detrimental weather conditions during the periparturient period might act alone or in combination as stressors. Since the two groups which calved 3 weeks apart showed similar changes, and since cs and body-weights fell within acceptable ranges for New Zealand pasture grazed dairy cows (MacDonald and Macmillan, 1993), it is unlikely that such factors contributed to the observed changes.

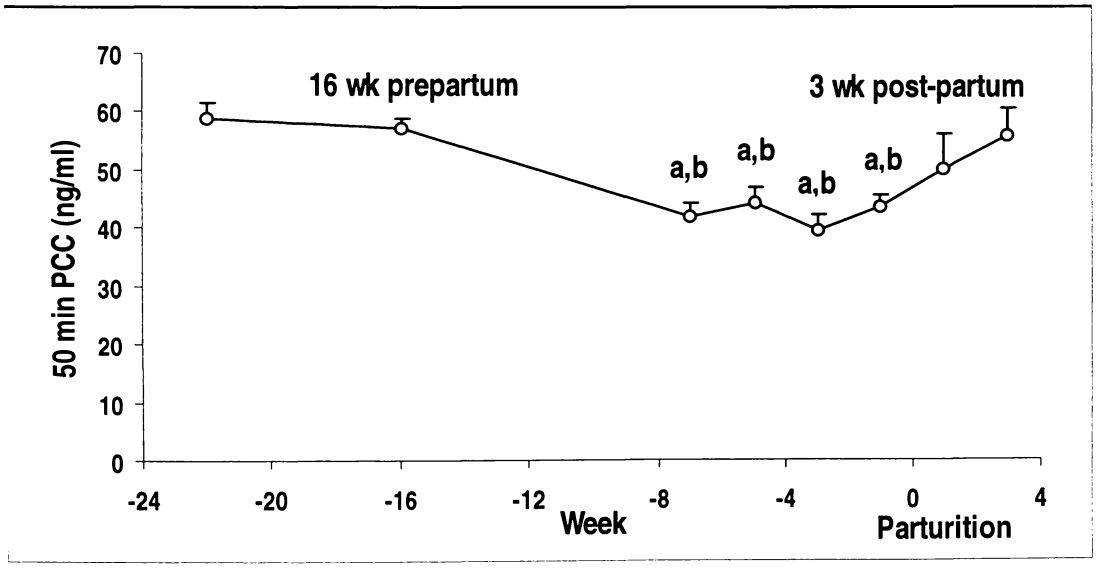


Figure 9.1: Plasma cortisol concentrations (PCC; ng/ml) at 50 min following administration of 0.05 mg ACTH to pregnant cows at various intervals during the third trimester of pregnancy and the early postpartum period. Week 0 is the week of parturition.

[a] subscript indicates significant difference ($P<0.05$) from week 16 prepartum.
[b] subscript indicates significant difference ($P<0.05$) from week 3 post-partum.

Theileria orientalis is considered non-pathogenic in New Zealand cattle (Mantkelow, 1984). While anaemia may have acted as a chronic stressor and altered responses to ACTH, the affected animals were clinically normal, and the anaemia was not severe until the post-parturient period. Examination of the data using RBC numbers as a covariate indicated that the low-grade anaemia had no significant influence on adrenocortical responses to ACTH. The

conclusion is that the observed changes are likely to be associated with changes due to advancing pregnancy.

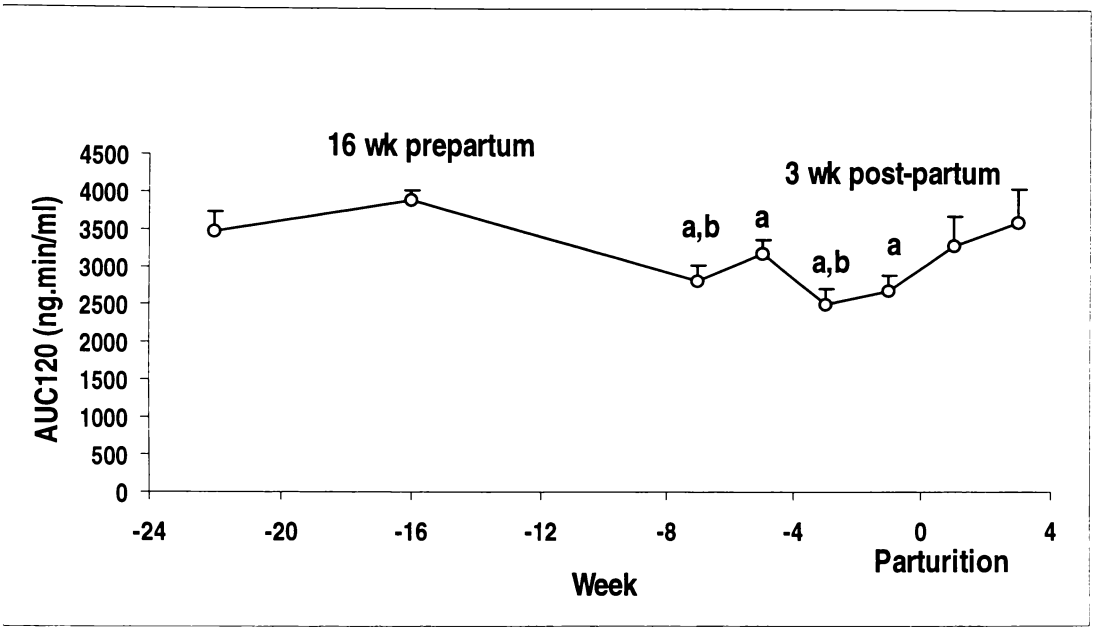


Figure 9.2: Area under the plasma cortisol concentration.time curve over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to pregnant cows at various intervals during the third trimester of pregnancy and the early postpartum period. Week 0 is the week of parturition.

[a] subscript indicates significant difference ($P<0.05$) from week 16 prepartum.
[b] subscript indicates significant difference ($P<0.05$) from week 3 post-partum.

Changes in the steroid 'milieu' during pregnancy might influence HPA axis function. Total PCC increases during pregnancy in the rat and in primates. Human and rat placental tissues contain high levels of 11β-hydroxysteroid dehydrogenase which metabolises cortisol to less active forms. This is thought to be a protective mechanism to reduce exposure of the foetus to maternal glucocorticoids (Monder and White, 1993). In sheep, increases in plasma progesterone levels during pregnancy may decrease feedback inhibition of the HPA axis leading to an increase in ACTH concentrations and in PCC (Keller-Wood *et al.*, 1988; Bell *et al.*, 1991). Both oestrogens and androgens have been implicated in the modulation of feedback mechanisms leading to alterations in levels of hypothalamic CRH (Bingaman *et al.*, 1994, Handa *et al.*, 1994). In contrast, cattle show a significant drop in basal PCC during the fourth month of pregnancy after which levels remained low apart from an acute increase in relation to parturition (Eissa and El-Belely, 1990).

The decline in adrenocortical responses to ACTH with advancing pregnancy suggests that there is a decrease in trophic drive through the axis. Increased plasma progesterone might

increase negative feedback and decrease trophic drive. Considering the critical role of glucocorticoids in the initiation of parturition in cattle, it is perhaps not surprising that the maternal adrenal cortex is suppressed in late gestation. Provision of a buffer in the event that the pregnant cow is exposed to an acute stressor would represent a short-term protection mechanism for the benefit of the foetus.

The role of the placenta in producing hormones which modulate both the maternal and foetal HPA axes has been partially elucidated. The human placenta may provide trophic support for the maternal adrenal cortex. The gene encoding the precursor for corticotropin-releasing hormone (CRH) is expressed in human placenta after mid-gestation and pro-opiomelanocortin-derived peptides including ACTH have been identified but their function may be autocrine rather than endocrine (Waddell, 1993). Immunocytochemical evidence of the presence of CRH and ACTH in sheep placenta has been obtained, but their activity is uncertain, and there appears to be little change in placental content of ACTH as a function of age. Increased progesterone levels in the media reduced the amount of CRH released from cultures of human placental tissue (Challis and Brooks, 1989).

The effects on the relationships between adrenocortical responses to ACTH and week prepartum associated with individual cows is interesting. Differences in responses to ACTH between individual cows could only be identified as changes in the *y*-axis intercept of the relationship between response variables and $\log_{10}$ dose of ACTH after several dose rates had been administered (Chapter 5). During pregnancy, effects associated with individual cows were readily observed with only one dose rate. In two of the three cows in which individual effects were seen, the rates of decline in adrenocortical responses to ACTH as pregnancy progressed were less than the mean; in the third animal, the rate of decline was greater. This suggests that individuals may exhibit different thresholds for activation of feedback mechanisms.

9.6 Conclusions

Adrenocortical responses to exogenous ACTH were suppressed during the third trimester of pregnancy in dairy cows. Responses showed a significant linear decline with advancing pregnancy, with responses for individual cows differing consistently.

CHAPTER 10

ADRENOCORTICAL SUPPRESSION FOLLOWING TREATMENT WITH DEXAMETHASONE

This chapter describes experiments which investigated some effects of treatment with selected dexamethasone formulations in cattle.

CHAPTER OUTLINE

10.1 Abstract

10.2 Introduction

10.3 Experiment 10.1 - Dexamethasone sodium phosphate in cows

- 10.3.1 Materials and methods
- 10.3.2 Results

10.4 Experiment 10.2 - Dexamethasone trimethyl-acetate in cows

- 10.4.1 Materials and methods
- 10.4.2 Results

10.5 Experiment 10.3 - Dexamethasone 21-isonicotinate in cows

- 10.5.1 Materials and methods
- 10.5.2 Results

10.6 Experiment 10.4 - Dexamethasone sodium phosphate in bulls and steers

- 10.6.1 Materials and methods
- 10.6.2 Results

10.7 Discussion

- 10.7.1 Adrenal response tests
  - 10.7.1.1 Persistence of activity of dexamethasone
  - 10.7.1.2 Hypothalamo-pituitary-adrenal axis suppression
- 10.7.2 Leucocyte counts

10.8 Conclusions

10.1 ABSTRACT

Adrenocortical responses to 0.05 mg of a synthetic adrenocorticotropic hormone ($ACTH_{1-24}$) administered intravenously, and circulating white blood cell (WBC) counts, were measured in cattle following a single intramuscular injection of selected dexamethasone formulations. Adrenocortical responses were measured as plasma cortisol concentrations (PCC) at 0, 50 and 120 min in relation to administration of ACTH, and as areas under the PCC.time curve over 120 min.

Responses to ACTH were suppressed following injection of dexamethasone sodium phosphate (20 mg) for at least 17, 9 and 16 days in cows, bulls (16 months) and steers, respectively. Leucocyte counts in cows were altered for up to 7 days. Dexamethasone trimethyl-acetate (20 mg) suppressed responses to ACTH for at least 71 days in cows, and WBC counts were altered for at least 4 days. Responses to ACTH were suppressed for up to 66 days in cows following injection of dexamethasone 21-isonicotinate (21 mg).

10.2 INTRODUCTION

Dexamethasone is 20 to 30 times more potent, is active at lower tissue concentrations and has a biological half-life at least 3 times longer than cortisol in humans (Melby, 1977). Several forms of dexamethasone are commonly administered to cattle for the induction of parturition. The solubility and specific pharmacological formulations of the dexamethasone salts can influence their metabolic clearance (Toutain *et al.*, 1985).

Treatment with dexamethasone can suppress the immune response and function of the hypothalamo-pituitary-adrenal (HPA) axis. The degree of suppression depends on the duration and dosage of treatment. Glucocorticoids altered numbers of circulating white blood cells (WBC) and these changes were used to monitor the effects of glucocorticoid administration in cattle (MacDiarmid and Cooper, 1982). Stimulation tests are required to identify suppressed HPA axis function, e.g., challenge of the adrenal cortex with low doses of ACTH is a sensitive test of adrenocortical suppression (Crowley *et al.*, 1991).

The principal aim of the experiments reported in this chapter was to measure changes in adrenocortical function following treatment with dexamethasone. The information derived from these measurements is of interest for two reasons:

a) assessment of risks associated with induction of premature parturition in dairy cows:

approximately 8% of the New Zealand dairy herd may be treated annually with long-acting synthetic glucocorticoid preparations for the purpose of inducing premature parturition (Bunny, 1992). This may alter the ability of treated cows to respond to stressors at parturition and during the early post-partum period; and,

b) assessment of suppressability of bovine adrenocortical function:

bulls exhibited lesser adrenocortical responses to ACTH than cows and steers (Chapter 6). This may be due to differences in steroidogenesis in the adrenal cortex, in negative feedback mechanisms, or in the level of trophic drive exerted from higher levels of the axis. A principal action of dexamethasone at the pituitary gland is to suppress trophic drive to the adrenal gland (Keller-Wood and Dallman, 1984). The degree of suppression and rate of recovery of adrenocortical responses to ACTH following injection of dexamethasone is an indication of the degree of support provided by the pituitary to the adrenal cortex (Fok *et al.*, 1991).

This chapter describes four experiments which examined HPA axis suppression following treatment with selected formulations of dexamethasone. Circulating WBC numbers were also monitored in two experiments. The aims of the experiments were to examine:

- the effect of three formulations of dexamethasone on adrenocortical responses to ACTH in parous, non-lactating, non-pregnant cows;
- the effect of two formulations of dexamethasone on circulating WBC in parous, non-lactating, non-pregnant cows; and
- the effect of dexamethasone on adrenocortical responses to ACTH in bulls and steers.

10.3 Experiment 10.1 - Dexamethasone sodium phosphate in Cows

10.3.1 Materials and methods

Experimental subjects:

Five parous, Holstein-Friesian (HF) cows, aged 3 to 6 years, were accustomed to frequent handling and to the sample collection routine. These cows were used previously for the

experiments described in Chapters 5 and 7. The cows were not lactating at the time of the experiment. They grazed ryegrass/clover pasture and were weighed every 2 weeks. Routine haematology and screening for liver function indicated that the animals were clinically healthy. The cows' oestrous cycles were synchronised with progesterone-releasing CIDR™ devices (Eazi-Breed CIDR-B, InterAg Pty. Ltd., Hamilton, NZ) and prostaglandin $F_{2\alpha}$ (Lutalyse, Upjohn; Macmillan and Peterson, 1993). The day following observation of the synchronised oestrus, a catheter was placed into a jugular vein of each cow to facilitate sampling. Cows were grazed close to the handling facilities, and were moved quietly to a yard before sample collection at the designated times on each sampling day.

The experimental treatments began 24 h after the catheterisation procedures. Cows were assigned randomly to either dexamethasone treatment (n = 4) or saline control (n = 1). On the first day of the experiment (day 0), blood samples were collected for WBC counts and an adrenal response test (ART) was performed. Following the final blood sample on that day, the cows were treated by intramuscular (im) injection with equal volumes of either physiological saline or 20 mg dexamethasone sodium phosphate (Dexadreson V, Pharmaco New Zealand, Auckland, NZ). Cows were subjected to ART and WBC counts on the following day (day 1), then at 2 to 4 day intervals until results returned to pre-defined limits (see below).

Adrenal response tests:

Each ART was made according to the field test developed previously (Chapter 5). All samples were collected and ACTH injections administered via the catheter. Immediately after a 10 ml blood sample was taken using a vacutainer containing lithium heparin (0 min), a dose of 0.05 mg $ACTH_{1\text{-}24}$ (Synacthen, CIBA-Geigy, Switzerland) was administered in saline to a total volume of 2 ml. Further blood samples were taken at 50 min and at 120 min after ACTH administration. The control cow received alternately ACTH or saline (2 ml) at each ART. This provided an estimate of the variation of both basal and stimulated adrenocortical functions during the time of the experiment. These tests continued until the results returned to within a 95% confidence interval established about the mean of the integrated response variable on day 0 (AUC120; see Statistical analyses). Each blood sample was held in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 3 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. Details of the assays are reported in Chapter 4.

WBC counts:

Total and differential WBC counts were performed on a 10 ml blood sample taken immediately prior to administration of ACTH. Each blood sample was placed in a vacutainer containing sodium EDTA and held in iced water. As soon as practicable, blood smears were prepared on microscope slides, and the samples, together with the smears, were submitted to a clinical pathology laboratory (Animal Health Laboratory, Ruakura, NZ) for analyses. Leucocyte counts continued until values did not differ significantly from pretreatment values.

Statistical analyses:

Results are reported as mean ± sem. Increments of plasma cortisol concentrations (PCC) over 0 min PCC were calculated at 50 min and 120 min (d50 min and d120 min PCC, respectively). Areas under the PCC.time curve for each animal at each ART were calculated by integration over 120 min following ACTH administration, using full data or data corrected for 0 min PCC (AUC120 and dAUC120, respectively). These values along with 0 min, 50 min and 120 min PCC were used as the response variables (Chapter 5).

Response variables for each sampling day were compared to day 0 values by paired Student's *t*-Test. Further analyses across time were made on an individual animal basis. The variability of response of the control animal was calculated as a coefficient of variation (CV) from the mean and standard deviation of all ART occasions in the experiment using 50 min and 120 min PCC and AUC120 as the response variables. The lower limit of the expected normal response for each cow was calculated from her Day 0 response and the CV of the response of the control cow. Animals were defined as "suppressed" if the value of the response variable was below this limit, e.g.,

CV of AUC120 for control cow = (560/3280) * 100
= 17%

Cow 6726: AUC120 on Day 0 = 3918

Allowable variation = (17%) * (3918)
= 666 ng.min/ml,

i.e., response was "suppressed" if AUC120 was below

3918 - 666 = 3252 ng.min/ml

10.3.2 Results

No symptoms of behavioural oestrus were observed during the course of the experiment. Cow live-weights are given in Table 10.1. Since many 0 min PCC were at the level of sensitivity of the assay following dexamethasone treatment, the incremental response variables were highly correlated to the variables from which they were derived (e.g., 50 min PCC *vs.* d50 min PCC; r = 0.89), and have not been reported further.

	0 min PCC	50 min PCC	120 min PCC	AUC120	Live-weight
Expt. 10.1	8.6 (3.6)	55.3 (5.9)	22.3 (3.0)	3280 (280)	476 (21)
Expt. 10.2	6.2 (2.0)	59.7 (4.1)	28.8 (3.1)	4026 (311)	494 (18)
Expt. 10.3	6.8 (1.5)	58.9 (1.7)	31.8 (4.0)	4006 (236)	507 (22)
Expt. 10.4 - Bull	5.8 (2.3)	26.0 (1.6)	7.4 (1.8)	1196 (106)	483 (5)
Expt. 10.4 - Steer	10.0 (4.4)	72.2 (4.0)	31.3 (1.1)	4624 (344)	483 (4)

Table 10.1: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min following administration of ACTH and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to control animals and live-weights [kg; mean (sem); Expts. 10.1 to 10.4].

Adrenal response tests:

Saline treatment of the control cow had no significant effect on PCC at any ART occasion. The control cow received ACTH on four occasions during the experiment and the means of response variables from these occasions are given in Table 10.1. The mean responses of the dexamethasone treated cows at each ART are given in Table 10.2. The mean 0 min PCC was different ($P<0.05$) from that of day 0 only on day 14. Means of both 50 min PCC and AUC120 differed ($P<0.05$) from those of day 0 from days 1 to 14, but neither were different on day 17 (Fig. 10.1). Mean 120 min PCC differed ($P<0.05$) from that of day 0 on days 5, 9 and 11.

Day	0 min PCC	50 min PCC	120 min PCC	AUC120
0	3.0 (1.0)	59.8 (2.3)	17.5 (3.7)	3843 (106)
1	0.8 (0.2)	48.0 (1.0)*	13.5 (4.8)	3277 (192)*
5	0.9 (0.4)	22.3 (3.0)**	1.9 (1.4)*	1318 (190)**
9	0.5 (0)	30.6 (1.7)*	5.1 (4.6)*	1966 (58)**
11	0.8 (0.3)	36.3 (2.6)**	4.8 (2.1)*	2269 (233)*
14	10.2 (1.2)*	45.4 (3.0)*	13.1 (1.3)	2216 (94)**
17	8.6 (3.2)	49.3 (2.1)	24.5 (1.3)	2996 (303)

Table 10.2: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH [mean (sem)] to cows on selected days following treatment with 20 mg dexamethasone sodium phosphate after ACTH challenge on day 0 (Expt. 10.1).

* and ** signify values which differ from day 0 values by paired Student's *t*-Test at levels of ($P<0.05$) and ($P<0.01$), respectively.

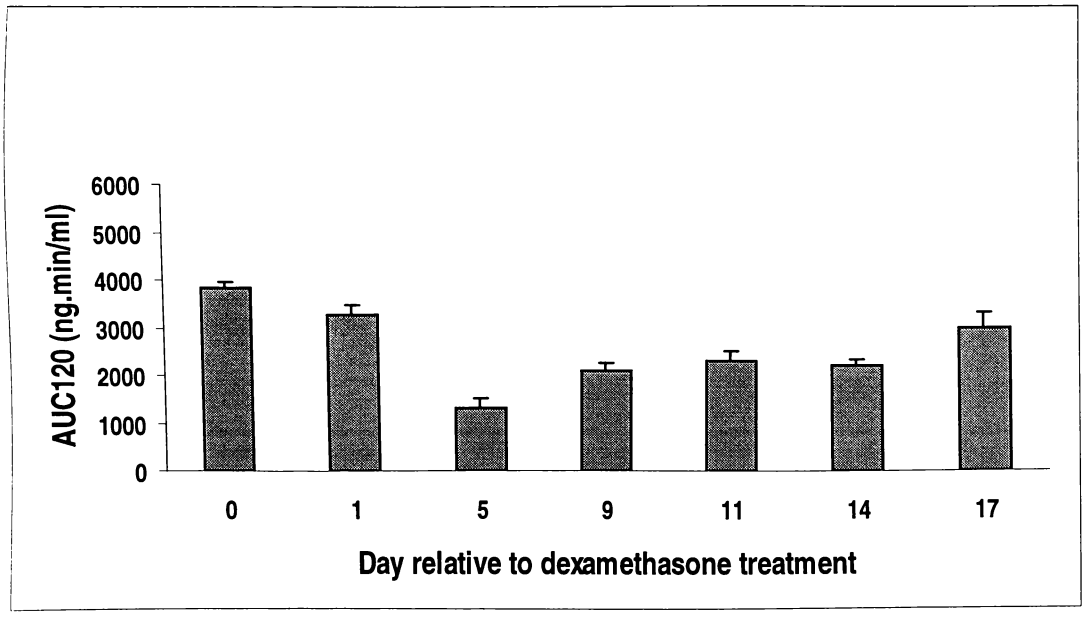


Figure 10.1: Areas under the plasma cortisol concentration.time curve over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to cows (n = 4) on selected days following treatment with 20 mg dexamethasone sodium phosphate after ACTH challenge on day 0 (Expt. 10.1).

Error bars represent sem.

All cows had suppressed values for the response variables of 50 min and 120 min PCC, and AUC120, by day 3. Times to recovery varied between individual cows. One cow was no longer suppressed by day 11 for any response variable; but 50 min PCC and AUC120 remained suppressed in two cows on day 17. The proportion of cows with suppressed 120 min PCC decreased more rapidly than for the other response variables - one individual had recovered by day 9 and all by day 17.

Leucocyte counts:

Leucocyte counts for the control cow did not fall outside the normal reference range (Animal Health Laboratory, Ruakura, NZ) on any day of sampling. Mean counts for the treated cows on each day of sampling are given in Table 10.3. Mean total WBC counts were different ($P<0.05$) from those on day 0 on days 1 to 5. Mean neutrophil numbers differed ($P<0.05$) on days 1 and 3. No eosinophils were detected in treated cows on day 1, and mean eosinophil numbers also differed ($P<0.05$) on day 7. The mean of the ratio of neutrophils to lymphocytes was different ($P<0.05$) to that of day 0 only on day 1. Mean lymphocyte numbers did not vary from day 0 on any sampling day.

Day	Total WBC	Neutrophils	Lymphocytes	Eosinophils	N:L ratio
0	7.5 (0.7)	2.2 (0.5)	4.3 (0.5)	0.8 (0.2)	0.5(0.1)
1	12.5 (2.2)*	9.7 (1.8)*	2.5 (0.5)	0*	4.2 (0.7)*
3	10.3 (0.9)*	4.6 (0.4)*	4.2 (0.3)	1.3 (0.3)	1.1 (0.1)
5	6.4 (0.5)*	2.2 (0.3)	3.4 (0.3)	0.6 (0.2)	0.7 (0.1)
7	6.5 (0.7)	1.6 (0.4)	4.2 (0.3)	0.5 (0.1)*	0.4 (0.1)

Table 10.3: Leucocyte counts [cells x 10^9/l; mean (sem)] in cows on selected days following treatment with 20 mg dexamethasone sodium phosphate on day 0 (Expt. 10.1).

n = 4 for dexamethasone treated cows.
* signifies values which differ from day 0 values by paired Student's *t*-Test at a level of ($P<0.05$).
No eosinophils were detected on day 1, hence no sem is reported.

10.4 EXPERIMENT 10.2 - DEXAMETHASONE TRIMETHYL-ACETATE IN COWS

10.4.1 Materials and methods

The five cows used in Expt. 10.1 were prepared in the same way for Expt. 10.2. Sampling and processing of samples were as described for Expt. 10.1. Jugular catheters were replaced when dislodged or blocked. CIDR™ devices were inserted on day 7 of the oestrous cycle, $PGF_{2\alpha}$ administered on day 10, and CIDR™ devices removed on day 20. This synchronisation procedure was repeated three times during the sampling period and oestrous behaviour by the cows was observed on days 22-23, days 44-45 and days 66-67 of the sampling period. No ART or WBC sampling was performed on any day when any cow was showing visible signs of oestrus.

Cows were assigned randomly to treatments of either saline (n = 1) or 20 mg dexamethasone trimethyl-acetate (Opticortenol 0.5%, CIBA-Geigy, NZ; n = 4). Treatments were given by im injection following collection of samples for ART on day 0. Cows were subjected to ART and WBC counts on days 1, 2, and 4, and then every third day until values returned to pre-defined limits calculated as for Expt. 10.1. The control cow received alternately ACTH or saline (2 ml) at each ART. Due to catheter failure some cows could not be sampled on 4 occasions (days 16, 22, 28 and 49). The data for these days were not included.

Statistical analyses of the data were as for Expt. 10.1.

10.4.2 Results

Cow live-weights are given in Table 10.1. As for Expt. 10.1, the incremental response variables were highly correlated to the variables from which they were derived and are not reported.

Adrenal response tests:

Saline treatment of the control cow had no significant effect on PCC at any ART occasion. Table 10.1 gives the means of the response variables from the 11 occasions during the

experiment in which the control cow received ACTH. The mean responses for the dexamethasone treated cows at each ART are given in Table 10.4. Plasma cortisol concentrations before administration of ACTH were below the sensitivity of the assay during much of the experiment. Means of all response variables were similar to those of day 0 on day 1; but means of both 50 min and 120 min PCC differed ($P<0.05$) from those of day 0 on days 2 to 58. Mean AUC120 varied ($P<0.05$) from day 0 on days 2 to 43 (Fig. 10.2). Plasma cortisol concentrations at 120 min after administration of ACTH were also below the sensitivity of the assay from days 7 to 31, and again on days 37 and 52.

All cows demonstrated suppression of 120 min PCC by day 2 following dexamethasone administration; by day 7, 50 min PCC and AUC120 were suppressed in all cows. Recovery of 50 min and 120 min PCC, respectively, were shown by two different cows by day 58. One cow showed recovery of AUC120 by day 49, even though 50 min and 120 min PCC were still suppressed. At the end of the sampling period on day 71, two cows still showed suppressed AUC120. Of these two cows, one demonstrated suppression of 50 min PCC while the other demonstrated suppression of AUC120.

Leucocyte counts:

Leucocyte counts for the control cow did not fall outside the normal reference range on any day of sampling. Mean counts for the treated cows on each day of sampling are given in Table 10.5. Mean total WBC counts differed ($P<0.05$) from day 0 on days 1 and 2. Mean neutrophil numbers and the mean ratio of neutrophils to lymphocytes differed ($P<0.05$) from those of day 0 on days 1 to 4. Mean lymphocyte and eosinophil numbers were not significantly different from day 0 on any sampling day.

Day	0 min PCC	50 min PCC	120 min PCC	AUC120
0	5.2 (4.6)	59.7 (4.9)	26.3 (1.8)	4030 (610)
1	0.6	62.8 (4.4)	30.4 (2.5)	4815 (217)
2	0.5	47.7 (5.2)*	9.1 (1.4)**	3182 (352)*
4	0.5	40.5 (5.6)**	6.3 (1.3)**	2649 (320)*
7	0.5	16.9 (1.3)**	0.5**	1011 (76)*
10	0.5	17.2 (1.5)**	0.5**	1043 (86)*
13	0.5	12.3 (2.9)**	0.6 (0.1)**	748 (168)*
19	0.5	7.4 (2.7)**	0.5**	435 (171)**
25	0.6	4.1 (1.3)**	0.5**	220 (102)*
31	0.5	1.9 (1.4)**	0.5**	89 (89)*
34	0.5	9.1 (2.7)**	6.2 (3.6)**	759 (283)*
37	5.4 (2.4)	1.9 (0.6)**	0.5**	-395 (276)**
39	3.8 (3.3)	4.0 (1.9)**	2.7 (2.1)**	-4.0 (269)*
43	0.5	13.8 (3.7)**	1.1 (0.6)**	854 (240)*
52	0.5	10.3 (7.5)*	0.5**	611 (455)
58	3.1 (1.5)	31.8 (4.9)*	7.8 (4.3)*	1898 (382)
63	6.5 (3.4)	34.4 (4.3)	19.4 (5.8)	2144 (231)
71	7.1 (3.9)	57.5 (7.8)	11.8 (2.1)*	3215 (239)

Table 10.4: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH [mean (sem)] to cows on selected days following treatment with 20 mg dexamethasone trimethyl-acetate after ACTH challenge on day 0 (Expt. 10.2).

n = 4 for dexamethasone treated cows.
* and ** signify values which differ from day 0 values by paired Student's *t*-Test at levels of ($P<0.05$) and ($P<0.01$), respectively.
Where no sem is reported, PCC were consistently below the level of sensitivity of the assay, i.e., an sem could not be calculated.

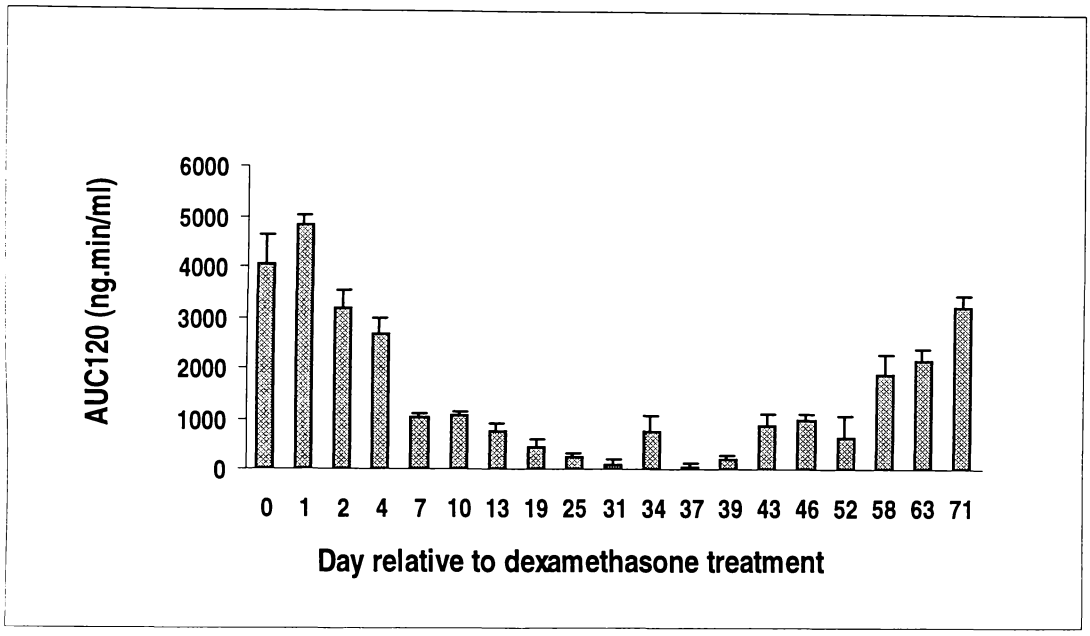


Figure 10.2: Areas under the plasma cortisol concentration.time curve over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to cows (n = 4) on selected days following treatment with 20 mg dexamethasone trimethyl-acetate after ACTH challenge on day 0 (Expt. 10.2).

Error bars represent sem.

Day	Total WBC	Neutrophils	Lymphocytes	Eosinophils	N:L ratio
0	6.9 (0.7)	1.9 (0.2)	4.4 (0.5)	0.5 (0.2)	0.4 (0.1)
1	10.2 (1.4)*	5.3 (0.9)*	4.5 (0.6)	0.2	1.2 (0.1)**
2	10.0 (0.9)*	5.5 (0.7)*	4.1 (0.5)	0.3 (0.1)	1.4 (0.2)*
4	8.5 (1.1)	4.3 (0.6)*	3.9 (0.5)	0.3 (0.1)	1.1 (0.1)*
7	7.7 (1.1)	3.5 (0.8)	4.0 (0.6)	0.2 (0.1)	0.9 (0.2)
10	7.4 (1.2)	3.4 (0.8)	3.8 (0.4)	0.1	0.9 (0.2)

Table 10.5: Leucocyte counts [cells x 10^9/l; mean (sem)] in cows on selected days following treatment with 20 mg dexamethasone trimethyl-acetate on day 0 (Expt. 10.2).

n = 4 for dexamethasone treated cows.
* signifies values which differ from day 0 values by paired Student's *t*-Test at a level of ($P<0.05$).
There was no variation in eosinophil numbers detected on day 1, hence no sem is reported.

10.5 EXPERIMENT 10.3 - DEXAMETHASONE 21-ISONICOTINATE IN COWS

10.5.1 Materials and methods

Four of the five cows used previously were prepared in the same way for Expt. 10.3. Sampling and processing of samples for ART were as described for Expt. 10.1 except that catheters were removed after sampling on day 14. Sample collections and injections of ACTH were carried out by coccygeal venipuncture thereafter. There was no assessment of WBC numbers. Oestrous synchronisation procedures were carried out as for Expt. 10.2.

Cows were assigned randomly to treatments of either saline (n = 2) or 21 mg dexamethasone 21-isonicotinate ester (Voren A.P., Boehringer Ingelheim, Germany; n = 2). Treatments were given by im injection following collection of samples for ART on day 0. Cows were subjected to ART on days 10, 14, 25, 48 and 62 following treatment after which time values had returned to the pre-defined limits calculated as for Expt. 10.1. One control cow received ACTH at each ART while the other received saline.

Statistical analyses of the data were as for Expt. 10.1.

10.5.2 Results

Cow live-weights are given in Table 10.1. Again the incremental response variables were highly correlated to the variables from which they were derived and are not reported.

Saline treatment of the control cow had no significant effect on PCC at any ART occasion. The other control cow received ACTH on 6 occasions during the course of the experiment and the means of the response variables from these occasions are given in Table 10.1. The mean responses for the dexamethasone treated cows at each ART are given in Table 10.6. The mean 0 min PCC did not differ statistically from day 0 on any test occasion. On day 10, the 0 min PCC was below the level of sensitivity of the assay. Only on day 10 was mean 50 min PCC different ($P<0.05$) from its day 0 value. Mean 120 min PCC and AUC120 varied ($P<0.05$) from day 0 values from day 10 to day 25 (Fig. 10.3).

Day	0 min PCC	50 min PCC	120 min PCC	AUC120
0	7.7 (2.7)	46.2 (1.8)	39.5 (3.0)	3426 (37)
10	0.5	10.1 (1.3)*	1.3 (0.8)*	640 (108)*
14	6.1 (0.1)	14.7 (1.6)	6.6 (1.2)*	532 (56)**
25	3.8 (0.8)	18.2 (4.8)	4.8 (0.6)*	896 (188)*
48	11.0 (1.2)	30.7 (14.6)	11.6 (5.8)	1207 (959)
62	7.9 (0.8)	49.4 (1.4)	23.3 (8.5)	3030 (306)

Table 10.6: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH [mean (sem)] to cows on selected days following treatment with 21 mg dexamethasone 21-isonicotinate after ACTH challenge on day 0 (Expt. 10.3).

n = 2 for dexamethasone treated cows.
* signifies values which differ from day 0 values by paired Student's *t*-Test at a level of ($P<0.05$).
Where no sem is reported, PCC were consistently below the level of sensitivity of the assay, i.e., an sem could not be calculated.

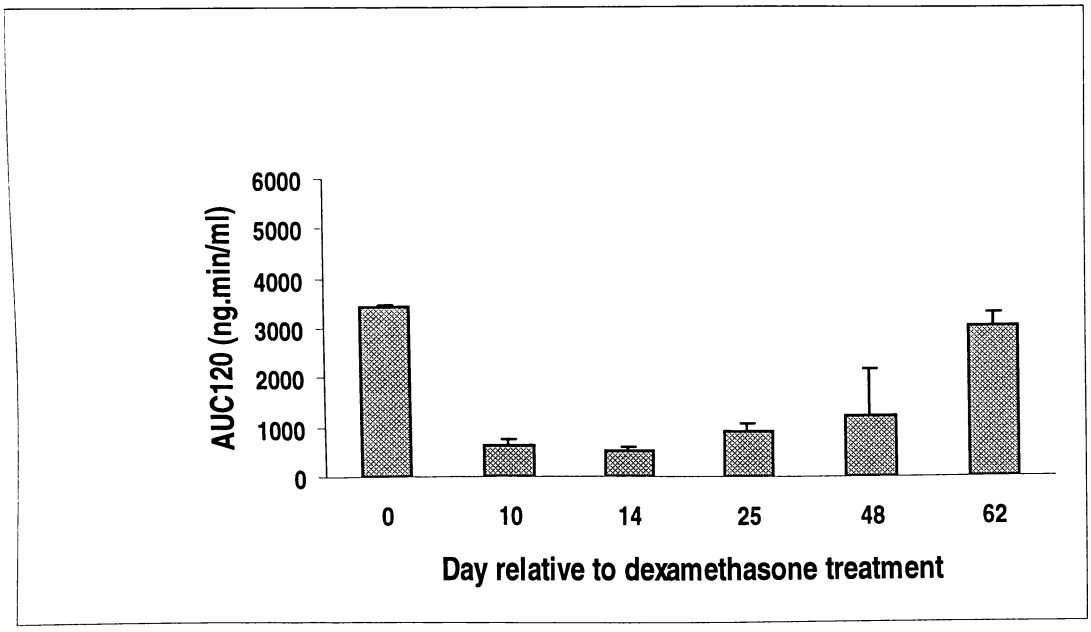


Figure 10.3: Areas under the plasma cortisol concentration.time curve over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to cows (n = 2) on selected days following treatment with 21 mg dexamethasone 21-isonicotinate after ACTH challenge on day 0 (Expt. 10.3).

Error bars represent sem.

Both cows showed suppression of 50 min and 120 min PCC and AUC120 from days 10 to 25, but recovery patterns differed for each cow. One cow showed recovery of 50 min PCC by day 48, and of 120 min PCC and AUC120 by day 62. By day 62 the other cow was no longer suppressed for 50 min PCC, but 120 min PCC and AUC120 were still suppressed at the end of the sampling period.

10.6 Experiment 10.4 - Dexamethasone sodium phosphate in Bulls and Steers

10.6.1 Materials and methods

Four HF steers, aged 20 months, and four Hereford bulls, aged 20 months, were used for the experiment. The steers had been castrated at 3 months of age and are described in Chapters 6 and 8. The bulls had been used as herd sires from 15 to 17 months of age, then had been rested during the 3 months prior to this experiment. The eight animals were managed as one group during this 3 month period, and were accustomed to handling in the facilities used. They were clinically healthy and were weighed at the start of the experiment. They grazed ryegrass/clover pasture close to the handling facilities and were moved quietly to a yard for sample collection at the designated times. Sampling and processing of samples for ART were as described for Expt. 10.1 except that sample collections and injections of ACTH were by coccygeal venipuncture.

Bulls and steers were assigned randomly within group to either saline (n = 1) or 20 mg dexamethasone sodium phosphate (n = 3). Treatments were given by im injection following collection of samples for ART on day 0. All animals were subjected to ART every third day thereafter until values for AUC120 had returned to pre-defined limits calculated as for Expt. 10.1.

Statistical analyses of the data were carried out within group as for Expt. 10.1.

10.6.2 Results

Animal live-weights are given in Table 10.1. The incremental response variables were highly correlated to the variables from which they were derived and are not reported.

Bulls:

The control bull received ACTH on 6 occasions during the course of the experiment and the means of the response variables from these occasions are given in Table 10.1. The mean responses of the dexamethasone treated bulls at each ART are given in Table 10.7. Mean 0 min PCC differed ($P<0.05$) from that of day 0 on days 3, 6 and 13. On days 3 and 6 the 0 min PCC were below the sensitivity of the assay. Mean 50 min PCC were different ($P<0.05$) from that of day 0 on days 3 and 9. The mean values for AUC120 and 120 min PCC did not differ statistically from their day 0 values (Fig. 10.4). On day 6, the 120 min PCC were below the sensitivity of the assay.

Only one bull showed suppression of 120 min PCC, and then only from days 3 to 9. In contrast, all animals showed suppressed 50 min PCC and AUC120 from days 3 to 9. Two bulls demonstrated recovery of these response variables by day 13, but one animal was still suppressed for both at the end of the sampling period. This was the same animal which demonstrated suppression of 120 min PCC.

Day	0 min PCC	50 min PCC	120 min PCC	AUC120
0	4.1 (0.8)	28.1 (5.9)	7.6 (7.1)	1563 (646)
3	0.5 *	7.5 (1.8)*	2.3 (1.1)	483 (149)
6	0.5 *	4.1 (2.8)	0.5	272 (133)
9	1.0 (0.5)	6.4 (1.6)*	0.6 (0.1)	290 (87)
13	1.0 (0.2)*	25.7 (1.3)	8.9 (2.4)	1761 (142)
16	8.0 (0.4)	32.3 (1.9)	11.5 (2.3)	1580 (194)

Table 10.7: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH [mean (sem)] to bulls on selected days following treatment with 20 mg dexamethasone sodium phosphate after ACTH challenge on day 0 (Expt. 10.4).

n = 3 for dexamethasone treated bulls.
* signifies values which differ from day 0 values by paired Student's *t*-Test at a level of ($P<0.05$).
Where no sem is reported, PCC were consistently below the level of sensitivity of the assay, i.e., an sem could not be calculated.

Steers:

The control steer also received ACTH on 6 occasions during the course of the experiment and the means of the response variables from these occasions are given in Table 10.1. The mean responses of the dexamethasone treated steers at each ART are given in Table 10.8. Plasma cortisol concentrations before administration of ACTH were below the sensitivity of the assay on day 3. Mean 0 min PCC were different ($P<0.05$) from day 0 on days 9 and 16. Mean 50 min PCC differed ($P<0.05$) from that of day 0 on days 3 and 6, while mean AUC120 were different ($P<0.05$) from days 3 to 13 (Fig. 10.4). The means of 120 min PCC did not vary statistically from the day 0 value on any sampling occasion.

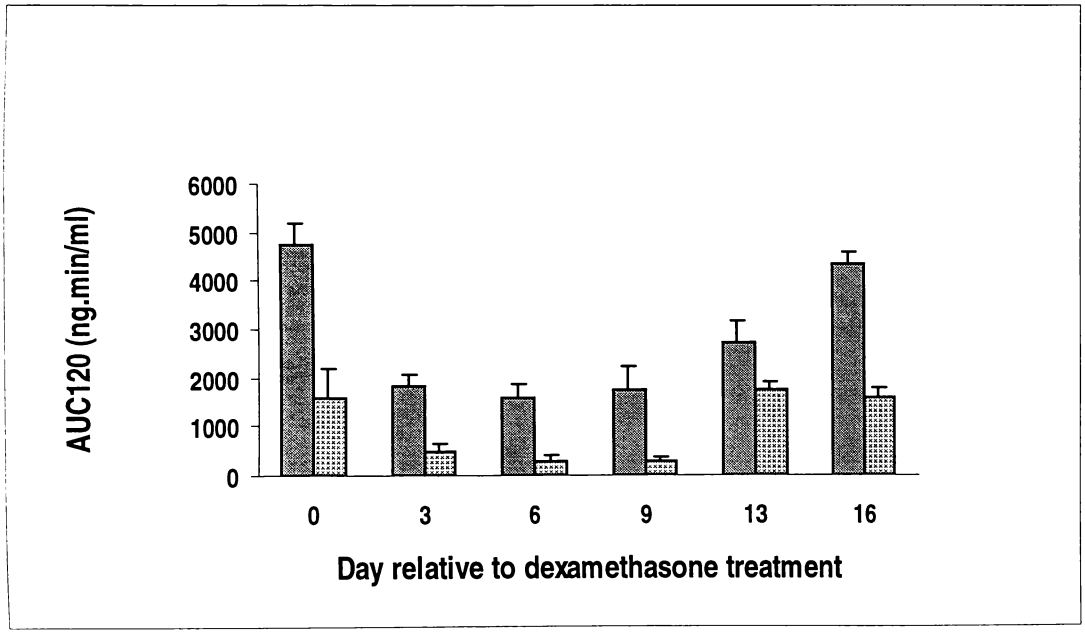


Figure 10.4: Areas under the plasma cortisol concentration.time curve over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH to bulls and steers (n = 3 each) on selected days following treatment with 20 mg dexamethasone sodium phosphate after ACTH challenge on day 0 (Expt. 10.4).

Legend:	solid columns	steers
	hatched columns	bulls

All steers showed suppression of 50 min and 120 min PCC, and AUC120 from days 3 to 9. One animal had recovered for all response variables by day 13, and all animals had recovered for 50 min PCC and AUC120 by day 16. Two steers still demonstrated suppression of 120 min PCC at the end of the sampling period.

Day	0 min PCC	50 min PCC	120 min PCC	AUC120
0	15.6 (2.8)	62.1 (8.0)	18.1 (4.7)	4748 (704)
3	0.5 *	27.1 (3.1)*	5.7 (2.2)	1837 (241)**
6	1.7 (0.8)	23.2 (2.9)**	4.2 (1.4)	1580 (194)**
9	1.4 (0.5)*	26.7 (6.7)	4.8 (1.5)	1749 (395)**
13	2.0 (1.1)	39.3 (7.1)	8.6 (3.8)	2709 (559)*
16	12.0 (2.2)*	58.3 (4.4)	15.8 (3.6)	4353 (439)

Table 10.8: Plasma cortisol concentrations (PCC; ng/ml) at 0, 50 and 120 min and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH [mean (sem)] to steers on selected days following treatment with 20 mg dexamethasone sodium phosphate after ACTH challenge on day 0 (Expt. 10.4).

n = 3 for dexamethasone treated steers.
* signifies values which differ from day 0 values by paired Student's *t*-Test at a level of ($P<0.05$).
Where no sem is reported, PCC were consistently below the level of sensitivity of the assay, i.e., an sem could not be calculated.

10.7 Discussion

10.7.1 Adrenal response tests

10.7.1.2 Persistence of activity of dexamethasone

Administration of dexamethasone to cattle altered their adrenocortical responses to ACTH in a manner which was dependent upon both the formulation of the drug and the time elapsed since treatment. Expts. 10.1 to 10.3 were carried out during a period of 9 months using the same animals, so that although seasonal conditions varied between experiments, comparisons of treatments are valid. The small size of treatment groups, especially in Expt. 10.3, and the variability between animals in the time to recovery resulted in failure to demonstrate statistical differences between pretreatment responses and responses near the end of sampling periods. Even though group differences could not be demonstrated, individual cows still showed

evidence of suppression when compared to their own pretreatment responses. Despite these difficulties in the analyses, prolonged suppression of the HPA axis of cows by two dexamethasone formulations commonly used for induction of parturition have been clearly demonstrated.

The results of Expt. 10.3 are in agreement with observations that adrenocortical responses to ACTH did not return to pretreatment levels until 52 days after im injection of a suspension of dexamethasone 21-isonicotinate (Toutain *et al.*, 1982). Persistence of dexamethasone in plasma and milk samples from cattle was measured using tritiated dexamethasone and radio-immunoassay techniques. Dexamethasone could not be detected in plasma 30 days after injection of a trimethyl-acetate formulation (McGowan *et al.*, 1975; Fairclough *et al.*, 1981). These results may reflect lack of sensitivity of the assays rather than clearance of the dexamethasone. Dexamethasone could not be detected in plasma following injection of a suspension of dexamethasone 21-isonicotinate despite evidence that the HPA axes of the cows were suppressed (Toutain *et al.*, 1982). Since dexamethasone is active at very low concentrations, measurement of adrenocortical responses to ACTH may be a more sensitive assessment of persistence activity than direct assay techniques.

Good pharmacological practise dictates that drug use should minimise undesirable side effects and should be only of sufficient duration to achieve the desired effect. Since all glucocorticoids bind to the same receptors (de Kloet, 1991), it is unlikely that more selective steroids will be developed. Adverse systemic effects might be reduced by selective delivery to the target organ, e.g., by inhaled steroids in the case of asthma. Continued use of synthetic glucocorticoids in dairy cattle may require similar mechanisms to increase their safety.

The statutory withholding period of a drug for animal use should indicate the time that residues may persist following treatment. In cattle, there are no withholding times for either dexamethasone sodium phosphate or dexamethasone trimethyl-acetate in milk and meat. The withholding time for dexamethasone 21-isonicotinate ester in milk is 4 days and in meat is 14 days (Meadows, 1990). Expts. 10.1 to 10.3 demonstrated that the period suppression of the HPA axis was longer than the respective withholding times for the drugs used, and suggest that the dexamethasone was not cleared within the time of the withholding period. If the persistence of HPA axis suppression is evidence of non-clearance, then one must question the

withholding periods accepted at present. The risk of entry of dexamethasone, which is metabolically active at low concentrations, into the human food chain, particularly in milk products, needs to be reassessed.

10.7.1.2. Hypothalamo-pituitary-adrenal axis suppression

Measurement of responses to ACTH challenge directly assesses adrenocortical function but provide only indirect information about the integrity of the HPA axis. Suppression of trophic drive reduces adrenocortical responsiveness to challenge. Adrenocortical responses to ACTH may return to the normal range before pituitary function is fully restored. Following challenge with corticotropin-releasing hormone, patients recovering from glucocorticoid suppression had PCC in the normal range but plasma ACTH concentrations were lower than normal. This suggested that the adrenal cortex may recover its functional reserve while the corticotroph is still suppressed (Schürmeyer *et al.*, 1985). A consistent pattern emerged in Expts. 10.1 to 10.3 where 120 min PCC was the response variable which recovered most slowly from the suppressive effects of dexamethasone. This response variable represents the ability of the adrenocortical cells to maintain steroid output following stimulation, and may reflect the processes whereby adrenocortical function is restored following loss of trophic drive.

The significance of HPA axis suppression in cattle requires further elucidation since bulls have been observed to have a lower adrenocortical response to ACTH (Chapter 6). The cows used in these experiments maintained their health despite the long-term effects of the preparations used, but their abilities to withstand challenge by stressors such as endotoxins or adverse environmental conditions were not assessed.

The duration of suppression of the HPA axis varied between individuals in all experiments. This might be due to differences at the injection site, in metabolic clearance of the drug, or in the ability of the HPA axis to overcome suppression. The exact site of deposition of the drug may influence the half-life of absorption, e.g., it may be delayed by injection into a fascial plane rather than into muscle tissue. Metabolic clearance of dexamethasone by cattle is slow compared to other species (Alvinerie *et al.*, 1988). Although mean half-life of elimination for dexamethasone from bovine plasma was reported to be 5.5 h, the range was 3.3 to 6 h (Toutain *et al.*, 1982). This explains some of the variability in persistence of activity between individuals; but the differences between animals were within a time-frame of days rather than

hours. Individual variations in numbers and plasticity of hypothalamic and hippocampal glucocorticoid receptors may also influence individual rates of recovery from HPA axis suppression (de Kloet, 1991).

The effects of dexamethasone sodium phosphate on adrenocortical responses to ACTH were similar in the steers and cows (Expts. 10.1 and 10.4). The duration of suppression appeared to be shorter in the bulls than in the steers; but this was biased by the statistical method used in the analyses, since the responses of the bulls were proportionately lower before dexamethasone treatment than those of the steers. Of more importance is the observation that responses to ACTH in the bulls were able to be suppressed by dexamethasone. This is evidence that trophic drive from higher levels of the HPA axis is important for maintenance of adrenocortical integrity in bulls, even though their adrenocortical responsiveness to ACTH is low.

10.7.2 Leucocyte counts

The changes in WBC numbers following dexamethasone treatment were similar to those previously reported with both cortisol and synthetic glucocorticoids in cattle (Convey *et al.*, 1971; Gwasdauskas *et al.*, 1980; MacDiarmid and Cooper, 1982). These changes were an insensitive measure of the duration of dexamethasone activity, being of shorter duration than the alterations in adrenocortical responses to ACTH. Functional changes in the WBC may have occurred over a longer period, and assessment of these may be a better measure of changes in leucocytes (Pruett *et al.*, 1987).

Measurement of WBC function may also be more relevant in the assessment of risk of treatment with long-acting preparations of dexamethasone such as are used for premature induction of parturition. An important function of the HPA axis is modulation of the immune system and suppression of cytokine responses which might be deleterious to the animal (Munck *et al.*, 1984). Following treatment, dexamethasone will exert a suppressive effect on the immune system for a period of time, thus protecting against excessive cytokine responses. A period of risk could occur should WBC function recover while HPA axis activity is still suppressed, thus preventing the animal from mounting an appropriate endogenous cortisol response in the event of exposure to a stressor.

10.8 CONCLUSIONS

Treatment of cows with selected dexamethasone preparations resulted in suppression of the HPA axis and also altered numbers of circulating WBC. Suppression of the HPA axis persisted for longer than the period that dexamethasone is reported to be detectable in circulation. This has cast some doubt on the validity of withholding periods for meat and milk following dexamethasone treatment of cattle. There were marked individual variations in the length of time that suppression persisted. Bulls demonstrated suppression of adrenocortical responses to ACTH, suggesting that trophic drive to the adrenal cortex from higher levels of the HPA axis is important for the maintenance of their adrenocortical function.

CHAPTER 11

ADRENOCORTICAL SUPPRESSION AND CHANGES IN PLASMA CONCENTRATIONS OF SELECTED HORMONES AND PEPTIDES DURING INDUCTION OF PARTURITION WITH DEXAMETHASONE

Adrenocortical suppression was studied following dexamethasone treatment to induce parturition in cows. Plasma concentrations of selected steroid hormones and peptides normally associated with periparturient changes were measured during induced parturition.

CHAPTER OUTLINE

11.1 Abstract

11.2 Introduction

11.3 Materials and Methods - Experiment 11.1 - Induced parturition

- 11.3.1 Experimental subjects and induction treatment
- 11.3.2 Adrenal response tests
- 11.3.3 Measurement of selected hormones and proteins
- 11.3.4 Statistical analyses

11.4 Results

- 11.4.1 Adrenal response tests
- 11.4.2 Plasma concentrations of selected hormones and proteins
- 11.4.3 Intervals from calving to first ovulation

11.5 Discussion

- 11.5.1 Adrenal response tests
- 11.5.2 Plasma indicators of lactogenesis and colostrogenesis
- 11.5.3 Relationship between oestrone sulfate and progesterone

11.6 Conclusions

11.1 ABSTRACT

Adrenocortical responses to ACTH and plasma concentrations of progesterone, oestrone sulfate, prolactin, α-lactalbumin and immunoglobulin G1 were measured in cows following the induction of parturition with dexamethasone. The induced cows were estimated to be between 32 and 37 weeks of gestation when the induction process was initiated. Adrenocortical responses to ACTH were suppressed from 6 days before parturition until at least 3 weeks after parturition. Plasma concentrations of the measured hormones and proteins followed a similar temporal pattern to those seen in spontaneously calving cows. Plasma α-lactalbumin concentrations were initially lower at the beginning of the induction period in cows which calved at <250 days of gestation than in cows which calved at ≥250 days, but all cows exhibited a similar peak at parturition. Plasma immunoglobulin G1 concentrations declined at a greater rate in cows which calved at <250 days of gestation than in cows which calved at a later stage of pregnancy or at full-term. The ratios between plasma concentrations of oestrone sulfate and progesterone showed an exponential increase as parturition approached. This ratio was lower in the cows which calved at <250 days of gestation than in cows which calved at ≥250 days, but was not related to incidences of abnormal parturition.

11.2 INTRODUCTION

The processes which culminate in natural parturition are initiated by increased activity of the foetal hypothalamo-pituitary-adrenal (HPA) axis. Administration of glucocorticoids during the last trimester of pregnancy can mimic the foetal changes to induce parturition (Jöchle, 1973). Induced calving treatments are regularly used by the majority of dairy herd owners in New Zealand with estimates of 8 to 11% of dairy cows being induced annually (Macmillan *et al.*, 1990; Bunny, 1992).

Dexamethasone preparations used for induction of parturition have been associated with persistent suppression of adrenocortical function for up to 60 days in non-pregnant dairy cows (Toutain *et al.*, 1982; Chapter 10). The duration of suppression of adrenocortical response to ACTH following induction of parturition with dexamethasone has not been measured.

Changes in plasma concentrations of progesterone, oestrone and 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (PGFM) following treatment with 20 mg dexamethasone trimethyl-acetate on about day 255 of pregnancy were similar to those seen during spontaneous parturition (Evans and Wagner, 1976; Edqvist *et al.*, 1981). Other studies have concentrated on optimising the efficacy of induction treatments rather than investigating the physiological changes which occur (MacDiarmid 1979; Day, 1979). An increase in plasma prolactin concentrations may be indicative of lactogenesis in the cow (Mepham, 1987). Plasma concentrations of α-lactalbumin (α-lac) increase concurrently with levels of lactose synthetase in mammary tissue during the prepartum period (McFadden *et al.*, 1987). Colostrogenesis is associated with a decrease in circulating immunoglobulin G1 (IGG1; Guy *et al.*, 1994). These hormones and proteins are indicators of colostrogenesis and lactogenesis. Measurement of their concentrations in plasma samples from periparturient cows will allow these processes to be compared between induced and spontaneously calving cows.

The aims of this experiment were:

- to measure the extent and duration of adrenocortical suppression during induction of parturition with dexamethasone; and,
- to measure plasma hormone concentrations of selected hormones and peptides, normally associated with periparturient changes, during induced parturition.

11.3 Materials and Methods

11.3.1 Experimental subjects and induction treatments

Induction of parturition was elected for 18 cows (4 Holstein-Friesian; HF; and 14 Jersey; J; aged from 3 to 8 years) from the herd at Dairying Research Corporation (DRC) No. 5 Dairy in 1993, because their expected calving dates were less than 6 weeks before the planned start of mating. These cows were used for the intensive studies of this experiment.

Cows were managed under a pasture grazing system with supplementation by pasture silage during the period between the end of their 1992/93 lactation and their subsequent parturition. Nutritional management aimed to increase the cows' mean body condition score (cs;

Macdonald and Macmillan, 1993) by 0.5 units per month from the end of the previous lactation, to achieve a mean cs at calving of 5.5 units. Cows were weighed and cs assessed every 2 weeks during the experiment. Live-weights were also recorded on the day after calving. Cows were treated *per os* daily with 10 gm elemental magnesium as magnesium chloride starting two days before induction treatment, and continuing until the end of the experiment. Routine haematology and screening for liver function occurred before induction treatment (14 July) and after calving (12 August). Serum magnesium and calcium levels were also screened before induction treatment. Plasma concentrations of β-hydroxy-butyrate (BOH) were measured weekly from 14 July to 18 August as an indicator of energy balance. Fig. 11.1 shows a time-line which relates the health testing to the other experimental procedures.

The induction procedure (Fig. 11.1) began with an intramuscular (im) injection of 21 mg dexamethasone 21-isonicotinate (Voren A.P., Boeringer Ingelheim, Germany) administered at 1200 h on 23 July following collection of blood samples. An injection of 0.5 mg cloprostenol (Estrumate, Pitman-Moore, Wellington, NZ) was administered im at 0600 h on 2 August. Each cow's udder was inspected daily and sprayed with an iodine-emollient teat sanitiser (Teat-Guard Plus, Ecolab, Hamilton, NZ) as prophylactic treatment against mastitis from the day of dexamethasone treatment.

A cow's last mating date followed by conceptus dating at pregnancy testing was used to calculate the gestational ages of the calves. This was adjusted for four calves following parturition based on the weight and crown-rump length of the calf (Roberts, 1971). Induced cows were divided into two groups according to the estimated gestational age of their calves - Group E: <250 days (n = 8), and Group L: ≥250 days (n = 10).

Five pregnant HF cows from the herd at the DRC No. 2 Dairy, which calved spontaneously at about the same time as the induced group were used to provide control information. These cows had been observed intensively throughout the last trimester of pregnancy (Calving Group 1, Chapter 9).

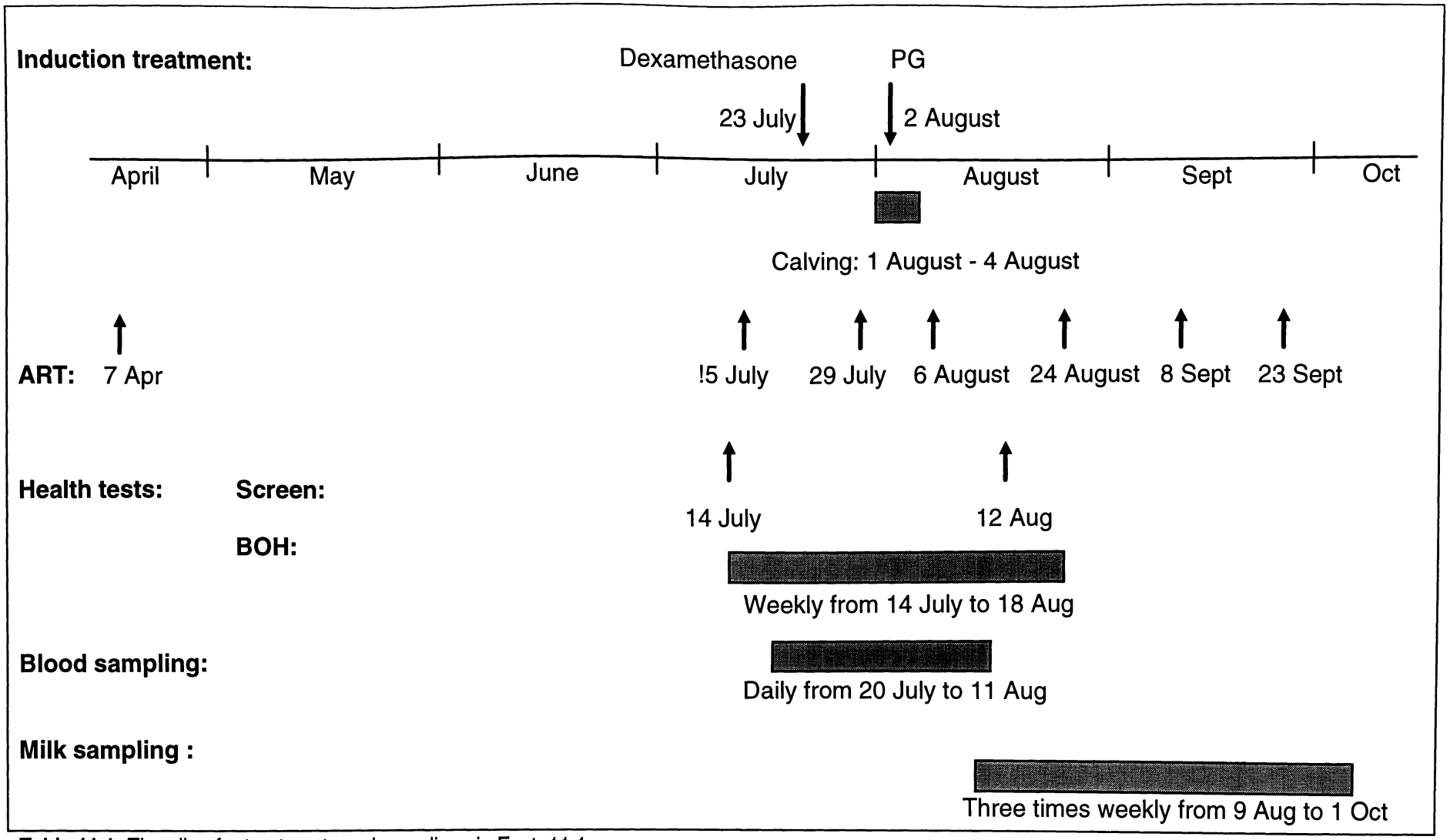


Table 11.1: Time-line for treatments and samplings in Expt. 11.1

Legend:

	PG	prostaglandin
	ART	adrenal response test
	BOH	♌-hydroxy-butyrate

Three non-pregnant HF cows, aged 4 to 6 years, which were not lactating, were used as control animals for adrenal response tests (ART). Results from these animals were intended to provide a link to data from experiments where adrenocortical suppression following dexamethasone was assessed in similar cows (Chapter 10). They were treated with dexamethasone and prostaglandins at the same times as the induced cows. They were managed with the pregnant cows until calving. After calving they were grazed as a separate group at a level to maintain their live-weights.

11.3.2 Adrenal response tests

Each ART was made according to the field test developed previously (Chapter 5). Blood sampling and ACTH injections were by coccygeal venipuncture. Immediately after a 10 ml blood sample was taken using a vacutainer containing lithium heparin (0 min), a dose of 0.05 mg $ACTH_{1-24}$ (Synacthen, CIBA-Geigy, Switzerland) was administered in saline to a total volume of 2 ml. Further blood samples were taken at 50 min and at 120 min after ACTH administration. Each blood sample was held in iced water, and centrifuged (2500 rpm for 20 min at RT°C) within 3 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. Details of the assays are reported in Chapter 4.

Cows were subjected to ART immediately before the end of their 1992/93 lactations (7 April). A second ART was on July 15 before induction treatment began, while ART 3 was on July 29, 6 days after the dexamethasone injection. The first post-partum ART was on 6 August which was between 2 and 5 days after parturition. Further tests were on 24 August, 8 September and 23 September which were approximately 3, 5 and 7 weeks post-partum, respectively (Fig. 11.1).

11.3.3 Measurement of selected hormones and proteins

Plasma samples were obtained from each cow daily, at 0900 h, from 3 days before the first induction injection through 7 days post-partum. The control cows were also sampled from 10 days before their expected day of calving until 7 days post-partum.

At each sampling, 20 ml blood was collected from the jugular vein into vacutainer tubes containing lithium heparin. Samples were placed in iced water and centrifuged within 1 h of collection (2500 rpm at RT°C). Plasma was aspirated and stored in aliquots at -20°C until analysed. All samples were assayed to determine plasma concentrations of prolactin, α-lac and IGG1. Plasma progesterone concentrations were measured only in samples from the induced cows. Samples assayed for oestrone sulfate were restricted to four cows selected at random from each of Groups E and L. Details of the assays are reported in Chapter 4.

Composite milk samples were collected at the morning milking from each cow on each Monday, Wednesday and Friday from 1 week after calving for 8 weeks (Fig. 11.1). At each sampling, 30 ml of milk was collected, mixed with 33 mg potassium dichromate (Merck, Germany) as a preservative, and stored at 4°C for no more than 14 days before analysis. Milk samples were assayed to determine progesterone concentrations. Details of the assays are reported in Chapter 4.

11.3.4 Statistical analyses

Results are reported as mean ± sem.

Adrenal response tests:

Increments of plasma cortisol concentrations (PCC) over 0 min PCC were calculated at 50 min and 120 min (d50 min and d120 min PCC, respectively). Areas under the PCC.time curve for each animal at each ART were calculated by integration over 120 min following ACTH administration, using full data or data corrected for 0 min PCC (AUC120 and dAUC120, respectively). These values along with 0 min, 50 min and 120 min PCC were used as the response variables (Chapter 5). Response variables were examined by analyses of variance (ANOVA) using a general linear model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania). The model examined for effects associated with treatment group and day of test, and for treatment group by day of test interactions. Data for ART 1 and 2 were compared with data for subsequent ART occasions by paired Student's *t*-Test.

Plasma concentrations of selected hormones and proteins:

Plasma hormone and protein concentrations were identified in relation to day of parturition. Mean plasma concentrations of each hormone and protein analysed were calculated for each day within each treatment group. Plasma concentrations of progesterone, prolactin, α-lac and IGG1 of the induced cows (Groups E and L) during the period from day -13 to day 2 were examined by repeated measures ANOVA using a GLM (SAS Institute Ltd., SAS Campus Drive, Cary, North Carolina), Data were examined for effects associated with day prepartum and stage of gestation at parturition, and for day prepartum by stage of gestation interactions. These analyses were repeated using data from day -7 to day 2 from all cows. The data were also examined for inter-relationships between plasma hormone and protein concentrations on each day separately, and then for all days from day -10 to day 0.

The ratio of oestrone sulfate to progesterone ([OS:P4]) was calculated for each cow on each day. These data were subjected to regression analyses using Minitab (version 8.2) to determine a prediction equation of best fit, examining for effects of day prepartum and stage of gestation at parturition. Data for cows which had experienced abnormal deliveries (parturient paresis or retained foetal membranes; RFM) were coded and their effect on the relationship of [OS:P4] and day were examined.

Interval from calving to first ovulation:

The day of first ovulation following parturition was determined for each cow from milk progesterone concentrations. Day of first ovulation was defined as being 5 days before the first day that milk progesterone concentrations were greater than 2.5 ng/ml (McDougall, 1994). These data were compared by ANOVA between Groups E and L for effects associated with stage of gestation at parturition.

11.4 RESULTS

Live-weights and condition scores of the induced cows at various intervals are given in Table 11.1. Results were within the normal reference ranges (Ruakura Animal Health Laboratory, Ruakura, NZ) for all haematological and liver function parameters as well as serum magnesium and calcium concentrations at the pre-induction tests. Plasma concentrations of BOH were

below the upper limit of the normal reference range (< 1.0 mmol/l) except at the sampling at 1 week post-partum (mean 1.7 ± 0.7 mmol/l; range 0.8 - 3.4 mmol/l *cf.* overall mean of 0.5 ± 0.1 mmol/l). This was associated with a decline in mean live-weight of 31 ± 13 and 61 ± 18 kg, and in mean cs of 0.6 ± 0.2 and 1.3 ± 0.3 units for J and HF, respectively (Table 11.1).

Date	Jersey		Holstein-Friesian	
	Live-weight	Condition Score	Live-weight	Condition Score
16 June 1993	408 (15)	4.9 (0.1)	507 (40)	5.3 (0.3)
14 July 1993	434 (13)	5.4 (0.1)	522 (41)	5.8 (0.3)
29 July 1993	437 (14)	5.5 (0.2)	533 (41)	6.3 (0.3)
Post-calving	424 (13)	-	505 (43)	-
12 August 1993	393 (14)	4.9 (0.1)	444 (27)	5.0 (0)

Table 11.1: Live-weights (kg) and condition scores[mean(sem)] of induced cows at selected intervals in relation to parturition.

Condition score units are on a scale of 1 to 5 (MacDonald and Macmillan, 1993).
Jersey (n = 14); Holstein-Friesian (n = 4).

One cow calved at 2100 h on 1 August, before the prostaglandin (PG) treatment was administered. The remaining cows calved between 0300 h on 3 August and 2100 h on 4 Aug. Time from PG treatment to calving was 40 ± 3 h (range: 21 to 63 h). Six cows required assistance at calving due to failure of the cervix and vulva to fully dilate (n = 2), or due to uterine inertia (n = 4). Six calves were male and 12 were female. Two calves were born dead and the remaining 16 calves were euthanased after birth. The mean estimated gestational ages of calves were 238.0 ± 3.8 days (range: 222 to 249 days) and 254.6 ± 1.2 days (range: 250-262 days) from cows in Groups E and L, respectively.

Two cows were diagnosed with mastitis on day 1 post-partum, and one cow developed mastitis 1 week later. These were treated successfully with intra-mammary antibiotics. One cow died suddenly of hypocalcaemia on the evening of August 5 (2 days post-partum).

Another cow was diagnosed with endometritis and treated with intra-uterine antibiotics from day 3 post-partum. Two cows were diagnosed as having RFM at 1 week after calving, but showed no clinical symptoms of illness due to the condition. They were treated with intra-uterine antibiotics after manual removal of the RFM.

11.4.1 Adrenal response tests

The incremental response variables of d50 min, d120 min PCC and dAUC120 were highly correlated to the variables from which they were derived (r = 0.97, 0.92 and 0.90, respectively) and have not been reported further.

All pregnant cows were within the expected range of values for pregnant cows in advanced lactation at ART 1 (Expt. 5.3). Since there was no significant effect associated with stage of gestation at parturition, data for the Groups E and L were pooled for further analyses. Mean AUC120 for the induced and non-pregnant groups at each ART are represented in Fig. 11.2. Adrenocortical responses to ACTH were significantly ($P<0.01$) influenced by ART occasion for all response variables measured ($P<0.01$).

Table 11.2 gives means of 50 min and 120 min PCC, and AUC120 for pregnant and non-pregnant groups at each ART. There was a significant effect of ART occasion ($P<0.001$) on 50 min and 120 min PCC, and on AUC120, which was similar in both pregnant and non-pregnant groups. There was a significant ($P<0.04$) effect associated with pregnancy status only for 0 min PCC, with the non-pregnant control animals having higher PCC before administration of ACTH. No pregnancy status by ART occasion interactions were detected indicating that the changes in adrenocortical function following dexamethasone treatment were independent of pregnancy status.

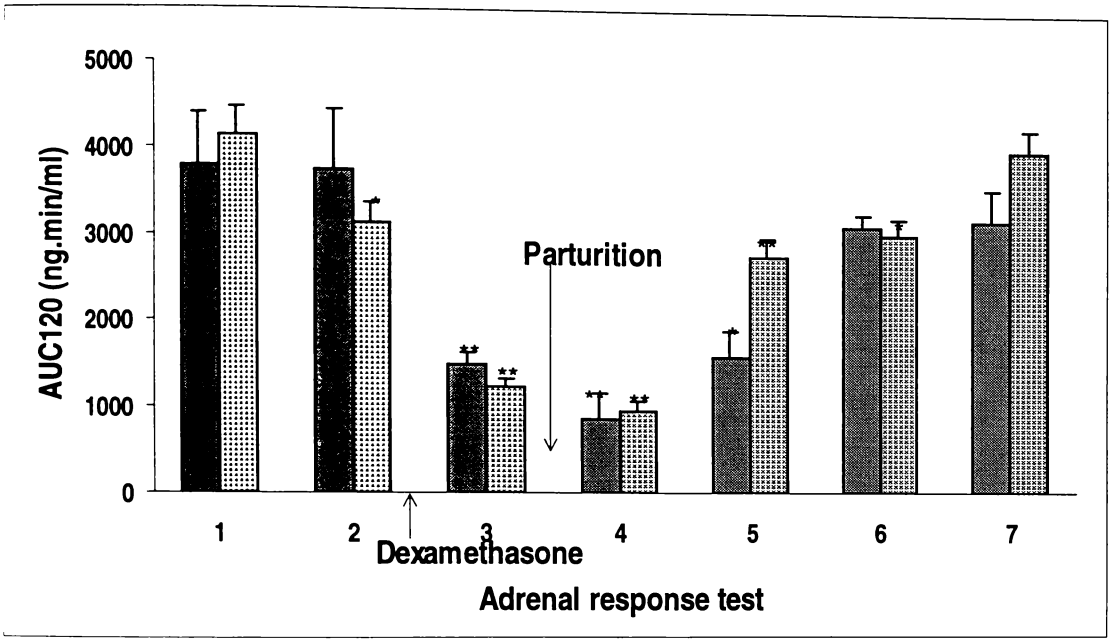


Figure 11.2: Areas under the plasma cortisol concentration.time curve over 120 min (AUC120; ng.min/ml)) following administration of 0.05 mg ACTH to induced and non-pregnant cows at selected intervals. Treatment of 21 mg dexamethasone 21-isonicotinate was administered 8 days after ART 2. Parturition occurred for induced cows between ART 3 and 4.

Legend: solid columns non-pregnant cows
cross-hatched columns induced cows
Error bars represent sem.
Refer to Fig. 11.1 for exact dates of ART.
* and ** signify values which differ within treatment group from ART 1 values by paired Student's *t*-Test at levels of ($P<0.05$) and ($P<0.01$), respectively.

Comparison of results within pregnancy groups between ART occasions (Table 11.2) indicated that responses in the pregnant cows were less ($P<0.001$) at ART 2 than at ART 1 when measured by both 50 min PCC and AUC120. At ART 3 and 4 (following dexamethasone treatment), means of 50 min and 120 min PCC as well as AUC120 were less ($P<0.01$) than at ART 1. At ART 5, means of both 50 min and 120 min PCC were still lower ($P<0.01$) than at ART 1. Mean AUC120 was also lower than at ART 1, but at a lesser level of significance ($P<0.05$). At ART 6, means of 50 min and 120 min PCC, and of AUC120 were all lower than at ART 1 to a level of significance of $P<0.05$. At ART 7, means of 50 min PCC and AUC120 were similar to those at ART 1; but mean 120 min PCC remained lower than at ART 1.

ART	Nonpregnant			Induced		
	50 min PCC	120 min PCC	AUC120	50 min PCC	120 min PCC	AUC120
1	59.8 (1.4)	38.4 (7.0)	3782 (607)	63.6 (2.6)	29.2 (3.5)	4139 (320)
2	65.5 (10.2)	27.3 (4.2)	3743 (691)	50.8 (2.4) **	28.8 (3.2)	3135 (241) *
3	25.0 (2.5) **	1.3 (0.8) **	1497 (146) **	19.7 (1.5) **	2.8 (0.6) **	1230 (93) **
4	14.9 (3.3) **	5.8 (3.2) **	874 (300) **	16.6 (2.3) **	7.4 (1.0) **	947 (133) **
5	29.4 (5.6) *	7.6 (2.2) **	1594 (295) *	44.1 (2.6) **	20.8 (2.9) *	2762 (213) **
6	53.3 (4.3)	13.0 (1.2) **	3100 (136)	53.4 (2.1) *	13.2 (2.1) *	3023 (189) *
7	55.0 (2.7)	8.3 (1.2) **	3184 (379)	63.7 (4.1)	17.0 (2.6) *	3991 (260)

Table 11.2: Plasma cortisol concentrations (PCC; ng/ml) at 50 and 120 minutes following administration of ACTH and integrated responses over 120 min (AUC120; ng.min/ml) following administration of 0.05 mg ACTH [mean (sem)] to induced and non-pregnant control cows at each adrenal response test (ART).

Non-pregnant (n = 3); Induced: ART 1 to 3 (n = 18), ART 4 to 7 (n = 17).
Dates of ART: ART 1 7 April ART 2 15 July
ART 3 27 July ART 4 6 August
ART 5 24 August ART 6 8 September
ART 7 23 September
Dexamethasone treatment was administered on 23 July. Parturition occurred between 1 August and 4 August.
* and ** signify values which differ from ART 1 values by paired Student's *t*-Test at levels of ($P<0.05$) and ($P<0.01$), respectively.

There was no difference between ART 1 and ART 2 in the response variables examined in the non-pregnant cows (Table 11.2). Thereafter the pattern of change in their adrenocortical responses to ACTH was similar to that seen in the induced cows; means of all response variables were lower ($P<0.05$) at the 3 ART following dexamethasone than at ART 1. Means of both 50 min PCC and AUC120 at ART 6 and 7 were similar to those at ART 1. The response variable of 120 min PCC was lower ($P<0.01$) at each of the last two ART when compared to the first.

11.4.2 Plasma concentrations of selected hormones and proteins

Mean plasma concentrations of the hormones and proteins measured on selected days are given in Tables 11.3 to 11.6. Plasma progesterone concentrations in the induced cows declined gradually by about 50% during the first 12 days of the sampling period (Table 11.3, Fig. 11.3). They fell rapidly following prostaglandin injection to be ≤1 ng/ml at day 1 prepartum, and remained low to the end of the sampling period. Plasma oestrone sulfate concentrations increased steadily after the dexamethasone treatment and reached the upper range of the assay by about day 7 prepartum (Table 11.3, Fig. 11.4). This peak was maintained until 1 to 2 days prepartum, after which plasma concentrations fell to be negligible by day 2 post-partum.

Day	Progesterone		Oestrone sulfate	
	Group E	Group L	Group E	Group L
-13	7.3 (0.5)	6.5 (0.6)	5.9 (2.4)	12.7 (14.9)
-7	6.8 (1.1)	5.9 (0.7)	33.8 (1.4)	35.0
-5	4.7 (1.0)	4.0 (0.4)	35.0	35.0
-2	2.8 (0.5)	2.2 (0.6)	18.0 (3.3)	33.4 (1.8)
0	0.6 (0.1)	0.5 (0.1)	13.7 (5.5)	20.7 (5.5)
2	1.1 (0.4)	0.4 (0.1)	0.7 (0.7)	3.9 (3.3)

Table 11.3: Plasma progesterone (ng/ml) and oestrone sulfate (pg/ml) concentrations [mean (± sem)] on selected days in cows induced to calve with dexamethasone and prostaglandin treatment. Day 0 is day of parturition.

Group E calved before 250 days of gestation; Group L calved on or after 250 days of gestation.
For plasma progesterone concentrations: Group E (n = 8); Group L (n = 10).
For plasma oestrone sulfate concentrations; both groups (n = 4).
Where no sem is reported, plasma oestrone sulfate concentrations were above the range of assay, i.e., an sem could not be calculated.

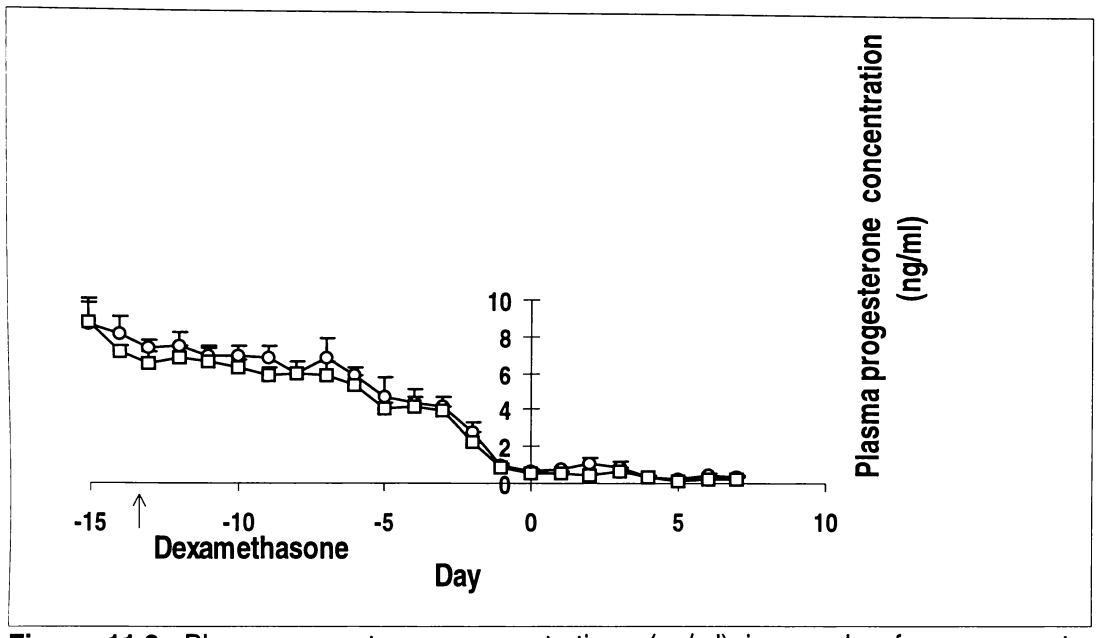


Figure 11.3: Plasma progesterone concentrations (ng/ml) in samples from pregnant and induced cows during the periparturient period.

Day 0 is day of parturition.
Error bars represent sem.
Legend: - O - Group E (calved at <250 days gestation)
- □ - Group L (calved at ≥250 days gestation)

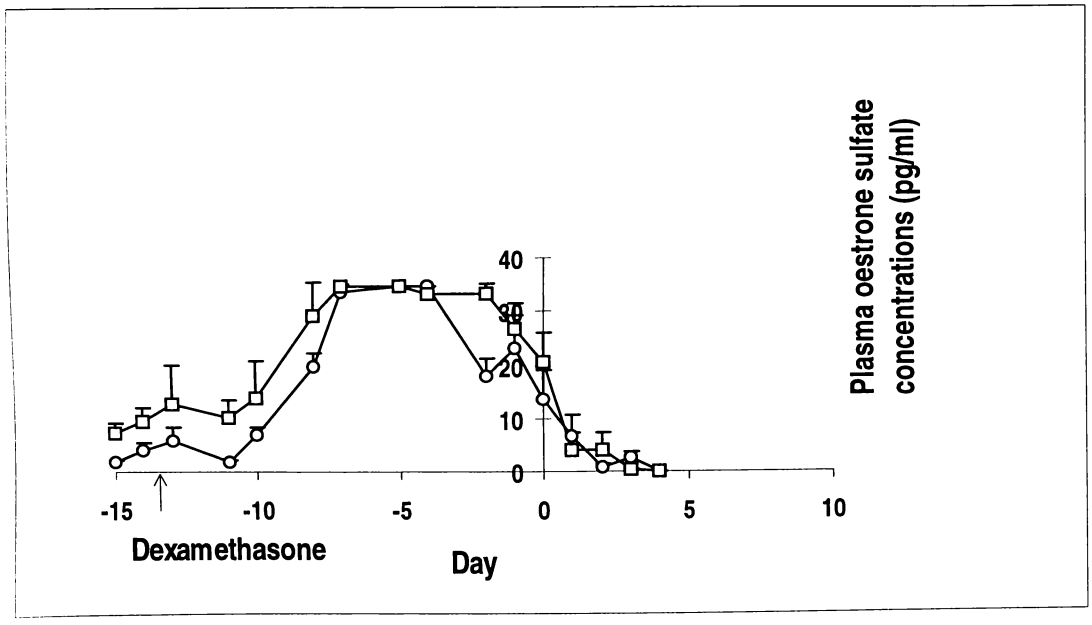


Figure 11.4: Plasma oestrone sulfate concentrations (pg/ml) in samples from pregnant and induced cows during the periparturient period.

Day 0 is day of parturition.
Error bars represent sem.
Legend: - O - Group E (calved at <250 days gestation)
- □ - Group L (calved at ≥250 days gestation)

Plasma prolactin concentrations showed great variability at the beginning of the sampling period. They demonstrated a periparturient peak before declining to previous levels (Table 11.4, Fig. 11.5). Plasma α-lac concentrations demonstrated a peak on the day of parturition (Table 11.5, Fig. 11.6) There was a steady decline in plasma IGG1 concentrations during the sampling period until parturition, after which time levels increased slightly (Table 11.6, Fig. 11.7). after a steady increase from low levels during the last week of gestation. They declined over 2 days following parturition, but remained higher than those levels seen at the start of the sampling period.

ANOVA:

Plasma concentrations of all the hormones and proteins measured were influenced by day prepartum ($P<0.001$). Examination of the data for Groups E and L from day -13 to day 2 showed no effects associated with stage of gestation at parturition for any hormone or protein measured. There was a significant interaction ($P<0.01$) between day of sampling and stage of gestation at parturition for plasma IGG1 concentrations. They were higher at the time of dexamethasone treatment then declined at a faster rate in Group E than in Group L (Table 11.6, Fig. 11.7). Plasma α-lac concentrations also showed a similar significant interaction ($P<0.05$) due to a tendency for plasma α-lac concentrations to increase earlier in Group L than in Group E (Table 11.5, Fig. 11.6).

Day	Group E	Group L	Control
-13	47.8 (14.0)	37.7 (11.3)	-
-7	14.7 (1.9)	29.2 (9.4)	58.1 (26.2)
-5	30.7 (11.3)	43.0 (14.3)	55.6 (22.5)
-2	35.7 (10.3)	90.0 (27.2)	111.2 (19.6)
0	121.7 (13.3)	87.5 (13.0)	90.6 (20.9)
2	51.1 (10.1)	51.7 (12.7)	65.0 (15.7)

Table 11.4: Plasma prolactin (ng/ml) concentrations [mean (± sem)] on selected days in relation to parturition on day 0.

Group E (n = 8) calved before 250 days of gestation; Group L (n = 10) calved on or after 250 days of gestation; control cows (n = 5) calved spontaneously.

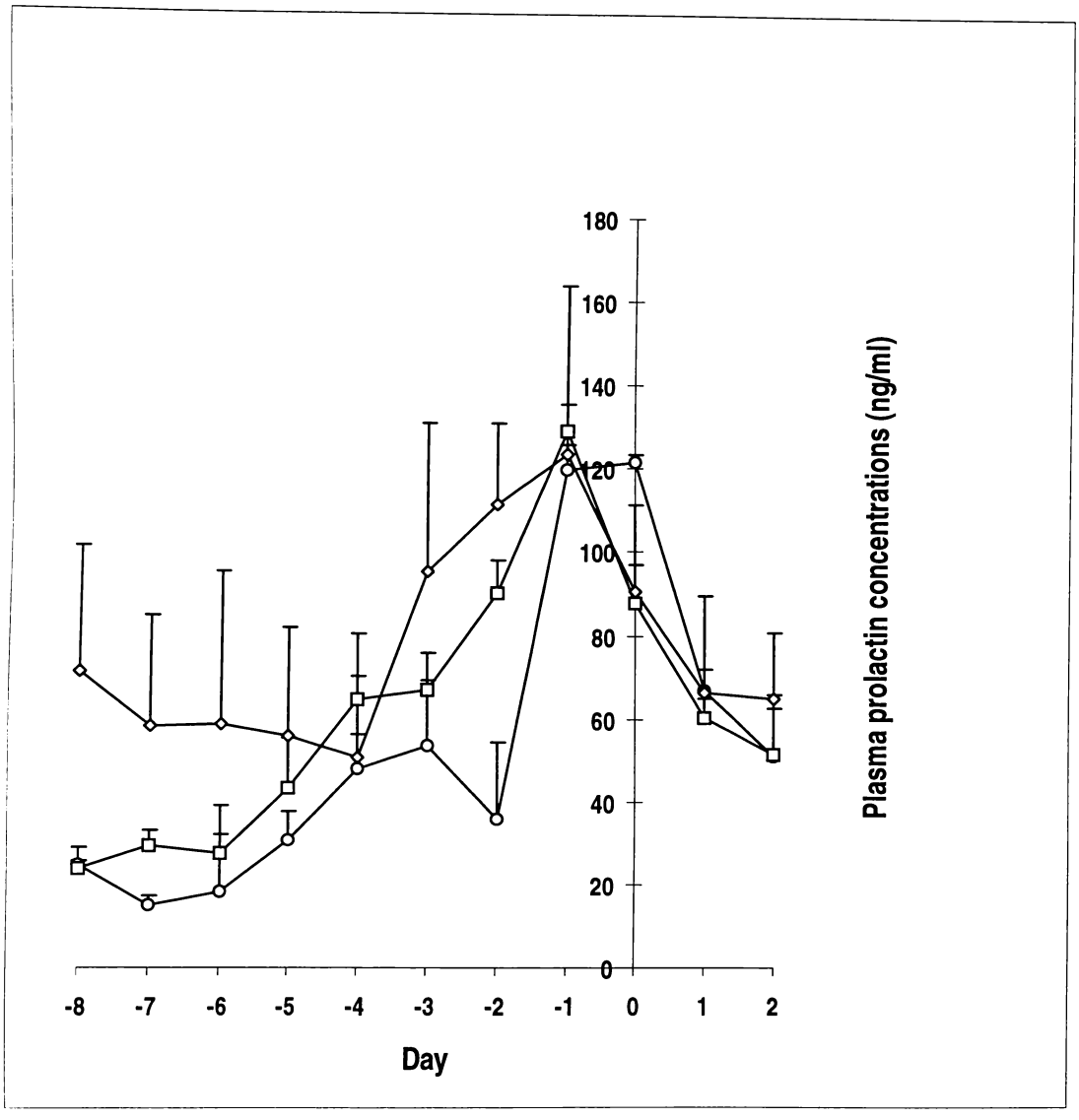


Figure 11.5: Plasma prolactin concentrations (ng/ml) in samples from pregnant and induced cows during the periparturient period.

Day 0 is day of parturition.
Error bars represent sem.
Legend: - O - Group E (calved at <250 days gestation)
- □ - Group L (calved at ≥250 days gestation)
- ♦ - Control group (calved spontaneously)

Day	Group E	Group L	Control
-13	13.1 (3.4)	29.8 (6.7)	-
-7	13.8 (4.3)	59.4 (12.5)	147.8 (50.4)
-5	27.7 (4.7)	172.6 (40.4)	219.7 (43.9)
-2	125.5 (15.9)	259.5 (54.3)	249.5 (40.3)
0	754.2 (279.7)	852.0 (129.3)	697.6 (128.5)
2	268.2 (71.4)	380.1 (115.0)	409.6 (98.1)

Table 11.5: Plasma α-lactalbumin (ng/ml) concentrations [mean (± sem)] on selected days of in relation to parturition on day 0.

Group E (n = 8) calved before 250 days of gestation; Group L (n = 10) calved on or after 250 days of gestation; control cows (n = 5) calved spontaneously..

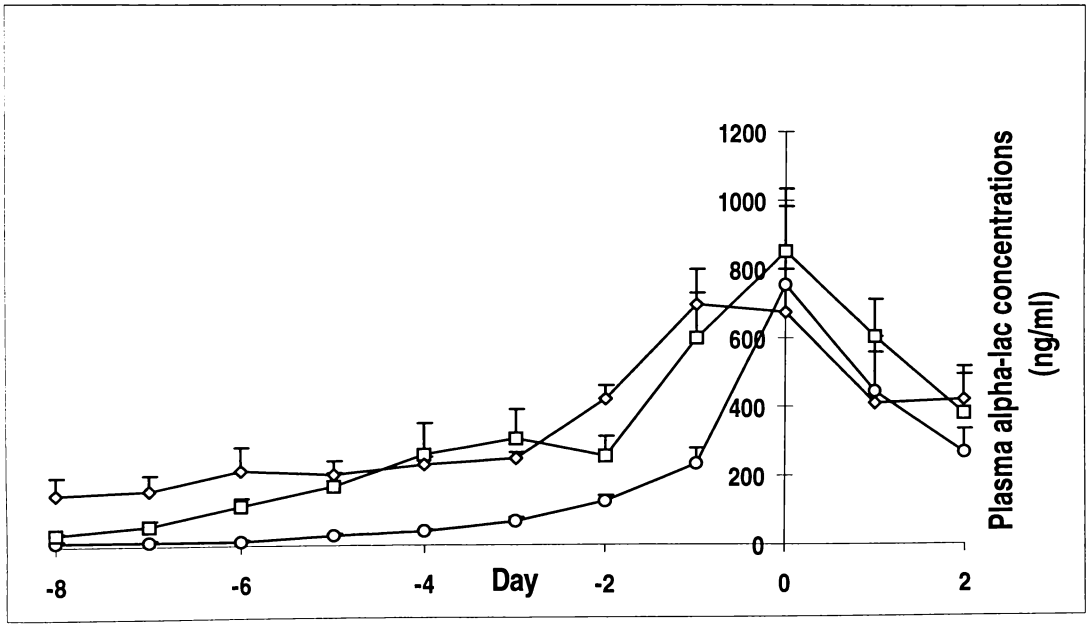


Figure 11.6: Plasma α-lactalbumin concentrations (alpha-lac; ng/ml) in samples from pregnant and induced cows during the periparturient period.

Day 0 is day of parturition.
Error bars represent sem.

Legend:
- O - Group E (calved at <250 days gestation)
- □ - Group L (calved at ≥250 days gestation)
- ◆ - Control group (calved spontaneously)

Analyses of the data set which comprised the plasma concentrations for all cows from day -7 to day 2 revealed a significant effect of stage of gestation at parturition ($P<0.03$) for α-lac (Table 11.5, Fig. 11.6). Plasma concentrations of α-lac tended to be lower among cows in Group E and only increased rapidly in the last 3 days before parturition. There was a significant interaction ($P<0.001$) between stage of gestation and day of sampling for IGG1 (Table 11.6, Fig. 11.7). This was due to the differences between Groups E and L as outlined above, while Group L was similar to the control group. Plasma IGG1 concentrations in the control cows were similar to those of Group L during the period examined.

Day	Group E	Group L	Control
-13	15.7 (1.0)	7.8 (1.3)	-
-7	13.0 (2.1)	6.4 (0.6)	5.1 (1.5)
-5	11.7 (2.0)	5.8 (0.7)	3.8 (1.2)
-2	8.0 (0.8)	4.1 (0.4)	3.2 (0.9)
0	6.3 (0.8)	3.6 (0.4)	4.8 (2.0)
2	4.8 (0.8)	5.0 (0.6)	6.7 (2.0)

Table 11.6: Plasma immunoglobulin G1 (mg/ml) concentrations [mean ($\pm$ sem)] on selected days of in relation to parturition on day 0.

Group E (n = 8) calved before 250 days of gestation; Group L (n = 10) calved on or after 250 days of gestation; control cows (n = 5) calved spontaneously.

Correlations between plasma hormone and protein concentrations:

There were occasional significant correlations between plasma hormone and protein concentrations when relationships were examined on a day by day basis, but these failed to demonstrate any consistent pattern. When data for all days were examined, the only significant relationship ($P<0.03$) was between progesterone and oestrone sulfate but it was not a close association ($r = 0.12$).

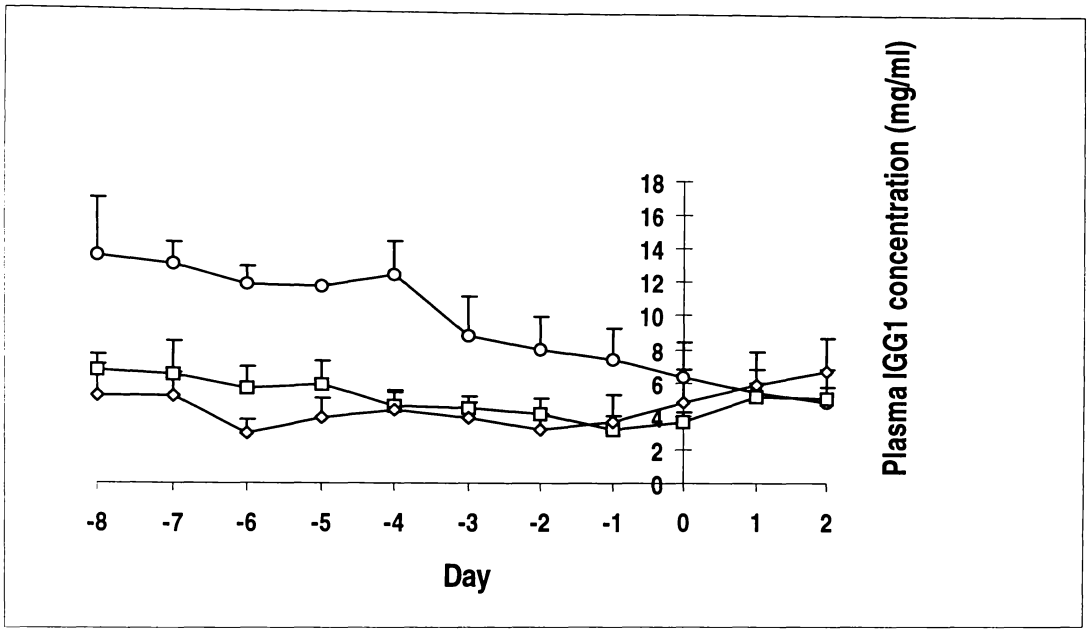


Figure 11.7: Plasma immunoglobulin G1 concentrations (IGG1; mg/ml) in samples from pregnant and induced cows during the periparturient period.

Day 0 is day of parturition.
Error bars represent sem.

Legend:
- O - Group E (calved at <250 days gestation)
- □ - Group L (calved at ≥250 days gestation)
- ◆ - Control group (calved spontaneously)

Oestrone sulfate:progesterone ratios:

The ratio of [OS:P4] increased exponentially as parturition approached. Means for selected days are represented in Table 11.7. These data showed a significant effect (P<0.001) associated with both treatment group and day of sampling. The ratio tended to increase as parturition approached, and was lower in Group E than in Group L.

There was a significant relationship between $\log_e$ [OS:P4] and day prepartum as described by the regression equation:

$\log_e$ [OS:P4]	=	3.54 +	0.293 day
se		± 0.15	± 0.016
P		<0.001	<0.001
	r = 0.77	P(regression)	<0.001

This relationship was not influenced by individuals which had experienced abnormal deliveries.

Day	[OS:P4]		$\log_e$ [OS:P4]	
	Group E	Group L	Group E	Group L
-13	0.9 (0.2)	2.4 (1.4)	-0.2 (0.2)	0.3 (0.5)
-7	6.7 (0.6)	9.3 (1.4)	1.9 (0.1)	2.2 (0.2)
-5	5.4 (1.2)	8.3 (0.9)	1.6 (0.2)	2.1 (0.1)
-2	15.0 (5.2)	27.1 (5.7)	2.5 (0.5)	3.2 (0.2)
-1	22.7 (3.7)	34.8 (7.9)	3.0 (0.2)	3.4 (0.3)

Table 11.7: Ratios of plasma concentrations of oestrone sulfate and progesterone ([OS:P4]) on selected days in relation to parturition on day 0.

(n = 4) for each group.

11.4.3 Intervals from calving to first ovulation

Mean time from calving to first ovulation was 25.1 ± 1.8 days (range: 14 to 40 days). This is within the usual range of time seen in dairy cows in herds in New Zealand (McDougall, 1994). There was no significant effect of stage of gestation at calving on time from calving to first ovulation.

11.5 Discussion

11.5.1 Adrenal response tests

At ART 1, all cows were within the normal range for both 50 min and 120 min PCC, and for AUC120. Adrenocortical responses to ACTH fell during the third trimester by a small, yet significant, amount in a similar manner to that observed previously (Chapter 9). Following dexamethasone treatment, adrenocortical responses to ACTH were suppressed. This was apparent by 6 days after the treatment at ART 3 (approximately 5 days before parturition), and also at ART 4 (immediately after calving). Suppression of responses was less prolonged in the cows which had been pregnant as they showed partial recovery of the integrated response

variable AUC120 at ART 5 (32 days after dexamethasone treatment). All response variables were lower at ART 5 than at ART 2 in the non-pregnant cows. This suggests that mechanisms contributing trophic drive to the adrenal cortex may be enhanced in early lactation. This may be due either to more rapid clearance of the dexamethasone because of the higher general metabolic activity of early lactation, or due to greater trophic drive via the pituitary gland from increased plasma concentrations of the pituitary secretagogues arginine vasopressin (AVP) or oxytocin during early lactation. The former has been shown to increase plasma ACTH concentrations and PCC following iv administration to cows (Carroll *et al.*, 1993; Jacob and Minton, 1993).

During recovery from adrenocortical suppression following dexamethasone treatment the response variable of 120 min PCC was the last to return to pretreatment levels for both groups. This is similar to the pattern of recovery seen in non-pregnant animals (Chapter 10). This may reflect the manner in which adrenocortical function is restored following dexamethasone treatment which reduced trophic drive from the pituitary.

11.5.2 Plasma indicators of lactogenesis and colostrogenesis

The pattern of changes in the various hormones and proteins measured as indicators of colostrogenesis and lactogenesis showed that the processes occurring were similar in both induced and full-term cows. Only plasma α-lac and IGG1 concentrations showed differences between treatment groups in the onset of the changes. The increases in plasma α-lac concentrations as parturition approached were delayed, and plasma IGG1 concentrations declined at a greater rate in the cows calving at less than 250 days of gestation than in the other cows. The milk serum protein α-lac comprises up to 4% of total protein in milk. It participates in lactose synthesis by creating a binding site for β-glucose, making it available to the enzyme galactosyl transferase. This enzyme is bound to the inner surface of the Golgi membranes in mammary cells. During lactation, α-lac is transported through the Golgi complex *en route* for secretion at the apical membrane, thus allowing it to exert its effect on lactose synthesis (Mepham, 1987). During the first stage of lactogenesis, the enzymes needed for synthesis of milk components appear in the cell and are secreted through the apical membrane. Some of these components may leak back from the alveoli into the circulation,

since tight junctions between mammary cells are not fully formed. As a result, measurements of the α-lac concentration in blood can provide information on the stage of mammary development (McFadden *et al.*, 1987). Comparison of plasma α-lac concentrations between the treatment groups suggest that the processes of mammary development were less advanced in the cows calving before 250 days of gestation than in the other cows.

Circulating levels of IGG1 decrease in association with colostrogenesis. In spontaneously calving dairy cows, this decrease begins at about 21 days prepartum and leads to a marked depletion of plasma IGG1 (Guy *et al.*, 1994). This pattern of transfer may have already begun in the cows in both Group L and the control group, as indicated by their lower plasma IGG1 concentrations at the beginning of the sampling period. The greater rate of decline of plasma IGG1 concentrations in Group E as compared to the other treatment groups implies an accelerated movement of IGG1 to the mammary glands of these cows in an effort to provide colostrum at birth.

Progesterone suppresses lactogenesis during advanced pregnancy and colostrum formation has been associated with the gradual decline in plasma progesterone concentrations as parturition is approached (Guy *et al.*, 1994). The failure to show significant relationships between plasma concentrations of the three hormones and the two protein indicators of lactogenesis and colostrogenesis implies that the mechanisms which initiate mammary secretion are complex.

11.5.3 Relationship between progesterone and oestrone sulfate in the periparturient period

The pattern of changes in plasma concentrations of progesterone and oestrone sulfate in induced cows are similar to those described in spontaneously calving cows. Plasma progesterone concentrations declined from about day 250 to day 282 of gestation, then fell sharply before delivery. Plasma oestrone sulfate concentrations increased gradually from day 230 to 250 of gestation, then rose rapidly (Fuchs *et al.*, 1992a). These patterns also occurred in the induced cows following dexamethasone treatment, but in an accelerated manner. Glucocorticoids alter circulating progesterone and oestrogen levels by direct effects on

placental tissue. Placental 17α-hydroxysteroid dehydrogenase and 17, 20 lyase activities increase following foetal glucocorticoid infusion in sheep. This results in an increased rate of conversion of progesterone to oestrogens, so that a decline in circulating progesterone levels occur simultaneously with a rise in oestrogen levels. In the goat, placental exposure to glucocorticoids results in increased levels of enzymes needed for the conversion of pregnenalone to oestrogens rather than increased 17, 20 lyase activity. These relationships are not well defined in the cow, but it is likely that some parallels exist with both sheep and goats (Flint *et al.*, 1979).

The molar ratio of oestrone and oestradiol-17β to progesterone has been correlated with oxytocin receptor concentrations in the endometrium and the foetal cotyledon during the final 7 to 10 days of pregnancy. Increases in concentrations of these receptors may be a key controlling factor for the release of prostaglandins E and $F_{2\alpha}$ during the initiation of parturition (Fuchs *et al.*, 1992 a and b). Inadequate PG production may be a factor in RFM. Cows which had RFM had a lower plasma PGFM concentration at parturition than cows which did not retain placentae (Chassagne and Barnouin, 1992).

Retained foetal membranes are an undesirable sequel to induction of parturition. They occur in 12-26% of induced cows, depending upon the treatment regime used (Welch *et al.*, 1977). The cows which showed uterine inertia, poor cervical dilation and/or RFM did not have different [OS:P4] as compared to the other cows. The calculations used may not be comparable with those of Fuchs *et al.* (1992 a and b) because the assay for oestrone sulfate had a maximum range of 35 pg/ml. Substantially higher oestrone sulfate concentrations probably occurred than were used in the calculations. Even given this limitation, the Group E cows demonstrated a lower [OS:P4] than Group L cows. This difference may be related to the immaturity of the placenta at the time of induction.

11.6 CONCLUSIONS

Adrenocortical responses to ACTH were suppressed following dexamethasone treatment to induce parturition in dairy cows. This suppression was profound during parturition and for up to 3 weeks post-partum. Evidence of suppressed adrenocortical function (reduced PCC at 120

min following administration of ACTH) was still present at 50 days post-partum. Induced cows may recover from adrenocortical suppression faster than non-pregnant non-lactating cows following similar dexamethasone treatment.

Changes in the selected hormones and proteins measured in the periparturient period were similar in both induced and spontaneously calving cows. A decline in plasma progesterone concentrations and simultaneous increase in plasma oestrone sulfate concentrations occurred soon after dexamethasone injection. Blood protein markers of colostrogenesis and lactogenesis, and plasma prolactin concentrations showed similar temporal relationships around parturition to those in spontaneously calving cows. The ratio between plasma levels of oestrone sulfate and progesterone demonstrated an exponential increase as time of parturition approached. Plasma concentrations of the hormones and proteins measured differed between cows which calved before 250 days of gestation and those which calved at a later stage of gestation by a greater lag time from injection of dexamethasone to an increase in plasma α-lac levels; a faster rate of decline of plasma IGG1 concentrations; and, a slower increase in the ratio between plasma levels of oestrone sulfate and progesterone.

CHAPTER 12

RESPONSES OF THE PITUITARY GLAND AND THE ADRENAL CORTEX TO CORTICOTROPIN-RELEASING HORMONE OR BACTERIAL ENDOTOXIN

This chapter describes two experiments involving stimulation of the hypothalamo-pituitary-adrenal (HPA) axis. For the first, plasma adrenocorticotropic hormone (ACTH) and cortisol concentrations (PCC) were measured following injection of bovine corticotropin-releasing hormone (bCRH); for the second, PCC and vital signs were measured following injection of a bacterial endotoxin.

CHAPTER OUTLINE

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12.2 Introduction
12.3 Experiment 12.1 - Responses to bovine corticotropin-releasing hormone in cows
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  12.3.2 Results
  12.3.3 Discussion
12.4 Experiment 12.2 - Responses to bacterial endotoxin in cows
  12.4.1 Materials and Methods
  12.4.2 Results
  12.4.3 Discussion
12.5 Conclusions

12.1 ABSTRACT

Responses by the hypothalamo-pituitary-adrenal (HPA) axis to injection of bovine corticotropin-releasing hormone (bCRH) or non-specific stimulation by a bacterial endotoxin were measured in adult dairy cows.

Expt. 12.1: Holstein-Friesian (HF) cows were treated by intravenous (iv) injection with a graded range of doses (6.25 to 25 μg per animal) of synthetic bCRH. Plasma concentrations of adrenocorticotropic hormone and cortisol (PAC and PCC, respectively) were measured. Time to peak PCC but not time to peak PAC was influenced by dose. PAC at 15 min and PCC at 30 min were influenced by dose of bCRH ($P<0.05$ for PAC, and $P<0.001$ for PCC). as were the integrated areas under the PAC.time curve (aAUC90; $P<0.001$). The areas under the PCC.time curve (cAUC90) were similar for each dose of bCRH. Regression analyses identified a linear relationship between aAUC90 and dose of bCRH, and a linear relationship between cAUC90 and $\log_{10}$ PAC at 15 min after treatment. Individual cows had a significant effect on these relationships. Measurement of PAC and PCC in plasma samples taken at 15 min intervals for 1 h following iv injection of a dose of 6.25 μg bCRH, is suggested as a test of pituitary-adrenal function in cattle.

Expt. 12.2: Vital signs and PCC were measured following iv injection of HF cows with a range of doses (3.125 to 12.5 μg per animal) of lipopolysaccharide of bacterial origin (LPS). Responses to LPS were highly variable between individuals and between dose occasions, but dose had a significant ($P<0.005$) effect on area under the PCC.time curve. Respiratory and heart rates altered following LPS treatment, with changes being more severe during earlier treatments. Rectal temperatures increased following treatment in a manner which varied by dose ($P<0.01$) and by cow ($P<0.05$). Changes in PCC following administration of LPS were not repeatable over time. Injection of LPS is not a suitable stressor for use on a repetitive basis in tests of HPA axis integrity.

12.2 INTRODUCTION

Measurement of PCC following administration of ACTH can be used to assess adrenocortical function, but provides no direct information about integrity of higher levels of the HPA axis. Altered adrenocortical responses to ACTH occur due to changes initiated by the adrenal cortex, or due to alteration in trophic drive from higher levels of the axis. Challenge with a pituitary secretagogue, typically corticotropin-releasing hormone (CRH), with measurement of plasma ACTH concentrations (PAC) and PCC, has been used to assess pituitary integrity (Schürmeyer *et al.*, 1985). Measurement of PAC and PCC following stimulation of the HPA

axis by a non-specific stressor, e.g., insulin-induced hypoglycaemia, has been demonstrated to provide information regarding the integrity of the axis as a whole (Schlaghecke *et al.*, 1992).

To understand the relationships between the pituitary and the adrenal cortex of cattle, it is necessary to develop techniques which can assess both integrity of pituitary-adrenal function and the ability of the HPA axis to respond to stimulation. These techniques would facilitate further investigation of physiological changes in adrenocortical function as have been described in previous chapters. The aims of Expt. 12.1 were:

- to examine the response of the pituitary gland and the adrenal cortex of dairy cows following intravenous (iv) injection of a range of doses of bCRH; and
- to determine an appropriate dose rate and sampling regime for bCRH challenge of the HPA axis of cattle.

The aim of Expt. 12.2 was to investigate the effect of iv injection of a lipopolysaccharide endotoxin (LPS) of bacterial origin by measurement of PCC and rectal temperatures and observation of vital signs. Although the original aim also included measurement of PAC, samples were not assayed for this when PCC were shown to vary inconsistently.

12.3 Experiment 12.1 - Responses to bovine Corticotropin-releasing Hormone in cows

12.3.1 Materials and Methods

Experimental subjects and treatment regime:

Four parous Holstein-Friesian (HF) cows aged 3 to 6 years, with a mean live-weight of 479 kg (range: 446 to 534 kg) were first trained to accept the experimental procedures. The cows were not lactating at the time of the experiment. Routine haematology and screening for liver function showed that the cows were clinically healthy during the experiment. During the training period cows were subjected to adrenal response testing. Results of these tests fell within the range established for similar cows (Chapter 5).

The cows' oestrous cycles were synchronised with progesterone-releasing CIDR™ devices (Eazi-Breed CIDR-B, InterAg Pty. Ltd., Hamilton, NZ) and prostaglandin $F_{2\alpha}$ (Lutalyse,

Upjohn; Macmillan and Peterson, 1993). Each animal was between day 5-17 of its oestrous cycle on any treatment day. An indwelling catheter was inserted into a jugular vein of each cow at least 24 h before treatment. The catheters were used for blood sample collection and for bolus injection of treatments. On each day of sampling, cows were restrained indoors in a head bail and were fed hay *ad lib.* Water was offered regularly.

Synthetic bCRH (Sigma Product No. C2671, Sigma Chemical Co., St. Louis, Missouri) was dissolved in physiological saline to a concentration of 12.5 μg/ml then measured into aliquots which were frozen at -20°C until required. Aliquots were thawed immediately before treatment and diluted as necessary to give doses of 6.25, 12.5 and 25 μg bCRH in a total volume of 2 ml physiological saline.

Sampling always began at 0830 h. Samples were taken at -30, 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 120, and 150 min, with administration of the treatment following the 0 min sample. Treatments were assigned randomly in a replicated four by four Latin square design. Each treatment, *viz.,* physiological saline (2 ml), 6.25, 12.5 or 25 μg bCRH was administered twice to each cow. The interval between treatments was at least 48 h.

At each sampling, 10 ml of blood was placed in each of 2 vacutainers containing either lithium heparin or sodium EDTA, and held in iced water. They were centrifuged (2500 rpm for 20 min at RT°C) within 2 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol and ACTH. Details of assays are reported in Chapter 4.

Statistical analyses:

Results are reported as mean ± sem. Since all cows had returned to pretreatment PAC and PCC by 90 min after treatment, this was used as the end-point for calculation of response variables. Peak PCC and PAC, and their respective times to peak (Ttp) were estimated for each cow at each dose and dose occasion using a computer-aided curve-fit programme (Kaleidagraph, Synergy Software, Reading, Pennsylvania). Since peak PAC and PCC occurred close to 15 and 30 min, respectively, after treatment, the plasma concentrations at these times were analysed as response variables. The integrated responses (areas under the PCC.time and PAC.time curves) for each cow at each treatment were calculated by integration

using full data from 0 to 90 min following administration of bCRH (cAUC90 and aAUC90, respectively).

The response variables of 15 min PAC, 30 min PCC, cAUC90 and aAUC90 as well as peaks and Ttp for both PCC and PAC were examined by analyses of variance (ANOVA) using a General Linear Model (GLM) with Minitab (version 8.2; Minitab Statistical Software, State College, Pennsylvania). These analyses examined for effects associated with dose, cow and occasion of treatment (i.e., 1st or 2nd), and for dose by cow interactions. The ratios of aAUC90 to cAUC90 for each cow at each treatment were calculated, and these data were examined by ANOVA using a GLM for effects associated with cow and dose, and for cow by dose interactions.

Data for all cows on all dose occasions were subjected to regression analyses using Minitab (version 8.2) to determine prediction equations of best fit. These analyses identified the statistical significance of for linear relationships to dose, $\log_{10}$ dose and individual cow, as well as relationships between the ACTH and cortisol response variables.

12.3.2 Results

Data were discarded from one cow (Cow 6305) at the first dose of 25 μg because PCC at -30 and 0 min were in excess of 45 ng/ml. There was no apparent reason for this, but the elevated PCC may have activated negative feedback mechanisms and interfered with the subsequent response to bCRH.

Saline control:

Mean PCC and PAC at 0 min did not differ statistically from those at -30 min on any other following administration of saline. Mean 0 min PCC and PAC for saline and for each dose of bCRH did not vary statistically (Table 12.1). Overall mean PAC and PCC for saline treated animals were 11.1 ± 1.5 ng/ml and 8.9 ± 2.0 pg/ml, respectively.

Dose bCRH	PAC 0min	PCC 0min	PAC peak	PCC peak	Time to PAC peak	Time to PCC peak *
0 (saline)	11.1 (1.5)	8.9 (2.0)	-	-	-	-
6.25 μg	13.2 (2.0)	13.0 (2.8)	115.1 (23.3)	45.3 (2.5)	15 (1)	24 (2)
12.5 μg	17.3 (5.0)	9.7 (2.5)	150.6 (26.3)	56.7 (6.2)	14 (1)	29 (2)
25 μg	9.7 (1.2)	8.0 (1.4)	218.9 (48.8)	56.9 (3.6)	17 (1)	36 (3)

Table 12.1: Mean plasma concentrations of cortisol (PCC; ng/ml) and ACTH (PAC; pg/ml) at 0 min and peak, and times to peaks (Ttp; min) following administration of a graded range of doses of bCRH (μg/500 kg) to cows [mean (sem)].

* represents significant differences within columns between doses of bCRH (not saline; $P<0.05$).
n = 8 for each value based on duplicated treatments in each of 4 cows.

Peak hormone concentrations and times to peak:

The mean PAC.time and PCC.time curves for each dose of bCRH are shown in Figures 12.1 and 12.2, respectively. Each dose produced a significant increase ($P<0.05$) in PAC and PCC. After the dose of 6.25 μg, PAC was different ($P<0.05$) from 0 min concentrations from 10 to 30 min post-treatment. PCC also varied ($P<0.05$), but from 10 to 40 min following treatment with 6.25 μg bCRH. With the dose of 12.5 μg bCRH, mean PAC and PCC were greater ($P<0.05$) from 10 min post-treatment until 30 and 70 min post-treatment, respectively. They also varied following the dose of 25 μg from 5 to 90 min for PAC, and from 10 to 100 min for PCC ($P<0.05$).

Peak hormone concentrations and Ttp are given in Table 12.1. Although peak PAC showed a tendency to increase with increasing dose, the changes were not statistically significant; nor did Ttp for PAC differ significantly between doses of bCRH. Peak PCC did not vary statistically with dose of bCRH administered, but Ttp for PCC showed a significant effect associated with dose ($P<0.03$).

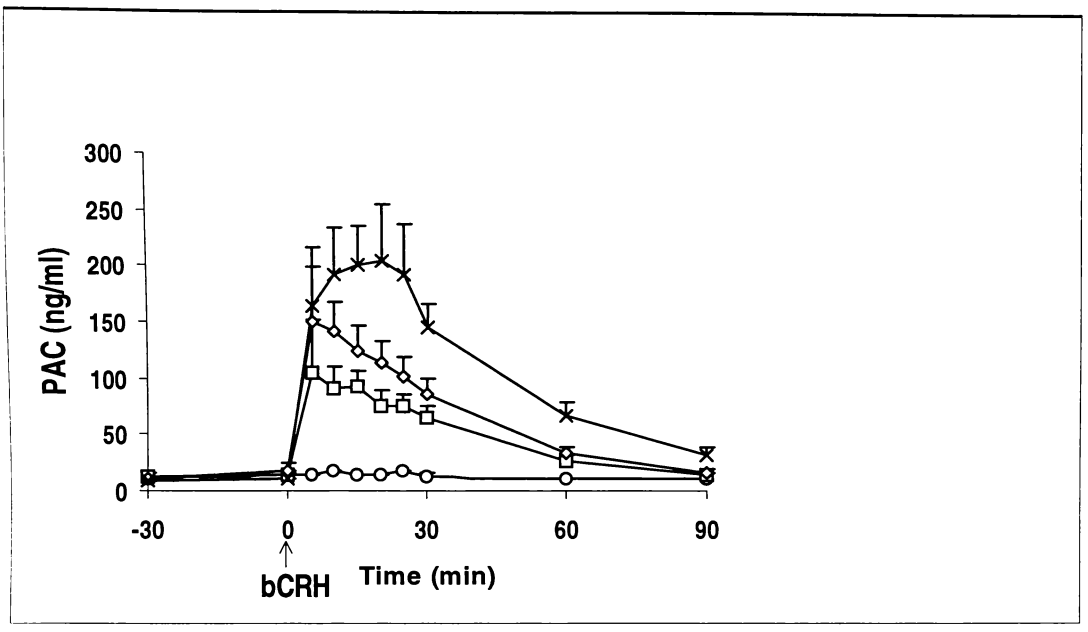


Figure 12.1: Mean plasma ACTH concentrations (PAC; pg/ml) following administration of a graded range of doses of bCRH to cows.

Legend: - O - saline - □ - 6.25 µg bCRH
-◆- 12.5 µg bCRH - x - 25 µg bCRH

n = 8 for each value based on duplicated treatments in each of 4 cows.

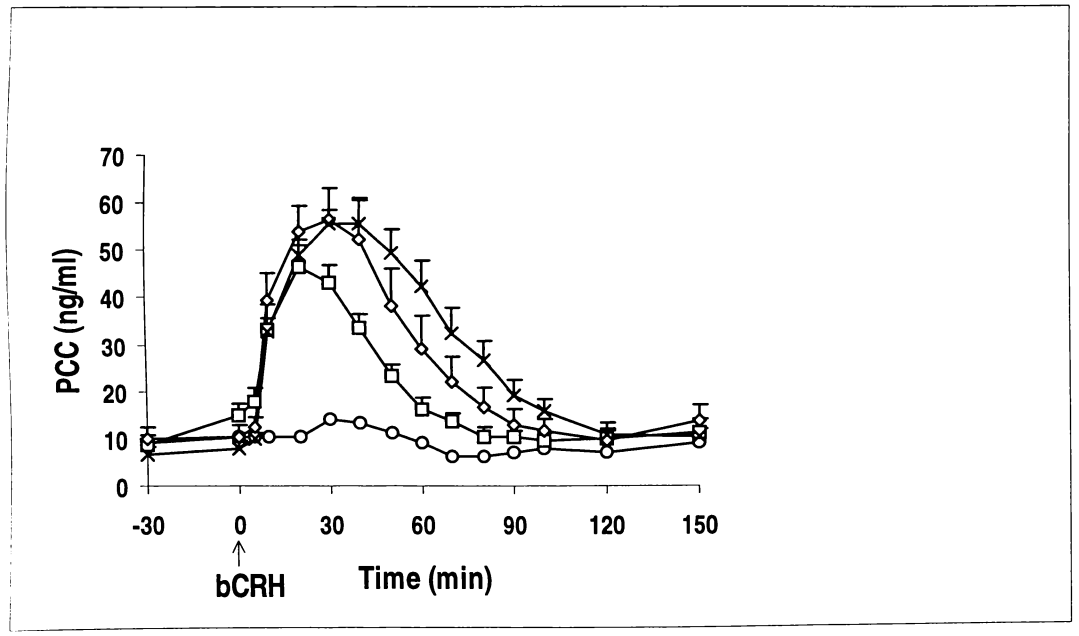


Figure 12.2: Mean plasma cortisol concentrations (PCC; ng/ml) following administration of a graded range of doses of bCRH to cows.

Legend: - O - saline - □ - 6.25 µg bCRH
-◆- 12.5 µg bCRH - x - 25 µg bCRH

n = 8 for each value based on duplicated treatments in each of 4 cows.

PAC at 15 min and PCC at 30 min:

There were significant effects associated with both dose (P<0.001) and cow (P<0.05) on 15 min PAC (Table 12.2). All doses of bCRH treatment increased PAC compared to saline, with responses to 25 μg being greater (P<0.01) than responses to 6.25 μg. There were also significant effects associated with both dose (P<0.001) and cow (P<0.03) on 30 min PCC (Table 12.2). By 30 min following treatment with 6.25 μg bCRH, PCC were greater than following saline; but less than that following the two higher doses for which 30 min PCC were similar.

Dose bCRH	PAC 15 min **	PCC 30 min **	aAUC90 **	cAUC90
0 (saline)	12.7 (2.7)	14.1 (2.3)	1050 (127)	960 (217)
6.25 μg	103.1 (16.5)	41.6 (3.5)	4676 (690)	2303 (147)
12.5 μg	130.4 (21.6)	57.1 (5.3)	6279 (974)	3149 (419)
25 μg	200.5 (35.8)	55.3 (3.2)	9807 (1817)	3505 (304)

Table 12.2: Plasma ACTH concentrations (PAC; pg/ml) at 15 min, plasma cortisol concentrations (PCC; ng/ml) at 30 min and areas under the PAC.time and PCC.time curves over 90 min (aAUC90; pg.min/ml and cAUC90; ng.min/ml, respectively), following administration of a graded range of doses of bCRH (μg/500 kg) to cows [mean (sem)].

* and ** represent significant differences within columns between doses of bCRH (not saline; P<0.05 and P<0.01, respectively).
n = 8 for each value based on duplicated treatments in each of 4 cows.

Integrated response variables:

Areas under the PAC.time and PCC.time curves (Table 12.2) showed significant differences associated with dose (P<0.001) and cow (P<0.03 for aAUC90; P<0.02 for cAUC90). Mean aAUC90 following administration of 6.25 μg bCRH was greater (P<0.01) than following saline, but less (P<0.05) than following the two higher doses of bCRH for which mean aAUC90 were similar. Mean cAUC90 were similar following each of the 3 doses of bCRH, but greater than that following saline. There were no significant effects associated with dose occasion or any cow by dose interactions for any of the response variables examined.

Ratio cAUC90:aAUC90:

Mean ratios ($x10^3$) of cAUC90 to aAUC90 for the four cows were 0.33 ± 0.05, 0.54 ± 0.09, 1.22 ± 0.10 and 0.37 ± 0.03. These mean ratios showed significant effects associated with individual cow (Cow 160; P<0.001) but not with dose, and no cow by dose interactions were detected.

Regression analyses:

There was a significant (P<0.001) relationship between aAUC90 and dose of bCRH (Fig. 12.3). One cow had significant effects on this relationship described by:

aAUC90	=	2437	+	329 dose	-	2988 (Cow 160)
se		± 774		± 52		± 1182
P		<0.01		<0.001		<0.02

r = 0.65 P(regression)<0.001

The equivalent relationship with dose as a logarithmic function was significant (P<0.04) but had r = 0.30.

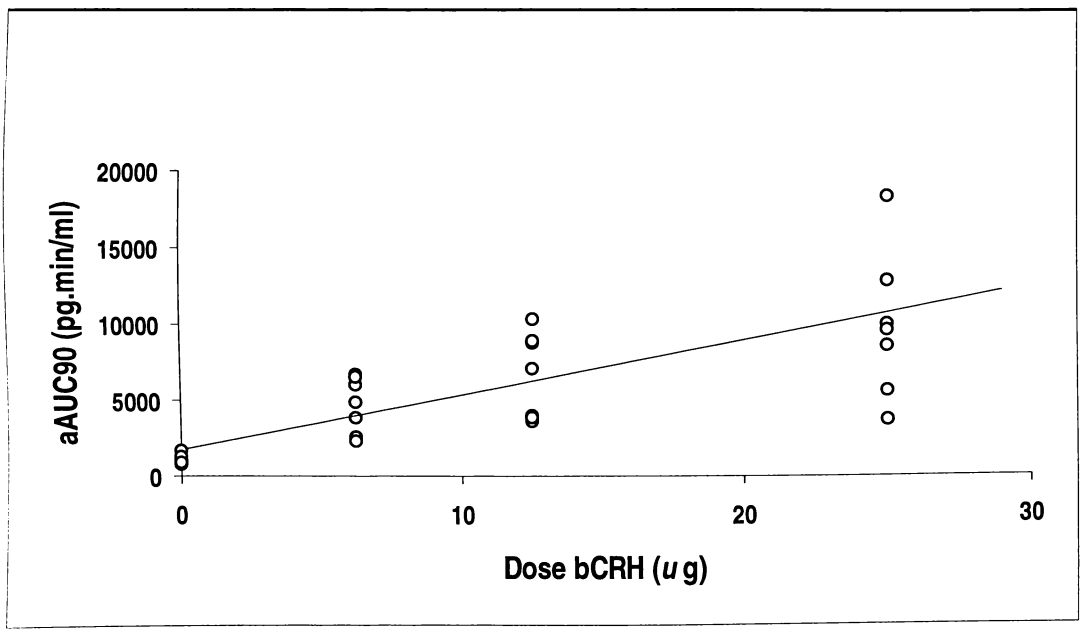


Figure 12.3: Areas under the plasma cortisol concentration.time curve over 90 min (cAUC90; ng.min/ml) plotted against $\log_{10}$ of plasma ACTH concentrations at 15 min (15 min PAC; pg/ml) for each cow at each dose following administration of a graded range of doses of bCRH to cows.

Refer to text for regression equation.

There was also a significant (P<0.001) relationship between cAUC90 and $\log_{10}$ 15 min PAC (Fig. 12.4). Dose of bCRH did not influence this relationship, but one cow again had significant effects. The relationship was described by:

cAUC90	=	- 798	+ 1379 $\log_{10}$(15 min PAC) +	43.2 dose	- 1390 (Cow 160)
se		± 583	± 387	± 21.2	± 315
P		ns	<0.01	< 0.06	<0.001

r = 0.76 P(regression)<0.001

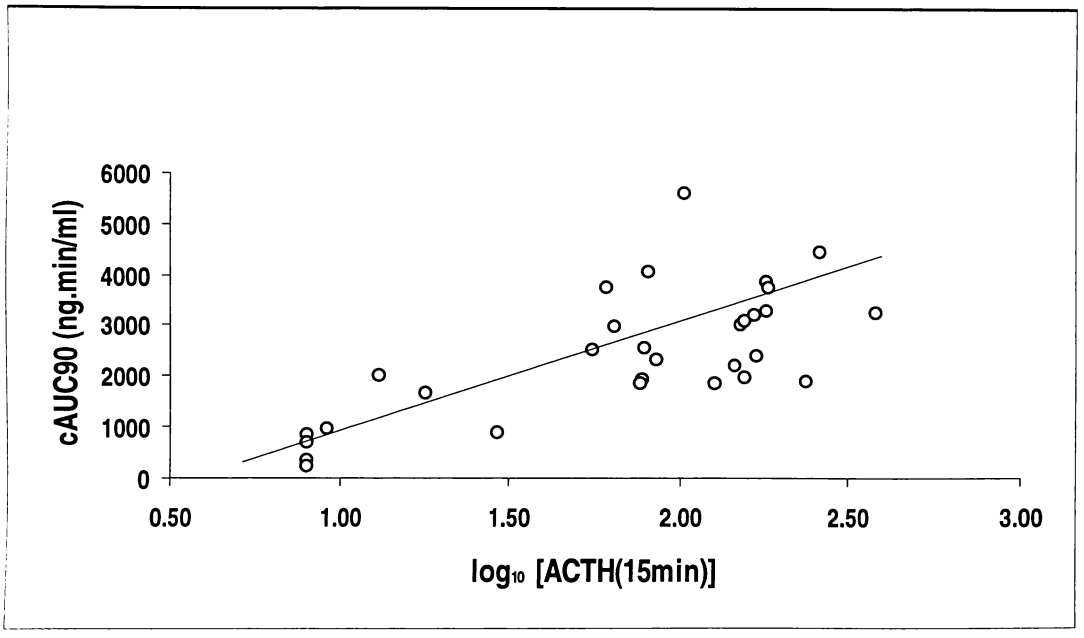


Figure 12.4: Areas under the plasma ACTH concentration.time curve over 90 min (aAUC90; pg.min/ml) following administration of a graded range of doses of bCRH to cows.

Refer to text for regression equation.

12.3.3 Discussion

All doses of bCRH produced a measurable increase in both PAC and PCC. Increases in PAC were of a shorter duration than of PCC. Changes in PCC followed a similar pattern as was seen following administration of ACTH in Experiments 5.1 and 5.2., where increasing doses producing similar peak PCC but increasing integrated responses. These observations are consistent with observations in other species that the integrated adrenocortical response to ACTH is proportional to dose (Keller-Wood *et al.*, 1983).

Mean PCC following 0.0125 mg synthetic $ACTH_{1\text{-}24}$ (Expt. 5.2) were similar to those seen following 12.5 μg bCRH. The response following administration of 6.25 μg bCRH (mean peak

PCC 45.3 ng/ml. Ttp 24 min; Table 12.1), is similar to the responses seen following restraint for commonly practised veterinary procedures. These resulted in three to six fold increases in PCC above basal levels which lasted up to 80 min (Alam and Dobson, 1986). This suggests that the changes in PAC after administration of 6.25 μg bCRH might tend towards the range of physiological responses to restraint stressors for adult dairy cows.

Isolation and characterisation of a corticotropin-releasing factor were first achieved using extracts of ovine hypothalami, and early experiments used ovine CRH to stimulate the pituitary-adrenal axis (Rivier *et al.*, 1982, Orth *et al.*, 1983). Ovine CRH has been suggested to produce prolonged responses in humans as compared to human CRH (Schürmeyer *et al.*, 1984). Ovine CRH administered to heifers at a dose of 0.3 μg/kg live-weight resulted in a peak PCC at 60 min following administration, and elevated PCC until 6 h post-treatment. Elevations in PAC also occurred up to 240 min post-treatment (Welsh *et al.*, 1985). These responses are far in excess of the responses recorded in Expt. 12.1 where the doses were in the range of 0.0125 to 0.5 μg/kg live-weight.

Responses to bCRH administered to cows at doses of 0.3 μg/kg live-weight have been described. Both PAC and PCC were elevated by 10 min following treatment, but actual levels and persistence of the elevations were not reported (Carroll *et al.*, 1993). The mean PAC in samples taken at 10 min intervals for 40 min following administration of a similar dose of bCRH were 242.6 pg/ml, with a corresponding PCC of 90.1 ng/ml (Jacob and Minton, 1993). This response compares favourably with the response to the dose of 25 μg (0.05 μg/kg live-weight) described in Experiment 12.1. Cows treated with 200 μg bCRH produced peak PAC of about 300 pg/ml at 15 min and peak PCC of about 80 ng/ml at 30 min following treatment (Abebe *et al.*, 1993). These reported responses are only marginally greater than responses to 25 μg bCRH in Expt. 12.1 despite the dose being 8 fold greater. This suggests that the range of doses used in Expt. 12.1 may have tended towards the physiological range. This conclusion is further supported by a linear rather than a logarithmic relationship between dose of bCRH (in the range used) and response measured as aAUC90. Extension of the range of doses above 25 μg might allow demonstration of a logarithmic dose response relationship. The lower end of the range probably approached a physiological response, but further testing of lower doses is needed to determine the sensitivity of the bovine pituitary gland to bCRH.

Responses measured by PAC and PCC displayed a constant relationship within individual cows. This is evidenced by the linear relationship between cAUC90 and (log_{10} 15 min PAC), and by the low variability of the ratio of cAUC90:aAUC90. The analyses identified significant effects associated with a particular cow. She was also shown to have a ratio cAUC90:aAUC90 of 1.22 compared to 0.33 to 0.57 for the other cows. This implies that she produced proportionately more cortisol in response to ACTH and suggests that her adrenocortical responsiveness was greater than that of the other 3 cows.

The results of this experiment have demonstrated that iv administration of bCRH will produce a consistent response, and suggests that it may be a useful technique for assessment of pituitary-adrenal function in cows. The dose of 6.25 μg produced a measurable increase in both PAC and PCC. Measurements of these hormones at 15 min intervals for 1 h following CRH administration may be suitable as a clinical test of pituitary-adrenal function.

12.4 Experiment 12.2 - Responses to Bacterial Endotoxin in Cows

12.4.1 Materials and Methods

Experimental subjects and treatment regime:

Four parous HF cows aged 4 to 7 years, with a mean live-weight of 569 kg (range: 543 to 605 kg) had been trained to accept the experimental procedures. The cows had been used previously (Expts. 5.1, 5.2, 7.1, 7.2, 10.1, 10.2 and 10.3) and were not lactating at the time of the experiment. Routine haematology and screening for liver function on three occasions (before, during and after the experiment) showed that the cows were clinically healthy.

The cows' oestrous cycles were synchronised with progesterone-releasing CIDR™ devices (Eazi-breed CIDR-B, InterAg Pty. Ltd., Hamilton, NZ) and prostaglandin $F_{2\alpha}$ (Lutalyse, Upjohn; Macmillan and Peterson, 1993). Each animal was between day 5-17 of its oestrous cycle on any treatment day. An indwelling catheter was inserted into a jugular vein of each cow at least 24 h before treatment. The catheters were used for blood sample collection

and for bolus injection of treatments. On each day of sampling, cows were restrained indoors in a head bail and were fed hay *ad lib.* Water was offered regularly.

A trichloro-acetic acid extract from *E. coli type O55:B5* (Sigma Product No. L4005, Sigma Chemical Co., St. Louis, Missouri). was prepared by dissolving in pyrogen-free Hank's balanced salt solution (HBSS) to a concentration of 12.5 μg/ml. The solution was filtered using an 0.2 μm needle filter and measured into aliquots which were frozen at -20°C until required. Aliquots were thawed immediately before treatment and diluted as necessary to give doses of 3.125, 6.25, and 12.5 μg LPS in a total volume of 2 ml HBSS.

Sampling always began at 0900 h. Samples were taken at -60, -30, and 0 min, with administration of the treatment following the 0 min sample. Thereafter samples were taken at 10 min intervals for 90 min, then at 30 min intervals until 6 h after treatment. Preliminary treatments of 12.5 μg LPS in 2 ml HBSS were given to each of 3 cows, with one further cow receiving HBSS as control, to ascertain whether the dose rates were sufficient to produce a safe but measurable response. Following this, treatments of 3.125, 6.25, and 12.5 μg LPS or control (HBSS) were assigned randomly in a replicated four by four Latin square design. Each treatment, *viz.,* HBSS (2 ml), 3.125, 6.25, or 12.5 μg LPS was administered twice to each cow. The interval between treatments was at least 48 h. Samples were not obtained from one cow (Cow 8720) on one occasion of HBSS treatment due to blockage of the catheter.

Since the pattern of PCC following treatments were highly variable, all cows were treated with a dose of 12.5 μg LPS at 48 h after the final treatments of the replicated Latin square. After a further 6 months had elapsed, all cows were again treated with 12.5 μg LPS. On this occasion treatments and blood-sampling used tail-vein venipuncture. Blood samples were collected immediately before, then at 60 min intervals following treatment, until 5 h had elapsed.

Samples were placed in vacutainers containing lithium heparin and held in iced water. They were centrifuged (2500 rpm for 20 min at RT°C) within 2 h of collection. Plasma was aspirated and stored at -20°C until assayed for cortisol. Details of assays are reported in Chapter 4.

Rectal temperature was measured every 30 min as were heart and respiratory rates. Regular clinical observations were made of the animals.

Statistical analyses:

Results are expressed as mean ± sem. The individual PCC.time curve for each cow at each treatment was plotted. A peak was defined to occur if PCC changed by greater than 20 ng/ml over the two preceding samples and if it was both preceded and followed by at least two samples at which PCC was less. Individual curves were classified as having single or double peaks.

The peak PCC, the time from treatment to an increase of >20 ng/ml, and the time from treatment to return of PCC below 20 ng/ml were calculated. Areas under the PCC.time curves from 0 to 360 min were calculated by integration (AUC360). Data were examined by ANOVA using a GLM for effects associated with dose, cow and treatment occasion (i.e., 1st to 4th dose).

The changes in rectal temperature from that of 0 min (ΔT; °C) were calculated for each cow at each treatment. These data were analysed by ANOVA using a GLM for effects associated with dose, cow and treatment occasion, and for dose by cow interactions.

Data for rectal temperature and PCC at the test carried out 6 months following the dose response treatments were compared with data from other occasions of treatment with 12.5 µg LPS by paired Students' *t*-Test.

12.4.2 Results

Plasma cortisol concentrations:

Mean PCC at -60 and -30 min did not vary from 0 min PCC. Overall mean PCC following treatment with HBSS was 4.6 ± 0.4 ng/ml. Data for one cow on one occasion of treatment with HBSS was excluded when she caught a leg in railing beside the bail just before the 0 min sample was taken.

The mean times, relative to treatment, for an increase in PCC of >20 ng/ml, as well as for time to peak PCC and time for PCC to return to <20 ng/ml are given in Table 12.3. Dose was the only variable which demonstrated effects associated with time to increase in PCC and time to return below 20 ng/ml PCC ($P<0.02$ and $P<0.01$, respectively). Time to increase in PCC was shorter ($P<0.05$) with the two higher doses of LPS than with the dose of 3.125 μg. Similarly time for PCC to return below 20 ng/ml was longer ($P<0.05$) with the two higher doses than with the lower dose.

Dose LPS	Time to increase *	Time to peak 1	Time to <20 ng/ml *
3.125 μg	72 (7)	90 (0)	180 (27)
6.25 μg	54 (4)	84 (3)	302 (34)
12.5 μg	54 (4)	101 (8)	309 (28)

Table 12.3: Time (min), relative to treatment, to an increase in plasma cortisol concentration (PCC) of >20 ng/ml, to peak PCC, and for PCC to return to <20 ng/ml [mean (sem)] following administration of a graded range of doses of bacterial lipopolysaccharide (LPS; μg/500 kg) to cows.

* represents significant differences within columns between doses of LPS ($P<0.05$).
n = 8 for each value based on duplicated treatments in each of 4 cows for 3.125 and 6.25 μg LPS; n = 15 based on four treatments in each of 3 cows, and 3 treatments in one cow for 12.5 μg LPS.

Table 12.4 gives the data for the first plasma cortisol peak and AUC360. Fig. 12.5 shows the mean PCC.time curves with single and double peaks following the dose of 12.5 μg LPS. The overall means for each dose, and the means for each cow are shown in Figure 12.6. These figures demonstrate the high degree of variability in adrenocortical responses following iv injection of LPS which was evident at all except the preliminary dose, e.g., Cow 7714 responded only to the highest dose of LPS, while cow 6752 had similar responses to both 6.25 and 12.5 μg doses. On four occasions cows exhibited a double PCC peak - once with a dose of 6.25 μg in cow 6726, and three times with the dose of 12.5 μg, being twice with cow 6752 and once with cow 8720. No double peaks were exhibited at any first treatment occasion.

Dose LPS	Peak PCC	AUC360 **	ΔT **

HBSS	-	1733 (413)	0.1 (0.2)
3.125 μg	54.0 (10.5)	4419 (1092)	0.5 (0.1)
6.25 μg	65.4 (7.3)	8416 (2230)	0.9 (0.1)
12.5 μg	69.7 (5.3)	13905 (2088)	1.5 (0.1)

Table 12.4: Peak plasma cortisol concentrations (PCC; ng/ml), areas under the PCC.time curve over 360 min (AUC360; ng.min/ml) and changes in rectal temperatures (ΔT; °C) following administration of a graded range of doses of bacterial lipopolysaccharide (LPS; μg/500 kg) or Hank's balanced salt solution (HBSS) to cows [mean (sem)].

** represents significant differences within columns between doses of LPS ($P<0.01$).

n = 7 for each value based on duplicated treatments in each of 3 cows and a single treatment in one cow for HBSS; n = 8 for each value based on duplicated treatments in each of 4 cows for 3.125 and 6.25 μg LPS; n = 15 based on four treatments in each of 3 cows, and 3 treatments in one cow for 12.5 μg LPS.

There were significant effects on AUC360 associated with dose of LPS ($P<0.005$) and treatment occasion ($P<0.04$), but not with cow. There were no cow by dose interactions detected. Individual integrated responses (AUC360) were more than two standard deviations below the mean AUC360 for each respective dose on 9 of the total 31 treatment occasions: twice at the dose of 3.125 μg LPS, 3 times at the dose of 6.25 μg, and 5 times at the dose of 12.5 μg LPS. Seven of these failures occurred during the 2nd round of the replicated treatments.

At the final dose of LPS (six months later) all cows demonstrated an increase ($P<0.01$) in PCC at each sampling time. Increases in mean PCC were less than those seen previously following administration of 12.5 μg LPS (30.6 ± 3.8 and 31.9 ± 1.8 ng/ml at 1 and 2 h following treatment, respectively).

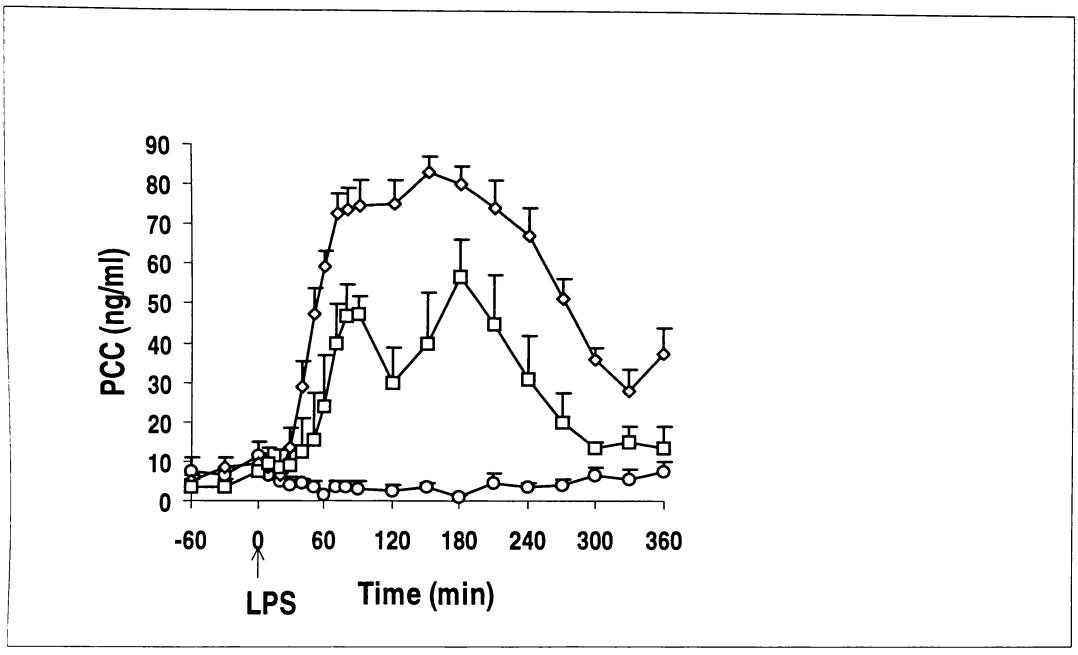


Figure 12.5: Mean plasma cortisol concentrations (PCC; ng/ml) following administration of Hank's balanced salt solution or 12.5 μg bacterial lipopolysaccharide (LPS) to cows. Responses to LPS are plotted according to whether the response produced was a single or double peak).

Legend:
- O - HBSS
- □ - double peak following 12.5 μg LPS
- ◆ - single peak following 12.5 μg LPS

n = 7 for HBSS representing duplicated treatments in 3 cows and a single treatment in one cow; n = 3 for single peak representing duplicated treatments in one cow and a single treatment in one cow; n = 12 for double peak representing four treatments in one cow, three treatments in two cows and a duplicated treatment in one cow.

Clinical changes:

The first clinical change evident was shivering which began within 30 min of treatment. Rumination ceased shortly after this. Rectal temperatures began to increase by 60 min following treatment at the two highest doses, and by 90 min at the dose of 3.125 μg (Fig. 12.7). All LPS treated cows showed symptoms of distress at the first two treatments regardless of dose - increased respiratory rate, grunting and open mouth breathing, increased heart rate, and sweating. At subsequent treatments, symptoms of distress were less severe. Clinical changes persisted for up to 2 h with recovery of normal respiration and rumination preceding the fall in both rectal temperature and PCC. On most treatment occasions by 2 to 3 h after treatment, cows accepted drinking water (up to 20 l).

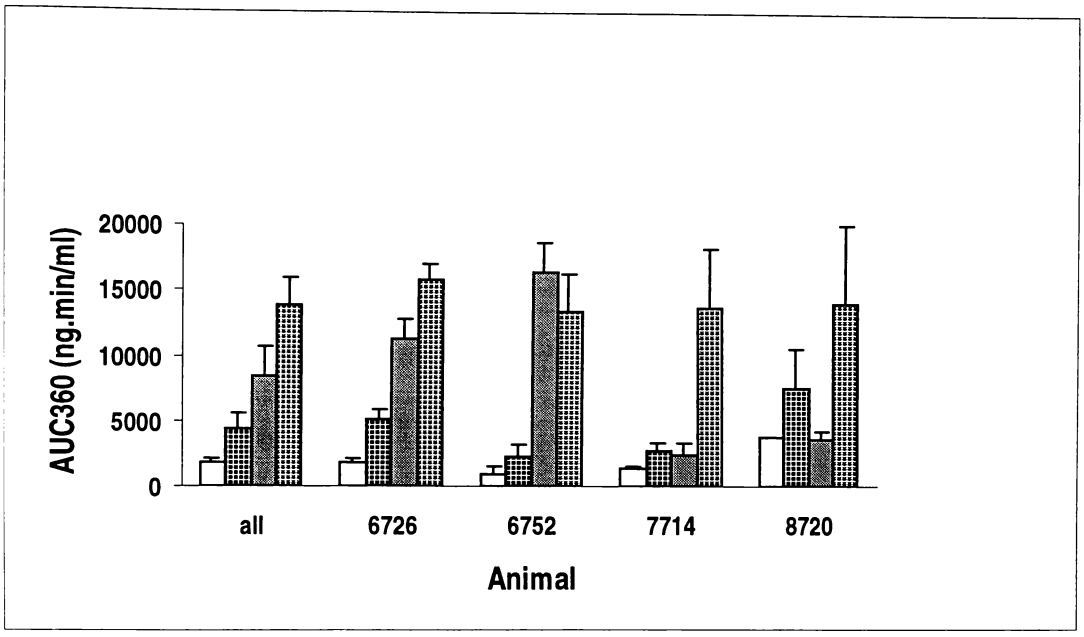


Figure 12.6: Areas under the plasma cortisol concentration.time curve over 360 min (AUC360; ng.min/ml) following administration of a graded range of doses of bacterial lipopolysaccharide to cows.

Legend:	open column	HBSS
	dark hatched	3.125 μg LPS
	solid column	6.25 μg LPS
	grey hatched	12.5 μg LPS

For columns representing all cows: n = 7 for HBSS based on duplicated treatments in each of 3 cows and a single treatment in one cow; n = 8 for 3.125 and 6.25 μg LPS based on duplicated treatments in each of 4 cows for; n = 15 for 12.5 μg LPS based on four treatments in each of 3 cows, and 3 treatments in one cow.
For columns representing Cows 6726, 6752 and 7714: n=2 for HBSS, and 3.125 and 6.25 μg LPS; n = 4 for 12.5 μg LPS.
For columns representing Cow 8720: n=1 for HBSS, n=2 for 3.125 and 6.25 μg LPS; n = 3 for 12.5 μg LPS.

There were significant effects on mean ΔT °C associated with both dose ($P<0.01$) and cow ($P<0.05$). Increments in rectal temperatures were greater at the higher doses (Table 12.4). Two cows experienced greater mean increments (0.9 ± 0.1 and 0.7 ± 0.1 °C) following 12.5 μg LPS than did the other two cows (both 0.4 ± 0.1 °C).

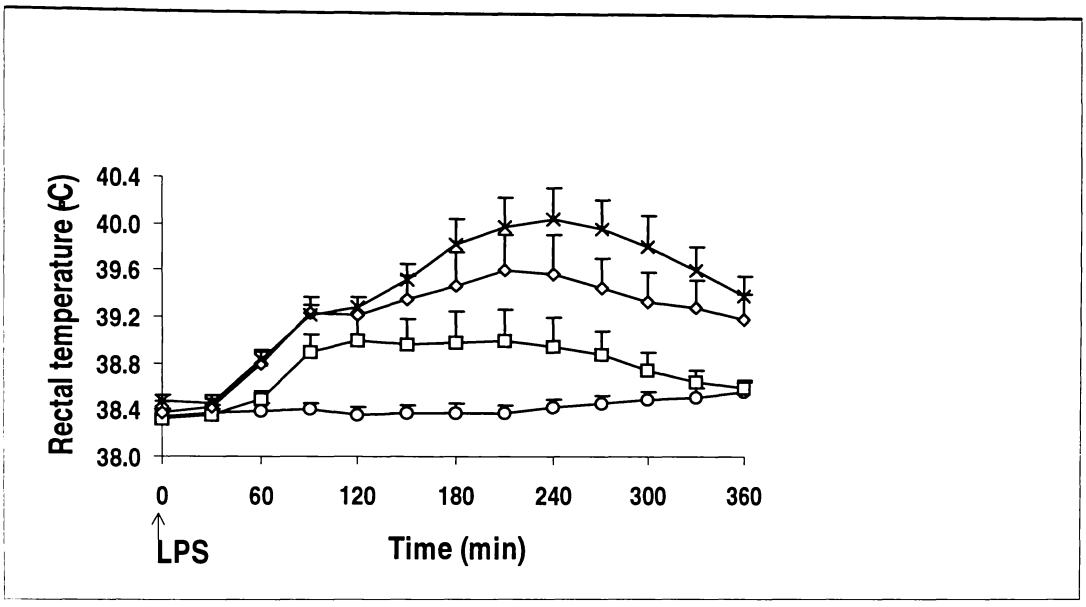


Figure 12.7: Rectal temperatures following administration of a graded range of doses of bacterial lipopolysaccharide to cows.

Legend: - O - HBSS - □ - 3.125 μg LPS
- ◆ - 6.25 μg LPS - x - 12.5 μg LPS

n = 7 for HBSS based on duplicated treatments in each of 3 cows and a single treatment in one cow; n = 8 for 3.125 and 6.25 μg LPS based on duplicated treatments in each of 4 cows for; n = 15 for 12.5 μg LPS based on four treatments in each of 3 cows, and 3 treatments in one cow.

Following the final dose of LPS, mean rectal temperature at 300 min following treatment was 40.2 ± 0.5 °C, which was not statistically different from mean temperatures measured following previous doses of 12.5 μg LPS. There was no clinical evidence of distress at this treatment.

12.4.3 Discussion

Changes in clinical signs and rectal temperatures were similar to those reported previously following administration of bacterial endotoxin to cattle. All temperature responses were monophasic, which had been identified as the common pattern following small doses of endotoxin (Lohuis *et al.*., 1988).

Changes in PCC following treatment were consistent with those seen following administration of 100 μg endotoxin intravenously. The endotoxin used was the same as that used in Expt. 12.1 (Jackson *et al.*, 1990). The pattern of responses to different doses suggests that the

responses of individual animals may be "all or nothing". There is also evidence that the animals become refractory to subsequent doses. The origin of this refractoriness is uncertain. Its incidence increased with time during the experiment suggesting that an immune tolerance may have developed. The reduced responses 6 months after the initial doses substantiate this. Pre-existence of a degree of immunity may have contributed to the initial variability in responses.

Refractoriness may also develop as a consequence of altered feedback mechanisms. The physiological process whereby bacterial endotoxin induces a response of the HPA axis is complex and incompletely understood. In mice, LPS has been shown to induce release of the cytokine interleukin 1 in a dose dependent manner from cells of the immune system and from Kuppfer cells of the liver (Rivier *et al.*, 1982). Interleukin 1 has a direct stimulatory effect on the adrenal cortex of rats at low doses, and at high doses will induce the release of CRH from the hypothalamus (Andreis *et al.*, 1991). The mechanism for this is uncertain, but probably involves prostaglandins since pretreatment with indomethacin, an inhibitor of prostaglandin synthesis, can block increases in hypothalamic CRH following LPS treatment (Rivier and Vale, 1991). Prostaglandins may also be responsible for the induction of fever by LPS (Watanabe *et al.*, 1991). The complex pathways through which the response is manifest allow multiple opportunities for modulation through alterations in negative feedback mechanisms.

The results of this experiment have demonstrated that iv administration of LPS to cows produces an inconsistent and unrepeatable response. This precludes its usefulness as a test of HPA axis integrity on a repetitive basis.

12.5 Conclusions

The pituitary gland and adrenocortical responses to a range of doses (6.25 to 25 μg) of synthetic bCRH in dairy cows has been defined. The integrated adrenocortical response measured as cAUC90 was proportional to the $\log_{10}$ of PAC at 15 min following treatment. The integrated PAC response demonstrated a linear relationship to dose of bCRH in the range of doses examined. Cows displayed a consistent ratio of cAUC90:aAUC90 and individual cow differences in sensitivity of the adrenal cortex to ACTH were identifed. A dose of 6.25 μg bCRH administered iv, with sampling at 15 min intervals for one hour following treatment is suggested as a method for clinical assessment of pituitary-adrenal integrity in cattle.

Adrenocortical responses and changes in rectal temperatures following iv administration of LPS were measured. In most cases PCC and rectal temperatures increased following treatment with LPS. On the first two occasions of treatment, but not on later occasions, cows exhibited marked distress. Responses showed a relationship to dose of LPS, but were highly variable both between cows and within cows, with repeated treatments. Injection of LPS is not a suitable stressor for use on a repetitive basis in tests of HPA axis integrity.

CHAPTER 13

GENERAL CONCLUSIONS

The overall aim of the research described in this thesis was to investigate regulatory mechanisms and physiological factors which influence adrenal cortex function in cattle. Adrenocortical responses to adrenocorticotropic hormone (ACTH) were shown to vary with changes in physiological state such as pregnancy, puberty and ageing. This suggested that factors such as endogenous gonadal steroids may modulate adrenocortical function in cattle. Changes following dexamethasone treatment were also studied.

The initial focus of the research described the responses of non-pregnant, non-lactating cows to a range of doses of a synthetic $ACTH_{1-24}$ administered by intravenous injection. The pattern of response was similar to that reported in other species. The adrenal cortices of these cows were demonstrated to be highly sensitive to ACTH. The integrated responses were proportional to dose, but the plasma cortisol peaks were independent of dose in the range studied. Individual animals displayed consistency of response.

The development of an adrenal response test (ART) which could be used to assess adrenocortical responses to ACTH in untrained animals in the field was instrumental for the further studies described. The protocol for ART used a low dose of ACTH - about one-fifth the rate usually employed for tests of adrenocortical reserve. A low dose was selected because it would be more sensitive in detecting changes in function. Even so, the selected dose may have sometimes been supra-physiological, but was used since the ART needed to be robust for field use.

Measurement of adrenocortical function in bulls and steers of various ages produced new information regarding the sexual dimorphism which occurs in cattle. Young bulls and steers were shown to have adrenocortical response patterns which were similar in shape to those described in cows; but the overall responses in bulls diminished about the time of puberty. Peak plasma cortisol concentrations were about 1.5 times greater in the steers than in the bulls by one year of age and responses in older bulls declined further. These findings have raised a

number of questions. Measurements of adrenocortical response to ACTH are a direct assessment of function of the adrenal cortex. They only provide indirect information regarding the integrity of pituitary function by the inference that ACTH is essential for the maintenance of steroidogenic machinery at a cellular level. The differences identified in bulls may be initiated by androgenic steroids firstly, because the changes begin at about the time of puberty, and secondly, because steers did not demonstrate the same changes. These changes could arise in several ways. Androgens may directly influence steroidogenesis at the level of the adrenocortical cells. Alternatively their effect may be at a higher level, either at the pituitary by altered negative feedback mechanisms, or via the hypothalamus by activation of androgen receptors in higher brain centres. Assessment of pituitary response to corticotropin-releasing hormone (CRH) will determine whether there is sexual dimorphism in aspects of pituitary secretion in cattle. Whether the effect is due to testosterone, some other androgenic steroid, or other influences of testicular origin also requires investigation.

Adrenocortical responses to ACTH were suppressed during the third trimester of pregnancy in cows. This is a further example of physiological modulation of adrenocortical function. Again, modulation could occur at several levels of the hypothalamo-pituitary-adrenal (HPA) axis. Factors of placental origin may also be involved.

Adrenocortical function following treatment of non-pregnant, non-lactating cattle with dexamethasone produced some surprising results. Cows demonstrated profound and prolonged suppression of adrenocortical function which lasted up to 17 days for highly soluble dexamethasone preparations and up to 2 months for formulations with low solubility. Although the nature of the pharmacological formulations and their release patterns from injection sites will influence the duration of the treatment effects, the extent of suppression was greater than expected. Young post-pubertal bulls and steers demonstrated suppression following treatment with soluble dexamethasone for a similar length of time to that observed in cows. Reductions in response to ACTH following dexamethasone treatment are mainly attributable to negative feedback inhibition of higher HPA axis centres, and consequent loss of trophic drive to the adrenal cortex. Evidence of suppression of adrenocortical responses to ACTH suggest that there is some trophic support from the pituitary in these bulls.

Rapid changes in plasma concentrations of selected hormones and peptides which are indicators of lactogenesis were seen in cows in which premature parturition was induced by dexamethasone. Cows, which were on average six weeks premature at the time of calving, underwent changes which were similar to those seen in spontaneously calving herd-mates. Several points of concern were raised in the induced cows. The persistent suppression of adrenocortical responses suggests that there may be persistent levels of dexamethasone. This implies that the recommended withholding periods for the drugs may be incorrect, and suggests that drug residues could be present in milk. Secondly, the rapid fall in plasma IGG1 concentrations in the more premature cows as accelerated colostrogenesis was initiated, suggests that their immune function may have been impaired. Whether or not these changes can alter susceptibility to disease is unknown. The induced cows recovered their ability to respond to ACTH in a shorter time than non-pregnant cows treated with the same dexamethasone formulation. This difference may be due to increased metabolic clearance of the dexamethasone, or due to increased trophic drive to the pituitary and adrenal cortex, possibly by arginine vasopressin. The need for a dexamethasone formulation with such persistent activity must be questioned - the changes leading to parturition were initiated rapidly and good pharmacological practise would dictate that the persistence of activity was not necessary. Improved formulations of dexamethasone for induction of parturition would be preferable.

Treatment of cows with a range of doses of bovine CRH allowed description of pituitary function as well as adrenocortical response as a function of pituitary stimulation. Endogenous $ACTH_{1-39}$ produced a similar pattern of response to the synthetic $ACTH_{1-24}$. These experiments also indicated that the dose of 6.25 μg bovine CRH produced a response which was very similar to physiological responses seen following exposure to acute stressors such as restraint. This preliminary work will be a valuable foundation for further investigation of changes in the adrenal cortex. Investigation of HPA axis function in cattle would also benefit from the development of a test using a controlled stressor to which animals could be exposed repetitively. Injection of bacterial lipopolysaccharide did not produce repeatable results, possibly due to changes in feedback mechanisms, precluding its use as a test of whole axis integrity.

Further work is required in the area of cortisol and ACTH clearance. Some changes seen in the results of ART were manifest as a delay in return of plasma cortisol concentrations to basal levels. This was evident following repetitive dosing with ACTH and may reflect increased steroidogenic capability of adrenocortical cells. A similar situation may occur during exposure to chronic stressors. Measurement of the metabolic clearance of ACTH and cortisol may identify the basis for these subtle changes.

The ability to mount a strong glucocorticoid response to stressor challenge may be less essential to survival in cattle than in other species. Contrary to popular belief from our current general knowledge of adrenocortical function, suppression of adrenocortical function in cattle was not entirely detrimental. The bulls and the dexamethasone treated animals remained healthy and were able to withstand the rigours of life. The higher levels of response seen in the female may reflect her more dynamic metabolic state, particularly during lactation. The lack of marked diurnal variations and the lower mean basal plasma cortisol concentrations also imply that the role of cortisol in cattle is rather different to that seen in other species. Whether this is a function of ruminants in general, or only cattle, requires further investigation. Ruminant physiology may favour alternative mechanisms, such as a sympathetic response, to assist the animal in coping with challenge and maintaining homeostasis.

In summary, the aims of the thesis have been achieved and have identified further areas which now require investigation. The overwhelming conclusions must be that some aspects of function of the adrenal cortex of cattle are unique. We cannot rely on extrapolation from other species alone if we are to fully understand the processes which enable their successful survival and production in the face of the many challenges to which they may be exposed.

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ADRENAL CORTEX RESPONSE TO ADRENOCORTICOTROPIC HORMONE IN DAIRY CATTLE

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ABSTRACT

Adult dairy cows were treated with a range of doses (0.0125mg to 0.4mg) of synthetic $ACTH_{1-24}$ and the plasma cortisol response was measured. Peak response was independent of dose. Higher doses of ACTH had a more prolonged effect such that the integrated response was dose dependent. Dose response curves were examined by regression analyses. Individual cows had a significant effect on y-axis intercept but not on the slope of the regression lines examined. A dose rate of 0.05mg ACTH iv was identified as a suitable dose rate for use in clinical assessment of adrenal cortex function, with 0, 50 and 120 min following ACTH being critical sampling times to identify the response.