H24/3708

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 17 August 2004.....
.....[redacted].....
Sec. Research Graduate School Committee

Erratum

Page 10 (Figure 2.2): This figure should show that noradrenaline, rather than adrenaline, is released from post-ganglionic neurons.

Page 58 (Figure 4.1a): The label on ordinate should be: Haematocrit (% of total blood volume).

Page 108: The first sentence of the legend to Figure 7.4 should be: Mean (±SEM) plasma concentrations of LH (ng/ml) in ewes (a) and in rams (b) during the first two hours of sampling on Day 0, the last two hours of the infusion of anti-GnRH antibodies, the first two hours of sampling on Day 1 and the first two hours of sampling on Day 2, which were both prior to treatment with the GnRH analogue.

The Effects of Isolation and Restraint Stress, and Cortisol, on the Responsiveness of the Anterior Pituitary to Gonadotrophin-Releasing Hormone in Rams and Ewes

Catherine Amelia Stackpole BSc (Biomedical)(Hons)

2004

Department of Physiology
Monash University

This thesis is presented for the degree of Doctor of Philosophy at Monash University

Table of Contents

Summary

Stress, or elevation in the plasma concentrations of cortisol, can be associated with a decrease in the responsiveness in the pituitary to stimulation by GnRH. There may also be sex differences and an effect of the sex steroids on the effects of these parameters on pituitary responsiveness. This thesis tested the unifying hypothesis that stress, or an increase in the plasma concentrations of the glucocorticoids, may reduce pituitary responsiveness to GnRH stimulation in a manner which is dependent upon the sex of the animal and the presence of the gonadal steroids. In the first of the four experimental chapters (Chapter 4) the hypothesis tested was that both LH pulse frequency in ewes, and LH pulse amplitude in rams would be affected by the infusion of either a high or low dose of cortisol. Gonadectomised ewes and rams received a 30 hour infusion of cortisol at two doses, which elevated plasma concentrations of cortisol to either 124±14ng/ml (low dose of cortisol) or to 260±28ng/ml or 227±22ng/ml during hours one to six and hours 24 to 30 of the infusion of the high dose of cortisol, respectively. LH pulse amplitude was suppressed during treatment with cortisol in both gonadectomised rams and ewes, however, the extent to which LH secretion was suppressed by cortisol was greater in rams than in ewes. In the experiments presented in Chapter 5, gonadectomised rams and ewes which had undergone hypothalamo-pituitary disconnection received an infusion of cortisol. The hypothesis tested was that elevated plasma concentrations of cortisol would be associated with a decrease in the amplitude of LH pulses detected in response to exogenous GnRH. Experiment 1 was conducted during the breeding season and plasma concentrations of cortisol were elevated to 154±19ng/ml. The amplitude of LH pulses detected in response to treatment with exogenous GnRH was suppressed in rams but not in ewes during the first six hours of the 30 hour infusion. Experiment 2 was conducted during the non-breeding season and plasma concentrations of cortisol were elevated to 382±31ng/ml in the first five and a half hours of the infusion, and to 218±10ng/ml during the last six hours of the infusion. There was no change in the amplitude of LH pulses secreted in response to exogenously administered GnRH during this cortisol infusion in ewes or rams. In Chapter 6, the hypothesis tested was that isolation and restraint stress may be associated with a decrease in pituitary responsiveness to GnRH. Gonadectomised ewes and rams that had undergone hypothalamo-pituitary disconnection were exposed to

five and a half hours of isolation and restraint stress during the breeding season or during the non-breeding season. There was no change in the amplitude of LH pulses secreted in response to exogenous treatment with GnRH during isolation and restraint stress imposed during the breeding season. In the non-breeding season, however, the amplitude of the first pulse, which occurred half an hour after the onset of isolation and restraint stress, was reduced in gonadectomised rams and ewes. A passive immunisation model was developed in Chapter 7 to further examine the effects of isolation and restraint stress on pituitary responsiveness. The endogenous secretion of GnRH was blocked by the administration of GnRH antiserum. The amplitude of LH pulses generated by the injection of a GnRH analogue, which was not affected by the antiserum, was then investigated during isolation and restraint stress in gonadectomised ewes and rams, and in gonadectomised ewes treated with oestrogen and gonadectomised rams treated with testosterone. There was no change in the amplitude of LH pulses during the imposition of isolation and restraint stress in this experiment. There was also no effect of the sex steroids on LH pulse amplitude. Overall, the amplitude of LH pulses was significantly smaller in rams than in ewes. Ewes displayed a significantly greater increase in the plasma concentrations of cortisol in response to isolation and restraint stress than rams. Cortisol secretion in response to the infusion of GnRH antiserum was greater in gonadectomised rams than in gonadectomised rams treated with testosterone. Collectively, the studies in this thesis suggest that there are likely to be mechanisms by which pituitary responsiveness to GnRH stimulation is reduced during stress. Sex differences in the effects of stress or elevated plasma concentrations of cortisol on LH secretion in response to GnRH may also exist, although no role for the sex steroids has been suggested by the studies presented in this thesis. It is possible, however, that there may be other factors which determine the extent to which pituitary responsiveness to GnRH may be suppressed during stress or elevated plasma concentrations of cortisol, such as season, the elevations in plasma concentrations of cortisol attained during treatment with cortisol or the imposition of stress, elevation in plasma concentrations of adrenaline and interplay between the sympathoadrenal axis and the hypothalamo-pituitary adrenal axis.

Preface

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution, and to the best of my knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

[redacted]

A list of publications arising from the work contained in this thesis is presented in Appendix 1.

Acknowledgements

Firstly and most importantly, I would like to thank my principal supervisor, Associate Professor Alan Tilbrook. His enormous professional and personal support has provided me with great opportunity for development on all levels throughout my candidature. I feel lucky to have had such a wonderful mentor. Secondly, I would like to thank Dr Anne Turner, whose support as co-supervisor has been of immense value to this work. I would also like to thank Professor Iain Clarke for his co-supervision and direction, in particular for his help with hypothalamo-pituitary disconnection and with the passive immunisation project.

An infinite amount of thanks must go to Michelle Ibbott, for her help with so many aspects of my PhD, especially for all the nights at Werribee. Most of all, I would like to thank her for her friendship throughout my time at Monash, you made it all so much more fun! Thank you to Bruce Doughton, Lynda Morrish, Adam Link and Karen Briscoe for their technical assistance with experiments. In particular to Bruce, for his guidance in conducting experiments, and to Bruce and Lynda, for so much help at some very odd hours. I would also like to thank fellow PhD student, Astrid Rivalland, and Dr Chris Scott for helping out so willingly when we were a pair of hands short.

I would like to thank Professor Fred Karsch and Kellie Breen from the Reproductive Sciences Program at the University of Michigan for their contribution to the experiment presented in Chapter 4. Thank you also to Kellie for her "hands-on" input into this study. It made the experiment very enjoyable!

For their support and advice with HPLC and catecholamine measurements, I would like to thank Dr Gavin Lambert and Ms Flora Socratous at the Baker Medical Research Institute. For their enormous assistance in the development of the protocol for enriching the concentration of immunoglobulin in serum, I would like to thank Professor Jim Goding, Dr Norm Mancuso and Mr Adnan Sali from the Department of Immunology and Pathology at the Alfred Hospital. I would also like to thank Mrs Alex Rao for her assistance in measuring antibody titres, in particular for the iodinated GnRH, and Dr Joe

Bertolini from CSL for the donation of equipment. Thanks to the proof readers – Michelle (again!), Lisa, Sheena, Sharon, Dave and Dad. This work was funded by grants from the NH&MRC and Monash University.

On a personal note, I would not have got through this without the support of many highly valued friends. To Ben, Ollie and Dan, thank you for being so hospitable and providing me with what has sometimes been a second home. Thanks to Mel and Powelly, who shared the experience with me and have moved on to new and exciting adventures, and to the rest of the "squad" for their friendship throughout the last eight years. In particular and extra-specially, I would like to thank my friend Mezzaluna, for knowing me right down to my very worst side, and still loving me enough to forgive me for it.

Finally, to David, who has always believed in me, and done everything to support me in every way possible. You are my rock! To my family, especially to Mum and Dad, who have and continue to give so much unconditional support, I love you all.

Thank You

Chapter 1 ~ General Introduction

Stress, which can be defined as a disruption to homeostasis, is associated with the activation of the hypothalamo-pituitary adrenal axis and the sympathoadrenal system, along with other physiological systems in a manner which is specific to the type of stressor imposed. Sex differences and the presence of the sex steroids may also be important in influencing the activation of these systems in response to stress.

Inappropriate stress responses are often associated with disorders of physiological systems. For instance, it is generally accepted that the imposition of stress is associated with a decrease in reproductive function including interruption of oestrus and decreased ovulation rates, as well as a delay and reduction in the LH surge. These effects of stress on reproductive end points may be due to interruption of the function of the hypothalamo-pituitary unit with a resultant decrease in the secretion of LH from the anterior pituitary. Such a decrease in the secretion of LH has been observed during stress in sheep and it is possible that it may arise from a decrease in GnRH secretion from the hypothalamus and/or a decrease in the responsiveness of the pituitary gland to stimulation by GnRH. While a potential effect of psychological and physiological stress on GnRH secretion is suggested, existing experimental models have not allowed changes in pituitary responsiveness to stress to be thoroughly and systematically investigated. Furthermore, it seems that sex and the sex steroids may be important in determining the effect of stress on the hypothalamus and/or pituitary.

It is likely that possible effects of stress on the hypothalamo-pituitary unit are mediated by systems that are activated in response to stress. In this regard, much research has focused on the glucocorticoids as potential mediators of the stress-induced decrease in the secretion of LH. Although a decrease in pituitary responsiveness during elevated plasma concentrations of the glucocorticoids is suggested, this cannot be concluded from previously published experimental models in which the effects at the hypothalamus and pituitary cannot be delineated. It is also likely that sex and the sex steroids influence the

effect of cortisol on the activity of the hypothalamus and/or pituitary. The general hypothesis tested in this thesis, therefore, was that stress, or an increase in the plasma concentrations of the glucocorticoids may reduce pituitary responsiveness to GnRH stimulation in a manner which is dependent upon sex and the presence of the gonadal steroids.

Chapter 2 ~ Review of the Literature

2.1. Stress

The concept of stress is one with which both the lay and scientific person is likely to be familiar. Although we essentially understand stress and the sensation of feeling stressed, historically, it has been difficult to apply a definition to this concept which allows its scientific investigation. The idea of a stable internal environment *(milieu interieur)* within an organism was first conceived by Claude Bernard (1813-1878), and this idea was later described as homeostasis by Walter Cannon (1871-1945). A commonly accepted definition of stress is that it is a disruption to homeostasis which occurs as a result of a stressful stimulus, or stressor (Chrousos & Gold 1992). It was Hans Selye who originally recognised that a neuroendocrine cascade was activated in response to a range of stressful stimuli. While the neuroendocrine response to these stimuli was directed at the re-establishment of homeostasis, inappropriate responses could also cause disease (Selye 1946).

The stress response is characterised by the biological changes which occur as a result of exposure to a stressor. Although it was previously thought that there was one generalised response to cope with a variety of stressful stimuli (Selye 1946), it is now believed that the stress response is modified according to the particular demands imposed upon the individual by the stimulus, and is therefore highly specific to each stressor (Mason 1968a; Mason 1968b; Mason 1975; Munck *et al.* 1984; Moberg 1985a). This theory is supported by evidence demonstrating that the neuroendocrine pathways activated by stress depend upon the stressor imposed (Canny *et al.* 1989; Vellucci & Parrott 1994; Adam & Findlay 1998; Van de Kar & Blair 1999; Dobson & Smith 2000; Tilbrook *et al.* 2000; Pacák & Palkovits 2001; Tilbrook *et al.* 2002a; Turner *et al.* 2002a). There are several factors which are thought to impact on whether an individual finds a stimulus stressful, as well as the intensity of the stress response to the stimulus. If an individual believes that a stressful stimulus can be overcome or managed, then the stimulus is less likely to be stressful. Additionally, if the stressor has been experienced before, the response to the stress may be smaller than if the stressor is novel (Munck *et al.* 1984; Sapolsky *et al.* 2000; Cook 2002). Other factors such as

genetics, age, sex and the presence of a peer may also determine the perceived stressfulness of a stimulus (Mason 1968a; Mason 1968b; Mason 1975; Moberg 1985a; Herbert 1987; Wingfield & Sapolsky 2003).

Among others, there are three classical responses to a stressor. These have been described as the behavioural response, the neuroendocrine response and the sympathoadrenal response (Moberg 1985a). Behavioural responses may include avoiding or moving away from a stressor, while the neuroendocrine and sympathoadrenal responses are physiological responses (Sections 2.1.1 and 2.1.2). From an experimental perspective, the measurement of plasma concentrations of hormones which are secreted due to activation of the neuroendocrine or the sympathoadrenal response are often used to define whether or not an individual is stressed. Typically, stressors are organised into a number of different categories. Psychological stressors include isolation, exposure to new environments or changes in status within a social group. Physical stressors include foot shock and immobilisation or restraint, although there may also be a psychological component to these stressors. Other stressors may be physiological in nature, such as exposure to extreme temperatures, food restriction or hypoglycaemia-induced by treatment with insulin. Immune stressors, such as treatment with endotoxin, can also be classed as physiological. In addition to defining the type of stressor, stressful stimuli are often classed as acute or chronic, according to their duration. Chronic stress is known to be associated with the onset of certain disease states (Sapolsky 1994). The role of acute stress in disrupting the functions of particular physiological systems is less clear.

2.1.1. The hypothalamo-pituitary adrenal axis

The hypothalamo-pituitary adrenal axis is a classical neuroendocrine axis activated during stress (Figure 2.1). Parvocellular neurones synthesising corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) are found in the paraventricular nucleus of the hypothalamus (Harbuz & Lightman 1992; Carrasco & Van de Kar 2003). Activation of these neurones occurs in response to inputs from other brain regions which detect and integrate signals from both internal and external stimuli. CRF and AVP neurones project to the external zone of the median eminence, a circumventricular organ superior to the hypophysial portal blood vessels, and release their neuropeptides into portal blood. CRF

and AVP then stimulate the activity of corticotroph cells found within the anterior pituitary. CRF is also released in other regions throughout the brain and may function as a neurotransmitter, while AVP is also secreted from the posterior pituitary and has a role in the maintenance of osmotic balance, fluid volume and vasoconstriction (Carrasco & Van de Kar 2003).

Peptides cleaved from the proopiomelanocortin (POMC) peptide are secreted from the corticotrophs in response to stimulation by CRF and AVP. These include adrenocorticotrophic hormone (ACTH) and the opioid, β-endorphin (Axelrod & Reisine 1984; Howlett & Rees 1987; Engler *et al.* 1989). CRF also causes an increase in the synthesis of the POMC peptide (Harbuz & Lightman 1992). Evidence from sheep suggested that AVP was the more potent secretagogue of ACTH than CRF (Familiari *et al.* 1989; Canny *et al.* 1999; Smith RF & Dobson 2002), although studies by McFarlane *et al.* (1995b) showed otherwise. Further studies in sheep showed that the actions of CRF to stimulate ACTH secretion were potentiated by AVP (Keller-Wood 1998).

ACTH has actions at the adrenal cortex to stimulate the production of the glucocorticoids. In the sheep and the human, the principal glucocorticoid secreted in response to ACTH is cortisol, while in the rat, the principal glucocorticoid is corticosterone. Traditionally, elevation of the plasma concentrations of the glucocorticoids in response to stress was primarily thought to mediate the physiological changes required to respond to a stressor, including promoting gluconeogenesis in the liver, catabolism of protein, decreasing glucose uptake in adipose tissue, promoting the effects of the sympathoadrenal system (Section 2.1.2) and causing cognitive changes resulting in increased mental alertness (Munck *et al.* 1984; Torpy & Chrousos 1996; Sapolsky *et al.* 2000; Kellendonk *et al.* 2002). The glucocorticoids are also well known clinically for their suppressive effects on the immune system (Munck *et al.* 1984). More recent reviews, however, now discuss the actions of glucocorticoids in several contexts, including actions which prepare the individual to mount a stress response in the future, actions which enhance the response to an existing stressor, and also actions which modulate the stress response to prevent it from causing damage to the individual (Munck *et al.* 1984; Sapolsky *et al.* 2000). Both basal concentrations of the glucocorticoids, and elevated concentrations observed during stress, may have a variety of

effects on the central nervous system to modulate the stress response, including maintaining its responsiveness to a novel stressor during periods already considered to be stressful, and dampening responses to repetitive stress (Munck *et al.* 1984; Sapolsky *et al.* 2000; Cook 2002).

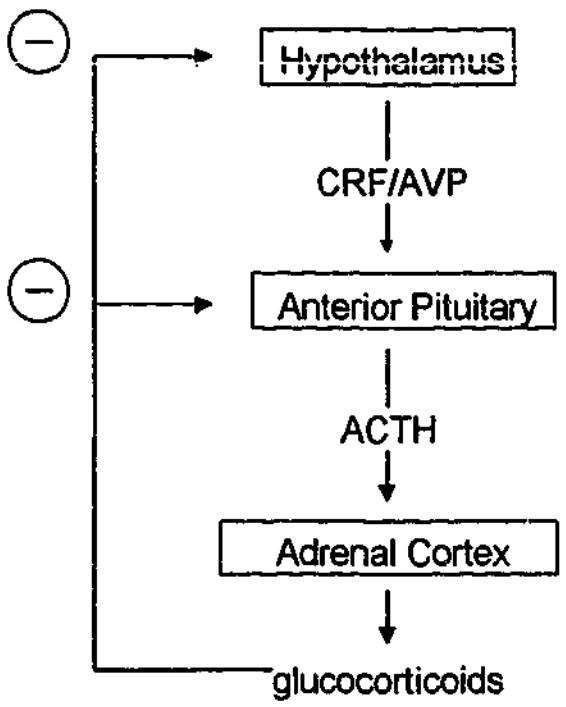


Figure 2.1

The secretion of the glucocorticoids by the hypothalamo-pituitary adrenal axis is stimulated by the secretion of ACTH from the pituitary. In turn, ACTH secretion is stimulated by the release of CRF and AVP from neurones located in the paraventricular nucleus of the hypothalamus. The glucocorticoids have feedback actions at the pituitary and the hypothalamus to regulate their own secretion.

Basal concentrations of the glucocorticoids have a circadian rhythm in diurnal species, being lowest in the afternoon and evening and highest in the early morning before activity commences (Fulkerson & Tang 1979; Dallman *et al.* 1991). The glucocorticoids have negative feedback effects both centrally and at the level of the pituitary to decrease their own secretion (Canny *et al.* 1989; Dallman *et al.* 1991; McFarlane *et al.* 1995a; Young 1995).

There are two types of glucocorticoid receptors. The mineralocorticoid receptor, or Type I receptor has a higher affinity for glucocorticoids than the glucocorticoid or Type II receptor (Reul & de Kloet 1985). Both receptor types are found in the brainstem and the hippocampus, which is thought to be the primary site of centrally mediated glucocorticoid feedback on the synthesis and secretion of CRF, as well as possibly on AVP (Young 1995; Kellendonk *et al.* 2002). Glucocorticoid receptors are also widely distributed throughout other areas of the brain and the body, including the pituitary, where they may mediate negative feedback effects on ACTH secretion (Kellendonk *et al.* 2002; Richards *et al.* 2003). It has been suggested that the mineralocorticoid receptor may mediate some of the actions of basal concentrations of the glucocorticoids, while the glucocorticoid receptor may mediate the actions of stress-like concentrations of the glucocorticoids and the elevated concentrations of the glucocorticoids observed during the peak of the circadian rhythm (Reul & de Kloet 1985). Feedback systems within the hypothalamo-pituitary adrenal axis are complicated by the fact that an individual is able to respond repeatedly to different stressors without an observable negative feedback effect on the secretion of the glucocorticoids. It is thought that there is a facilitative effect of the stress response on the hypothalamo-pituitary adrenal axis, which allows it to be repeatedly activated in response to stress (Dallman *et al.* 1991; Smith & Dobson 2002). This may be a result of actions of the glucocorticoids within the limbic system (Cook 2002). Other studies suggest that the sites and mechanisms of negative feedback may differ depending on the stressor imposed (Canny *et al.* 1989; Dobson & Smith 2000). In addition to regulatory factors within the hypothalamo-pituitary adrenal axis, factors external to the axis are also thought to play an important role in the generation of the stress response. Hypothalamo-pituitary adrenal responses to stress are attenuated during lactation in rats (Lightman & Young 1989; da Costa *et al.* 2001), and it has been suggested that this may be due to an increase in the actions of oxytocin within the central nervous system (Neumann *et al.* 2000; Nomura *et al.* 2003). Sex and the presence of the gonadal sex steroids are also thought to be important in determining the magnitude of the stress response (Section 2.1.4).

Activation of the hypothalamo-pituitary adrenal axis has been observed in many species in response to a number of different types of stressors. In sheep, activation of the hypothalamo-pituitary adrenal axis has been measured in response to isolation and restraint

(Tilbrook *et al.* 1999a; Turner *et al.* 2002a), electric foot shock (Polkowska & Przekop 1988; Cook 2002), audiovisual stress (barking dog stress) (Canny *et al.* 1989; Engler *et al.* 1989; Komesaroff *et al.* 1998; Cook 2002; Turner *et al.* 2002c), transport stress (Parrott *et al.* 1994; Dobson *et al.* 1999), wetting stress (Parrott *et al.* 1994), insulin-induced hypoglycaemia (Canny *et al.* 1989; Dobson & Smith 2000; Turner *et al.* 2002a) and endotoxin (Coleman *et al.* 1993; Battaglia *et al.* 1997; Battaglia *et al.* 1998; Dadoun *et al.* 1998). Similar responses have been observed in response to a variety of psychological, physical and physiological stressors in primates (Sapolsky 1985; Norman & Smith 1992; Norman *et al.* 1994; Xiao *et al.* 2002), and in rats (Taché *et al.* 1978; Taché *et al.* 1980; Ariznavarreta *et al.* 1989).

2.1.2. The sympathoadrenal system

Activation of the sympathoadrenal system occurs in response to stress and results in the secretion of the catecholamines, adrenaline and noradrenaline (Figure 2.2). The synthetic pathway of the catecholamine hormones is demonstrated in Figure 2.3. The sympathoadrenal system is principally responsible for the increased activity of the cardiovascular system associated with exposure to stress. At the spinal cord, preganglionic neurones synapse with postganglionic neurones using acetylcholine as the neurotransmitter. The postganglionic neurones innervate various regions throughout the body, such as the heart and skeletal muscle (Goldstein 1987). Other preganglionic neurones do not synapse at the spinal cord but directly innervate the chromaffin cells of the adrenal medulla, and use acetylcholinergic neurotransmission to stimulate the secretion of the catecholamine hormones into the peripheral circulation. Adrenaline is the principal catecholamine released from the adrenal medulla. Noradrenaline is released in small amounts from the adrenal medulla, and also from the postsynaptic neurones (Axelrod & Reisine 1984; Goldstein 1987). The activity of the preganglionic neurones is regulated by inputs from several regions of the brain, including the hypothalamus, the ventrolateral medulla, the anteroventral third ventricle region and the limbic system (Goldstein 1987). Furthermore, a variety of interactions between the hypothalamo-pituitary adrenal axis and the sympathoadrenal axis occur, which regulate the actions and responses of each axis (Axelrod & Reisine 1984; Sapolsky *et al.* 2000). Increases in plasma concentrations of adrenaline have been observed in prepubertally castrated rams exposed to isolation stress, simulated transport stress and wetting stress (Parrott *et al.* 1994). In contrast, a small non-significant

increase in plasma concentrations of noradrenaline was observed in response to isolation but not in response to transport or a wetting stress (Parrott *et al.* 1994). In primates, cold stress has been shown to increase plasma concentrations of noradrenaline and adrenaline (Peyrin *et al.* 1981). Increased plasma concentrations of the catecholamines have also been reported in humans after surgical stress (Udelsman & Chrousos 1988). In rats, increases in the enzymes in the synthetic pathway for noradrenaline and adrenaline have been observed in response to swimming, electric shock and insulin. Handling and immobilisation of rats have also been shown to cause an increase in the plasma concentrations adrenaline and noradrenaline (Axelrod & Reisine 1984).

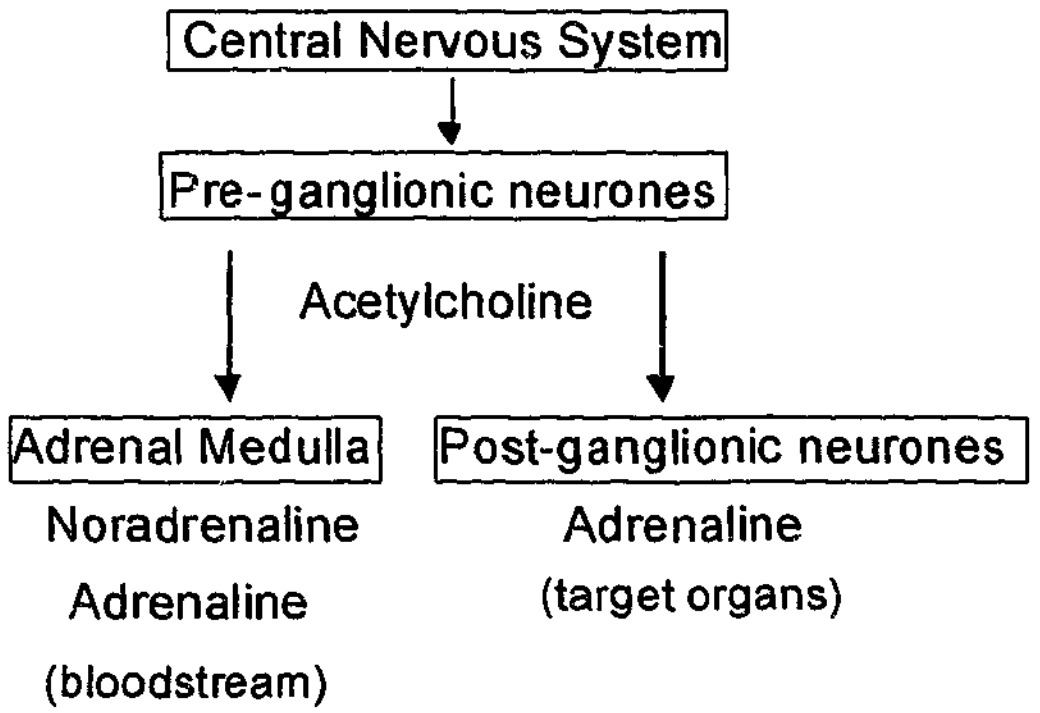


Figure 2.2

The catecholamines, noradrenaline and adrenaline, are secreted from the adrenal medulla in response to stimulation by acetylcholine from preganglionic neurones. The pre-ganglionic neurones are activated in response to a stressor detected by the central nervous system. The pre-ganglionic neurones also synapse with post-ganglionic neurones which release noradrenaline and affect various target organs.

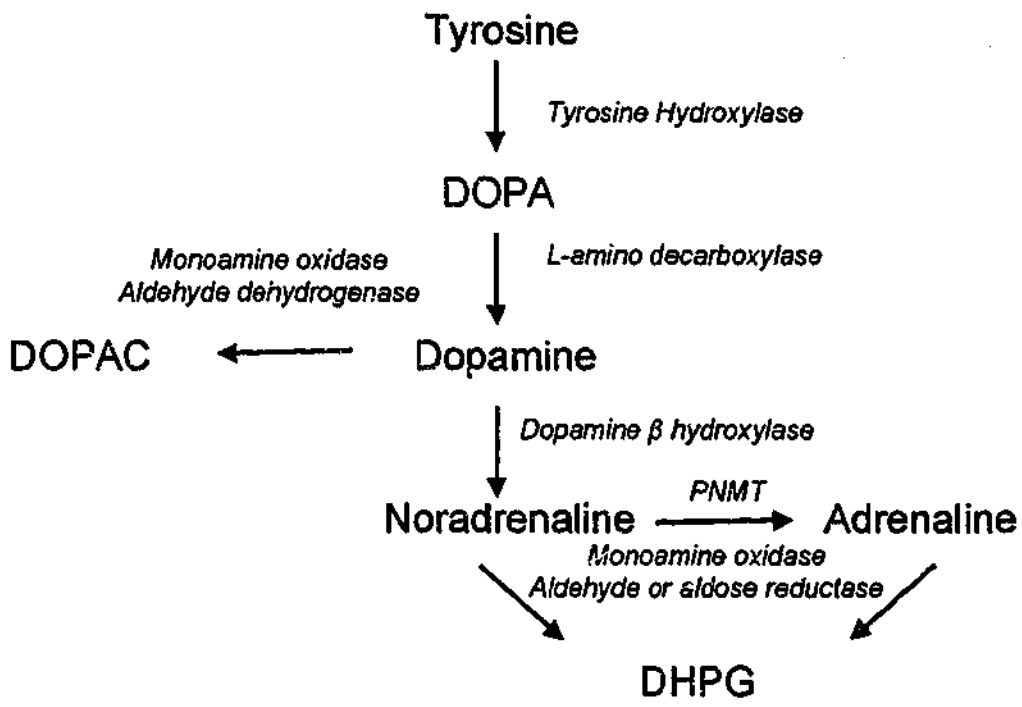


Figure 2.3

Dopamine, noradrenaline and adrenaline are synthesised from tyrosine after it is converted to 3,4 dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. Dopamine is metabolised to 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase and aldehyde dehydrogenase. Noradrenaline is converted into adrenaline by phenylethanolamine-N-methyltransferase (PNMT). Both adrenaline and noradrenaline are metabolised into 3,4 dihydroxyphenylglycol (DHPG) by monoamine oxidase and aldehyde or aldose reductase (Sundberg 1995; Eisenhofer *et al.* 2001).

2.1.3. Other systems activated by stress

Many other physiological systems are activated by a range of stressors, although they are not a focus of this thesis. The opioid peptides are secreted from a range of sites in the body, and their secretion increases in response to various stressors (Howlett & Rees 1987). As well as activating the hypothalamo-pituitary adrenal axis, endotoxin has been shown to stimulate the release of proinflammatory cytokines, such as tumour necrosis factor-α and interleukin-1, and prostaglandins (Wright *et al.* 1990; Raetz *et al.* 1991; Freudenberg *et al.* 1993; Bertok 2003). Increased prolactin secretion during stress has also been observed in prepubertally castrated rams exposed to simulated transport stress (Parrott *et al.* 1994) or endotoxin (Coleman *et al.* 1993), and in rats exposed to ether stress (Ajika *et al.* 1972; Krulich *et al.* 1974), handling stress (Krulich *et al.* 1974; Euker *et al.* 1975) or immobilisation (Du Ruisseau *et al.* 1978). Oxytocin concentrations within the hypothalamus have been shown to increase after exposure to a social stress in male rats (Engelmann *et al.* 1999) and

may have an inhibitory effect on the hypothalamo-pituitary adrenal axis in both males and females (Neumann *et al.* 2000).

2.1.4. Sex differences in the stress response and the effect of the gonadal steroids

Various studies have demonstrated sex differences in the response of the hypothalamo-pituitary adrenal axis and sympathoadrenal system to stress, and suggest that these differences may be due, in part at least, to the influence of the gonadal steroids. In gonadectomised sheep, females demonstrated a larger increase in cortisol secretion in response to isolation and restraint stress than males. In response to insulin-induced hypoglycaemia, however, the male cortisol response was larger, suggesting that sex differences in the stress response may depend upon the nature of the stressor (Turner *et al.* 2002a). *In vitro* studies in gonadectomised male and female sheep showed that males had higher concentrations of AVP in the median eminence while there was no effect of sex on the ACTH response to stimulation by AVP and/or CRF *in vitro* (Canny *et al.* 1999). Adrenal weight in females was larger than in males, as was cortisol secretion from cultures of adrenal cells treated with ACTH.

The function of the hypothalamo-pituitary adrenal axis may also be regulated by the presence of the sex steroids at the level of the hypothalamus and pituitary. Komesaroff *et al.* (1998) showed that elevated plasma concentrations of ACTH and cortisol secreted in response to a barking dog or insulin-induced hypoglycaemia were reduced in ovariectomised ewes treated with oestrogen in comparison to ovariectomised ewes receiving no oestrogen treatment. Further comparisons made between ewes in the luteal or the follicular phase of the oestrous cycle (Section 2.2.4) showed that ACTH and cortisol responses to the barking dog stress and insulin-induced hypoglycaemia were lower in the follicular phase, when oestrogen levels are higher, than in the luteal phase in response to both stressors (Komesaroff *et al.* 1998). These data suggest that oestrogen may have a suppressive effect on the response of the hypothalamo-pituitary adrenal axis to stress. ACTH responses to hypotension, however, were shown to be lower in chronically ovariectomised ewes when compared to acutely ovariectomised ewes, suggesting that the effect of oestrogen may be stressor specific (Pecins-Thompson & Keller-Wood 1994). Therefore, in situations where oestrogen increases the hypothalamo-pituitary adrenal

response to stress, it may be that some neural pathways which stimulate activation of the hypothalamo-pituitary adrenal axis may be positively regulated by oestrogen. Alternatively, oestrogen may inhibit the activity of neurones which inhibit stimulation of the hypothalamo-pituitary adrenal axis. Other neural pathways involved in the regulation of the hypothalamo-pituitary adrenal axis may be either independent of the actions of oestrogen or be regulated by oestrogen in a manner which results in a decreased response of the hypothalamo-pituitary adrenal axis to stress. Further studies showed that the responses to CRF or AVP or both in combination were not changed by ovariectomy in ewes, suggesting that the site of sex steroid modulation of the hypothalamo-pituitary adrenal axis was not at the pituitary and was more likely to be in the brain (Pecins-Thompson & Keller-Wood 1994). Comparisons made between gonadectomised and gonad intact rams and ewes further support a role of the sex steroids in modulating activity of the hypothalamo-pituitary adrenal axis. The concentrations of AVP and CRF in the median eminence were lower in gonad intact animals than in gonadectomised animals, suggesting a possible suppressive effect of the sex steroids in rams and ewes on AVP and CRF synthesis (Canny *et al.* 1999). A suppressive effect of the androgens on the hypothalamo-pituitary adrenal axis in the ram is also suggested by Hileman *et al.* (1996), who showed that POMC messenger ribonucleic acid (mRNA) expression in the pituitary was reduced by testosterone treatment in gonadectomised male sheep.

In humans, sex differences in response to a psychological stressor may differ to that in sheep, with men showing greater cortisol responses than women (Seeman *et al.* 2001). These results were reversed, however, when subjects were in an older age group. These age-related changes may be in line with the decrease in plasma concentrations of the sex steroids observed in older people, and may explain the differences in the results obtained from studies in gonad intact humans and gonadectomised sheep. Studies in humans support evidence from sheep that stress responses are higher in the luteal phase than in the follicular phase. Imposition of exercise stress on women was shown to generate ACTH and AVP responses that were higher in the mid luteal than in the follicular phase (Altemus *et al.* 2001), again suggesting a role of the sex steroids in modulating hypothalamo-pituitary adrenal responses to stress. Further studies by Roca *et al.* (2003) showed that progesterone treatment in women was associated with an increase in AVP, ACTH and cortisol in

response to exercise stress, suggesting that progesterone may stimulate increased activity of the hypothalamo-pituitary adrenal axis. No changes in the ACTH responses to insulin-induced hypoglycaemia were observed in women across the menstrual cycle (Long *et al.* 2000), suggesting that as in sheep, the effects of the sex steroids on stress responses may be stressor-specific.

Reviews of studies in rats suggest that the hypothalamo-pituitary adrenal response to psychological and physical stress in the female is generally larger than that of the male (Handa *et al.* 1994; Young 1995; Patchev & Almeida 1998). In contrast to sheep and humans, studies suggest that oestrogen may increase the secretion of AVP, ACTH, corticosterone and oxytocin in response to stress, and an oestrogen response element has been detected on the CRF gene (Young 1995; Patchev & Almeida 1998). Changes in the reactivity of the hypothalamo-pituitary adrenal axis have been observed throughout the oestrous cycle in rats, but these are in the opposite direction to that observed in sheep and humans. For example, in ovariectomised rats exposed to insulin-induced hypoglycaemia, corticosterone responses to the stressor were increased when rats were treated with oestrogen (Li *et al.* 2003). In line with studies in humans, treatment with progesterone further increased the response to hypoglycaemia, suggesting that progesterone may positively regulate the hypothalamo-pituitary adrenal response to stress in these species. Evidence from rats suggests that oestrogen may contribute to differences in the reactivity of the hypothalamo-pituitary adrenal axis by altering negative feedback interactions within the axis. A larger number of glucocorticoid and mineralocorticoid receptors are found in the female rat hippocampus than in the male. Sex differences in glucocorticoid receptor mRNA expression, but not mineralocorticoid receptor mRNA expression, were no longer observed after ovariectomy in rats (Patchev & Almeida 1998), suggesting that the sex difference in glucocorticoid receptors, but not mineralocorticoid receptors, may be maintained by the sex steroids. Sex differences in mineralocorticoid receptors may be a result of differences in organisational patterns in the brain caused by exposure to the sex steroids during development. Nevertheless, these findings suggest that glucocorticoid negative feedback at basal concentrations may be stronger in the female rat, and that the female sex steroids may affect negative feedback at stress-like concentrations of the glucocorticoids. Several studies in rats suggest that the effect of the androgens in

decreasing the hypothalamo-pituitary adrenal axis response to stress is through a central mechanism rather than a change in pituitary sensitivity to CRF (Handa *et al.* 1994; Patchev & Almeida 1998). Androgen receptors are found in the medial preoptic area, the bed nucleus of the stria terminalis and the lateral septum, and all of these areas have projections to the paraventricular nucleus. Androgen receptors are also themselves found in the PVN but do not appear to be colocalised with CRF (Handa *et al.* 1994).

Sex differences in the sympathoadrenal system have been observed in the sheep and the rat. Studies in sheep show that the secretion of adrenaline and noradrenaline can be regulated by the sex steroids, as adrenaline and noradrenaline responses to insulin-induced hypoglycaemia were lower during the follicular phase than the luteal phase (Komesaroff *et al.* 1998). In response to stress induced by a barking dog, however, there was no difference in the adrenaline and noradrenaline responses between the follicular and luteal phases of the oestrous cycle (Komesaroff *et al.* 1998). As with the hypothalamo-pituitary adrenal axis, these data suggest that some pathways involved in stimulation of the catecholamines in response to stress may be sensitive to the sex steroids, whereas others may be sex-steroid independent. Reviews of studies in the rat suggest that sympathoadrenal responses to stress vary with the oestrous cycle. The sympathoadrenal system may also be less responsive to stress in the female than in the male, and more sensitive to inhibitory stimuli in the female (Hinojosa-Laborde *et al.* 1999).

2.1.5. The relationship between stress and disease

Hans Selye was the first to suggest that stress could cause disease (Selye 1946), and it is now well known that chronic or inappropriate stress responses lead to abnormalities in many physiological systems including growth and development, the immune system and cardiovascular function (Sapolsky 1994). Stress has also been associated with decreases in reproductive function, the focus of this thesis.

2.2. Reproduction

Reproduction is an optimistic process. That is, it presupposes that the internal and external environment of an organism are in a condition to support the growth and development of offspring (Wingfield & Sapolsky 2003). Consequently, a healthy reproductive system is thought to be a key indicator of the overall health of an organism (Moberg 1985b). In

sheep, reproduction is influenced by environmental stimuli and therefore shows a seasonal pattern of activity (Lincoln & Short 1980). During the non-breeding season, ewes are described as being in anoestrus.

2.2.1. The hypothalamo-pituitary gonadal axis

The reproductive process in males and females is controlled by the hypothalamo-pituitary gonadal axis. This neuroendocrine axis is responsible for the production of the gametes, the synthesis and secretion of the sex steroids, the maintenance of secondary sexual characteristics and contributes to the development and maintenance of sexual behaviour.

2.2.2. Gonadotrophin releasing hormone secretion from the medial preoptic area and hypothalamus

Gonadotrophin releasing hormone (GnRH) is a neuropeptide of 10 amino acids. In both sexes, the secretion of GnRH into the hypophysial portal blood vessels can be thought of as the primary stimulus of this neuroendocrine axis, although GnRH neurones themselves are under regulation by hormonal feedback and inputs from various internal and external environmental stimuli (Moenter *et al.* 1990; Hamada *et al.* 1996; Skinner & Herbison 1997). Neurones secreting GnRH are dispersed throughout the preoptic area and anterior hypothalamus. In the sheep, they are found principally in the septal area, medial preoptic area and the organum vasculosum of the lamina terminalis (Polkowska *et al.* 1980; Dees *et al.* 1981; Lehman *et al.* 1986). GnRH neurones project to the median eminence. The greatest density of nerve terminals have been identified in the rostral area of the external zone of the median eminence (Polkowska *et al.* 1980; Dees *et al.* 1981).

GnRH is a stimulus for the secretion of the gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH). The gonadotrophins are secreted from the gonadotroph cells of the anterior pituitary and are exposed to GnRH by way of the hypophysial portal blood vessels (Clarke 1996). In both sexes, the gonadotrophins act at the gonads to stimulate production of the sex steroids and the glycoprotein hormone, inhibin, and regulate the processes required for production of the gametes.

2.2.3. The secretion of GnRH and LH is pulsatile

In male and female sheep, GnRH and LH are secreted in a pulsatile pattern (Clarke & Cummins 1982). Pulses of GnRH are secreted at regular intervals due to the coordinated release of GnRH from GnRH neurones. The mechanisms by which GnRH neurones

coordinate their activity is unknown but may involve an inherent phasic property of the cell (Barry 1979; Wetsel *et al.* 1992; Terasawa *et al.* 1999) and/or connections between GnRH neurones at the level of the cell body or the terminal (Lehman *et al.* 1986; Lehman *et al.* 1988; Martinez de la Escalera *et al.* 1992). Nevertheless, it is well accepted that the secretion of one pulse of LH arises due to stimulation by a pulse of GnRH (Clarke & Cummins 1985). In addition, there is an inverse relationship between the frequency of GnRH pulses and the amplitude of LH pulses (Clarke 1996).

2.2.4. Reproduction in the female

In females, cyclic hormonal interactions of the hypothalamo-pituitary ovarian neuroendocrine axis characterise the oestrous cycle of the ewe and other domestic species, and the menstrual cycle in higher primates. Ovulation in the sheep is regulated by the endocrine interactions of the oestrous cycle which are represented schematically in Figure 2.4. Development of an ovarian follicle for ovulation occurs during the follicular phase of the cycle in response to stimulation by FSH (Hunter *et al.* 1988; McNeilly *et al.* 1991). In the early stages of the follicular phase, low levels of oestrogen are secreted by the developing follicle in response to stimulation by LH (Moor 1977). During the breeding season, these low levels of oestrogen exert negligible negative feedback effects on the secretion of GnRH and LH. During the non-breeding season, however, oestrogen has a strong negative feedback action on the secretion of GnRH. As the follicular phase progresses, LH secretion rises, stimulating a gradual increase in the secretion of oestrogen from the maturing follicles. It is believed that, at some time in the late follicular phase, oestrogen reaches a threshold concentration. At this level, oestrogen exerts a positive feedback and triggers a surge in the secretion of GnRH (Clarke & Cummins 1985; Clarke & Tilbrook 1999). The surge in GnRH, in combination with an increase in pituitary sensitivity, triggers a surge in the secretion of LH (Clarke *et al.* 1989; Clarke 1995a; Clarke 1995b). During this time, a pulsatile pattern in the secretion of LH is no longer detectable.

The surge in LH causes the ovulation of at least one mature follicle from the ovary. The rising levels of oestrogen also induce behavioural oestrus which typically lasts 20 to 36 hours (Driancourt *et al.* 1993; Clarke 1996). If fertilisation occurs during this period,

pregnancy may develop. If no fertilisation occurs, however, the luteal phase of the cycle commences.

After ovulation, luteinisation of granulosa and theca cells of the follicle occurs. The resulting structure, called the corpus luteum, secretes the high concentrations of the sex steroid progesterone which characterise the luteal phase of the cycle. A small amount of oestrogen is also produced (Driancourt *et al.* 1993). Progesterone has negative feedback actions at the hypothalamus to decrease the frequency of GnRH pulses during this phase of the cycle (Karsch *et al.* 1987; Clarke 1995a; Caraty & Skinner 1999a). Exposure to progesterone during the luteal phase is also necessary for the display of oestrous behaviour in sheep (Fabre-Nys *et al.* 1994), and the amplitude of the GnRH surge is increased by a period of progesterone priming (Caraty & Skinner 1999b).

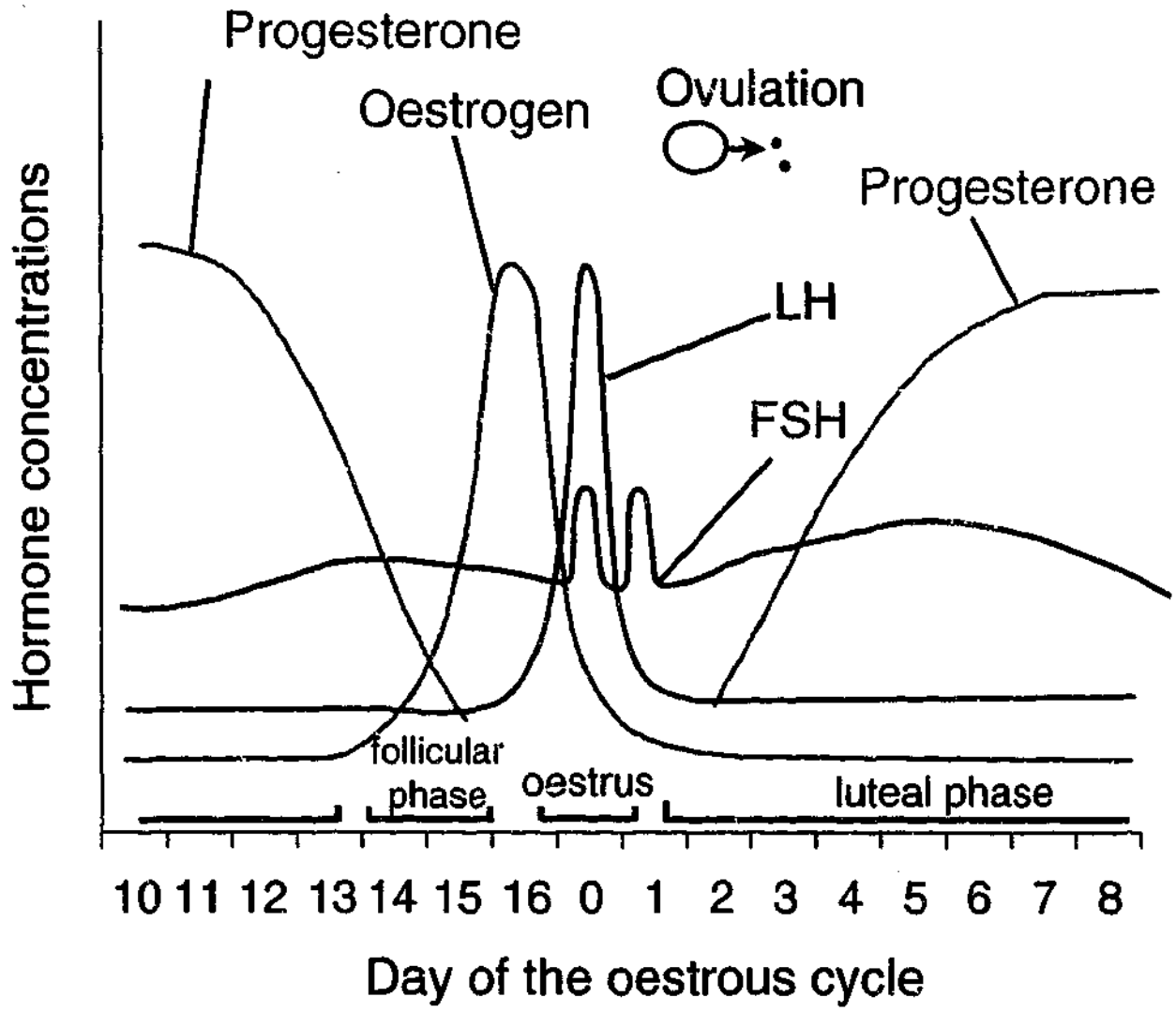


Figure 2.4

A schematic representation of the major events of the oestrous cycle of the ewe. The commencement of oestrus is marked by a surge in luteinising hormone (LH) and a small increase in follicle stimulating hormone (FSH) secretion, which are both triggered by the high concentration of oestrogen in the late follicular phase. The LH surge stimulates the ovulation of at least one mature follicle from the ovary. A high concentration of progesterone and a low concentration of oestrogen are typical of the luteal phase of the oestrous cycle. See text for further details. (Adapted from Turner (1998) with permission).

2.2.5. Reproduction in the male

In the ram, the gonadotrophins stimulate the process of spermatogenesis and the production of testosterone within the testis (Tilbrook & Clarke 2001). The production of mature sperm occurs within the seminiferous tubules of the testis as a result of complex interactions with sertoli cells which line the tubule. The function of the sertoli cells is stimulated by FSH (Maddocks *et al.* 1995). Sertoli cell function is also dependent on

testosterone which is produced by leydig cells which lie outside the seminiferous tubules and respond to stimulation by LH. Testosterone also has an important role in supporting the structure and function of accessory sex organs, such as the prostate and seminal vesicles, and in the expression of male behaviour necessary for reproduction to occur (Crichton *et al.* 1991).

Negative feedback effects of the glycoprotein hormone, inhibin, and testosterone, regulate the hypothalamo-pituitary testicular axis. Inhibin is produced by the sertoli cells and has negative feedback effects on FSH secretion from the gonadotrophs (Tilbrook & Clarke 2001). Testosterone may synergise with inhibin in its negative feedback effects (Tilbrook *et al.* 1993). Negative feedback effects of testosterone occur at the hypothalamus to decrease the frequency of GnRH pulses (Jackson *et al.* 1991; Tilbrook *et al.* 1991; Hileman *et al.* 1996). Effects of testosterone on pituitary responsiveness to GnRH are minimal in the breeding season but significant in the non-breeding season (Tilbrook *et al.* 1993). In target tissues, testosterone is reduced to the more potent androgen, 5α-dihydrotestosterone, or aromatised to form oestrogen. The relative role of each of these hormones in regulating the activity of the hypothalamo-pituitary testicular axis is unclear, although evidence suggests that all three are potentially important in negative feedback (Tilbrook & Clarke 1995; Tilbrook & Clarke 2001).

2.3. The Effect of Stress on the Reproductive System

There are several contexts within which the suppression of reproduction due to stress can be discussed. In the farming of domestic animals, stress can occur as a result of animal husbandry procedures, leading to a decrease in fertility (Moberg 1991; Dobson & Smith 1998; Dobson *et al.* 2001). Within the human context, stress can be caused by a variety of lifestyle factors and lead to temporary or permanent infertility. From an evolutionary perspective, it has traditionally been considered that the suppression of the reproductive system during times of stress prevents the investment of energy in breeding when the chances of successful reproduction are not optimal. Thus, a selective advantage is gained for the adult individual in that the chances of survival are increased by the conservation of resources during a stressful period. There is also a selective benefit to the species in that there is an increase in the potential for survival for the offspring if its development and

maturation occurs during a period when conditions are optimal (Moberg 1985a). Recently, this theory has been expanded upon by Wingfield and Sapolsky (2003) who have suggested that, under conditions when opportunities for reproduction are low, it is evolutionarily beneficial for the reproductive system to be resistant to stress. Examples include species which are semelparous, ageing individuals in which further opportunities for reproduction are already limited, species in which stress is induced by competition for access to females, and species which have a very short breeding season (Wingfield & Sapolsky 2003).

Studies on the effects of stress on reproduction vary widely in the choice of stressor imposed and the animal model used. It is likely that both variables are important in determining the effect of stress on the reproductive parameter that is measured. As a stressor that is primarily psychological and physical in nature (isolation and restraint stress) has been used in the experiments presented in this thesis (Chapter 6 and Chapter 7), an attempt has been made to focus on studies using similar stressors in this review. Stressors which are principally physiological, such as an immune stress or insulin-induced hypoglycaemia, activate many physiological systems. Therefore, effects on reproduction induced by these stressors are more difficult to interpret in the context of classical physiological systems activated by stress.

The effect of stress on reproduction has been reviewed and investigated extensively (Moberg 1985b; Liptrap 1993; Dobson & Smith 1995; Dobson & Smith 1998; Dobson & Smith 2000; Tilbrook *et al.* 2000; Tilbrook *et al.* 2002a; Wingfield & Sapolsky 2003). In more recent times, it has become apparent that sex has an important role in determining how the reproductive system is affected by stress. The endocrine environment provided by the gonadal steroids also seems to be important in determining the effect of stress on the reproductive system (Ferin 1999; Tilbrook *et al.* 2000; Tilbrook *et al.* 2002a).

2.3.1. The effect of stress on reproductive end points

Behavioural oestrus and ovulation in females have been measured many times as reproductive endpoints and the timing of these two events so that they are coincidental is important for successful reproduction. In ewes, stress caused by daily wetting for six hours throughout an entire oestrous cycle and then for approximately 25 days after mating was

associated with a decrease in mean ovulation rate and an increase in embryo loss, but no delay in the onset of oestrus (Griffiths *et al.* 1970). Further studies by Doney *et al.* (1976) showed that imposition of a similar stress during the late follicular phase of the oestrous cycle again did not delay the onset of behavioural oestrus, but increased the time interval between the onset of behavioural oestrus and the occurrence of the LH surge. There was also a decreased ovulation rate. Finally, in a study comparing the effects of the same stress imposed during the luteal phase, the late follicular phase or throughout one continuous oestrous cycle, an increased incidence of delayed oestrus was observed in ewes that had been exposed to the stress during the late follicular phase or throughout the entire oestrous cycle, but not in ewes who had been exposed to the stress during the luteal phase. A decrease in ovulation rate was also observed in ewes which had been exposed to stress during the late follicular phase. The authors suggested that inconsistencies between the studies in the effect of the stressor imposed during the follicular phase on the onset of oestrus may have been due to variations in intensity of the stressor, or in the relative tameness of the animals used (Doney *et al.* 1976). More importantly, however, this work also suggests that the timing of the imposition of the stressor in relation to the oestrous cycle may be important in determining the capacity of the stressor to delay ovulation (Doney *et al.* 1973). Changes in the response of the hypothalamo-pituitary adrenal axis to stress are observed throughout the oestrous cycle (Section 2.1.4). This may alter the resistance of the reproductive system to stress throughout the cycle. Alternatively, the imposition of the stressor may be more detrimental to the developing follicle during the follicular phase rather than in the luteal phase. Stress imposed during the follicular phase may lead to a reduction in the production of oestrogen and therefore a delay in the onset of oestrous behaviour and the LH surge.

Extensive work in ewes using transport as a stressor provides evidence of an effect of stress on the LH surge. Phogat *et al.* (1999) showed that in ewes exposed to transport stress, there was a delay in the onset of the oestradiol-induced LH surge. The duration of the surge was also decreased. This observation was dependent on the timing of the stress relative to treatment with oestradiol. Further studies examining the effects of transport stress on the LH surge showed that in intact ewes with synchronised oestrous cycles, or in ovariectomised ewes with artificial follicular phases created by the exogenous

administration of oestrogen and progesterone, there was a delay in the LH surge during imposition of four hours of transport stress (Dobson *et al.* 1999). In both groups, there was also a decrease in the maximum plasma concentration of LH measured during the LH surge. In ovariectomised ewes with an artificial follicular phase, however, the delay in the LH surge was significantly less than that observed in intact ewes, and there was a bigger decrease in the LH surge in ovariectomised ewes with an artificial follicular phase than in the intact ewes (Dobson *et al.* 1999). These studies suggest that the effect of stress on the reproductive axis is highly sensitive to the endocrine environment provided by the sex steroids. In support of this, transport stress delayed the onset, but not the amplitude, of the oestradiol-induced-LH surge in ewes that were 14 days postpartum (Smart *et al.* 1994). This effect was not observed, however, in ewes that were 28 days postpartum. In cows, stress has been shown to delay the onset of the LH surge (Stoebel & Moberg 1982b), and to increase the time from calving to conception (Dobson & Smith 2000). In rats, restraint stress imposed for 3 days and commencing on day 1 of diestrus, was associated with a delay in the expected detection of oestrus by 3 days (Euker & Riegle 1973).

The effects of stress on reproductive end points such as oestrous behaviour, the LH surge, ovulation in females and spermatogenesis in males, may be due to changes in the regulation of the reproductive system by the hypothalamo-pituitary unit. Although changes in the activity of the gonads are also possible under conditions of stress, they are not the focus of this thesis and will not be discussed in detail. Studies investigating the effect of stress on the hypothalamo-pituitary unit have examined changes in concentrations of LH as an indicator of changes in reproductive function. It is possible that perturbations in LH secretion during the ovulatory cycle in females may result in a disruption to follicular development, and ultimately ovulation and oestrous behaviour, as discussed above. In the same way in males, disturbances in LH secretion from the hypothalamo-pituitary unit may result in disturbances in spermatogenesis and reproductive behaviour, although the amount of experimental evidence supporting this is much less than that found for females.

2.3.2. The effect of stress on plasma concentrations of LH

Numerous studies in sheep have shown that stress has the capacity to decrease mean plasma concentrations of LH. In gonadectomised rams and ewes, there was a significant

decrease in mean plasma concentrations of LH during isolation and restraint stress (Tilbrook *et al.* 1999a; Turner *et al.* 2002a). This effect was not observed in gonad intact rams and ewes, however (Turner *et al.* 2002a), suggesting that the effect of this stressor on the reproductive axis is dependent upon the sex steroids. Confinement stress has also been shown to decrease plasma concentrations of LH in ovariectomised ewes (Rasmussen & Malvern 1983). The degree of suppression, however, was decreased after two days of repeated exposure to the stressor which, the authors suggest, was due to habituation of the animals to the stressor.

In female Rhesus Macaques, restraint stress imposed in the follicular phase caused a significant decrease in mean plasma concentrations of LH, while the same stressor imposed during the luteal phase had no effect (Norman *et al.* 1994). In another study, however, when a combined physical, psychological and surgical stress was imposed on Rhesus Macaques in the luteal phase of the ovulatory cycle, there was a decrease in plasma concentrations of LH and progesterone, with no effect observed on ovulation (Xiao *et al.* 2002). Similar to studies on the effect of wetting stress on the onset of oestrus in ewes (Doney *et al.* 1973), these results suggest that the reproductive system may be more sensitive to exposure to a principally psychological and physical stressor during the follicular phase than during the luteal phase. This may not be true, however, for stressors such as a surgical stress, which have a more physiological component. Alternatively, decreases in the plasma concentrations of LH during the luteal phase may not necessarily result in negative effects on oestrus and ovulation, or may result in impairment of oestrus behaviour, but not ovulation. Further investigation of the effects of the combined physical, psychological and surgical stress on LH secretion in Rhesus Macaques showed that the stressor was also associated with a decrease in mean LH and progesterone concentrations in the cycle following that in which the stressor was imposed (Xiao *et al.* 2002). This suggests that the effects of a stressor imposed during one ovulatory cycle may also compromise subsequent ovulatory cycles. In male Rhesus Macaques, the imposition of restraint stress has been associated with a significant decrease in mean plasma concentrations of LH and testosterone (Norman & Smith 1992).

In rats, it seems that acute stress may cause an acute increase in plasma concentrations of LH. Exposure to ether in ovariectomised females (Ajika *et al.* 1972) and in gonad intact males (Krulich *et al.* 1974; Euker *et al.* 1975), restraint or an intraperitoneal injection of saline in gonad intact males (Krulich *et al.* 1974), handling stress, serial blood sampling and serial anaesthesia in males (Euker *et al.* 1975) and forced muscular exercise in gonad intact females (Du Ruisseau *et al.* 1978), have all been associated with an acute increase in the plasma concentrations of LH. The physiological significance of an acute increase in plasma concentrations of LH is unknown. Ferin (1999) suggests, however, that an inappropriate increase in LH may be just as detrimental to reproductive end points as an inappropriate decrease in LH. In some studies, the change in LH appears to be biphasic, as restraint or an intraperitoneal injection of saline was shown to be associated with an acute increase in LH, followed by a significant decrease in LH in male rats (Krulich *et al.* 1974). A similar response has been observed in female rats during forced muscular exercise (Du Ruisseau *et al.* 1978). Further to this, Euker *et al.* (1975) showed that the decline in LH was more rapid in castrated rats than in male gonad intact rats, suggesting the possibility of an effect of testosterone on the effect of stress on the secretion of LH. Other studies which have examined the effect of a chronic stress on reproduction in the rat report a corresponding decrease in LH. Six hours of immobilisation stress was associated with a decrease in plasma concentrations of LH in female rats. A significant effect of this stressor, or of forced exercise, was not observed in male rats (Du Ruisseau *et al.* 1978), suggesting a sex difference in the effect of this stressor on LH. This is not supported, however, by studies by other investigators which showed that restraint stress was associated with a decrease in plasma concentrations of LH in male rats (Taché *et al.* 1980; Ariznavarreta *et al.* 1989; Gonzaléz-Quijano *et al.* 1991).

As the secretion of LH from the pituitary is regulated by stimulation by GnRH secretion from the preoptic area and hypothalamus, and also by the responsiveness of the pituitary gland itself to stimulation by GnRH, it is likely that stress-induced alterations in LH secretion may arise from changes in hypothalamic and/or pituitary function. In recent years, efforts have been focused on dissecting the sites of action at which the stress response causes a change in the secretion of LH. Direct measurement of GnRH as an indicator of changes in hypothalamic function and measurement of LH in response to the

exogenous stimulation of GnRH are two approaches that have been used to identify hypothalamic or pituitary sites of action. Another useful approach in delineating the sites of action at which the stress response suppresses LH is the analysis of the pulsatile secretion of LH. As the frequency of LH pulses is determined by the frequency of GnRH pulses, changes in this parameter during the imposition of stress are thought to be indicative of changes in the frequency of GnRH pulses and, therefore, a hypothalamic site of action. Changes in LH pulse amplitude, however, may occur as a result of changes in the amplitude of GnRH pulses from the hypothalamus and/or a change in the responsiveness of the gonadotroph to stimulation by GnRH, and therefore may indicate a site of action at the hypothalamus and/or the pituitary.

2.3.3. The effect of stress on the secretion of GnRH

In the sheep, an effect of psychological or physical stress on GnRH secretion from the hypothalamus has been suggested by studies of the pulsatile secretion of LH. In gonadectomised rams treated with testosterone, ovariectomised ewes, ovariectomised ewes treated with oestrogen and ovariectomised treated with progesterone, LH pulse frequency was reduced during isolation and restraint stress (Tilbrook *et al.* 1999a). No changes in LH pulse frequency were observed in gonadectomised rams and ovariectomised ewes treated with a combination of oestrogen and progesterone, highlighting both a sex difference and an effect of the sex steroids on the susceptibility of LH pulse frequency to stress.

Studies in rats suggest a biphasic response of GnRH secretion to acute stress. After 20 or 45 minutes of restraint stress imposed on adult male rats, there was a decrease in the hypothalamic content of GnRH measured in tissue homogenate (López-Calderón *et al.* 1989). Given the time after the onset of the stressor, and the corresponding increase in plasma concentrations of LH, these results suggest that there was an acute increase in the secretion of GnRH during stress. Chronic restraint stress, however, was associated with a decrease in plasma concentrations of LH and a corresponding decrease in hypothalamic content of GnRH, suggesting a decrease in the hypothalamic drive of LH secretion (López-Calderón *et al.* 1989). Other studies have also demonstrated a reduced hypothalamic content of GnRH after exposure to isolation and restraint stress (Gonzalez-Quijano *et al.*

1991). Other types of stressors have been shown to have an effect on GnRH secretion in sheep, primates and rats (Section 2.3.5).

2.3.4. The effect of stress on anterior pituitary responsiveness to GnRH

Although previous studies have examined changes in the pituitary responsiveness to GnRH during stress, interpretation of these data are limited by the fact that the studies use an *in vivo* model in which uncontrolled, endogenous stimulation of the pituitary still occurs. Thus, changes in responsiveness of the pituitary gland may also be due to a change in endogenous GnRH secretion. This is also true of measurements made of LH pulse amplitude. Nevertheless, changes in the responsiveness of the pituitary gland to exogenous GnRH stimulation have been suggested in several species during the imposition of physical or physiological stressors. Rams exposed to restraint stress showed decreased secretion of LH in response to treatment with exogenous GnRH (Matteri *et al.* 1984). It seems likely that there may be a sex difference by which this stressor impacts on pituitary sensitivity, as it was shown that isolation and restraint stress reduced the LH response to a single injection of GnRH in gonadectomised rams but not ewes (Tilbrook *et al.* 1999a). Transport stress may also induce changes in pituitary responsiveness to GnRH as Dobson *et al.* (1999) showed that LH secretion was reduced in response to exogenous GnRH in ewes during transport.

Although not definitive, decreases in LH pulse amplitude may also suggest changes in pituitary responsiveness to GnRH. LH pulse amplitude was unaffected in gonadectomised ewes or gonadectomised ewes treated with progesterone, but was reduced in ewes treated with oestrogen or oestrogen and progesterone (Tilbrook *et al.* 1999a), indicating that the presence of oestrogen may determine changes in LH pulse amplitude during isolation and restraint stress. Decreases in LH pulse amplitude during isolation and restraint stress were also observed in gonadectomised rams but not in gonadectomised rams treated with testosterone (Tilbrook *et al.* 1999a), suggesting an effect of testosterone on the parameters of pulsatile LH secretion affected by stress. Inherent sex differences, independent of the sex steroids, are also suggested by this study (Tilbrook *et al.* 1999a). A further role for the sex steroids was suggested in a study by Phogat *et al.* (1999), which showed that in intact ewes in the follicular phase, there was a decrease in LH pulse amplitude during transport

stress which was not observed when ewes were ovariectomised and treated with oestrogen and progesterone to induce a synthetic follicular phase, again highlighting that the sex steroids may be important in determining the effect of stress on the parameters of pulsatile LH secretion.

Species differences in the effect of psychological stress on pituitary responsiveness may exist. In the male wild baboon, there was no change in the pituitary response to challenge with GnRH during the imposition of six hours of isolation stress, suggesting no effect of the stressor on pituitary secretion of LH. There was, however, a decrease in the measured bioactivity of LH, suggesting that although plasma concentrations of LH remained unchanged, there was some modification in LH which reduced its bioactivity (Sapolsky 1985). The mechanisms by which this may occur are unknown. In contrast, in rats which had been exposed to four days of restraint stress, the LH response to GnRH was significantly higher in stressed than in non-stressed animals (López-Calderón *et al.* 1989), suggesting an increase in pituitary responsiveness to GnRH.

2.3.5. Physiological stressors and their effects on the hypothalamo-pituitary gonadal axis

Several physiological stressors have been shown to have inhibitory effects on the reproductive axis. In the ewe, hypoglycaemia induced by treatment with insulin was associated with a delay in the onset of the LH surge (Dobson & Smith 2000). In castrated rams with or without oestrogen treatment, insulin-induced hypoglycaemia reduced LH pulses (Adam & Findlay 1998). Hypoglycaemia has also been shown to be associated with a decrease in LH secretion in ovariectomised Rhesus Macaques (Heisler *et al.* 1994). A hypothalamic effect of hypoglycaemia is suggested by a study in monkeys, which showed that insulin-induced hypoglycaemia was associated with a decrease in the electrical activity in GnRH neurones (Chen *et al.* 1996). This is supported by a study in oestrogen treated rats which showed a decrease in LH pulse frequency during hypoglycaemia (Cates *et al.* 1999). There may be species differences in the mechanisms by which the effects of hypoglycaemia on LH secretion are mediated. In Rhesus Macaques in the follicular phase (Lado-Abeal *et al.* 2002), and in ovariectomised monkeys that were also restrained (Heisler *et al.* 1993), opioid antagonists did not reverse the effect of insulin-induced hypoglycaemia on LH secretion, suggesting that in primates, opioids may not have a role in mediating the

suppression of LH in response to this stressor. Several studies suggest that in rats, however, the effect of insulin-induced hypoglycaemia to reduce LH secretion is mediated by opioids. In gonadectomised male rats, insulin-induced hypoglycaemia was associated with a decrease in the secretion of LH, an effect which was reversed by treatment with an opioid antagonist (Goubillon & Thalabard 1996). Similar results have been obtained in ovariectomised rats (Cagampang *et al.* 1997).

It is not clear whether the sex steroids modulate changes in the response of the reproductive system to insulin-induced hypoglycaemia. In castrated rams, the presence of oestrogen had no effect on the suppression of LH pulses which occurred during hypoglycaemia (Adam & Findlay 1998). Ovariectomised Rhesus monkeys, however, have been shown to be more sensitive to the decrease in GnRH pulse generator activity induced by insulin-induced hypoglycaemia than ovary-intact monkeys or ovariectomised monkeys receiving oestrogen replacement (Chen *et al.* 1992). In ovariectomised rats, insulin-induced hypoglycaemia had no effect on LH secretion, but was associated with a suppression in LH secretion when the rats received oestrogen treatment (Cagampang *et al.* 1997). In contrast, the results of Li *et al.* (2003) showed that insulin-induced hypoglycaemia was associated with a suppression of LH secretion in ovariectomised rats. This group also showed that the effect of hypoglycaemia was potentiated by oestrogen, and further potentiated by oestrogen and progesterone. In the oestrogen-treated ovariectomised rat, removal of the area postrema was associated with an inhibition of the suppressive effect of insulin-induced hypoglycaemia on LH secretion (Cates & O'Byrne 2000), suggesting that this area may be important for mediating the effect of the stressor itself, and/or the sensitising effects of oestrogen.

The administration of bacterial endotoxin, or lipopolysaccharide, is another stressor which has been used to investigate the effects of stress on reproduction. In ewes, administration of endotoxin inhibited GnRH pulse amplitude and LH pulse frequency, suggesting a hypothalamic site of action of this stressor (Battaglia *et al.* 1997). LH pulse frequency was also reduced during treatment with endotoxin in prepubertally castrated rams (Coleman *et al.* 1993). Further studies showed that in ovariectomised ewes in which an artificial follicular phase was created by treatment with oestrogen and progesterone, endotoxin

treatment was associated with the prevention of the GnRH surge (Breen *et al.* 2004). Endotoxin treatment was also associated with a suppression in the amplitude of LH pulses arising from treatment with exogenous GnRH in anoestrous ewes treated with progesterone (Williams *et al.* 2001), while *in vitro* studies showed endotoxin was associated with an increase in the secretion of LH from dispersed sheep anterior pituitary cells (Coleman *et al.* 1993). In combination, these results suggest that immune stressors, such as endotoxin, may have both hypothalamic and pituitary sites of action. Further studies using ovine pituitary cells showed that the effect of endotoxin on the pituitary may be mediated by interleukin 1α and 1β (Braden *et al.* 1998). In female Rhesus monkeys, treatment with lipopolysaccharide and interleukin 1α was associated with a decrease in the plasma concentrations of LH. The effects of lipopolysaccharide on LH, however, were not reversed by an interleukin 1α receptor antagonist, and were reversed by an opiate antagonist, suggesting that, in primates, the effects of lipopolysaccharide may be mediated by the opioids but not interleukin 1α (Xiao *et al.* 2000). Central effects of immune stressors on LH secretion are also suggested in rats, as LH pulse frequency was suppressed in rats treated with lipopolysaccharide (Watanobe & Habu 2003).

2.4. Possible Mediators of the Effect of Stress on Reproduction

Any of the numerous systems which are activated during the response to a stressor have the potential to trigger a suppression of reproductive function. As the focus of this thesis is the role of the glucocorticoids, and to a lesser extent, the sympathoadrenal system in the stress-induced suppression of LH, these systems have been considered in detail in the following sections.

2.4.1. The hypothalamo-pituitary adrenal axis

The association between reproductive dysfunction and an increase in the activity of the hypothalamo-pituitary adrenal axis implicates this endocrine system as a suppressor of reproduction during stress. This association has been demonstrated in many species including sheep (Battaglia *et al.* 1999; Tilbrook *et al.* 1999a; Williams *et al.* 2001; Debus *et al.* 2002; Turner *et al.* 2002a), bulls (Welsh & Johnson 1981) and primates (Loriaux & Nieman 1990; Berga 1995; Meczekalski *et al.* 2000). The effects of the hypothalamo-pituitary adrenal axis on reproduction have been extensively reviewed in domestic species (Moberg 1991; Dobson & Smith 1995; Turner *et al.* 2002b), non-rodent species (Tilbrook *et al.* 2000;

Tilbrook *et al.* 2002a), humans (Loriaux & Nieman 1990) and rodents (Rivier & Rivest 1991). It is possible that elevation of the plasma concentrations of the glucocorticoids, ACTH, AVP and/or CRF may mediate any suppressive effects of the hypothalamo-pituitary adrenal axis on the reproductive system.

2.4.2. CRF and AVP as inhibitors of the reproductive system

It is unclear whether CRF and AVP are involved in the stress-induced suppression of reproduction, particularly through centrally mediated mechanisms. In ovariectomised ewes, intravenous or intracerebroventricular treatment with CRF did not affect LH secretion (Clarke *et al.* 1990). The combined administration of CRF and AVP also had no effect on LH secretion. These results are supported by those of Caraty *et al.* (1997), which showed that there was no effect of CRF on LH secretion in ovariectomised ewes, but in ovariectomised ewes treated with oestrogen and progesterone, there was a decrease in LH pulse frequency. In contrast, Naylor *et al.* (1990) found that intracerebroventricular administration of CRF into ovariectomised ewes was associated with an increase in LH pulse frequency. Direct infusion of CRF into the cerebral ventricles of castrated rams has also been shown to cause an increase in mean plasma concentrations of LH and an increase in LH pulse amplitude (Tilbrook *et al.* 1999b). An increase in LH secretion in response to CRF has also been observed in castrated rams treated with testosterone (Caraty *et al.* 1997). This effect was independent of whether or not the rams were treated with testosterone and independent of whether or not AVP was also administered. In ovariectomised Rhesus monkeys, intravenous infusion of CRF was associated with a significant decrease in concentrations of LH (Gindoff & Ferin 1987; Olster & Ferin 1987; Xiao & Ferin 1988; Xiao *et al.* 1989). Contemporaneous treatment with the opioid antagonist naloxone prevented the suppression in LH secretion (Gindoff & Ferin 1987). Further studies showed that this effect was still observed in ovariectomised Rhesus Macaques that were adrenalectomised, suggesting that the effect of CRF was not mediated by the resulting increase in cortisol (Xiao *et al.* 1989). The effect was dependent, however, on the plasma concentrations of cortisol achieved with cortisol replacement therapy in these animals, suggesting that basal concentrations of cortisol may play an important role in determining the ability of CRF to affect plasma concentrations of LH. In contrast to ovariectomised Rhesus Macaques, there was no effect of an intravenous infusion of CRF on LH secretion

in Macaques in the luteal or follicular phase of the cycle (Norman 1994), suggesting that the gonadal status or presence of the sex steroids is also an important determinant of the effect of CRF on LH secretion. However, in ovariectomised Rhesus Macaques, there was a decrease in the frequency of the electrical activity of GnRH neurones after treatment with CRF (Williams *et al.* 1999), suggesting that there may be an effect of CRF on LH secretion in ovariectomised Macaques and that this is mediated through changes in the activity of GnRH neurones in the hypothalamus. Studies in humans also suggest that CRF and AVP may suppress reproductive function at the hypothalamic level (Ferin 1999).

In male rats, *in vitro* studies showed that treatment with CRF reduced the release of GnRH from the mediobasal hypothalamus and the median eminence (Gambacciani *et al.* 1986), suggesting that there may also be an effect of CRF on GnRH secretion in the rat. This is supported by a study in female rats which showed that treatment with CRF decreased the basal concentrations of GnRH measured in hypophysial portal blood and also blocked the GnRH surge (Petraglia *et al.* 1987). Further studies suggested that GnRH secretion may have been sensitised to the actions of CRF by the presence of oestrogen (Petraglia *et al.* 1987). Other evidence from the rat also shows that a direct inhibitory neural pathway from the paraventricular nucleus of the hypothalamus to the testis is activated by CRF (Lee *et al.* 2002; Selvage & Rivier 2003), suggesting a direct mechanism by which CRF may decrease testicular function and reduce plasma concentrations of testosterone during stress.

AVP may also have a role in the stress-induced suppression of LH secretion, although further investigation is required to look at sex differences and the role of the sex steroids in each species. In rams, administration of AVP alone had no effect on plasma concentrations of LH (Tilbrook *et al.* 1999b). In ovariectomised Rhesus Macaques, however, LH secretion was reduced during intracerebroventricular administration of AVP (Shalts *et al.* 1994; Heisler *et al.* 1994). Further studies suggested that AVP may have a role in mediating the hypoglycaemia-induced suppression of LH secretion (Heisler *et al.* 1994). In ovariectomised rats, intracerebroventricular administration of AVP was associated with a decrease in LH pulse amplitude, but no change in LH pulse frequency. This effect was blocked by central administration of an AVP antagonist and only occurred in rats that were also treated with oestrogen (Cates *et al.* 1999), suggesting a sex steroid dependent

mechanism of action in the female rat. The administration of AVP antiserum into ovariectomised female rats exposed to ether reduced the increase in LH secretion which occurred in response to the stressor (Ono *et al.* 1985), suggesting that AVP may have a role in mediating the effect of this stressor on LH secretion. These results were supported by another study in ovariectomised female rats which showed that AVP secretion stimulated basal LH secretion but blocked the LH surge (Salisbury *et al.* 1980).

2.4.3. ACTH as an inhibitor of the reproductive system

There is strong evidence for a role of ACTH in mediating the stress-induced suppression of reproduction. ACTH delayed the oestradiol-induced LH surge in ewes, although this effect was dependent upon the time that ACTH was administered in relation to oestradiol treatment (Dobson *et al.* 1988). ACTH has also been shown to reduce LH secretion in response to exogenously administered GnRH in intact ewes (Dobson *et al.* 1988) and in gonadectomised rams (Tilbrook *et al.* 1999a), suggesting an effect on pituitary responsiveness. Studies in anoestrous ewes showed that the observable effect of ACTH on pituitary responsiveness was dependent upon the timing of the treatment with GnRH in relation to treatment with ACTH. That is, when ACTH was administered 3 hours, but not 30 minutes before treatment with GnRH, there was a decrease in pituitary LH secretion in response to GnRH (Dobson *et al.* 1988), raising the possibility that the effects of ACTH on LH secretion may be through a cortisol dependent mechanism (Section 2.4.4). However, when intact rams of mixed breed were treated with ACTH and then with GnRH, it was shown that ACTH reduced the LH response to GnRH treatment and that this still occurred regardless of whether or not the animals were adrenalectomised (Rivier & Rivest 1991), suggesting that the effect of ACTH is not mediated by cortisol. In heifers, infusion of ACTH for three days during the follicular phase of the oestrous cycle caused a delay in the onset of the LH surge and behavioural oestrus (Stoebel & Moberg 1982a). Basal LH and oestrogen concentrations were also suppressed in these animals, while progesterone concentrations were increased. In contrast, Li and Wagner (1983) showed that ACTH treatment given to intact heifers during the luteal phase of the cycle had no effect on basal concentrations of LH. In this model, however, ACTH did reduce the pituitary response to GnRH. These results are supported by Matteri & Moberg (1982a), who showed that there was no effect of ACTH on basal LH secretion but there was a decrease in the LH response

to treatment with GnRH after treatment with ACTH. An effect of ACTH on pituitary responsiveness to GnRH, however, was not observed in bovine pituitary cell culture (Padmanabhan *et al.* 1983). In steers and bulls, ACTH treatment had no effect on LH secretion in response to GnRH (Barnes *et al.* 1983). Testosterone secretion was reduced, however, in bulls treated with ACTH, but not in bulls treated with ACTH and GnRH, suggesting a direct effect of ACTH or cortisol on the testis. In ovariectomised Rhesus monkeys, infusion of ACTH had no effect on the pulsatile secretion of LH (Xiao & Ferin 1988). In male Rhesus monkeys, repeated but not acute administration of ACTH caused a decrease in basal concentrations of LH, with no corresponding decrease in plasma concentrations of testosterone (Hayashi & Moberg 1987). There was also no change in LH secretion in response to exogenously administered GnRH, while there was a decrease in testosterone secretion in response to GnRH after both acute and chronic treatment with ACTH.

2.4.4. The glucocorticoids

Although there is a large body of research investigating glucocorticoid-induced suppression of the reproductive system, the effects of glucocorticoids on reproduction are not clearly understood. The elevation of plasma concentrations of glucocorticoids during stress makes them a candidate for involvement in the stress-induced interruption of reproductive function. The possibility that they induce suppression of LH secretion at the hypothalamo-pituitary adrenal unit is reviewed below. While changes in gonadal function during cortisol treatment are suggested in the bovine ovary (Kawate *et al.* 1993), bovine testis (Barnes *et al.* 1983), male Rhesus monkeys (Hayashi & Moberg 1987) and in male rats (Welsh *et al.* 1982), they are not considered in detail in the following review.

2.4.5. The effects of the glucocorticoids on reproductive endpoints

Treatment of ewes with cortisol from days 13 to 17 of the oestrous cycle (Doney *et al.* 1976), or with cortisol for five days either during the luteal or follicular phases, or for ten days during both phases (MacFarlane *et al.* 2000), has been shown to delay the LH surge. In ewes, the increase in serum concentrations of oestradiol which occurs after the removal of progesterone was blocked or reduced by treatment with cortisol. The LH surge was also blocked or delayed in these animals (Daley *et al.* 1999a). In heifers, cortisol treatment for three and a half days during the follicular phase of the oestrous cycle inhibited the onset of

oestrous behaviour and delayed the LH surge, while causing an increase in basal concentrations of LH (Stoebel & Moberg 1982a). Nevertheless, neither treatment with the synthetic glucocorticoid, dexamethasone, nor cortisol, for 90 hours during an artificially induced follicular phase, blocked the oestrogen-induced LH surge in the anoestrous ewe (Moberg *et al.* 1981). Phillips & Clarke (1990) found that chronic dexamethasone treatment during the luteal and follicular phase had no significant effect on ovulation or on ovulation rates in ewes where ovulation occurred naturally during a synchronised oestrous cycle, or in ewes treated with pregnant mare serum gonadotrophin to induce ovulation. It is possible that differences in the endocrine manipulations undertaken to generate LH surges in these animal models account for the differences reported in the literature. Cortisol treatment successfully resulted in a delay in the LH surge in ewes and heifers when their oestrous cycles were synchronised with either progesterone or a synthetic progestin (Doney *et al.* 1976; Stoebel & Moberg 1982a; MacFarlane *et al.* 2000; Daley *et al.* 1999a), but not in ewes where the LH surge was induced by treatment with oestrogen only (Moberg *et al.* 1981). Furthermore, the use of dexamethasone may not be a reliable model for studying the stress-induced suppression of LH as dexamethasone is a synthetic glucocorticoid and has different binding affinity for the glucocorticoid and mineralocorticoid receptors than the endogenous glucocorticoids (de Kloet *et al.* 1975). Nevertheless, in rats receiving twice daily injections of dexamethasone on one or both days of diestrus, or on the second day of diestrus and the first day of proestrus, ovulation was delayed (Baldwin & Sawyer 1974).

2.4.6. The effects of glucocorticoids on plasma concentrations of LH

Effects of elevated concentrations of the glucocorticoids on the hypothalamo-pituitary unit have been identified through measurement of changes in plasma concentrations of LH. In ovariectomised ewes, there was a decrease in mean serum concentrations of LH during treatment with cortisol in the non-breeding season (Debus *et al.* 2002). Phillips & Clarke (1990), however, showed that in ovariectomised ewes treated with dexamethasone in either the breeding or the non breeding season, there was no change in mean plasma concentrations of LH, or on mean LH pulse frequency or LH pulse amplitude. In rams castrated before puberty, there was a suppression in basal LH during continuous infusion of cortisol for seven days (Daley *et al.* 1999b). In postpubertal bulls, after a single injection with dexamethasone there was an immediate decrease in mean plasma concentrations of

LH and testosterone for two days following the injection (Thibier & Rolland 1976). This dose of dexamethasone was approximately 10 times higher than the highest dose used by Phillips & Clarke (1990). In adrenalectomised heifers, there was no change in the basal secretion of LH during treatment with cortisol during the luteal phase of the oestrous cycle (Li & Wagner 1983).

Elevated plasma concentrations of the glucocorticoids may also be capable of suppressing LH secretion in primates. In women, there was a decrease in mean serum concentrations of LH during 8 to 10 days of cortisol treatment commencing during the late follicular phase of the menstrual cycle. There was no effect of the treatment on LH in the luteal phase of the following cycle and no disturbance in the onset of the following menses (Saketos *et al.* 1993). Samuels *et al.* (1994), however, found that in six men and in four women in the early follicular phase of their menstrual cycle, infusion of cortisol at two doses for 24 hours had no effect on mean LH or any of the LH pulse parameters that were also measured. In the wild male baboon, dexamethasone administration was associated with a decrease in plasma concentrations of testosterone (Sapolsky 1985). It is possible that this effect may be a result of actions of dexamethasone on the hypothalamo-pituitary unit. In castrated, male Rhesus monkeys, a prolonged infusion of cortisol has been shown to reduce mean serum concentrations of LH (Dubey & Plant 1985). This effect was also observed in gonad intact male Rhesus monkeys in which cortisol significantly lowered basal concentrations of LH and basal concentrations of testosterone (Hayashi & Moberg 1987).

In rodents, it seems possible that sex differences or the presence of the sex steroids, is important in determining glucocorticoid-induced suppression of LH. In female rats, during chronic treatment with cortisol, the mean serum concentration of LH was suppressed (Ringstrom *et al.* 1992). This was accompanied by disruption of the changes in vaginal cytology commonly observed throughout the oestrous cycle. In ovariectomised, immature rats, investigation of the effects of various natural and synthetic glucocorticoids on serum LH showed that cortisol decreased serum LH in a dose-dependent manner (Brann *et al.* 1990). Infusion of dexamethasone had a biphasic effect on LH concentrations with an increase and then decrease in LH concentrations observed as the dose of dexamethasone increased. Another synthetic glucocorticoid, triamcinolone acetonide, increased LH

concentrations at all doses. Further studies showed that the effects of triamcinolone acetonide were dependent on whether or not the rat had been previously treated with oestrogen. In gonad intact male rats, cortisol or corticosterone implants had no effect on basal secretion of LH, whereas in castrated males, during treatment with either cortisol or corticosterone, there was a suppression in mean basal plasma LH concentrations (Ringstrom & Schwartz 1985; Suter *et al.* 1988).

Several studies have examined the effects of the glucocorticoids on the increases in LH that are observed after gonadectomy. In a ten day period after ovariectomy, ewes given a concurrent treatment of oestradiol and cortisol showed no post-ovariectomy increase in mean plasma concentrations of LH or in LH pulse frequency. This was not observed in animals receiving either cortisol or oestradiol, suggesting that cortisol may affect the negative feedback actions of oestrogen (Breen *et al.* 1999). At 11.5 hours after castration in rats, Ringstrom & Schwartz (1985) showed that the post castration increase in LH was dependent upon the presence of the adrenal glands, and that in the absence of the adrenals, the increase in LH with the withdrawal of negative feedback by testicular steroids did not occur. These animals displayed responsiveness to GnRH which was the same as non-adrenalectomised animals, leading the authors to suggest that the adrenal glands were necessary for the increase in hypothalamic activity observed after castration, but not for the increase in pituitary responsiveness. These findings suggest that basal concentrations of cortisol may have an important role in normal feedback interactions within the hypothalamo-pituitary gonadal axis.

Some studies using an adrenalectomised animal model suggest that cortisol is not responsible for the stress-induced suppression of LH. Immobilisation stress imposed for six days caused a decrease in plasma concentrations of LH in male rats which was more pronounced when the animals were adrenalectomised (Taché *et al.* 1980). This study suggests that the glucocorticoids do not mediate the effects of this stressor on LH secretion, and that either stress-induced or basal concentrations of the glucocorticoids may have protective effects on the reproductive system. These effects may be in line with other non-suppressive actions of the glucocorticoids, as suggested by Sapolsky *et al.* (2000). In adult male rats, the suppression of LH due to restraint stress was not prevented by

adrenalectomy. Treatment with metyrapone, a glucocorticoid synthesis inhibitor, increased the inhibitory effect on LH, while dexamethasone blocked the inhibitory effects. These results suggest that something attenuated by the glucocorticoids, such as CRF, AVP or ACTH was mediating the suppressive effects of LH, but not the glucocorticoids themselves (López-Calderón *et al.* 1987).

Several studies suggest that the glucocorticoids do not mediate the effects of insulin-induced hypoglycaemia on LH secretion. Insulin-induced hypoglycaemia caused a delay in the LH surge which was reversed by treatment with the opioid antagonist, naloxone, but not the glucocorticoid receptor antagonist RU486 (Dobson & Smith 2000). van Vugt *et al.* (1997) showed that treatment of ovariectomised monkeys with metyrapone had no effect on LH in the basal state, or on the suppression of LH induced by hypoglycaemia, suggesting that cortisol or the resulting increase in ACTH in its absence was not necessary for basal LH secretion and that cortisol did not mediate the suppression in LH response to hypoglycaemia. Similar results have been demonstrated when investigating the cortisol mediated effects of immune stressors. In the sheep, the effects of endotoxin were not reversed by treatment with metyrapone (Debus *et al.* 2002). Watanobe & Habu (2003) showed that the effects of lipopolysaccharide on the pulsatile parameters of LH secretion were potentiated in adrenalectomised animals and reversed with cortisol administration, suggesting that cortisol was protective against the effects of lipopolysaccharide on LH in ovariectomised rats.

As for the effect of stress on LH secretion (Section 2.3), further studies have been required to delineate between actions of the glucocorticoids at the hypothalamus or at the pituitary. In many species, including the sheep, the exact sites of action are not known. Changes in hypothalamic activity have been demonstrated during treatment with glucocorticoids, although changes in the response of the pituitary to GnRH during stress or elevation of the glucocorticoids are the focus of this thesis.

2.4.7. The effects of the glucocorticoids on the secretion of GnRH

Most studies which have investigated changes in the secretion of GnRH during exposure to elevated concentrations of the glucocorticoids have examined changes in LH pulse

frequency as an indicator of changes in the frequency of GnRH pulses. Decreases in LH pulse frequency during treatment with glucocorticoids have been observed in prepubertally castrated rams treated with oestradiol, but not in the absence of oestradiol (Daley *et al.* 1999b). In contrast, there was no change in LH pulse frequency during treatment with dexamethasone in ovariectomised ewes with or without oestrogen replacement in the breeding and non-breeding season (Phillips & Clarke 1990). Moberg *et al.* (1981), however, found that treatment with neither dexamethasone nor cortisol blocked the oestrogen-induced LH surge in the anoestrous ewe, suggesting that glucocorticoids do not block the positive feedback mechanisms of oestrogen at the hypothalamus which result in the induction of a GnRH surge. In women with functional hypothalamic amenorrhea, high concentrations of cortisol were associated with a decreased LH pulse frequency, suggesting that elevated concentrations of cortisol may reduce the activity of GnRH neurones (Berga *et al.* 1997). Studies examining changes in hypothalamic activity in the presence of elevated concentrations of cortisol have shown that LH pulse frequency was suppressed during treatment with cortisol in women during the follicular phase of the cycle (Saketos *et al.* 1993). In contrast, no effect of cortisol on LH pulse frequency has been found in men and in women on day 3 to 5 of the menstrual cycle (Samuels *et al.* 1994).

Possible mechanisms by which the glucocorticoids may affect hypothalamic function have been demonstrated in rats. In male rats and in female rats which are intact, ovariectomised or ovariectomised and treated with oestrogen, GnRH neurones have been identified in the hypothalamus which express glucocorticoid receptor immunoreactivity (Ahima & Harlan 1992). The number of cells expressing immunoreactivity increased with ovariectomy but this increase was reversed with oestrogen treatment suggesting that oestrogen may play an important role in regulating the expression of the glucocorticoid receptor. In addition, a glucocorticoid response element has been identified in the promoter region of the human GnRH gene which suggests that cortisol may be able to act directly on GnRH neurones in the human (Radovick *et al.* 1990). These studies provide the biochemical and neuroanatomical framework to support a possible mechanism by which the glucocorticoids can directly affect GnRH secretion.

2.4.8. The effects of the glucocorticoids on anterior pituitary responsiveness to GnRH

The possibility that elevated circulating concentrations of the glucocorticoids can suppress the LH secretory response to GnRH has been considered, however studies in this area have not been extensive and have not focused on the role of sex and the sex steroids in mediating the possible effects of glucocorticoids on the pituitary. As with studies on stress, interpretation of the data in this area is mostly limited to animal models in which endogenous GnRH stimulation of the pituitary still occurs. Therefore, effects of the glucocorticoids which appear to be at the pituitary may be complicated by the fact that there are possible effects of the glucocorticoids on the hypothalamic secretion of GnRH. In the sheep, it is unclear whether changes in pituitary responsiveness occur during stress as a result of an increase in the plasma concentrations of the glucocorticoids. During chronic dexamethasone treatment in the breeding or the non-breeding season, there was no change in the LH response to GnRH in ovariectomised ewes with or without oestrogen replacement (Phillips & Clarke 1990). Nangalama & Moberg (1991), however, found that in pituitary cell cultures generated from castrated rams, there was a suppression in the secretion of LH in response to GnRH after four, six or eight hours of cortisol treatment, although basal LH secretion was significantly increased during cortisol treatment. In dairy heifers (Matteri & Moberg 1982a), and in adrenalectomised heifers in the luteal phase of the cycle (Li & Wagner 1983), there was no change in the LH response to treatment with exogenous GnRH under conditions of elevated concentrations of cortisol. In male Rhesus monkeys treated with cortisol for five days, there was no change in LH secretion in response to exogenous GnRH (van Vugt *et al.* 1997). In women receiving dexamethasone and treated with GnRH on day 7 or day 14 after ovariectomy, a decrease in LH secretion in response to GnRH was observed in comparison to women receiving a placebo (Melis *et al.* 1987). In women suffering from chronically elevated concentrations of cortisol (Cushing's Syndrome), however, no decreases in the LH response to treatment with GnRH were reported when compared to women with normal menstrual cycles (Lado-Abeal *et al.* 1998). In castrated and in gonad intact male rats, there was a decrease in the secretion of LH in response to exogenously administered GnRH during treatment with cortisol or corticosterone (Suter *et al.* 1988). Similar results have also been observed in female rats in proestrus treated with dexamethasone (Baldwin & Sawyer 1974). Ringstrom & Schwartz (1985) showed that there was a suppression in the LH response to GnRH stimulation in

castrated and adrenalectomised or castrated male rats, but not in gonad intact male rats during cortisol treatment. These data suggest that, in male rats, the ability of the glucocorticoids to suppress pituitary responsiveness may be dependent on the sex steroids.

Many studies have examined changes in LH secretion *in vitro* in response to GnRH after exposure to elevated concentrations of glucocorticoids. Care must be taken when interpreting these data, however, as Li & Wagner (1983) found that there was no change in the responsiveness of the pituitary to GnRH during treatment with hydrocortisone *in vivo*, whereas the secretion of LH in response to GnRH from dispersed bovine pituitary cells was reduced during cortisol exposure. These results suggest that the performance of the gonadotrophs *in vitro* may not necessarily be a faithful representation of that observed *in vivo*. Padmanabhan *et al.* (1983) used bovine pituitary cell cultures from animals in the luteal phase of the cycle to show that the responsiveness of the pituitary to GnRH was reduced after treatment with cortisol or dexamethasone, but not progesterone. There was no effect of the glucocorticoids, however, on the basal secretion of LH. Furthermore, a decrease in pituitary responsiveness was observed in pituitary cells that were not pretreated with GnRH, but in pituitaries that had been treated or 'primed' with GnRH, there was no effect of cortisol on basal LH secretion or LH secretion induced by GnRH. In cultures of porcine anterior pituitary cells, there was no change in basal LH secretion, but a reduction in GnRH stimulated LH secretion was observed during treatment with cortisol (Li 1994). These studies suggest in domestic species, that cortisol may suppress the secretion of LH through a mechanism which interrupts the GnRH stimulated secretion of LH. In contrast, in cultures of female rat pituitary cells, Suter & Schwartz (1985b) showed that the basal secretion of LH was decreased during the first 48 hours of incubation by cortisol and corticosterone while the maximal secretion of LH which could be stimulated by GnRH was unaffected by the presence of the glucocorticoids. In cultures of dispersed pituitary cells from the male rat, there was no change in the basal secretion of LH or FSH, or in LH secretion in response to a maximally stimulating dose of GnRH (Suter & Schwartz 1985a). Ringstrom & Schwartz (1985) showed that there was a suppression in the LH response to GnRH stimulation in castrated and adrenalectomised or castrated male rats, but not in gonad intact male rats during cortisol treatment. These data suggest that, in male rats, the ability of the glucocorticoids to suppress pituitary responsiveness may be dependent on the

sex steroids. It is possible that sex differences in the mechanisms by which the glucocorticoids can suppress LH in rats may account for the discrepancies reported in the literature.

2.4.9. The sympathoadrenal system

There are few data on the effects of the sympathoadrenal system on the hypothalamo-pituitary gonadal axis, and most studies have been conducted in rats. One study in ovariectomised and anoestrous ewes, however, showed that intravenous treatment with noradrenaline in a non-stressed condition was able to suppress basal LH secretion and LH secretion in response to treatment with exogenous GnRH (Deaver & Dailey 1982). Basal LH secretion from pituitary tissue of castrated ram lambs was unaffected by incubation with adrenaline or noradrenaline (Swartz & Moberg 1986). Furthermore, LH secretion in response to treatment with GnRH was increased in the presence of adrenaline and noradrenaline. Further investigation suggested that the effects of adrenaline were mediated through a β_2 adrenergic receptor. In normal male rats, subcutaneous injection of adrenaline was associated with an acute increase in plasma LH, followed by a decrease in LH with respect to controls at 60 and 120 minutes (Krulich *et al.* 1974). In female rats treated with oestrogen, the suppressive effects of insulin-induced hypoglycaemia on LH secretion were reversed by adrenomedullectomy, suggesting a role for the catecholamines in mediating these stress-induced effects (Cagampang *et al.* 1997). In the male rat, restraint induced a decrease in plasma concentrations of LH and an increase in corticosterone. In rats that were subject to adrenomedullectomy, however, restraint stress did not cause a decrease in LH. The injection of a β-adrenergic blocker during stress also attenuated the inhibition of LH, suggesting a role for adrenaline in stress-induced suppression of LH (Ariznavarreta *et al.* 1989). In female rats, infusion of adrenaline, but not noradrenaline or dopamine, blocked spontaneous LH release and ovulation. LH release in response to GnRH was not affected by adrenaline, suggesting a hypothalamic site of action of the adrenaline (Blake 1976).

2.5. Conclusion

While stress is likely to interrupt reproductive function by disrupting LH secretion from the pituitary, current *in vivo* models do not allow the delineation of a hypothalamic or pituitary

site of action of the effects of stress on LH secretion. Systems activated during stress, such as the hypothalamo-pituitary adrenal axis and sympathoadrenal system, are likely to mediate the effects of stress on the reproductive hypothalamo-pituitary unit, but once again, new animal models are required to delineate between hypothalamic and pituitary sites of action. Further investigation is also required to determine the effect of sex and the presence of the sex steroids on the responsiveness of the reproductive hypothalamo-pituitary unit to stress and the systems activated during stress.

Chapter 3 ~ General Materials and Methods

3.1. Animals

The experiments presented in this thesis were conducted at Prince Henry's Institute of Medical Research Biological Resource Centre in Werribee, Victoria, Australia (38°S). Romney Marsh sheep were used in all experiments. The breeding season for Romney Marsh sheep at this facility is considered to be from January until May (mid-summer until the end of autumn) (Bremner *et al.* 1984). Experiments were performed in the breeding or non-breeding season, as specified in each chapter. In between experimental periods, sheep were maintained in fields at the facility. During experimental periods, sheep were kept indoors with exposure to natural light. In the experiments described in Chapter 5 and during the control periods of the experiments described in Chapter 6, sheep were housed in individual, adjacent pens arranged in groups of three in a room holding a total of six sheep. Each pen was 880mm x 420mm x 1380mm. In the experiments described in Chapter 4, during the imposition of stress in Chapter 6 and also during Chapter 7, sheep were held in individual, adjacent pens that were arranged in bays of six. The pens were 940mm x 420mm x 1380mm. Water was available throughout all experiments and animals were fed chaff twice daily. All animal procedures were conducted with prior institutional ethical approval under the requirements of the Australian Prevention of Cruelty to Animals Act 1986 and the NH&MRC/CSIRO/AAC *Code of Practice for the Care and Use of Animals for Scientific Purposes.*

3.2. Surgical Techniques

To induce anaesthesia for surgery, sheep received an intravenous injection of Thiobarb (10mg/kg)(Jurot Pty Ltd, Rutherford, Australia). Animals were then layed on their side and intubated using a rubber tube inserted into the trachea. Anaesthesia was maintained using Halothane (Inhalation Anaesthetic Halothane B.P., Veterinary Companies of Australia Pty. Ltd., Artarmon, Australia) and oxygen. Nitrous oxide was used to deepen anaesthesia when necessary. The mixture of gases was administered using an anaesthetic machine (Halothane Fluotec 3, Cyprane Keighley, Yorkshire, UK) (Anaesthetic Apparatus TM41,

Commonwealth Industrial Gases Ltd, Rocklea, Australia) and inhaled through the rubber tubing.

To clean the incision site for surgery, the area was shaved, scrubbed with detergent, sprayed with 70% alcohol and then treated with iodine. Sheep were treated with an intramuscular injection of antibiotic (1ml/10kg) (Terramycin LA, Troy Laboratories, Smithfield, Australia). Between two and three mls of the same antibiotic was also administered at the incision site before suturing. All surgery was performed under aseptic conditions. An analgesic (2mg/kg) (Rimadyl, Pfizer, Mulgrave, Australia) was administered to all animals while under anaesthesia.

3.2.1. Ovariectomy

Ewes were anaesthetised and prepared for surgery as described above. An abdominal incision was made next to the ventral midline of the abdomen. The peritoneal cavity was opened by making an incision along the midline. The reproductive tract was located using palpation and exteriorised. Each ovary was identified and the broad ligament severed. The blood supply to the ovary was then occluded using suture and the ovary was removed. The reproductive tract was restored and the peritoneum sutured using a blanket stitch. The skin was then sutured using a running stitch.

3.2.2. Castration

Rams were anaesthetised and prepared for surgery as described above (Section 3.2). A midline scrotal incision was made. Subcutaneous connective tissue was separated and the testes were exposed using blunt dissection. Blunt dissection was also used to separate the blood vessels of the pampiniform plexus and the cremaster muscle. The isolated blood vessels and those within the cremaster muscle were occluded using suture. Both the blood vessels and the cremaster muscle were severed and cauterisation was used to stop bleeding. After both testes were removed, the excess portion of the scrotal sac was removed. Cauterisation was used to stop bleeding from cutaneous blood vessels in the remaining tissue and the wound was sutured using a running stitch.

3.2.3. Hypothalamo-pituitary disconnection

Hypothalamo-pituitary disconnection was performed according to a procedure previously described (Clarke *et al.* 1983). Animals were prepared for surgery as described above

(Section 3.2). After anaesthesia was induced, sheep were placed in a prone position and the head stabilised with the use of a stereotaxic frame. A transnasal, transphenoidal route was taken using a high speed drill to tunnel through the sphenoid bone along the midline to the pituitary. The dura mater was opened to expose the portal pituitary vessels, the pituitary stalk and the median eminence. A lesion was then created in the external zone of the median eminence and the nerve terminals removed by suction. The superior hypophyseal arteries were left intact. A piece of aluminium foil was placed in the resulting lesion to prevent any re-establishment of connections from the median eminence to the hypophyseal-portal blood vessels. Gelfoam® (Pharmacia and Upjohn Company, Kalamazoo, MI) and dental acrylic (Densply International Inc, Milford, De) were used to seal the cavity created and the wound was closed.

3.2.4. Catheterisation

As specified in the materials and methods section of each experimental chapter, either an indwelling jugular catheter or silastic tubing was used to cannulate the jugular vein. To insert an indwelling jugular catheter (Dwellcath, Tuta Laboratories, Lane Cove, Australia) the sheep was held upright in a sitting position. The area over the vein was shaved and sprayed with 70% alcohol. An incision was made over the vein and the catheter inserted into the vein. A 100cm manometer line (Portex Ltd., Kent, UK) filled with heparinised saline (100K units/L) was attached to the catheter and sealed with a three-way tap (Indoplas, Sydney, Australia) or a four-way tap (Becton Dickinson, Helsinborg, Sweden). To stabilise the position of the catheter, Leukoplast tape (Beiersdorf, Hamburg, Germany) was applied to the exposed end of the catheter to form a tab. This tab was then sutured to the skin at the site above the vein. Throughout the experimental periods, the manometer line was fastened to wool on the back of the sheep or to wires above the pen using elastic bands. To keep the catheter and manometer line patent, they were flushed regularly with heparinised saline (100K units/L) (Pharmacia and Upjohn, Bentley, Australia).

Silastic tubing (Biocorp Australia Pty Ltd, Melbourne, Australia) was used for cannulation when access to the jugular vein was required for periods longer than one week. These catheters were used in conjunction with an automated system to administer intravenous doses of GnRH (Section 3.4). To catheterise using this method, animals were anaesthetised

and prepared for surgery as described in Section 3.2 and layed on their side on the surgical table. Sterile, 200mm pieces of silastic tubing were marked at the 140mm length, filled with heparinised saline (100K units/L) and clamped at one end. An incision was made in the skin over the vein. A 16 gauge needle was used to puncture the jugular vein. The silastic tubing was then threaded through the needle and into the vein to a length of 60mm. The needle was removed, leaving the silastic tubing in place. The tubing was clamped to the skin while an incision was made on the midline at the back of the neck, approximately 140mm from the incision site over the jugular vein. A trocar was used to tunnel subcutaneously from the site of this incision to that over the vein. The remaining length of silastic was then threaded through the trocar and externalised through the incision at the back of the neck. The trocar was then removed, leaving the silastic tubing in place. A three-way tap was attached to seal the silastic tubing. A tab was made using Leukoplast and this was sutured to the skin at the back of the neck to fasten the catheter. A suture was also placed in the incision above the jugular vein to aid healing. After surgery, a 1000mm manometer line was attached to the three way tap at the back of the neck. During experiments, the line was held in place by fastening it to the back of the sheep, or to overhead lines using elastic bands. The manometer line and silastic tubing were flushed daily with heparinised saline (100K units/L) to keep them patent.

3.3. Collection of Blood Samples

To take a blood sample, a 10ml syringe was used to withdraw heparinised saline in the manometer line, as well as approximately three mls of blood. A second 10ml syringe was then used to withdraw a further five mls of blood. This was the sample used for analysis. It was immediately placed in a blood collection tube. The initial sample taken to clear the manometer line of saline was then injected back into the manometer line to limit decreases in haematocrit throughout experiments. A 20ml syringe was then used to flush the manometer line and catheter with approximately seven mls of heparinised saline (50K units/L). After centrifuging the blood samples at 3,000rpm for 10 minutes at 4°C, the plasma was harvested and stored at -20°C until ready for analysis. In the experiments presented in Chapter 4, Chapter 5, the first experiment presented in Chapter 6 and in the pilot study presented in Chapter 7, 10ml collection tubes containing lithium heparin gel (Sarstedt Australia, Technology Park, South Australia) were used to collect blood samples.

In the second experiment presented in Chapter 6 and in the experiment presented in Chapter 7, empty blood collection tubes were used (Sarstedt Australia, Technology Park, South Australia). A glutathione inhibitor which consisted of 3% glutathione (Sigma, St. Louis, MO) in 9.5% ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma, St Louis, MO) was added to the tubes prior to the collection of blood samples to inhibit oxidation of the catecholamines and as an anti-clotting factor.

3.4. GnRH Treatment

In Chapter 5 and Chapter 6, hypothalamo-pituitary disconnected rams and ewes were treated with intravenous injections of GnRH (Auspep, West Melbourne, Australia) every two hours for at least five days prior to the experimental period. In the second experiment presented in Chapter 5, and in both experiments presented in Chapter 6, 125ng of GnRH was administered during each injection (2.25ml). In the first experiment presented in Chapter 5, 250ng of GnRH was administered during each injection (2.25ml). The injections were administered by an automated pump which was connected to 50ml glass syringes filled with a solution of GnRH. A timer connected to the pump controlled the administration of GnRH so that it occurred over a six minute period every two hours. The pump and syringes were kept at 4°C. They were connected to a 1000mm manometer line, which was connected to the silastic catheter, by a 200mm manometer line (Portex Ltd., Kent, UK). The manometer lines were connected to each other by a four way tap. On sampling days, injections of GnRH were delivered by hand directly through the 1000mm manometer line to the jugular catheter in a 5ml volume. The lines were then flushed with 10ml of heparinised saline (50K units/L).

3.5. Isolation and Restraint Stress

Isolation and restraint stress was imposed on rams and ewes according to the procedure described by Tilbrook *et al* (1999a). During the control periods of the experiments, the sheep were maintained in individual, adjacent pens (for dimensions see Section 3.1). The sheep had aural, visual and limited physical contact. To impose isolation and restraint stress, each sheep was removed from its pen and moved to another pen which was partially covered by hessian and had the dimensions of 940mm x 420mm x 1380mm (Figure 3.1). Once in the pen, the sheep was harnessed to the side of the pen so that only its head could move freely. The pen was then fully covered with hessian so that the sheep no longer had

visual or physical contact with other sheep. The hessian covered pens were arranged so that there were no sheep in the immediately adjacent pens to further limit contact. The sheep remained in aural contact throughout the experiment. At the end of the experiment, the sheep were removed from the pens covered in hessian and moved back to their original pens for any further study during control periods.

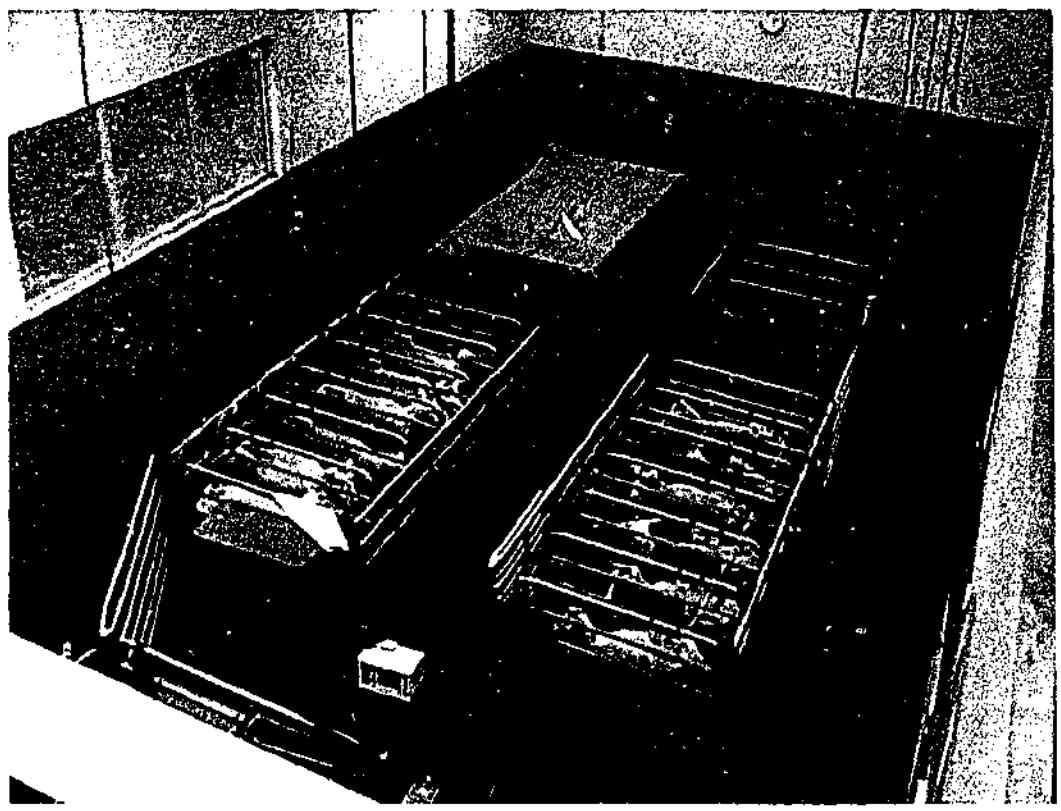


Figure 3.1

Isolation and restraint stress was imposed on rams and ewes by moving them from pens in which they were adjacent and opposite to other sheep to pens which were covered by hessian.

3.6. Radioimmunoassays

3.6.1. LH radioimmunoassay

The concentration of ovine LH (oLH) in the plasma was determined using a procedure based on the protocol described by Lee *et al.* (1976). The assay was conducted using an egg-white phosphate buffered saline (PBS) buffer (5% egg white in 0.02M PBS) at pH 7.4. The standard used in the assay was NIH LH S18 (NIDDK, Torrance, CA). An oLH

antibody, raised in the rabbit (NIDDK, Torrance, CA), was used at a final dilution of 1:840,000. ^{125}I-oLH was prepared using 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Sigma, St Louis, MO) according to the procedure previously described (Salacinski *et al.* 1981). ^{125}I-oLH is a competitive ligand for the oLH antibody. Both the plasma from blood samples and ^{125}I-oLH were incubated with the oLH antibody overnight at room temperature. The final volume in the assay tubes was 600µl. The ligand-antibody complex formed was precipitated with goat anti-rabbit serum at a final concentration of 1:1600 and normal rabbit serum used at a final concentration of 1:16,000. Goat anti-rabbit serum was added after the first overnight incubation and the assay was incubated again at 4°C overnight. The tubes were then centrifuged (Beckman, J-6B centrifuge, Beckman Coulter, Fullerton, CA) at 3000rpm for 30 minutes to separate the bound and unbound ligand and aspiration was used to remove the supernatant. The tubes were counted for two minutes on a gamma counter (Crystal™ Multi Detector RIA System, United Technologies Packard, Downers Grove, IL) which calculated the standard curve and value of the LH concentration in unknown samples. In the experiments presented in Chapter 4 and Chapter 7, Prince Henry's Institute of Medical Research Radioimmunoassay program was used to calculate the standard curve and concentration of LH in the unknown samples.

3.6.2. Extracted cortisol radioimmunoassay

In the experiments presented in Chapter 6, chemical separation was used to extract cortisol found in plasma for analysis by radioimmunoassay. In 12x75mm glass tubes, 100µl of distilled water and two mils of dichloromethane was added to plasma samples and the tubes vortexed twice for one minute, with a five minute interval between each vortex. "Blank tubes" containing only distilled water or dichloromethane were also included. The organic phase (one mil) was then removed, placed in 55x12mm plastic tubes and dried down under air at 37°C. The sample was then reconstituted in 0.5M phosphate buffer (pH 7.4) and bovine γ-globulin (Calbiochem, Darmstadt, Germany) at a final concentration of 32µg/100µl for radioimmunoassay. In order to measure the recovery of the extraction process, 5µl of ^{3}H-cortisol ([1,2,6,7-^{3}H] cortisol, Pharmacia Upjohn, Northamptonshire, UK) was placed in a glass tube and dried down under air. This was then reconstituted in one mil of sheep plasma. An equal amount of the sheep plasma was aliquoted into glass tubes in triplicate, for extraction as for the samples, and into plastic assay tubes in triplicate.

The recovery was calculated by comparing the amount of ^{3}H-cortisol measured in tubes that had gone through the extraction process to that in those that had not gone through the process.

The final assay volume was 400µl. Cortisol standards were prepared from a stock (Sigma Chemical Company, Castle Hills, Australia) and serially diluted in ethanol. Cortisol antibody (#3368, Bioquest Ltd., North Ryde, Australia) was used at a working dilution of 1:32,000, the competitively binding ligands for which were endogenous cortisol in the sample and the radioactively labeled cortisol.

The assay was incubated at 4°C overnight and 22% polyethylene glycol 6000 (BDH Laboratory Supplies, Poole, England) was used to precipitate the antibody-ligand complexes formed. Tubes were centrifuged at 3000rpm for 15 minutes at 4°C. The supernatant was aspirated and the pellet resuspended in 200µl of assay buffer. Aqueous Counting Scintillant (one ml) (Amersham, Arlington Heights, IL) was added to all tubes which were subsequently counted in a beta counter (LS 5000TA, Beckman Coulter, Fullerton, CA). The beta counter calculated the standard curve of the assay and the cortisol concentration in the unknown samples.

3.6.3. Unextracted cortisol radioimmunoassay

In the experiments described in Chapter 4, Chapter 5 and Chapter 7, an unextracted cortisol radioimmunoassay was performed according to the procedure described by Broadbear *et al.* (2004). The standard used in the assay was hydrocortisone H-4001 (Sigma Chemical Company, Castle Hills, Australia) which was stored in ethanol at -20°C. The final volume in each tube was 500µl and the assay buffer used was 0.1% BSA, 0.73% sodium citrate, 0.69% $NaH_2PO_4.H_20$ (pH 3.0). Cortisol labeled with ^{125}I (Amersham Pharmacia Biotech Ltd., Chalfont St Giles, UK) was a competitive ligand for cortisol antiserum which was used at a final concentration of 1:1,400,000. Both radioactively labeled cortisol and the unlabelled cortisol in the samples were incubated with antibody overnight at 4°C. The antibody-ligand complex was precipitated using 22% polyethylene glycol 6000 and the assay centrifuged at 4000rpm for 30 minutes to separate the bound and unbound ligand. The supernatant was removed by aspiration. The assay was counted using a gamma counter. In

Chapter 7, Prince Henry's Institute of Medical Research Radioimmunoassay Program was used to calculate the amount of cortisol in unknown samples.

3.7. High Performance Liquid Chromatography with Electrochemical Detection

The plasma concentrations of DOPA, adrenaline, noradrenaline and DHPG were measured using high performance liquid chromatography with coulometric detection (Model 5100A coulometric detector, Environmental Sciences Associates, MA) after extraction using alumina adsorption, according to a previously described procedure (Medvedev *et al.* 1990). Blood samples for analysis were collected into tubes containing EGTA and reduced glutathione as described in Section 3.3 and stored at -70°C until ready for assay.

To extract the catecholamines and their metabolites, approximately 10mg of aluminium oxide (Merck, Darmstadt, Germany) and 400µl of Tris HCL buffer (15.125% Tris, 2.5% EDTA, pH 8.6) was added to each sample. 6-fluoronoradrenaline (2ng) (Sigma Chemical Company, Castle Hills, Australia) was used as an internal standard. Samples were mixed using an orbital shaker for 20 minutes and then centrifuged for 30 seconds using a bench top 'nicrofuge. The supernatant was discarded and the alumina was washed with 1.9% $NaHCO_3$ and then distilled water. Catecholamines were removed from the aluminium oxide by vigorous shaking in acid (80% 0.2M acetic acid and 20% 0.2M phosphoric acid) for 15 minutes. External standard solutions containing one nanogram of DOPA, adrenaline, noradrenaline and DHPG were also extracted.

After alumina adsorption, the catecholamines collected in acid were separated using high performance liquid chromatography (25cm Altex Ultrasphere column, ODS 4.6mm x 25cm, 5µm particle size, Beckman Instruments Inc, Carlsbad, CA). The mobile phase used was 0.5% acetonitrile (0.1M $NaH_2PO_4.H_20$, 0.05mM ethylene diamine tetraacetic acid, 0.16mM octanesulfonic acid, 0.5% HPLC grade acetonitrile) at pH 3.3 and was delivered at a flow rate of 1.2ml/min. Prior to use, the mobile phase was degassed by vacuum filtration through a 0.2µm millipore membrane. A guard cell operating at -0.35V was used for oxidation of the extracted components. The operating potentials at detector 1 (+0.35V) and detector 2 (-0.30V) allowed measurements of the catecholamines to be made as a result

of their oxidation at detector 2. Compounds were identified by their retention behaviour compared to that of authentic standard solutions.

3.8. Statistical Analysis of Data

In each study, the parameters of LH pulses stimulated by endogenous GnRH secretion (Chapter 4 and Chapter 7) or exogenous treatment with GnRH (Chapter 5 and Chapter 6) were measured and statistically analysed using the computer software SPSS 10.0 for Windows. Under the condition of endogenous GnRH secretion, pulses of LH were detected using a modification of the protocol previously described by Karsch *et al.* (1987). Accordingly, a pulse of LH was identified when the difference in plasma concentrations of LH between one sample and that preceding it was greater than three standard deviations of the mean of the first sample, and when this was followed by a progressive decline in plasma concentrations of LH consistent with the half-life of LH in sheep of 29 minutes (Geschwind & Dewey 1968). The pre-pulse nadir concentration was the concentration of LH in the sample immediately preceding the LH pulse. Under the condition of exogenous GnRH secretion, the baseline concentration of LH was defined as the concentration of LH in the sample taken 10 minutes before treatment with exogenous GnRH. In both cases, the amplitude of the pulse was defined as the difference between the peak concentration of LH measured during the occurrence of a pulse, and the pre-pulse nadir or baseline concentrations of LH measured before the pulse. The frequency of LH pulses was also measured in studies where there was endogenous secretion of GnRH. It was defined as the number of pulses occurring per hour. Mean plasma concentrations of LH and cortisol were also measured. They were defined as the mean of all measurements of either LH or cortisol made within a particular period. Mean plasma concentrations of DOPA, adrenaline, noradrenaline and DHPG were measured in the second experiment presented in Chapter 6 and in the experiment presented in Chapter 7. In this case, mean plasma concentrations were compared by examining the plasma concentrations at each point in time rather than during a period of sampling. Mean plasma concentrations of cortisol were also treated this way in Chapter 7.

In each chapter, data were analysed by a Repeated Measures Analysis of Variance. Post-hoc comparisons were made by determining least significant differences. The within and

between subjects factors for each analysis are listed in each chapter. Before analysis, the variance in the data was tested for homogeneity of variance using a Univariate Repeated Measures Analysis of Variance and Levene's Test of Equality of Error Variances. If the variance was not homogenous, the data were transformed using a square root or log transformation, as specified in each chapter.

Chapter 4 ~ Does Cortisol Suppress LH Pulse Amplitude in Gonadectomised Rams and Ewes?

4.1. Introduction

A previous study from this laboratory showed that isolation and restraint stress imposed on gonadectomised ewes and rams was associated with a suppression in mean plasma concentrations of LH (Tilbrook *et al.* 1999a). Although mean plasma concentrations of LH were equally suppressed in both sexes, there were sex differences in the parameters of LH secretion that were affected by the stressor. LH pulse frequency was suppressed during the isolation and restraint stress in gonadectomised ewes, while LH pulse amplitude was suppressed in gonadectomised rams (Tilbrook *et al.* 1999a). The differences in the parameters of LH secretion affected suggest that there may be sex differences in the sites of action at which mediators of the stress response suppress the secretion of LH. As LH pulse frequency was suppressed in ewes, a hypothalamic site of action is likely be involved. An effect of LH pulse amplitude in rams suggests that the site of suppression of LH secretion may be at the hypothalamus or the pituitary (Section 2.3.2).

Plasma concentrations of cortisol are commonly elevated during stress and there is evidence to suggest that cortisol suppresses mean plasma concentrations of LH (Section 2.4.6). Conflicting evidence exists as to whether there is an effect of cortisol at the hypothalamus and/or at the pituitary to influence LH secretion (Section 2.4.7 and 2.4.8). Furthermore, it is unknown if there are sex differences in the effect of cortisol on the secretion of LH. In this study, the hypothesis tested was that both LH pulse frequency and LH pulse amplitude would be affected by the infusion of either a high or low dose of cortisol, suggesting that cortisol may induce mechanisms at the hypothalamus and/or the pituitary to reduce the secretion of LH. Furthermore, it was hypothesised that cortisol may suppress LH pulse frequency in ewes, and LH pulse amplitude in rams, further supporting the idea that there may be sex differences in the sites of action at which stress interrupts the secretion of LH.

4.2. Materials and Methods

4.2.1. Animals

The experiment was conducted during the breeding season using adult rams and ewes that had been gonadectomised (Section 3.2.1 and 3.2.2) at least three months prior to the experiment. On the day prior to the experiment, animals were fitted with an indwelling jugular catheter (Section 3.2.4) into each jugular vein. One catheter was used for the infusion of saline or cortisol, while the other catheter was used for the collection of blood samples (Section 3.3).

4.2.2. Experimental procedure

Gonadectomised ewes (n=6) and rams (n=6) were infused with saline (1ml/50kg/hr), a low dose of cortisol (12.5mg/50kg/hr) (Solucortef, HSA, Sydney, Australia), which elevated plasma concentrations of cortisol to those observed during the imposition of fully characterised stressors, or a high dose of cortisol (25mg/50kg/hr) for 30 hours. The infusions were administered using MS 16A syringe drivers (Smiths Medical Australia Pty Ltd, Gold Coast, Australia). The experiment was conducted three times so that every animal received every treatment in a randomised order. Prior to the commencement of the infusion, there was a control period of six hours when no treatment was given (hours -6 to 0). Blood samples were collected (Section 3.3) at 10 minute intervals throughout the control period, and throughout the first six hours (hours 0-6) and the final six hours (hours 24-30) of the infusion. Radioimmunoassays were used to measure the plasma concentrations of LH in all the samples taken, and the plasma concentrations of cortisol in samples collected at half hourly intervals. The haematocrit of each animal was measured every two hours throughout the sampling periods.

4.2.3. Radioimmunoassays

Assays for LH and cortisol were conducted as described in Sections 3.6.1 and 3.6.3 respectively. The mean sensitivity of the LH assay was 0.5ng/ml (n=13). The intra-assay coefficient of variation was 12% at 3.5ng/ml, 10% at 7.4ng/ml and 9.3% at 12ng/ml. The inter-assay coefficient of variation was 8.8% at 3.9ng/ml, 8.7% at 8.0ng/ml, 7.6% at 12ng/ml and 14% at 26ng/ml. The mean (±SEM) sensitivity of the cortisol radioimmunoassay was 2.0±0.5ng/ml (n=6). The intra-assay coefficient of variation was

9.4% at 9.7ng/ml and 7.3% at 117ng/ml. The inter-assay coefficient of variation was 16% at 14ng/ml and 1.8% at 125ng/ml.

4.2.4. Statistical analysis

The mean pre-pulse nadir LH concentration, the frequency of LH pulses, the mean amplitude of LH pulses, mean plasma concentrations of LH and the mean plasma concentrations of cortisol were measured (Section 3.8) during the control period, hours 1-6 of the infusion period, and hours 24-30 of the infusion period. As considerable time is required for the effects of steroid hormones to be displayed, data for the first hour after the commencement of the infusion were omitted from the statistical analysis of all parameters. Within subjects factors for the repeated measures analysis of variance were the treatment given during the infusion (saline, low dose of cortisol or high dose of cortisol) and the period of sampling (hours -6 to 0, 1 to 6 or 24 to 30) while the between subjects factor was sex. Transformation was not applied to any set of data.

4.3. Results

4.3.1. Haematocrit

Haematocrit levels were not compromised by the sampling schedule in any animal on any of the sampling days (Figure 4.1a-c). Due to a technical error, no measurements of haematocrit were made 36 hours after the commencement of blood sampling on the day of the second infusion (Figure 4.1b).

4.3.2. Mean plasma concentrations of cortisol

There were no sex differences in the mean plasma concentrations of cortisol, so data from each sex have been combined (Figure 4.2). There were also no significant changes in the mean (±SEM) plasma concentrations of cortisol (14±0.1ng/ml) during the periods of no infusion, or during the saline infusion (Figure 4.2a). During the infusion of the low dose of cortisol (12.5mg/50kg/hr), the mean (±SEM) plasma concentrations of cortisol achieved were 124±14ng/ml (Figure 4.2b). There was no significant difference between the mean plasma concentrations achieved during hours 1 to 6 of the infusion and those achieved during hours 24 to 30 of the infusion. During hours 1 to 6 of the infusion of the high dose of cortisol (25mg/50kg/hr), the mean (±SEM) plasma concentrations of cortisol achieved were 260±28ng/ml (Figure 4.2c). This was significantly higher than the mean (±SEM)

plasma concentrations of cortisol achieved during hours 24 to 30 of the infusion (227±22ng/ml). The plasma concentrations of cortisol achieved during the infusion of the high dose of cortisol were significantly greater ($p<0.05$) than the plasma concentrations of cortisol achieved during the infusion of the low dose of cortisol.

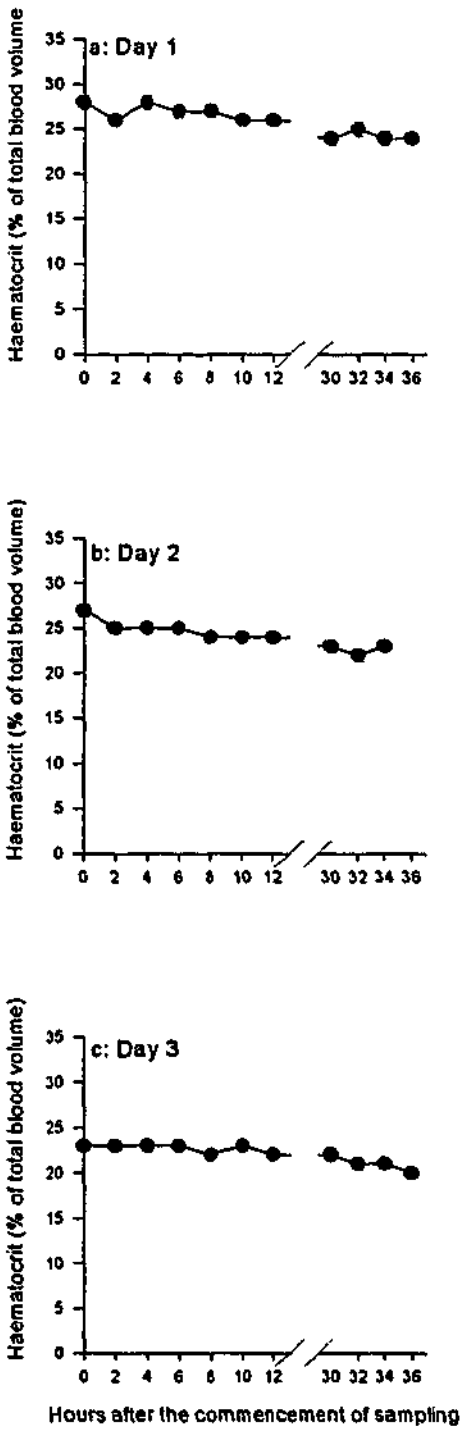


Figure 4.1

Mean (±SEM) haematocrit values in all sheep during the periods of sampling on the day of the first (a), second (b) and third (c) infusion. There were no meaningful significant differences over time. No measurements of haematocrit were made 36 hours after the commencement of blood sampling on the day of the second infusion (Figure 4.1b).

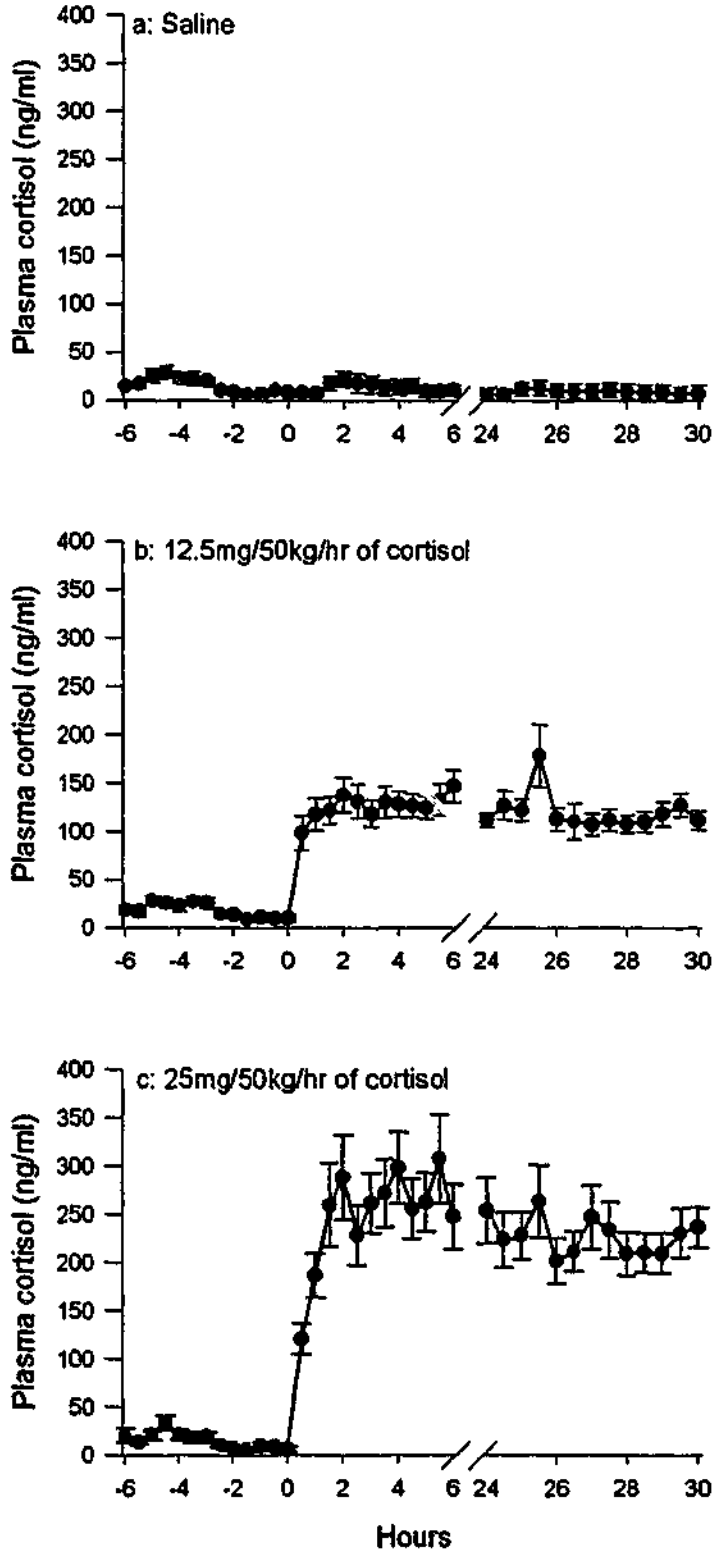


Figure 4.2

Mean (±SEM) plasma concentrations of cortisol (ng/ml) in rams and ewes (combined) during a control period (hours -6 to 0) and during the infusion of saline (a), 12.5mg/50kg.hr of cortisol (b) or 25mg/50gk/hr (c). Significant differences are described in the results text.

4.3.3. Mean plasma concentrations of LH

In both ewes and rams, mean plasma concentrations of LH during hours 1 to 6 and hours 24 to 30 of the infusion of either the high or low dose of cortisol were significantly lower ($p<0.05$) than the plasma concentrations of LH during the control periods or during the saline infusion (Figure 4.3a). Furthermore, in rams, the mean plasma concentrations of LH during hours 24 to 30 of the infusion of both doses of cortisol were significantly lower ($p<0.05$) than the mean plasma concentrations of LH observed during hours 1 to 6 of the same infusion (Figure 4.3b). Sex differences in the mean plasma concentrations of LH were observed during the periods of cortisol infusion at either the low or high dose. Mean plasma concentrations of LH were significantly lower ($p<0.05$) in rams during hours 1 to 6 and hours 24 to 30 of the infusion of either dose of cortisol than in ewes. Mean plasma concentrations of LH were also significantly lower in rams during hours -6 to 0 which occurred prior to the infusion of a high dose of cortisol. During hours 1-6 of the saline infusion, mean plasma concentrations of LH were higher ($p<0.05$) in rams than in ewes (Figure 4.3c).

4.3.4. Mean pre-pulse nadir concentrations of LH

In ewes, pre-pulse nadir concentrations were significantly reduced during hours 1 to 6 ($p=0.06$) and hours 24 to 30 ($p<0.05$) of the infusion of the low dose of cortisol (Figure 4.4a). During the infusion of the high dose of cortisol, pre-pulse nadir concentrations of LH were significantly reduced ($p<0.05$) during both periods. Furthermore, the pre-pulse nadir concentrations of LH during hours 24 to 30 of the infusion were lower ($p=0.06$) than those measured during hours 1 to 6 of the infusion. In rams, pre-pulse nadir concentrations of LH were significantly reduced ($p<0.05$) during both periods of the infusion of both the low and high doses of cortisol. Additionally, the pre-pulse nadir concentrations of LH during hours 24 to 30 of each infusion were significantly lower ($p<0.05$) than hours 1 to 6 of the infusion (Figure 4.4b). Ewes had significantly higher ($p<0.05$) pre-pulse nadir concentrations than rams during all periods in which cortisol was infused (Figure 4.4c). Ewes also had significantly higher ($p<0.05$) pre-pulse nadir concentrations during hours -6 to 0 on the day of the high cortisol infusion. Rams had significantly higher ($p<0.05$) pre-pulse nadir concentrations of LH during hours 1 to 6 of the saline infusion.

4.3.5.Mean LH pulse amplitude

In ewes, LH pulse amplitude was significantly ($p<0.05$) suppressed during infusion of the high dose of cortisol (Figure 4.5a). There was no effect, however, of the low dose of cortisol on LH pulse amplitude in ewes. In rams, LH pulse amplitude was significantly reduced ($p<0.05$) during hours 24 to 30 of the infusion of the low dose of cortisol, but not during hours 1 to 6 of the infusion (Figure 4.5b). During the infusion of the high dose of cortisol, LH pulse amplitude was reduced during hours 1 to 6 of the infusion ($p=0.058$), and also during hours 24 to 30 of the infusion ($p<0.05$). In addition, LH pulse amplitude in rams was significantly lower ($p<0.05$) during hours 24 to 30 of the infusion of the high dose of cortisol, than during hours 1 to 6 of the infusion. During the infusion of both the high and low doses of cortisol, mean LH pulse amplitude in rams was significantly lower ($p<0.05$) than in ewes (Figure 4.5c). Mean pulse amplitude was also significantly higher ($p<0.05$) in rams than in ewes during hours -6 to 0 prior to the infusion of the high dose of cortisol.

4.3.6. Mean LH pulse frequency

In ewes, mean LH pulse frequency was significantly lower ($p<0.05$) during hours 24 to 30 than during the control period on that day (Figure 4.6a). There was no decrease in LH pulse frequency during the infusion of the low dose of cortisol in ewes. In rams, LH pulse frequency was significantly decreased ($p<0.05$) during hours 24 to 30 of the infusion of the low dose of cortisol (Figure 4.6b). During the infusion of the high dose of cortisol, LH pulse frequency was significantly decreased ($p<0.05$) during hours 1 to 6 and hours 24 to 30 of the infusion. There was no significant difference in LH pulse frequency between these two periods. During hours 1 to 6 of the infusion of the high dose of cortisol, and hours 24 to 30 of the infusion of both a high and low dose of cortisol, LH pulse frequency was significantly higher ($p<0.05$) in ewes than in rams (Figure 4.6c).

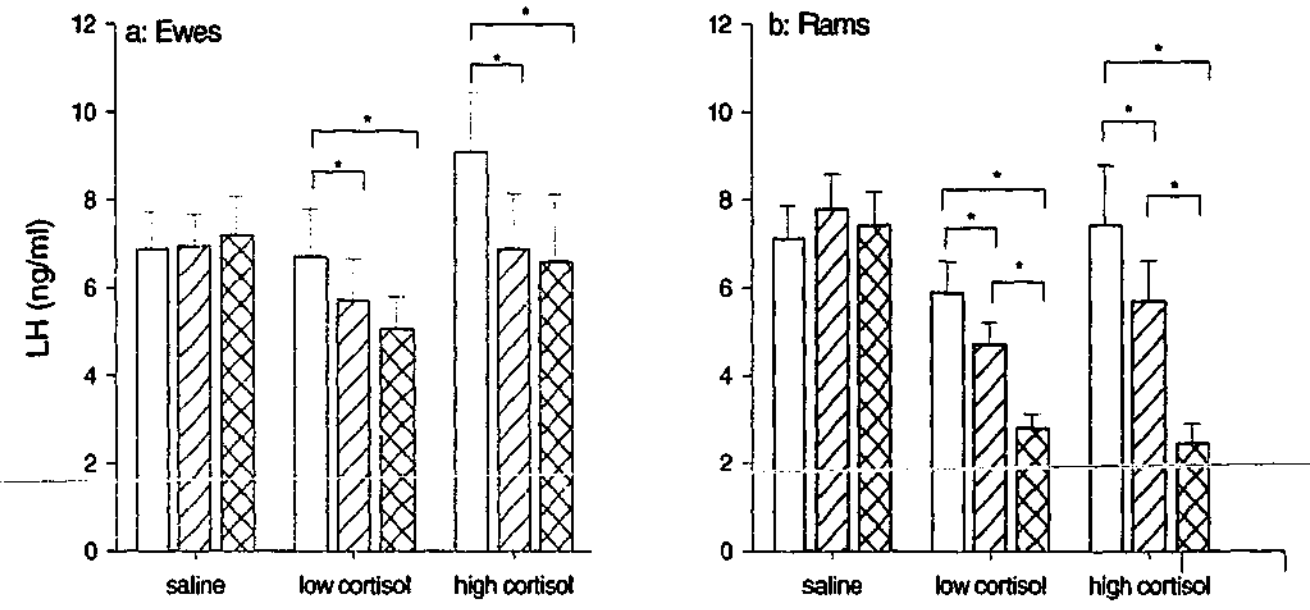


Figure 4.3

Mean (±SEM) plasma concentrations of LH (ng/ml) in gonadectomised ewes (a), and gonadectomised rams (b). Significant differences (* $p<0.05$) between periods on a given day are indicated. Mean (±SEM) plasma concentrations of LH in gonadectomised ewes (green) and gonadectomised rams (blue) are compared in (c). Significant differences (*$p<0.05$) between the sexes at any period are indicated. Hours -6 to 0 are indicated by plain bars, hours 1 to 5 are indicated by diagonally striped bars and hours 24 to 30 are indicated by hatched bars.

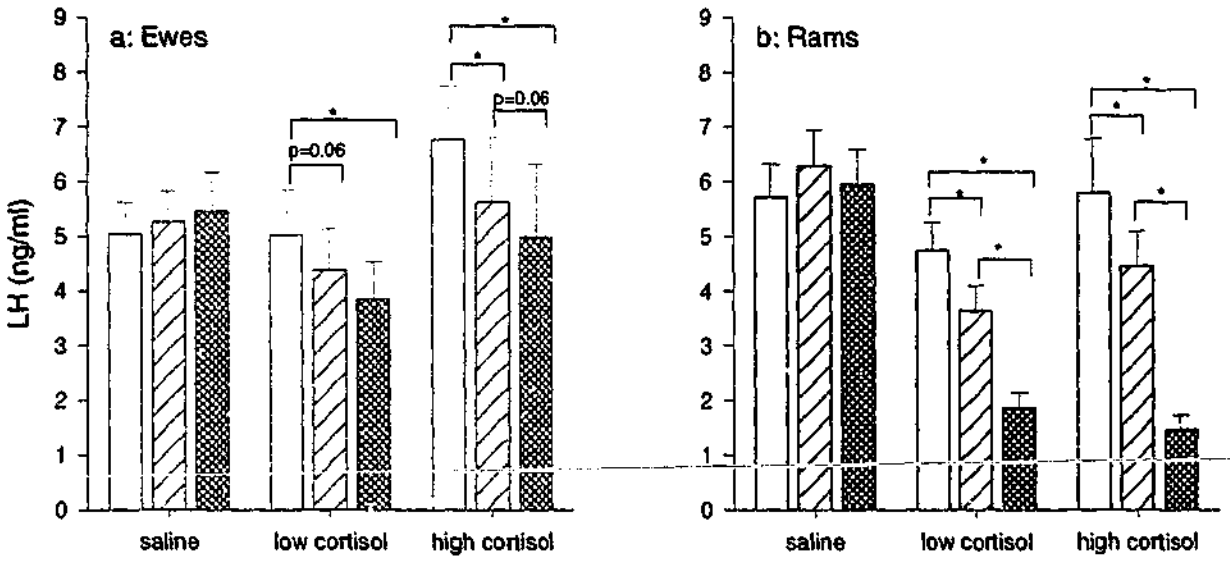


Figure 4.4

Mean (±SEM) pre-pulse nadir concentrations of LH (ng/ml) are shown in gonadectomised ewes (a), and in gonadectomised rams (b). Significant differences (*p<0.05 unless otherwise specified) between periods on a given day are indicated. Mean (±SEM) pre-pulse nadir concentrations of LH in gonadectomised ewes (green) and gonadectomised rams (blue) are compared in (c). Significant differences (* p<0.05) between the sexes at any period are indicated. Hours -6 to 0 are indicated by plain bars, hours 1 to 5 are indicated by diagonally striped bars and hours 24 to 30 are indicated by hatched bars.

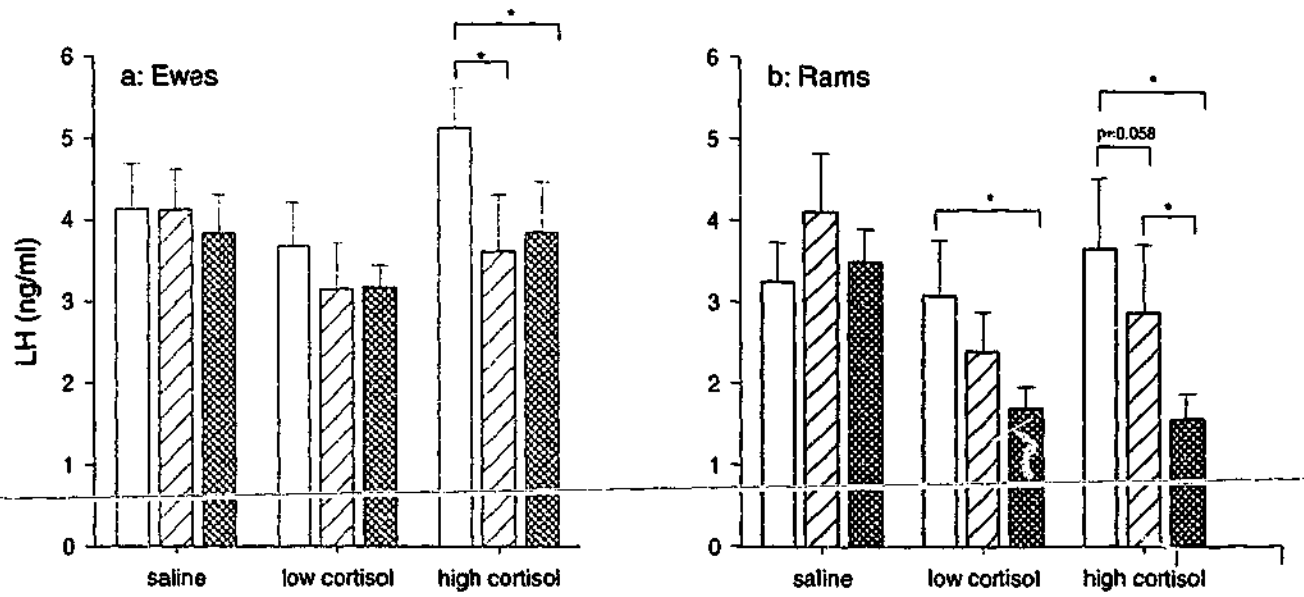


Figure 4.5

Mean (±SEM) LH pulse amplitude (ng/ml) in gonadectomised ewes (a), and in gonadectomised rams (b). Significant differences (* $p<0.05$ unless otherwise specified) between periods on a given day are indicated. Mean (±SEM) LH pulse amplitude in gonadectomised ewes (green) and gonadectomised rams (blue) are compared in (c). Significant differences (* $p<0.05$, # $p=0.06$) between the sexes at any period are indicated. Hours -6 to 0 are indicated by plain bars, hours 1 to 5 are indicated by diagonally striped bars and hours 24 to 30 are indicated by hatched bars.

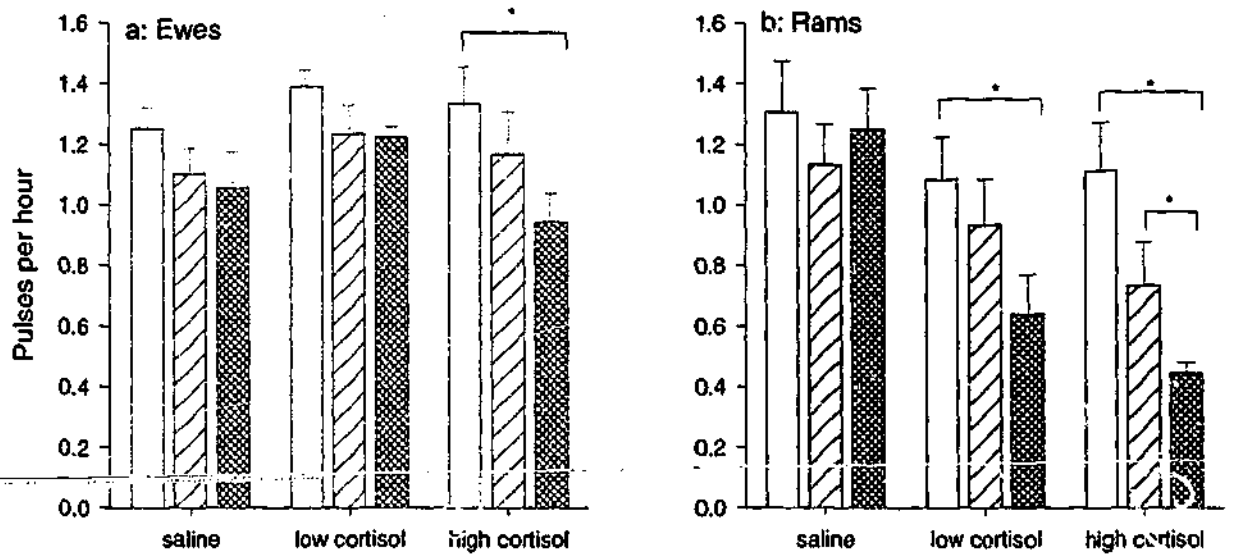


Figure 4.6

Mean (±SEM) LH pulse frequency (pulses per hour) in gonadectomised ewes (a), and in gonadectomised rams (b). Significant differences (* $p<0.05$) between periods on a given day are indicated. Mean (±SEM) LH pulse frequency in gonadectomised ewes (green) and gonadectomised rams (blue) are compared in (c). Significant differences (* $p<0.05$) between the sexes at any period are indicated. Hours -6 to 0 are indicated by plain bars, hours 1 to 5 are indicated by diagonally striped bars and hours 24 to 30 are indicated by hatched bars.

4.4. Discussion

These data show that plasma concentrations of cortisol at 124ng/ml or 260ng/ml were associated with a decrease in mean plasma concentrations of LH in gonadectomised rams and ewes. They confirm findings in castrated rams (Daley *et al.* 1999b) and ovariectomised ewes (Debus *et al.* 2002; Breen & Karsch 2004) which also demonstrated a decrease in the plasma concentrations of LH during periods of elevated cortisol. Similar results have also been obtained in castrated male rats (Ringstrom & Schwartz 1985; Suter *et al.* 1988) and castrated and gonad intact male Rhesus monkeys (Dubey & Plant 1985; Hayashi & Moberg 1987). Mean pre-pulse nadir concentrations of LH were also significantly reduced in rams and ewes, again demonstrating an effect of elevated concentrations of cortisol on plasma concentrations of LH. As both the mean plasma concentrations of LH and the mean pre-pulse nadir concentrations of LH were significantly lower in males than in females during both periods in which sampling occurred during the infusion of both doses of cortisol, this suggests, for the first time, that there may be a sex difference in the responsiveness of LH secretion to elevated concentrations of cortisol.

As for the analysis of mean plasma concentrations of LH, a sex difference in the extent to which LH pulse amplitude is reduced during elevated plasma concentrations of cortisol is suggested by the observation that during the infusion of both doses of cortisol, LH pulse amplitude was significantly lower in rams than in ewes. Furthermore, LH pulse amplitude was only reduced during the infusion of the higher dose of cortisol in ewes, whereas both doses of cortisol were associated with a decrease in LH pulse amplitude in rams. These data suggest that in both males and females, cortisol may have actions directly at the pituitary to reduce the responsiveness of the gonadotrophs to GnRH. Although several *in vivo* studies in domestic species do not support this hypothesis, it cannot be dismissed in the sheep as these studies were performed in heifers (Matteri & Moberg 1982b; Li & Wagner 1983), or in ewes with dexamethasone as the glucocorticoid administered (Phillips & Clarke 1990). *In vitro* studies have demonstrated an effect of cortisol treatment on LH secretion in response to GnRH in pituitary cell cultures from castrated rams (Nangalama & Moberg 1991). Alternatively, the reduction in LH pulse amplitude during infusion with cortisol may have been due to a decrease in the amplitude of GnRH pulses. Although LH pulse amplitude was suppressed in both sexes, underlying sex differences in the mechanisms by

which this was induced may exist. For example, a decrease in LH pulse amplitude in rams may be due to an effect at the pituitary, while in ewes it may be due to actions at the hypothalamus, or vice versa.

In addition to changes in LH pulse amplitude, changes in LH pulse frequency were also observed during infusion with both doses of cortisol in both sexes. These data suggest that there are mechanisms which involve hypothalamic effects of cortisol on the frequency of GnRH pulses, which result in a reduction in the frequency of LH pulses. Sex differences in LH pulse frequency observed during hours 24 to 30 of the infusion of the low dose of cortisol and hours 1 to 6 and 24 to 30 of the infusion of a high dose of cortisol support the idea that LH pulse frequency in the male may be more readily suppressed by elevated plasma concentrations of cortisol than in the female. This is also supported by the fact that a significant difference of LH pulse frequency in the female was only observed during the infusion of the high dose of cortisol, whereas in the male, both doses of cortisol were successful in suppressing LH pulse frequency. Changes in LH pulse frequency have previously been demonstrated in ovariectomised ewes when plasma concentrations of cortisol were elevated to 160ng/ml, but not to 60ng/ml (Debus *et al.* 2002), supporting the results from the present study that the effects of cortisol in LH pulse frequency in ewes are dose dependent. The results are in contrast, however, to those of Daley *et al.* (1999b) which demonstrated that LH pulse frequency was reduced during cortisol treatment in prepubertally castrated rams receiving oestradiol treatment, but not in rams receiving no oestradiol treatment. The mean (±SEM) serum concentrations of cortisol recorded in these animals was 68±8ng/ml. Thus, differences in the plasma concentrations of cortisol achieved may explain the different results. Alternatively, differences in the time of castration may also be important as in the current study, rams were castrated in adulthood rather than prior to the onset of puberty.

The observation that in males, mean plasma concentrations of LH and mean pre-pulse nadir concentrations of LH were further suppressed during hours 24 to 30 of the infusion than hours 1 to 6 of the infusion, suggests that the duration of the cortisol exposure may be important in determining the effect of this treatment on LH secretion. The time-dependent mechanism by which cortisol may induce these effects is unknown. A similar

effect was observed on LH pulse amplitude during the infusion of the high dose of cortisol in the male, suggesting that the effect of cortisol on LH increases with time.

As plasma concentrations of cortisol are elevated during stress, it may be possible that elevated concentrations of cortisol are responsible for the changes in plasma concentrations of LH observed during stress. Sex differences in the parameters of LH secretion that were affected by isolation and restraint stress have been demonstrated previously (Tilbrook *et al.* 1999a). LH pulse frequency was suppressed during isolation and restraint stress in ewes, while LH pulse amplitude was suppressed in rams, although plasma concentrations of LH were equally suppressed in each sex. Based on the findings in the current study, it is possible that cortisol may be a mediator of these effects, particularly in rams where LH pulse amplitude was suppressed during plasma concentrations of cortisol which were elevated to a smaller extent than that observed in the present study. The decrease in LH pulse frequency during isolation and restraint stress in ewes may be less likely to be due to the sole actions of cortisol as LH pulse frequency was not significantly reduced during infusion of the low dose of cortisol in the current study, and these plasma concentrations of cortisol were higher than those achieved during isolation and restraint stress (Tilbrook *et al.* 1999a).

In conclusion, these results suggest that LH secretion is depressed by elevated concentrations of cortisol, and that sex differences, which are also dose dependent, exist in the parameters of pulsatile LH secretion that are affected. The reduction in LH pulse amplitude observed in both rams and ewes suggests that cortisol may suppress pituitary responsiveness to GnRH. This requires further investigation, however, in a model where the confounding effects of hypothalamic influence over the pituitary can be removed.

Chapter 5 ~ Does Cortisol Inhibit Pituitary Responsiveness to GnRH in Hypothalamo-Pituitary Disconnected Ewes and Rams?

5.1. Introduction

Activation of the hypothalamo-pituitary adrenal axis during stress implicates the glucocorticoids as one of many possible mediators of the stress-induced suppression of LH (Section 2.4.4). The experiment presented in Chapter 4 of this thesis showed that the administration of cortisol was associated with suppressed mean plasma concentrations of LH in rams and ewes. As changes in LH pulse frequency and LH pulse amplitude were detected in rams and ewes, it is possible that elevated concentrations of cortisol may have actions at both the hypothalamus and/or pituitary which result in the suppression of the secretion of LH. Further studies are required however, to delineate whether the possible effects of cortisol on LH pulse amplitude are due to a direct effect on pituitary responsiveness, or due to an effect on the amplitude of GnRH pulses.

At present, it is unclear whether pituitary responsiveness to GnRH is suppressed during periods of elevated plasma concentrations of cortisol in sheep. In ovariectomised ewes, dexamethasone treatment had no effect on the responsiveness of the pituitary gland to stimulation with exogenous GnRH (Phillips & Clarke 1990). In another study, however, LH pulse amplitude was reduced by treatment with cortisol and this was independent of changes in the secretion of GnRH (Breen & Karsch 2004). Given the results obtained in the study presented in Chapter 4, it is possible that a naturally occurring glucocorticoid may be able to suppress the response of the pituitary to GnRH, and that sex differences may exist in the site of action at which elevated concentrations of the glucocorticoids suppress LH pulse amplitude. Sex differences in the sites of action of stress on LH secretion were suggested by (Tilbrook *et al.* 1999a), who showed that although mean plasma concentrations of LH were equally suppressed during isolation and restraint stress in rams and ewes, the imposition of the stressor was associated with a decrease in LH pulse

frequency in gonadectomised ewes and a decrease in LH pulse amplitude in rams. Sex differences in the extent to which parameters of LH secretion are suppressed during elevated concentrations of cortisol were also suggested by the data in Chapter 4, again highlighting the possibility that sex differences exist in the sites of action at which cortisol is acting to suppress the secretion of LH. Despite these studies, it remains to be definitively demonstrated that cortisol is capable of decreasing LH secretion by mechanisms that influence the responsiveness of the anterior pituitary to GnRH in males and females. The hypothalamo-pituitary disconnected sheep model can be utilised to determine direct pituitary actions without confounding effects from hypothalamic inputs (Section 3.2.3). Therefore, in the experiments described in this chapter, the hypothalamo-pituitary disconnected sheep model was used to test the hypothesis that cortisol may suppress LH pulse amplitude by a direct effect on pituitary responsiveness to GnRH. Furthermore, the hypothesis that there may be sex differences in the sites of action by which cortisol suppresses LH pulse amplitude was also tested. The first experiment was conducted using the same dose of cortisol as was used for the low dose in Chapter 4. As no effect of this dose of cortisol was observed in ewes, and ewes were more resistant to the effects of cortisol in Chapter 4, Experiment 2 was conducted as a repeat of Experiment 1, except that plasma concentrations of cortisol were elevated even further during the Experiment 2. It was hypothesised that there would be an effect of cortisol on pituitary responsiveness in ewes during this treatment. As Chapter 4 suggested that the effects of cortisol on LH secretion were also time dependent, the effect of a short (five and a half hours) or prolonged (29.5h) infusion of each dose of cortisol on the response of the pituitary gland to GnRH was also investigated.

5.2. Materials and Methods

5.2.1. Animals

The first experiment presented in this chapter was conducted during the breeding season (Section 3.1), while the second experiment was conducted during the non-breeding season. Both experiments used adult rams and ewes that had been gonadectomised between 2 and 24 months prior to the experiment and underwent hypothalamo-pituitary disconnection between 1 week and 24 months prior to the experiment. Routine measurement of plasma concentrations of LH in hypothalamo-pituitary disconnected rams and ewes showed that

they were undetectable, confirming the hypogonadal status of the animals. At least five days prior to each experiment, the animals were fitted with an indwelling jugular catheter (Section 3.2.4) for treatment with GnRH (Section 3.4) and the collection of blood samples (Section 3.3). One day prior to the experiment, animals were fitted with a second indwelling jugular catheter (Section 3.2.4) for the infusion of cortisol or saline.

5.2.2. Experimental procedure

Experiment 1

Experiment 1 was conducted using one group of gonadectomised ewes (n=5) and one group of gonadectomised rams (n=6) during the breeding season. It was conducted in two replicates with two ewes and three rams in the first replicate and three ewes and three rams in the second replicate. The experiment was divided into three sampling periods (Figure 5.1). The initial period was a control period of five hours where there was no treatment. After the control period, an infusion of cortisol (12.5mg/50kg/h) or heparinised saline (1ml/50kg/h) commenced and lasted for 30 hours. The second sampling period occurred during the first six hours of the infusion, and the third sampling period occurred during the last five hours of the infusion. A cross-over design was used in the experiment so that it was repeated seven days later with every animal ultimately receiving a cortisol and a saline infusion.

All animals were treated with intravenous injections of GnRH every two hours for five days prior to the commencement of the experiment and continuing until the end of the experiment. Prior to the experiment, and between experimental days, the injections were administered intravenously by an automated pump (Section 3.4). At the beginning of the experiment, the pump was stopped immediately prior to 0400h. At 0400h, and every two hours until the end of the saline or cortisol infusion, the animals were given a hand-delivered intravenous injection of GnRH (250ng in 5ml saline). The first three injections were given prior to the first period of the experiment to stabilise the pituitary response to GnRH and three injections of GnRH occurred in each of the three periods of the experiment. Blood samples (5ml) were taken from the jugular vein at -10, 5, 10, 20, 30, 40 and 60 minutes relative to each GnRH injection occurring within the three periods of the experiment. The plasma was harvested for measurement of plasma concentrations of LH and cortisol. The concentration of LH was measured in all samples collected, while the

concentration of cortisol was measured in the samples collected at -10, 30 and 60 minutes relative to each GnRH injection.

Experiment 2

Experiment 2 was conducted using one group of gonadectomised rams (n=6) and one group of gonadectomised ewes (n=6) in the non-breeding season. The experiment was conducted in three replicates consisting of three ewes and one ram in the first replicate, two rams and two ewes in the second replicate, and three rams and one ewe in the third replicate. The experimental design and sampling periods were the same as for Experiment 1, except that the infusion of cortisol commenced five and a half hours (rather than five hours) after the control period commenced, and the first sampling period after the start of the infusion was five and a half hours rather than six hours. The total infusion period was 29.5 hours. Cortisol was infused at the same rate as for Experiment 1 except that an initial loading dose of 1mg/kg was given to further elevate plasma concentrations of cortisol. In Experiment 2, each injection of GnRH consisted of 125ng/ml rather than 250ng/ml and two hand-delivered injections of GnRH commencing at 0600h were given prior to the commencement of the sampling periods.

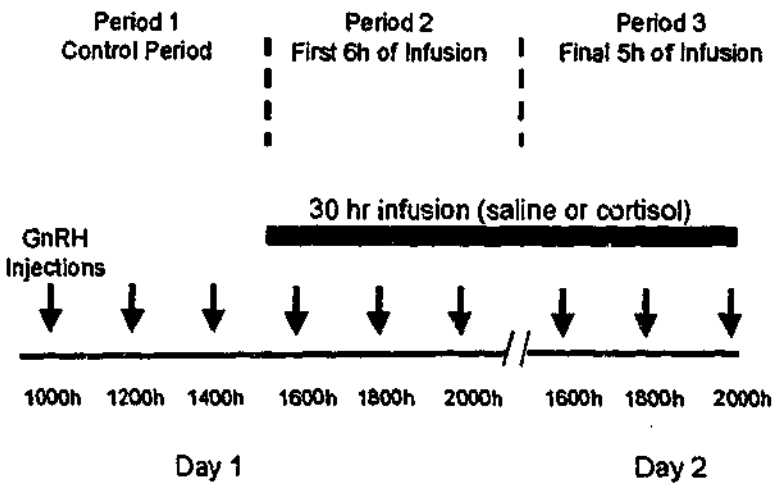


Figure 5.1

Schematic representation of the experimental procedure for Experiment 1. Injections of GnRH were given every two hours and are represented by the arrows. The period of infusion of saline or cortisol is represented by the black bar. The experiment was divided into three periods; a control period of five hours where there was no treatment, a second period which explored the short term effects of cortisol occurring during the first six hours of the infusion, and the third period which investigated the effect of prolonged elevation of cortisol during the last five hours of the infusion.

5.2.3. Radioimmunoassays

The plasma concentrations of LH were measured using the LH radioimmunoassay (Section 3.6.1). In Experiment 1, the mean (±SEM) sensitivity of the assay was 0.50±0.02ng/ml (n=6). The intra-assay coefficient of variation was 7.0% at 3.3ng/ml, 11% at 5.3ng/ml, 7.5% at 6.5ng/ml and 9.9% at 17.7ng/ml. The inter-assay coefficient of variation was 10.9% at 3.5ng/ml, 11.3% at 6.0ng/ml, 12.5% at 7.9ng/ml and 6.7% at 9.1ng/ml. In Experiment 2, the mean (±SEM) sensitivity of the assay was 0.9±0.05ng/ml (n=6). The intra-assay coefficient of variation was 11% at 3.3ng/ml and 14% at 6.8 and 9.0ng/ml. The inter-assay coefficient of variation was 12% at 3.8ng/ml, 7.5% and 10.9ng/ml and 11% at 23.1ng/ml.

Plasma concentrations of cortisol were measured using an unextracted cortisol radioimmunoassay (Section 3.6.3). In the first experiment, the mean sensitivity of the assay was 1.8±0.8ng/ml (n=3). The intra-assay coefficient of variation was 11% at 17.5ng/ml and 12% at 122ng/ml. The inter-assay coefficient of variation was 3.2% at 8.3ng/ml and 2.1% at 65ng/ml. In the second experiment, the mean sensitivity of the assay was 2.5±0.8ng/ml (n=5). The intra-assay coefficient of variation was 9% at 42ng/ml and 10% at 12ng/ml. The inter-assay coefficient of variation was 9% at 12ng/ml, 3% at 39ng/ml and 11% at 85ng/ml.

5.2.4. Statistical analysis

The amplitude of LH pulses, baseline concentrations of LH and mean plasma concentrations of LH and cortisol were calculated (Section 3.8) during the period preceding the infusion, as well as during the first and last periods of sampling during the infusion. Data were compared using a repeated measured analysis of variance with sex as a between subjects factor. The mean hormone concentrations were analysed with day and sampling period as within subjects factors. LH pulse amplitude and baseline concentrations of LH were analysed with day and GnRH injection as within subjects factors. Homogeneity of variance was assessed for each set of data. A square root transformation was applied to data describing mean plasma concentrations of LH in Experiment 2. Transformation by $\log_{10}$ was applied to data describing the mean basal concentrations of LH in Experiment 2 and the mean plasma concentrations of cortisol in Experiment 1.

5.3.Results

5.3.1. Mean plasma concentrations of cortisol

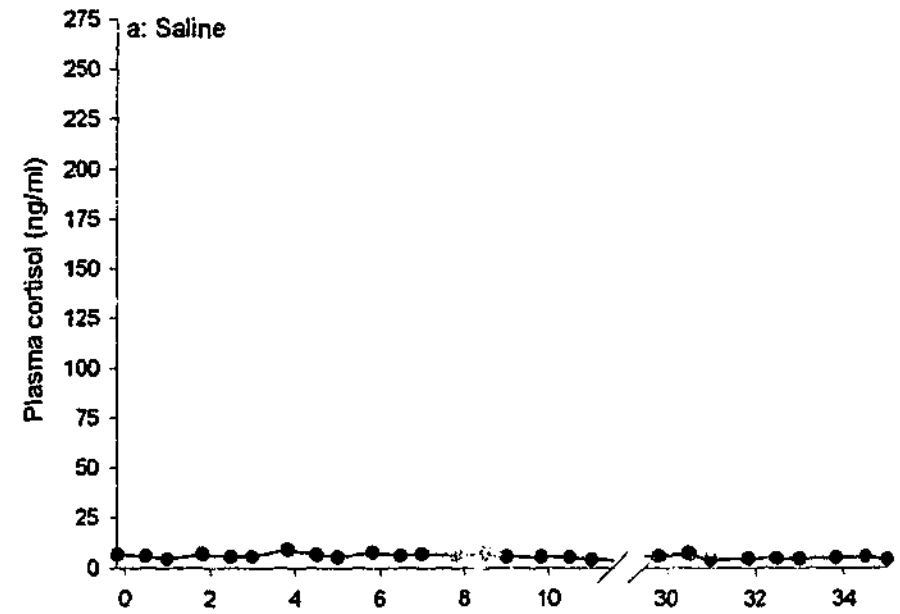
The mean (±SEM) plasma concentrations of cortisol during the control periods on both experimental days were not significantly different to those during the infusion of saline in Experiment 1 (5.6±0.5ng/ml; Figure 5.2) and Experiment 2 (8±1ng/ml; Figure 5.6). In both experiments, the mean plasma concentrations of cortisol during the first and last five and a half hours of the infusion were significantly higher ($p<0.01$) than basal concentrations. During the cortisol infusion in Experiment 1, the mean (±SEM) plasma concentration of cortisol achieved was 154±19ng/ml. There were no significant differences between the plasma concentrations of cortisol achieved in the first six hours and the final five hours of the infusion. In Experiment 2, mean (±SEM) plasma concentrations of cortisol in the first five and a half hours of the infusion (382±31ng/ml) were significantly higher ($p<0.01$) than the concentrations of cortisol during the last five and a half hours of the infusion (218±10ng/ml). There were no significant differences between the sexes in the basal concentrations of cortisol or in the concentrations achieved during the infusion periods in either experiment so the data were combined for analysis.

5.3.2. Parameters of LH secretion

In Experiment 1, there were no significant differences in mean plasma concentrations of LH, or baseline concentrations of LH between control periods or saline infusion and the periods of cortisol infusion in ewes (Figure 5.3) or rams (Figure 5.4). There were no consistent significant differences in LH pulse amplitude in ewes between control periods and the period of cortisol infusion (Figure 5.3). In rams, the amplitude of the LH pulses occurring during the first six hours of the cortisol infusion was significantly reduced ($p<0.05$) (Figure 5.4). The amplitude of these pulses was also significantly lower ($p<0.05$) in rams than in ewes (Figure 5.5). There were no consistent significant changes in LH pulse amplitude in rams during the infusion of saline.

In Experiment 2, there were no significant changes in mean plasma concentrations of LH, baseline concentrations of LH or in LH pulse amplitude during the infusion of saline or cortisol (Figure 5.7). There were also no sex differences in any of the parameters of LH

secretion that were measured, so data from rams and ewes have been combined in Figure 5.7.



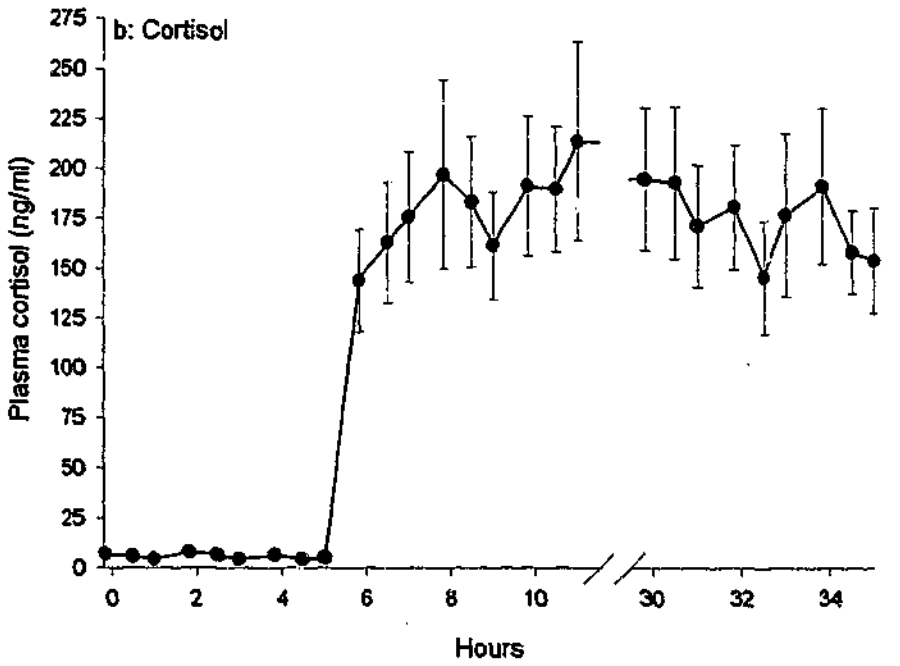
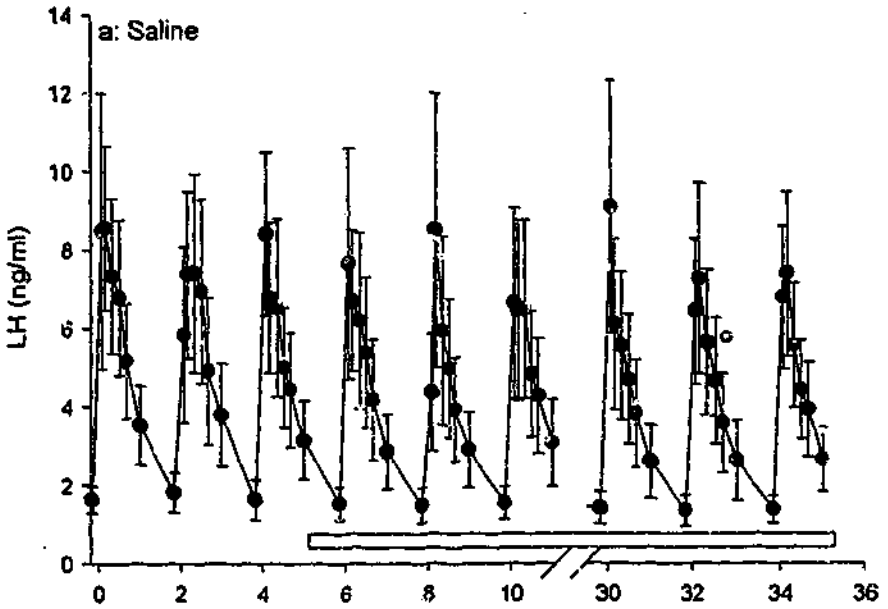


Figure 5.2

Mean (±SEM) plasma concentrations of cortisol (ng/ml) in rams and ewes (combined) prior to and during an infusion of saline (a) or an infusion of cortisol (12.5mg/50kg/hr) (b) in Experiment 1. Plasma concentrations of cortisol were significantly elevated ($p<0.05$) during the infusion of cortisol. There were no differences between the plasma concentrations of cortisol achieved during the first six hours or the last five hours of the infusion.



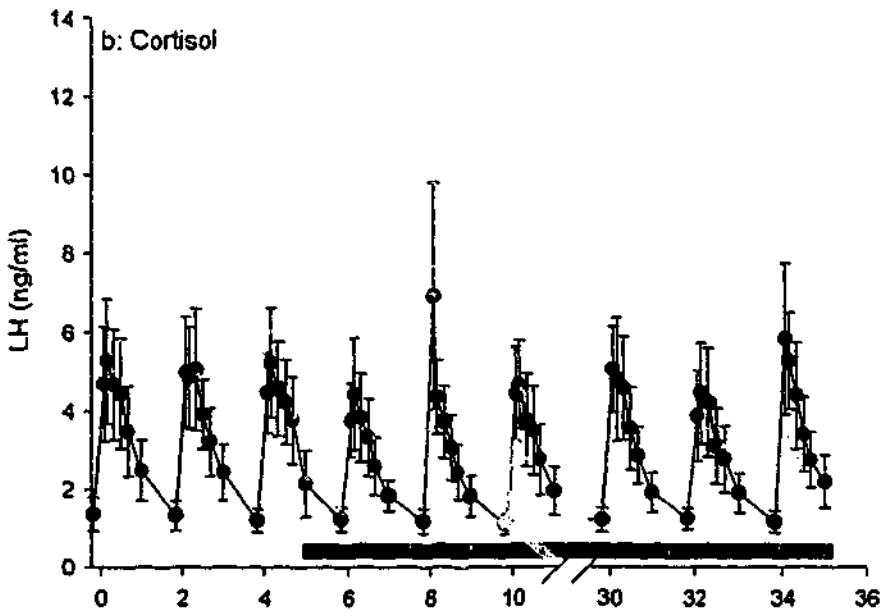
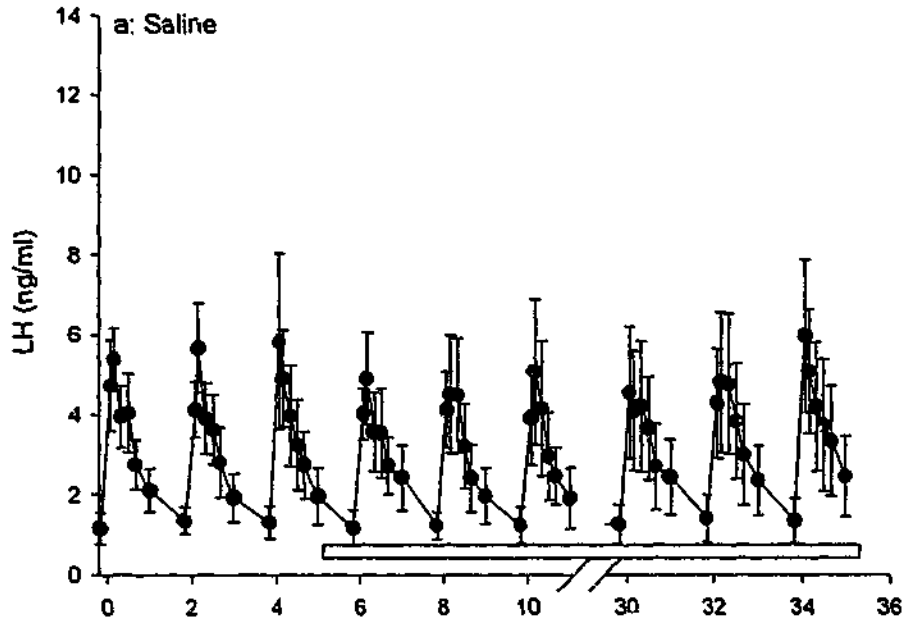


Figure 5.3

Mean (±SEM) plasma concentrations of LH (ng/ml) in ewes prior to and during the infusion of saline (a) or cortisol (b) in Experiment 1. Bars indicate the infusion of saline (open bar) or cortisol (black bar). There were no significant differences in mean plasma concentrations of LH, mean baseline concentrations of LH or LH pulse amplitude between periods.



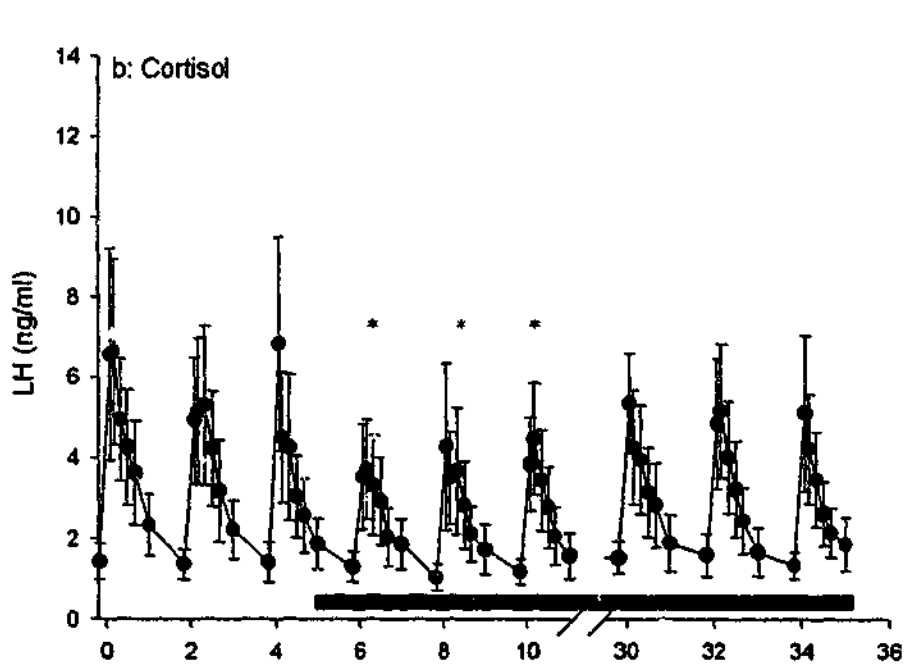
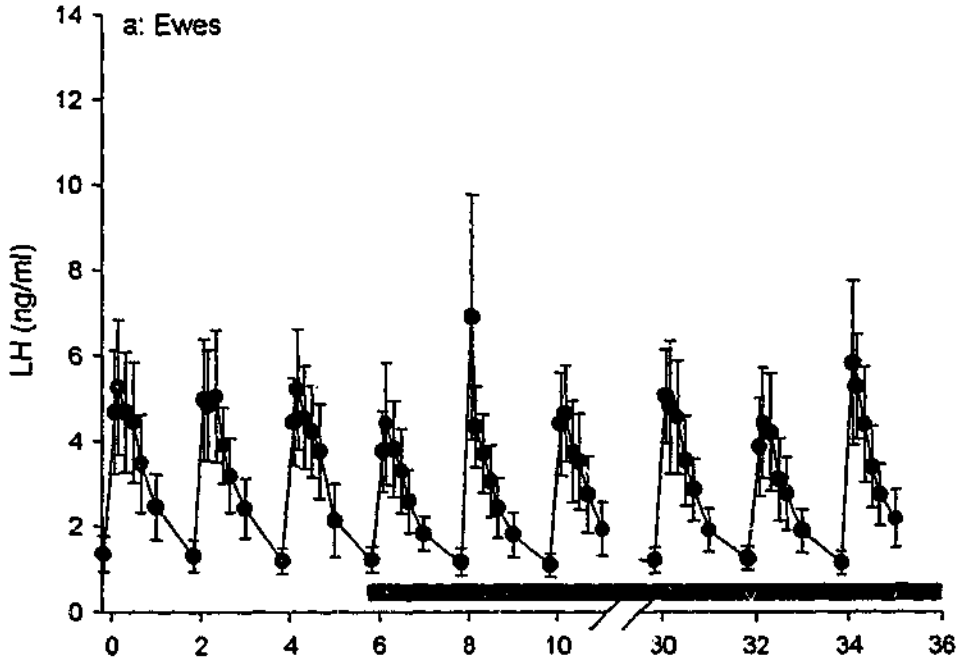


Figure 5.4

Mean (±SEM) plasma concentrations of LH (ng/ml) in rams prior to and during an infusion of saline (a) or cortisol (b) in Experiment 1. Bars indicate the infusion of saline (open bar) or cortisol (black bar). The amplitude of the LH pulses occurring during the first five hours of the cortisol infusion was significantly reduced (* $p<0.05$) compared to the preceding control period. There were no significant differences in mean plasma concentrations of LH or mean baseline concentrations of LH between periods.



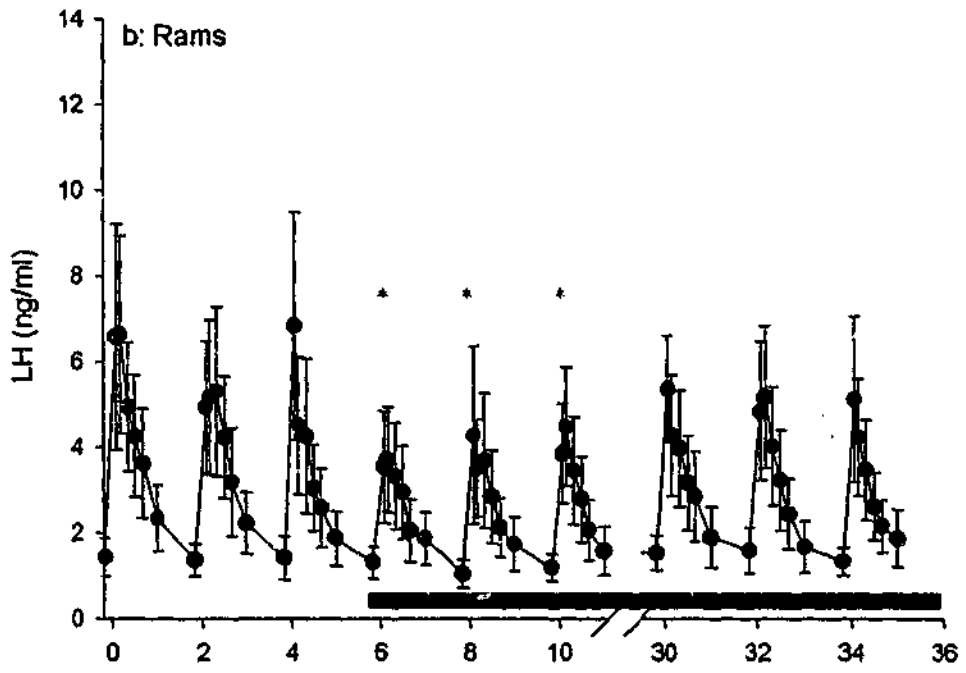


Figure 5.5

Mean (±SEM) plasma concentrations of LH (ng/ml) in ewes (a) or rams (b) prior to and during an infusion of cortisol in Experiment 1. The period of infusion is indicated by the black bar. The amplitude of the LH pulses during the first five hours of the cortisol infusion was significantly smaller (* $p<0.05$) in rams than in ewes.

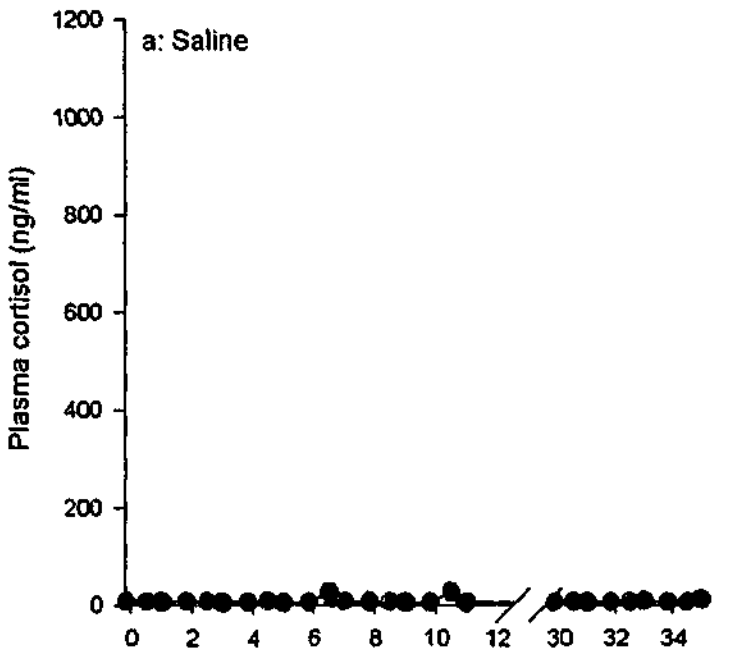
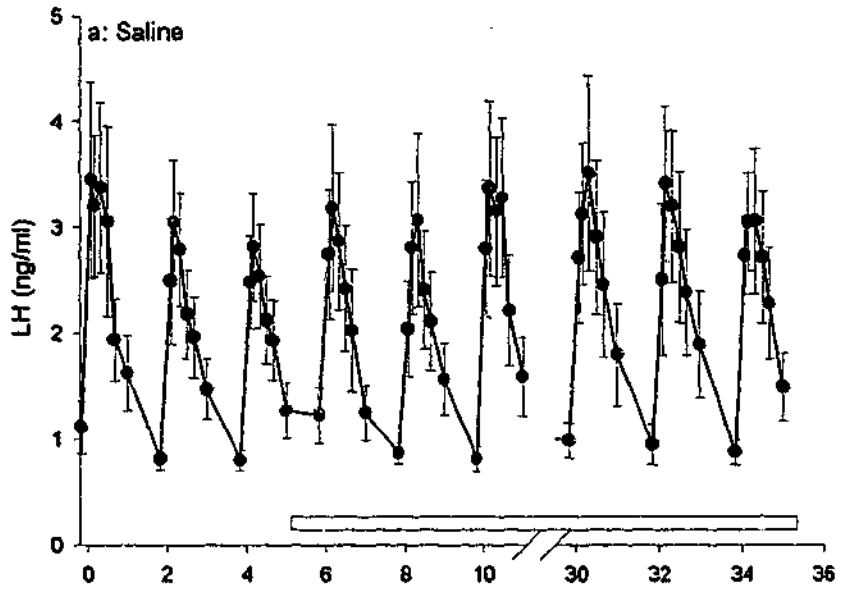


Figure 5.6

Mean (±SEM) plasma concentrations of cortisol (ng/ml) in rams and ewes (combined) prior to and during an infusion of saline (a) or an infusion of cortisol (12.5mg/50kg/hr after a loading dose of 1mg/kg)(b) in Experiment 2. Plasma concentrations of cortisol were significantly elevated ($p<0.05$) during the infusion of cortisol. In addition, the plasma concentrations of cortisol achieved during the first five and a half hours of the infusion were significantly higher ($p<0.05$) than those achieved during the final five hours of the infusion.



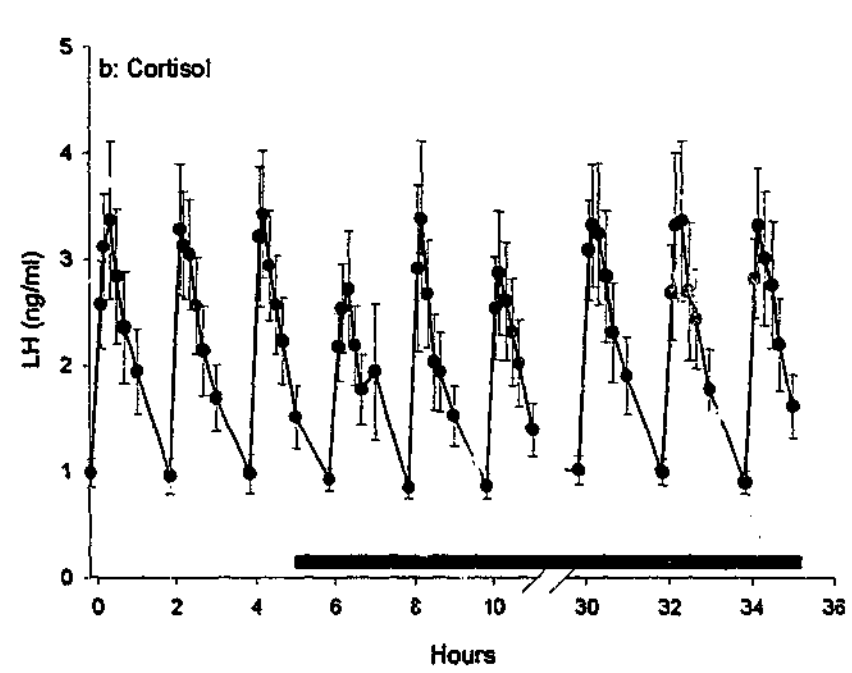


Figure 5.7

Mean (±SEM) plasma concentrations of LH (ng/ml) in rams and ewes (combined) before or during an infusion of saline (a) or cortisol (b). Bars indicate the infusion of saline (open bar) or cortisol (black bar). There were no significant differences in mean plasma concentrations of LH, baseline concentrations of LH or pulse amplitude between periods.

5.4. Discussion

These results show that, in hypothalamo-pituitary disconnected, gonadectomised Romney Marsh rams during the breeding season, LH pulse amplitude was reduced during an infusion of cortisol that raised plasma concentrations to 150ng/ml. The results suggest that, in rams, cortisol may have direct pituitary actions that result in a decrease in pituitary responsiveness to GnRH. No direct actions of cortisol on pituitary responsiveness to GnRH were observed in ewes, suggesting that cortisol does not have direct pituitary actions in ewes and that there may be a sex difference in the mechanisms by which cortisol suppresses LH secretion. Furthermore, the effect of cortisol on pituitary responsiveness in rams to GnRH may be seasonally dependent or dependent upon the concentrations to which plasma concentrations of cortisol are elevated.

Results from the experiment presented in Chapter 4 of this thesis showed that LH pulse amplitude was reduced in rams when plasma concentrations of cortisol were elevated to concentrations of 120ng/ml and higher. In conjunction with data from Experiment 1, these results suggest that cortisol may have direct actions at the pituitary to cause a reduction in responsiveness to GnRH. Previous studies in gonadectomised rams showed a decrease in LH pulse amplitude during isolation and restraint stress (Tilbrook *et al.* 1999a). It is possible that this reduction in LH pulse amplitude occurred due to a decrease in pituitary responsiveness as a result of elevated concentrations of cortisol. Furthermore, a decrease in the LH response to GnRH has been observed in rams treated with ACTH (Matteri *et al.* 1984; Tilbrook *et al.* 1999a). This may also be attributed to an increase in plasma concentrations of cortisol and may not be as a result of the direct actions of ACTH. This hypothesis could be tested by administering ACTH to animals in which endogenous cortisol secretion had been blocked by treatment with the glucocorticoid synthesis inhibitor, metyrapone, or by adrenalectomy. The present results are supported by studies in bulls (Chantaraprateep & Thibier 1978) and rats (Baldwin & Sawyer 1974; Ringstrom & Schwartz 1985; Rosen *et al.* 1988; Suter *et al.* 1988) which have also shown a decrease in pituitary responsiveness to GnRH during elevated plasma concentrations of cortisol in males. Furthermore, in cultures of pituitary cells from castrated rams, cortisol was able to suppress LH secretion in response to GnRH (Nangalama & Moberg 1991). Collectively,

these studies, and the present results, provide a strong case for a direct effect of cortisol on pituitary responsiveness in males.

Sex differences in the sites of action by which cortisol suppresses the secretion of LH are suggested by the finding that cortisol did not suppress pituitary responsiveness to GnRH in ewes in either experiment. Although plasma concentrations of LH, and LH pulse amplitude, were suppressed in ewes during periods of elevated concentrations of cortisol in Chapter 4, it is possible that these effects were not due to an effect of cortisol directly at the pituitary gland. Rather, cortisol may act at the hypothalamus in ewes to cause a reduction in GnRH pulse amplitude, which is then transduced into a decrease in LH pulse amplitude and this is supported by findings from (Phillips & Clarke 1990). In contrast, however, (Breen & Karsch 2004) found that LH pulse amplitude in response to the injection of exogenous GnRH was decreased during treatment with cortisol in ovariectomised ewes with an oestrogen-induced negative-feedback clamp on the secretion of endogenous GnRH. Studies in humans (Melis *et al.* 1987) and rats (Baldwin & Sawyer 1974; Ringstrom *et al.* 1992) are in contrast to the current findings and suggest that in females, pituitary responsiveness to GnRH may be decreased during periods of elevated concentrations of glucocorticoids.

In Experiment 2, there was no effect of cortisol on pituitary responsiveness to GnRH secretion in rams or in ewes. These findings are unexpected in light of the findings from Experiment 1, but the differences observed may be due, in part at least, to the different cortisol treatments used. The method of administration of cortisol was designed to cause a rapid increase in cortisol to concentrations similar to that observed during the higher dose of cortisol in Chapter 4 (260ng/ml). The plasma concentrations of cortisol achieved in Experiment 2 were much greater than this and it is possible that down-regulation of cortisol receptors may have occurred. It is also possible that there is an effect of season on the ability of cortisol to reduce pituitary responsiveness to GnRH. Metabolic activity is seasonally dependent (Clarke 2001). Melatonin secretion is also seasonal in sheep (Brook & Marshall 1996), and melatonin receptors are known to be in close proximity to the gonadotrophs (Helliwell & Williams 1992). It is possible, therefore, that seasonally

dependent factors may regulate the actions of cortisol on pituitary responsiveness to GnRH.

In conclusion, these results suggest that elevated concentrations of cortisol may directly alter pituitary response to GnRH in gonadectomised rams, but not in gonadectomised ewes. This suggests that the effect of stress on the amplitude of LH pulses in rams may be as a direct result of cortisol actions at the pituitary. In ewes, however, the reduction in LH pulse amplitude observed previously during elevation of cortisol may be due to an action of cortisol on the amplitude of GnRH pulses, rather than a direct action at the pituitary. It is possible that substances other than cortisol cause a decrease in pituitary responsiveness during stress. Further work is required before these substances can be identified.

Chapter 6 ~ Does Stress Inhibit Pituitary Responsiveness to GnRH in Hypothalamo-Pituitary Disconnected Ewes and Rams?

6.1. Introduction

The secretion of LH has been shown to decrease under conditions of stress (Section 2.3). The effect may be sex-dependent, since isolation and restraint stress caused a decrease in LH pulse frequency in gonadectomised ewes but a decrease in LH pulse amplitude in gonadectomised rams (Tilbrook *et al.* 1999a). Differences in the sites of action where mediators of the stress response are able to suppress the secretion of LH may account for these sex differences.

Changes in LH pulse amplitude may be due to a decrease in the amplitude of GnRH pulses from the hypothalamus, or a change in the responsiveness of the pituitary gland to GnRH. Changes in the responsiveness of the pituitary gland to GnRH during stress have been suggested in several studies (Section 2.3.4). The idea that there are sex differences in the mechanisms by which stress affects LH secretion was supported by findings reported in Chapter 4. These findings demonstrated that there were sex differences in the extent to which LH secretion was reduced under elevated concentrations of cortisol. The findings in Chapter 5 also support this hypothesis, as they showed that pituitary responsiveness to LH secretion may be suppressed under conditions of elevated concentrations of cortisol in rams but not in ewes.

It is possible that in addition to the hypothalamo-pituitary adrenal axis, there may be other systems activated by stress which affect pituitary responsiveness to GnRH. The hypothalamo-pituitary disconnected sheep provides a robust model for the *in vivo* study of pituitary function in isolation from regulation by the hypothalamus (Section 3.2.3). In addition to the removal of endogenous GnRH input, the hypothalamic control of ACTH and cortisol is also rendered inoperable. Therefore, exposure to psychological stressors

such as the stress of a barking dog (Engler *et al.* 1988), or isolation and restraint stress, will not cause activation of the hypothalamo-pituitary adrenal axis. Consequently, any changes in pituitary responsiveness to GnRH during a stress response are therefore likely to be due to other systems that respond to stress. One such example is the sympathoadrenal system which has been shown to be activated by stress and may have a role in the stress-induced suppression of LH secretion (Section 2.4.9).

In addition to sex, it seems feasible that season may influence the impact of stress on the response of the pituitary to GnRH. It is not known whether the physiological systems activated during stress are altered by seasonal factors in mammals, but there is evidence for seasonal differences in basal and stress-induced secretion of the glucocorticoids in birds, reptiles and amphibians (Romero 2002). Furthermore, there may be seasonal differences in the role of cortisol in the suppression of LH secretion (Chapter 5). Consequently, the experiments presented in this chapter tested the hypothesis that isolation and restraint stress suppresses the responsiveness of the pituitary gland to exogenous GnRH and that this response is altered by sex and season.

6.2. Materials and Methods

6.2.1. Animals

Adult rams and ewes were used in two experiments. Experiment 1 was conducted during the breeding season, while Experiment 2 was conducted during the non-breeding season. In both experiments, the rams and ewes were gonadectomised at least six weeks prior to the commencement of the experiment (Sections 3.2.1 and 3.2.2). All animals underwent hypothalamo-pituitary disconnection between two weeks and nine months prior to the commencement of the experiment (Section 3.2.3). The animals were fitted with an indwelling jugular catheter (Section 3.2.4) eight days prior to the commencement of the experiment for the injection of GnRH (Section 3.4) and the collection of blood samples (Section 3.3). Animals were housed in individual pens for one week prior to the beginning of the experiment.

6.2.2. Experimental Procedure

Experiment 1

A cross-over design was used in Experiment 1. There were two groups consisting of one group of gonadectomised rams (n=6) and one group of gonadectomised ewes (n=5). The experiment was conducted in two replicates, so that three rams and three ewes were included in one replicate of the experiment and three rams and two ewes were in the other replicate. There were three experimental days in each replicate with two days between each experimental day. Each experimental day was divided into two periods. The first period on each day was a control period. Days 1 and 3 were designated control days and no stress was imposed during the second period on these days. Isolation and restraint stress was imposed during the second period on Day 2 (Section 3.5).

For one week prior to the commencement of the experiment, and between experimental days, the animals were treated with an intravenous injection of GnRH by an automated pump every two hours (Section 3.4). On each experimental day, the pump was stopped immediately prior to 0600h. At 0600h, and every two hours until 2000h, the animals were given a hand-delivered injection of GnRH (125ng/5ml saline). Two GnRH injections were given prior to the commencement of the first period on each experimental day and three injections were given in each of the two subsequent periods. Blood samples (5ml) were taken from the jugular vein at -10, 5, 10, 20, 30, 40 and 60 minutes relative to each experimental GnRH injection, beginning at 0950h and continuing until 2100h. The plasma was harvested for the measurement of the concentrations of LH and cortisol. Whereas the concentrations of LH were measured in all the samples collected, the concentrations of cortisol were measured in the samples collected at -10, 30 and 60 minutes relative to each GnRH injection and in all the samples taken in the first hour after the commencement of isolation and restraint stress. Beginning at 1530h on Day 2, isolation and restraint stress was imposed for five and a half hours (until 2100h).

Experiment 2

In Experiment 2, the design and procedure of Experiment 1 was repeated, except that additional blood samples were taken –10, -5, 2, 5, 10, 15, 20 and 25 minutes relative to the onset of the stress and at the same time on the control days. The samples were collected in non-heparinised tubes containing glutathione oxidation inhibitor (Section 3.3). The plasma

concentrations of adrenaline, noradrenaline, DOPA and DHPG were measured in the additional samples. In addition, catecholamine concentrations were measured in samples taken one hour before, and one and two hours after the onset of stress. As in Experiment 1, LH was measured in samples taken at -10, 5, 10, 20, 30, 40 and 60 minutes relative to each injection of GnRH. Cortisol was measured in samples taken approximately every hour throughout the experiment and in samples taken approximately every half an hour for two hours after the onset of isolation and restraint stress.

6.2.3. Radioimmunoassays

Plasma LH concentrations were determined using a LH radioimmunoassay (Section 3.6.1). The mean (±SEM) sensitivity of the assay was 0.70±0.05ng/ml (n=13). The intra-assay coefficient of variation was 7% at 15ng/ml and 7% at 36ng/ml. The inter-assay coefficient of variation was 11% at 8ng/ml and 9% at 11ng/ml. Concentrations of cortisol in plasma were measured using an extracted cortisol radioimmunoassay (Section 3.6.3). The mean (±SEM) sensitivity of the assay was 1.6±0.06ng/ml (n=14). The intra-assay coefficient of variation was 6% at 19ng/ml and 8% at 164ng/ml. The inter-assay coefficient of variation was 9% at 8ng/ml and 9% at 21ng/ml.

6.2.4. Catecholamine detection

Adrenaline, noradrenaline, DOPA and DHPG were measured in plasma samples according to the procedures described in Section 3.7. The sensitivity of this procedure was 15pg/ml.

6.2.5. Statistical analysis

The parameters measured were LH pulse amplitude, baseline concentrations of LH and mean plasma concentrations of LH and cortisol (Section 3.8). All data were analysed using repeated measures analysis of variance with sex as the between subjects factor. LH pulse amplitude and baseline LH concentrations were analysed with day and pulse as within subjects factors. The mean concentrations of LH and cortisol were analysed with day and period as within subject factors. The concentrations of adrenaline, noradrenaline, DOPA and DHPG were analysed with day and time as within subject factors. For each set of data, the homogeneity of variance was assessed. Square root transformation was applied to the data describing mean plasma concentrations of LH and cortisol in Experiment 1 and plasma concentrations of DOPA in Experiment 2. Log_{10} transformation was applied to

data describing LH pulse amplitude and plasma concentrations of adrenaline and noradrenaline in Experiment 2. For the adrenaline data, one unit was added to each data point before log transformation.

6.3. Results

6.3.1. Mean plasma concentrations of cortisol

In Experiments 1 and 2, there were no significant changes in plasma concentrations of cortisol when isolation and restraint stress was imposed. In Experiment 1, the mean (±SEM) concentrations of cortisol were 5.9±1.3ng/ml on Day 1, 5.6±0.6ng/ml on Day 2 and 4.0±0.5ng/ml on Day 3. In Experiment 2, the mean (±SEM) plasma concentrations of cortisol were 7.2±1.0ng/ml, 7.9±1.3ng/ml and 7.1±1.1ng/ml on Days 1, 2 and 3 respectively. There were no significant differences between the sexes so the data for males and females are combined.

6.3.2. Plasma concentrations of the catecholamines

Adrenaline

In rams, plasma concentrations of adrenaline were significantly ($p<0.05$) greater than pre-stress concentrations from 2 to 180 minutes after the onset of isolation and restraint stress (Figure 6.1). Plasma concentrations of adrenaline in the samples taken 60, 10 and 5 minutes before the onset of stress on Day 2 were not significantly different from each other. There were no consistent significant changes in the plasma concentrations of adrenaline on Day 1 or Day 3 in rams.

In ewes, plasma concentrations of adrenaline were significantly ($p<0.05$) greater than pre-stress concentrations from 2 to 25 minutes after the commencement of the isolation and restraint stress (Figure 6.1). At 60 and 180 minutes after the onset of stress, the plasma concentrations of adrenaline were similar to those in the pre-stress period. There were no significant changes in the plasma concentrations of adrenaline in the period before isolation and restraint stress and there were no consistent significant differences in the plasma concentrations of adrenaline on Day 1 or Day 3 in ewes. Plasma concentrations of adrenaline did not differ significantly in a consistent manner between rams and ewes.

Noradrenaline, DOPA and DHPG

There were no significant changes in noradrenaline concentrations when isolation and restraint stress was imposed, however, at all time points, the plasma concentrations of noradrenaline in ewes were significantly ($p<0.05$) higher than those in rams (Figure 6.2). Neither isolation and restraint stress nor sex significantly influenced plasma concentrations of DOPA (Day 1: 1.5±0.8, Day 2: 1.6±0.7, Day 3: 1.4±0.7 ng/ml) or DHPG (Day 1: 1.0±0.2, Day 2: 1.2±0.3, Day 3: 1.1±0.3 ng/ml) in any consistent manner.

6.3.3. Parameters of LH secretion

In Experiment 1, conducted during the breeding season, there were no significant changes in the LH pulse amplitude, mean LH concentrations or the nadir preceding each GnRH injection when isolation and restraint stress was imposed (Figure 6.3). During the non-breeding season (Experiment 2) there was a significant ($p<0.05$) effect of isolation and restraint stress on LH pulse amplitude (Figure 6.4). On Day 2, the amplitude of the fourth LH pulse (the first pulse during isolation and restraint stress) was significantly ($p<0.05$) less than that of the third pulse (the pulse immediately preceding the onset of stress). There were no effects of isolation and restraint stress on mean plasma concentrations of LH or on the nadir preceding each GnRH injection. There were no significant differences between the sexes so the data for rams and ewes are combined in Figure 6.3 and Figure 6.4.

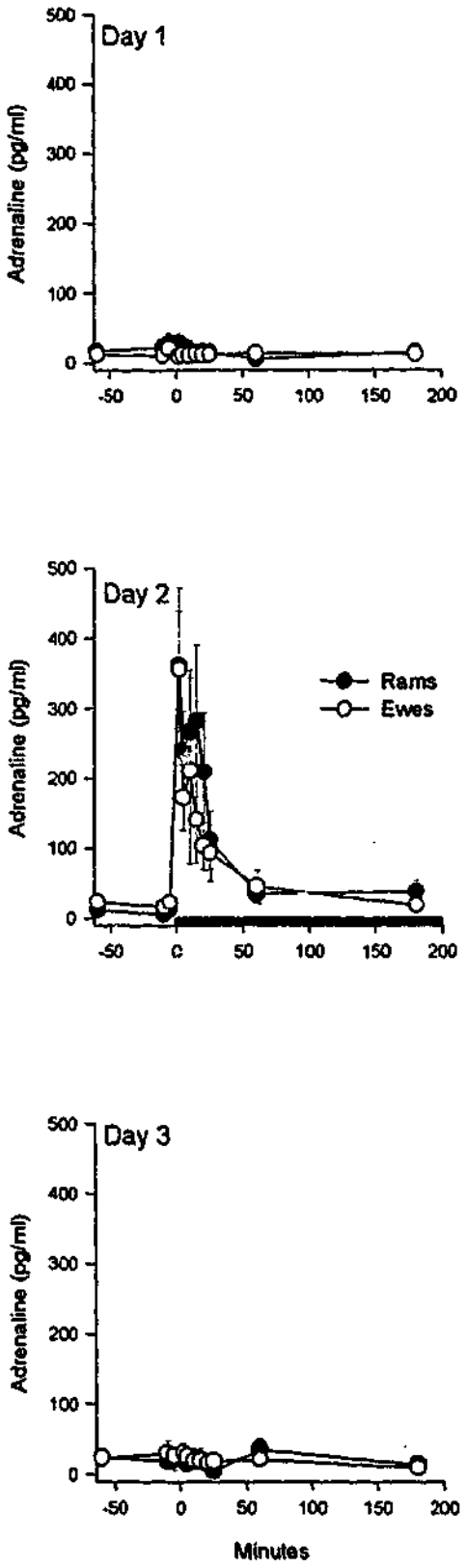


Figure 6.1

Mean (±SEM) plasma concentrations of adrenaline (pg/ml) in rams (closed circles) and ewes (open circles) on Day 1, 2 and 3 of Experiment 2. The period of isolation and restraint stress is indicated by the black bar. Plasma concentrations of adrenaline were significantly higher ($p<0.05$) than prestress concentrations of adrenaline from 2 to 180 minutes after the onset of stress in rams, and from 2 to 25 minutes after the onset of stress in ewes. There were no consistent significant differences in the plasma concentrations of adrenaline between rams and ewes.

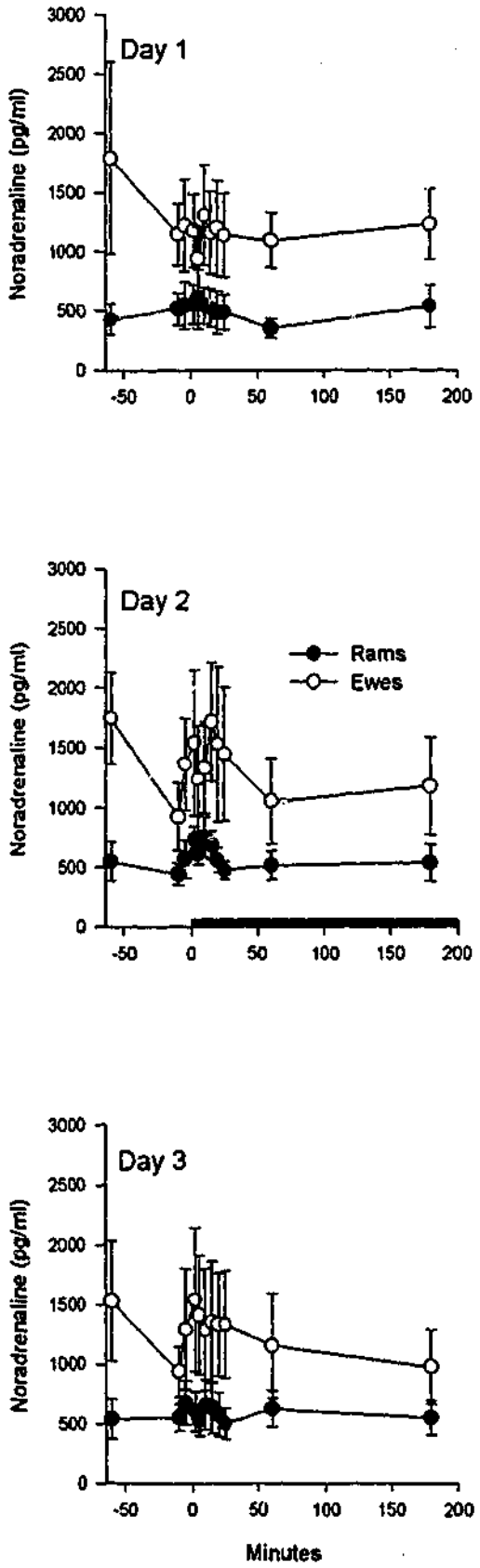


Figure 6.2

Mean (±SEM) plasma concentrations of noradrenaline (pg/ml) in rams (closed circles) and ewes (open circles) on Days 1, 2 and 3 of Experiment 2. The period of isolation and restraint stress is indicated by the black bar. Plasma concentrations of noradrenaline were consistently significantly ($p<0.05$) higher in ewes than in rams but were not influenced significantly by isolation and restraint stress.

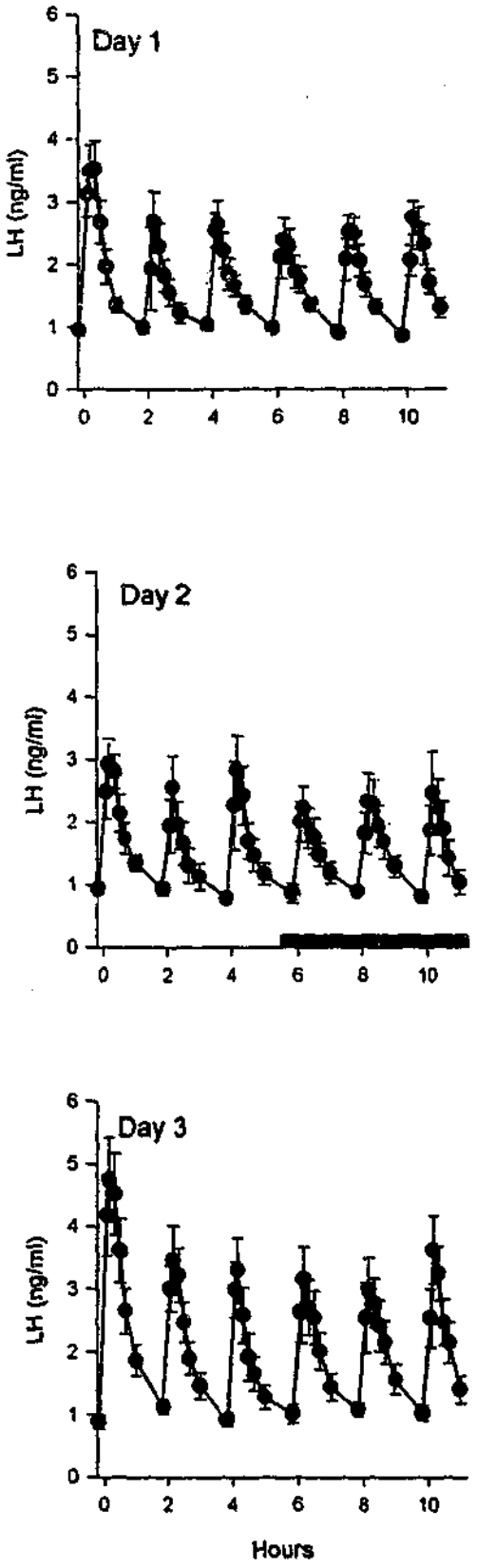


Figure 6.3

Mean (±SEM) plasma concentrations of LH (ng/ml) for rams and ewes (combined) in Experiment 1, conducted during the breeding season. Isolation and restraint stress was imposed during the second period on Day 2 and is represented by the black bar. There were no consistent significant differences in any of the parameters of LH that were measured between the pre-stress and stress periods on Day 2.

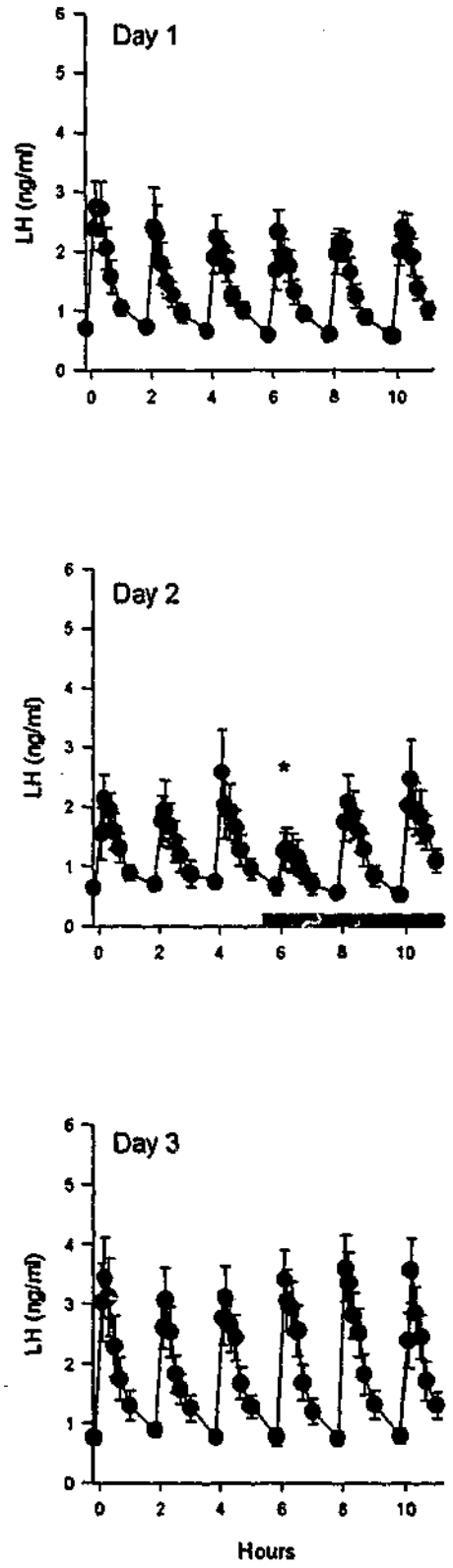


Figure 6.4

Mean (±SEM) plasma concentrations of LH (ng/ml) for rams and ewes combined in Experiment 2, conducted during the non-breeding season. Isolation and restraint stress was imposed during the second period on Day 2 and is represented by the black bar. *The amplitude of the first LH pulse that occurred during isolation and restraint stress (fourth pulse on Day 2) was significantly ($p<0.05$) lower than that for the preceding, pre-stress pulse (third LH pulse on Day 2).

6.4. Discussion

The results from this study show that stress may act directly at the pituitary gland to reduce its ability to secrete LH in response to exogenous GnRH during the non-breeding season but not during the breeding season in sheep. Thus, alteration in the pituitary responsiveness to GnRH may be one mechanism by which stress can affect reproduction in sheep but this appears to vary with season. Previous work from our laboratory, conducted during the breeding season, showed that there was a decrease in LH pulse amplitude, and a decrease in the LH response to an injection of GnRH during isolation and restraint stress in gonadectomised rams, but not in gonadectomised ewes (Tilbrook *et al.* 1999a). The present data suggest that there is no decrease in pituitary responsiveness to GnRH in the breeding season when the pituitary gland is surgically isolated from the brain and given repeated injections of GnRH. The reason for the different findings in these studies is not known but it may be due, in part at least, to the different animal models that were used. In hypothalamo-pituitary disconnected sheep, there is no stress-induced activation of the hypothalamo-pituitary adrenal axis with consequent increases in plasma concentrations of ACTH and cortisol whereas, in hypothalamo-pituitary intact animals, the hypothalamo-pituitary adrenal axis is able to respond to a stressful stimulus. Therefore, the changes in the LH response of the pituitary due to stress during the breeding season in rams seen in our previous study may have been due to the actions of ACTH and/or cortisol. Indeed, infusion of ACTH into adrenalectomised and adrenal intact rams during the breeding season has been shown to cause a decrease in the LH response to exogenous GnRH Fuquay & Moberg (1983) and Matteri *et al.* (1984) have also shown that infusion of ACTH into rams caused a decrease in the LH response to GnRH. Furthermore, cortisol appears to be capable of decreasing in pituitary responsiveness to GnRH by a direct pituitary action (Chapter 5).

The mechanisms by which stress decreased the LH response of the pituitary to GnRH during the non-breeding season are unknown. Nevertheless, it appears that ACTH and cortisol were not involved as the hypothalamo-pituitary adrenal axis of these animals was rendered inoperable due to the disconnection of the hypothalamic inputs to the pituitary gland. This disconnection was confirmed in our animals as there was no significant increase in cortisol during isolation and restraint stress. The decrease in LH responsiveness

to GnRH was only observed after the first GnRH injection given half an hour after the commencement of the stress, and was not observed after the second and third injections given 2.5 and 4.5 hours after commencement of the stress, respectively. These results suggest that there was an immediate, short term effect of isolation and restraint stress on pituitary responsiveness in these animals. The mediators of this rapid effect have not been identified but it is possible that adrenaline is involved as there was a contemporaneous increase in adrenaline secretion in both rams and ewes during isolation and restraint stress. This hypothesis has not been tested. In contrast to the results of (Niezgoda *et al.* 1993), who showed an increase in noradrenaline concentrations in response to isolation and restraint stress in ewes, we found no significant increase in peripheral plasma concentrations of noradrenaline during isolation and restraint stress in Experiment 2. It is also possible that neural modulation of secretory cells of the pituitary may be a mechanism by which short-term changes in pituitary responsiveness can be regulated as synapses between nerve fibres and secretory cells of the anterior pituitary have been identified in the dog and the rat (Ju 1999), and in particular on gonadotrophs in the rat (Liu *et al.* 1996). Other systems which are activated during stress include the opioidergic system but it is unclear whether opioids are able to decrease the pituitary responsiveness to GnRH during stress (Blank *et al.* 1986; Horton *et al.* 1990). Studies have suggested that the opioids may cause a decrease in the secretion of LH in rams in a manner which is dependent on both season and the presence of testosterone (Lincoln *et al.* 1987; Jackson & Kuehl 2000).

Collectively, these studies suggest that the mechanisms by which stress decreases LH secretion are dependent on seasonal factors. Previous studies from our laboratory have shown that there is no seasonal change in the release of LH from the pituitary in response to GnRH (Tilbrook *et al.* 1991), although there are seasonal differences in the negative feedback effects of inhibin and testosterone on the secretion of the gonadotrophins (Tilbrook *et al.* 1999a). Seasonal factors may be able to alter the susceptibility of the reproductive axis to stress. The mechanisms by which this may occur are unknown. Melatonin secretion is known to increase during periods of shortening day length in sheep (Brook & Marshall 1996) and melatonin receptors have been identified in the pars tuberalis of the ovine pituitary (Helliwell & Williams 1992), a site which is rich in gonadotrophs. The pars tuberalis remains with the pituitary in the hypothalamo-pituitary disconnected

sheep (Clarke *et al.* 1983) and seasonal differences in endocrine feedback effects at the pituitary have previously been demonstrated in the ram using this model (Tilbrook *et al.* 1993). There are also seasonal changes in metabolic activity between seasons (Clarke 2001), and the hypothalamo-pituitary adrenal axis response to stress has been shown to be different in fat and thin sheep (Tilbrook *et al.* 2002b). Consequently, it seems feasible that metabolic activity may also be important in regulating the susceptibility of the pituitary LH response to GnRH during stress.

In this study, there were no sex differences in the maximal concentrations of circulating adrenaline during isolation and restraint stress, although the duration of the response was longer in rams than in ewes. There was a sex difference in the basal plasma concentrations of noradrenaline as the plasma concentrations of noradrenaline were consistently higher in ewes than in rams. In contrast, a previous study in sheep (Turner *et al.* 2002c) found no sex differences in basal or stress-induced concentrations of central noradrenaline. There are sex differences in the response of the hypothalamo-pituitary adrenal axis to stress and this varies with the type of stressor and is influenced by the presence of the sex steroids (Handa *et al.* 1994; Turner *et al.* 2002a). Evidence also exists for sex differences in the activation of the sympathoadrenal system in response to stress. For example, it has been shown that the sympathetic nervous system is less reactive in women than in men (Hinojosa-Laborde *et al.* 1999). Weinstock *et al.* (1998), however, found that, in rats, the female sympathoadrenal system was more sensitive to foot shock and a novel environment than that of males. Despite the differences between males and females in activation of various stress systems, and the findings described in Chapter 5 that cortisol suppressed pituitary responsiveness to GnRH in rams but not ewes, these results indicate that there is no difference between gonadectomised male and female hypothalamo-pituitary disconnected sheep in the impact of isolation and restraint stress on the release of LH in response to GnRH.

In summary, these studies show that changes in pituitary responsiveness to GnRH occur during isolation and restraint stress in hypothalamo-pituitary disconnected rams and ewes in the non-breeding season but not during the breeding season. It is likely that this effect is due to mechanisms that are independent of increases in the secretion of ACTH and cortisol. It is possible that members of the sympathoadrenal system are involved in

decreasing the pituitary responsiveness to GnRH but this possibility has not been explored. Further work is needed to investigate the mechanisms by which stress decreases pituitary responsiveness and the effect of seasonal factors on these mechanisms. The development of a model which blocks endogenous GnRH secretion without disrupting the stress response will further aid work in this area.

Chapter 7 ~ The Effect of Stress on Pituitary Responsiveness to an Analogue of GnRH in Ewes and Rams Passively Immunised Against Native GnRH: Effects of the Sex Steroids.

7.1. Introduction

The imposition of isolation and restraint stress has been associated with a decrease in LH pulse amplitude in gonadectomised rams and in gonadectomised ewes treated with oestrogen or oestrogen and progesterone (Tilbrook *et al.* 1999a). Transport stress has also been associated with a decrease in LH pulse amplitude in ewes (Dobson *et al.* 1999). Furthermore, elevated plasma concentrations of cortisol have been associated with a decrease in LH pulse amplitude in gonadectomised rams and ewes (Chapter 4). It is possible, therefore, that elevated plasma concentrations of cortisol may mediate the effect of isolation and restraint stress on LH pulse amplitude in gonadectomised rams. Cortisol may also mediate the effect of isolation and restraint stress on LH pulse amplitude observed in ewes treated with oestrogen.

It is unknown as to whether the decrease in LH pulse amplitude observed during isolation and restraint stress or increased plasma concentrations of cortisol is due to a decrease in the responsiveness of the pituitary gland to GnRH. This hypothesis is supported by results from Chapter 5 which showed that elevated plasma concentrations of cortisol were associated with a decrease in pituitary responsiveness to GnRH in hypothalamo-pituitary disconnected rams. Other studies in gonadectomised ewes also suggest that elevated plasma concentrations of cortisol may cause a decrease in pituitary responsiveness (Breen & Karsch 2004), although this finding is not supported by studies in the hypothalamo-pituitary disconnected ewe (Chapter 5).

Although the hypothalamo-pituitary disconnected sheep model permits the investigation of changes in pituitary responsiveness to GnRH, the use of this model may be limited when investigating the direct impact of stress on pituitary responsiveness to GnRH because the

hypothalamo-pituitary adrenal axis of these animals cannot be activated in response to stress. Therefore, decreases in pituitary responsiveness due to a stress-induced increase in cortisol secretion cannot be detected. An alternative approach to studying changes in pituitary responsiveness to GnRH in the hypothalamo-pituitary disconnected model, is to eliminate endogenous GnRH through passive immunisation. Pulsatile LH secretion can then be re-established by injecting an analogue of GnRH that is not recognised by the GnRH antiserum. This model allows direct assessment of events that occur at the level of the pituitary to affect LH secretion while leaving other hypothalamo-pituitary endocrine axes, such as the hypothalamo-pituitary adrenal axis, intact. This approach was used in the current chapter to test the hypothesis that changes in pituitary responsiveness to a GnRH analogue occur during the imposition of isolation and restraint stress and the simultaneous increase in the plasma concentrations of cortisol which occur as a result of stress. As the imposition of isolation and restraint stress has been associated with a decrease in LH pulse amplitude in gonadectomised rams and in gonadectomised ewes treated with oestrogen or oestrogen and progesterone (Tilbrook *et al.* 1999a), it was hypothesised that in the animal model used in the current study, a decrease in pituitary responsiveness to a GnRH analogue would be observed during isolation and restraint stress in gonadectomised rams and in gonadectomised ewes treated with oestrogen, but not in gonadectomised rams treated with testosterone or in gonadectomised ewes receiving no steroid treatment.

7.2. Materials and Methods

7.2.1. Animals

Rams and ewes were gonadectomised (Section 3.2.1 and 3.2.2) at least two months prior to the pilot study or the main experiment. On the day prior to the pilot study or the experiment, animals were fitted with an indwelling jugular catheter (Section 3.2.4) into each jugular vein. One catheter was used for the infusion of GnRH antiserum, while the other catheter was used for the collection of blood samples and the injection of a GnRH analogue.

7.2.2. Generation of GnRH antiserum

Antibodies to endogenous GnRH were generated by actively immunising 10 adult, gonad intact rams against GnRH. The antigen consisted of 1mg GnRH conjugated to bovine serum albumin using glutaraldehyde (1mg GnRH:4mg bovine serum albumin:0.625mg

glutaraldehyde) (Figure 7.1) prepared in 0.01M PBS according to the procedure previously described by Caraty *et al.* (1984). The conjugate was then emulsified in Montanide Marcol (Tall Bennett Group, Mona Vale, Australia) in a ratio of two parts conjugate to three parts Montanide Marcol before being injected subcutaneously at four sites under the forelimbs or the hind limbs. Animals were initially immunised by being injected once a week for four weeks. Booster injections were then given approximately monthly. Antibodies were harvested by collecting blood from rams approximately three times per week during the first, second and third week after the booster injection. The serum was collected and centrifuged for 25 minutes at 3000rpm and stored at -4°C until ready for use. As a control, plasma from rams which had not been immunised against GnRH was also collected and processed in the same manner as plasma from immunised rams.

Antibody titres were checked by measuring the amount of binding to GnRH radioactively labeled with I^{125} using 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril. The antibody-ligand complex was incubated in 0.5% bovine serum albumin phosphate buffer (pH 7.4) for 48 hours before being precipitated with donkey anti-sheep serum at a final concentration of 1:500 and normal sheep serum at a final concentration of 1:4500, along with 10% polyethylene glycol 6000. Measurement of the binding in the plasma harvested showed that it was approximately 20% at a 1/1000. After the enrichment process (Section 7.2.3), this increased to approximately 20% at 1/10,000 in the final solution. Binding to I^{125}-GnRH was displaced by native GnRH, but not by the GnRH analogue, des-gly^{10} GnRH ethylamide (Auspep, West Melbourne, Australia).

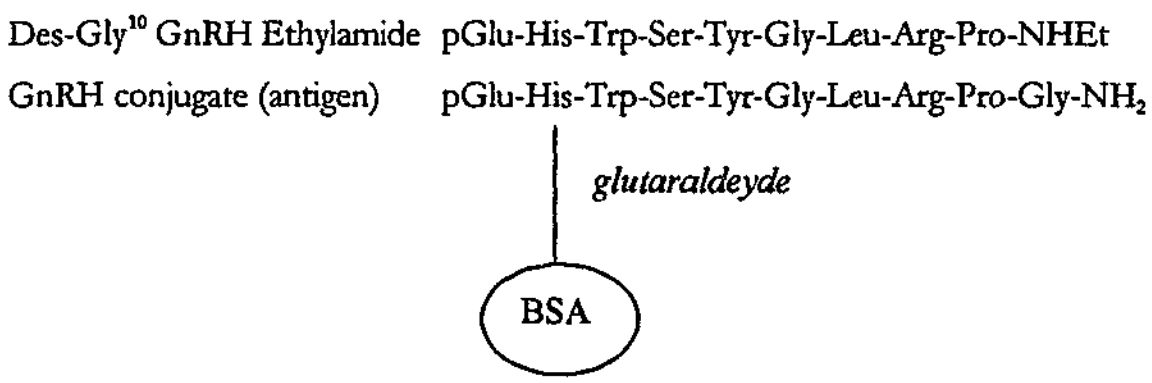


Figure 7.1

The amino acid sequence of the GnRH analogue des-gly[10]GnRH ethylamide and the native GnRH peptide are shown. The conjugation of BSA to the histidine residue at the N-terminus of the peptide means that the C terminus of the native peptide is exposed, so antibodies formed are directed to this site, and do not cross-react with the GnRH analogue.

7.2.3. Enrichment of plasma concentrations of GnRH antibodies

For use in the experimental model, the concentration of immunoglobulin in the plasma was enriched using anion exchange chromatography. Equipment for this procedure was kindly donated by Dr. Joe Bertolini (CSL, Melbourne, Australia). This process removed unwanted protein from the serum harvested and concentrated GnRH antibodies in the serum. The harvested serum was filtered through filter paper and diluted with 37.7mM Tris buffer (pH 7.4) to give a final salt concentration of 110mM and a final Tris concentration of 10mM. The diluted serum solution was loaded onto a column with a volume of approximately 1.5L (a diameter of approximately 5 cm and length of approximately 77 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden) containing diethylaminoethyl sepharose beads. The column was controlled using the Pharmacia LKB-Controller LCC-501 Plus (Pharmacia Fine Chemicals, Uppsala, Sweden) and pressure within the column was maintained using two pumps (Pharmacia LKB-Pump P-500, Uppsala, Sweden). The column was equilibrated using a Tris buffer (10mM Tris, 110mM NaCl, ph7.4) and loaded with a maximum of one litre of serum at a time. Fractions were collected when protein was detected by UV absorbance (Pharmacia LKB UV-MII UV Monitor, Uppsala, Sweden). To identify the fractions containing immunoglobulin, SDS polyacrylamide gel electrophoresis was used

(Section 7.2.4). Fractions containing immunoglobulin were pooled and concentrated using a Pall Centramate™ Lab Tangential Flow System with a 100kDa Omega™ membrane (Pall Corporation, Cheltenham, Australia). Protein bound to the column was stripped using an elution buffer (1M NaCl, 20mM piperazine, pH 5.5) and the column re-established using the equilibration buffer.. Changes in salt concentration were detected using a Pharmacia Biotech Conductivity Monitor (Pharmacia Fine Chemicals, Uppsala, Sweden). The final protein concentrations in the GnRH antiserum solution and in the control solution were measured using the Pierce Protein Kit Assay (Pierce, Rockford, Il).

7.2.4. SDS polyacrylamide gel electrophoresis

To visualise immunoglobulin, protein samples were separated by gel electrophoresis using sodium dodecyl sulphate (SDS) as a reducing agent. Polyacrylamide gels (13% polyacrylamide) were poured using the BIO-RAD Mini Protean® 3 gel electrophoresis system (BIO-RAD laboratories, Hercules CA) according to the manufacturers instructions. Gels were run at 30 amperes and 300V for approximately half an hour. To visualise protein bands, gels were stained with 0.25% Coomassie Blue R-250 in 40% methanol and 10% glacial acetic acid. To estimate protein sizes, molecular weight markers were also run in each gel (K494 protein molecular weight markers, Amresco, Solon, OH).

7.2.5. Pilot experiment

To test the success of the GnRH antiserum in suppressing LH secretion, two gonadectomised ewes and two gonadectomised rams were infused with 85ml of the GnRH antiserum over a period of four hours. Two gonadectomised rams and two gonadectomised ewes were also infused with 85ml of the control serum which had been treated in the same way. The serum was infused using MS 16A syringe drivers. Blood samples were taken every 10 minutes for three hours prior to the commencement of the infusion and during the infusion. To investigate the duration of the suppression of LH secretion, blood samples were taken every 10 minutes for two hours, 19 hours and 43 hours after the commencement of the infusion. Blood samples were also taken every 10 minutes for four hours at 70 hours after the commencement of the infusion. Plasma concentrations of LH were measured in all samples collected during the pilot study.

7.2.6. Experimental procedure

Four groups of animals were used in the experiment. They included; gonadectomised rams that received a twice daily injection of one ml of peanut oil (n=6), gonadectomised rams that received a twice daily injection of one ml of testosterone proprionate (6mg/ml peanut oil) (Sigma, St Louis, MO) (n=6); gonadectomised ewes that received an empty 2cm subcutaneous implant (n=6) and gonadectomised ewes that received a 2cm subcutaneous implant filled with oestradiol (Sigma, St Louis, MO) (n=6). All treatments commenced six days prior to the beginning of the experiment. These steroid treatments have been used previously (Tilbrook *et al.* 1999a). The experiment was conducted in two replicates so that half the animals from each group were used in the first replicate and the remainder of the animals were used in the second replicate. On Day 0 of the experiment, blood samples were taken at 10 minute intervals for eight hours. No treatment was given for the first three hours of the experiment. Animals were then infused with 85mls of GnRH antiserum over five hours. On Day 1 of the experiment, blood samples were taken every 10 minutes for two hours. Each animal then received six injections of 75ng of the GnRH analogue, given at two hourly intervals. This dose of the analogue was used as previous trials showed that it generated LH pulses with an amplitude similar to those observed in Chapter 4. Blood samples were taken –10, 5, 10, 20, 30, 40 and 60 minutes relative to the injection of the GnRH analogue. The first three injections occurred during the control period (Period 1), while the second three injections occurred during the imposition of isolation and restraint stress (Period 2) (Section 3.5). Additional blood samples were taken –20, -10, 2, 5, 10, 15, 20 and 25 minutes relative to the onset of isolation and restraint stress. On Day 2, the same experimental procedure was repeated as for Day 1, except that no isolation and restraint stress was imposed.

7.2.7. Radioimmunoassays

Plasma LH concentrations were determined in all samples taken during the experiment (except for the additional samples taken during stress) using a LH radioimmunoassay (Section 3.6.1). The sensitivity of the assay was 0.5ng/ml (n=16). The intra-assay coefficient of variation was 7% at 3.2ng/ml, 11% at 5.3ng/ml, 7% at 6.5ng/ml at 10% at 12.7ng/ml. The inter-assay coefficient of variation was 13% at 2.9ng/ml, 10% at 5.4ng/ml, 9% at 7.1ng/ml and 9% at 18.7ng/ml.

Concentrations of cortisol in plasma were measured in samples taken every half an hour on Day 0, and in samples taken approximately every half an hour on Day 1 and Day 2. Measurements were made using an extracted cortisol radioimmunoassay (Section 3.6.3). The mean (±SEM) sensitivity of the assay was 3.1±0.03ng/ml (n=7). The intra-assay coefficient of variation was 11% at 17.5ng/ml and 12% at 121ng/ml. The inter-assay coefficient of variation was 13% at 10.2ng/ml and 15% at 61ng/ml.

7.2.8.Catecholamine detection

Adrenaline, noradrenaline, DOPA and DHPG were measured in the additional samples taken during the onset of isolation and restraint stress and 60 minutes before and 60, 90 and 180 minutes after the commencement of isolation and restraint stress using alumina adsorption with electrochemical detection (Section 3.7). The sensitivity of this procedure was 15pg/ml.

7.2.9. Statistical analysis

In determining the efficacy of the model, repeated measures analysis of variance was used to compare the mean plasma concentrations of LH measured during the first three hours prior to the infusion on Day 0, the last four hours of the infusion on Day 0, the first two hours on Day 1 and the first two hours on Day 2. The within subjects factor was period and the between subjects factors were steroid treatment and sex. Data were transformed using the square root function to improve the homogeneity of the variance. A chi-square analysis was used to compare the proportions of animals which did not display pulsatile secretion of LH during these periods.

To assess the effect of isolation and restraint stress on pituitary responsiveness, the amplitude of LH pulses detected in response to treatment with the GnRH analogue, baseline concentrations of LH preceding each pulse and the mean plasma concentrations of LH during Period 1 (control) and Period 2 (isolation and restraint stress) on Day 1 and Day 2 were calculated and statistically analysed using a repeated measures analysis of variance. The within subjects factors were day and injection number for LH pulse amplitude and baseline concentrations, and day and period for mean plasma concentrations of LH. The between subjects factors were steroid treatment and sex. Data describing LH pulse amplitude were transformed using the square root function, while baseline

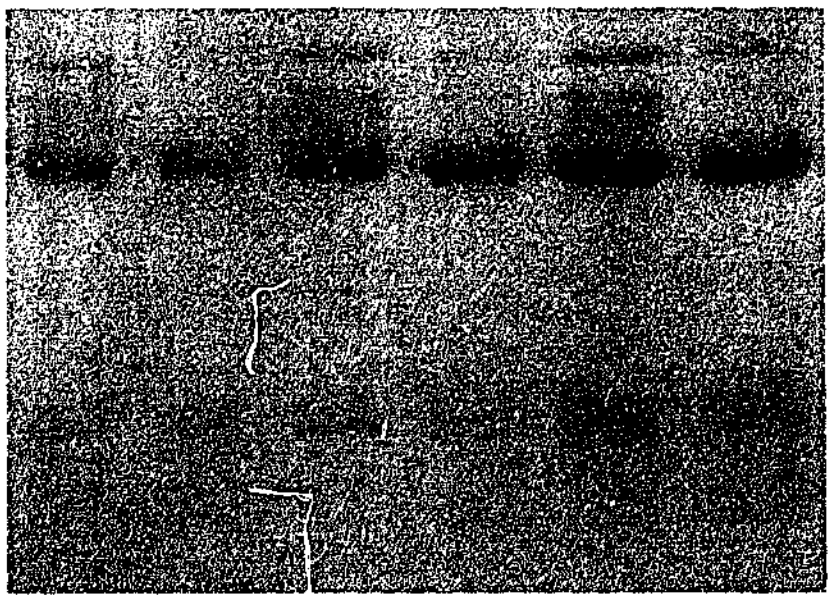
concentrations of LH and mean plasma concentrations of LH were log_{10} transformed to improve the homogeneity of the variance.

Plasma concentrations of cortisol on Day 0 were analysed using a repeated measures analysis of variance. The within subjects factor was time and the between subjects factors were steroid treatment and sex. Data were transformed using the square root function. To examine the effect of isolation and restraint stress on plasma concentrations of cortisol and the catecholamines, data from Day 1 and Day 2 were analysed using a repeated measures analysis of variance. The within subjects factors were day and time and the between subjects factors were steroid treatment and sex. Log_{10} transformation improved the homogeneity of the variance in the data describing adrenaline. Log_{10} transformation was also applied to the noradrenaline data.

7.3. Results

7.3.1. Pilot study

The final protein concentration in the enriched antiserum and control serum was determined to be approximately 230mg/ml. Figure 7.2 shows that the composition of the GnRH antiserum and the control serum was similar. The infusion of the control serum had no effect on the pulsatile secretion of LH in any animal (Figure 7.3). The infusion of GnRH antiserum, however, resulted in a subsequent suppression of the secretion of LH in all animals. This suppression persisted at 19 and 43 hours after the commencement of the infusion. By 70 hours, however, the pulsatile secretion of LH had been re-established in all animals (Figure 7.3).



lane	1	2	3	4	5	6
	1nl GnRH antibody solution	1nl control solution	2.5nl GnRH antibody solution	2.5nl control solution	10nl GnRH antibody solution	10nl control solution

Figure 7.2

SDS polyacrylamide gel showing the similarity in the concentration of proteins present in the enriched GnRH antiserum and the control solution. In lanes 1 and 2, 1nl of the GnRH antibody solution and the control solution was loaded, respectively. In lane 3 and 4, 2.5nl of the solutions were loaded, in lane 5 and 6, 10nl of the solutions were loaded.

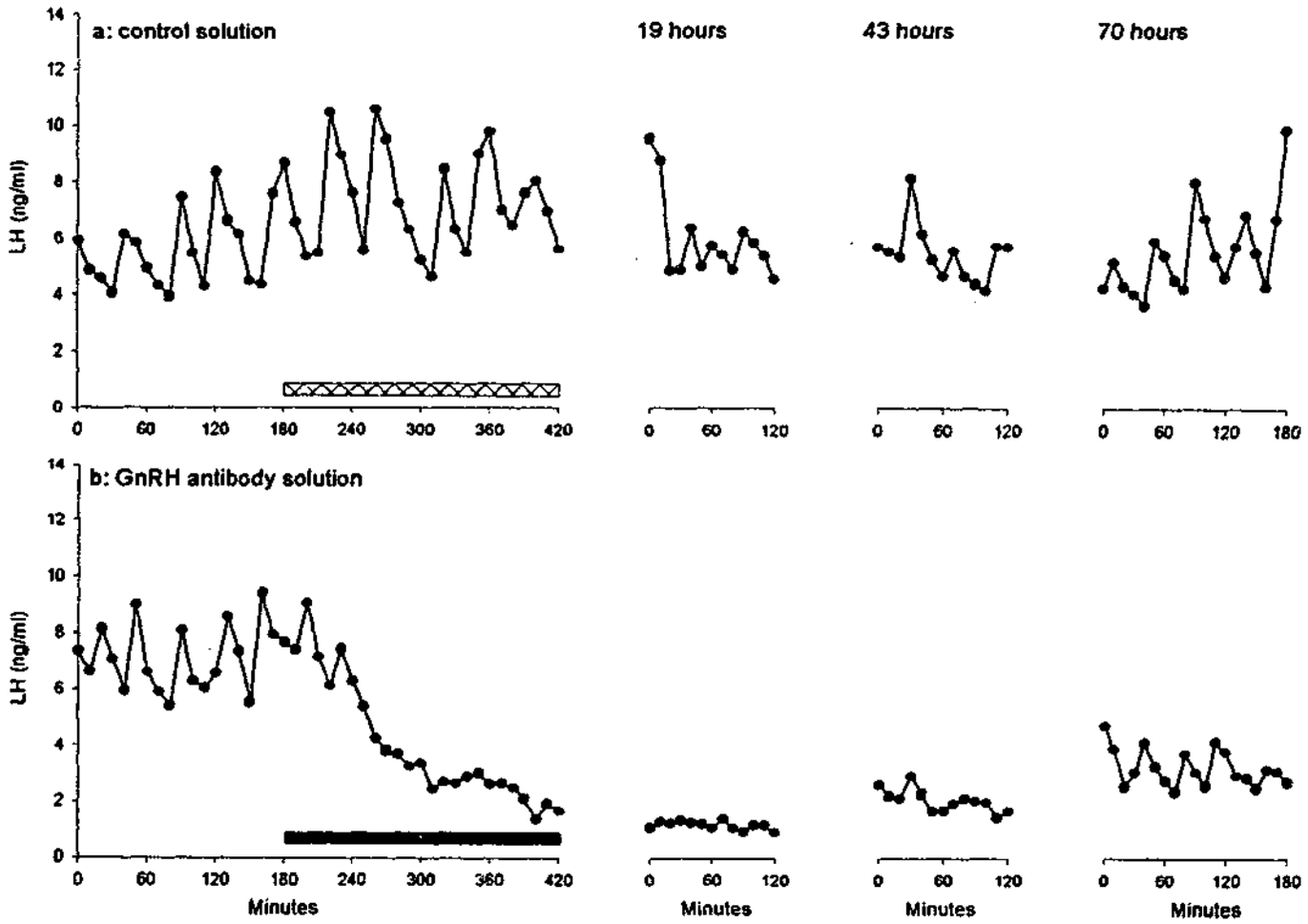
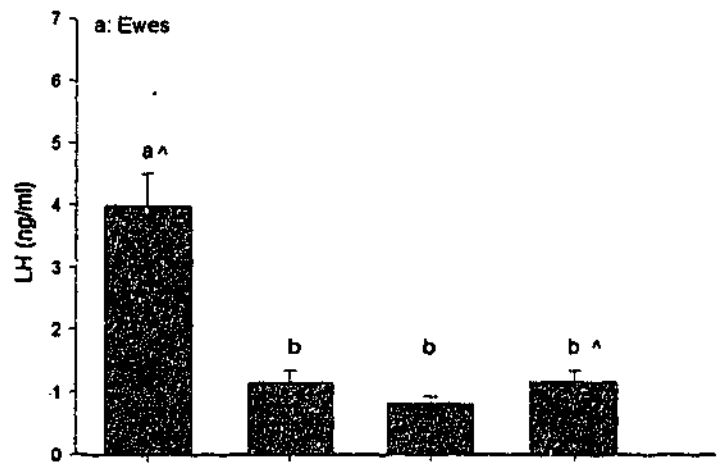


Figure 7.3

Plasma concentrations of LH (ng/ml) in a ewe before, during and after an infusion of control serum (a), or GnRH antiserum (b). The infusion of the control serum (hatched bar) did not affect the pulsatile secretion of LH. The infusion of GnRH antiserum (black bar) resulted in a suppression of the secretion of LH. This suppression persisted at 19 and 43 hours after the commencement of the infusion. By 70 hours, however, the pulsatile secretion of LH had been re-established.

7.3.2. GnRH antiserum-induced suppression of LH in experimental animals

During the experiment, mean (±SEM) plasma concentrations of LH in ewes and rams were significantly reduced ($p<0.05$) during the final two hours of the infusion of antiserum on Day 0, and in the initial two hours of sampling on Day 1 and Day 2 of the experiment (Figure 7.4). Ewes had significantly lower ($p<0.05$) plasma concentrations of LH than rams prior to the infusion of the GnRH antiserum, and in the two hours of sampling on Day 2 of the experiment. Furthermore, chi-square analysis confirmed that the antiserum was successful in abolishing LH pulses ($\chi^2_{3df}=60.7$; $p<0.0001$)



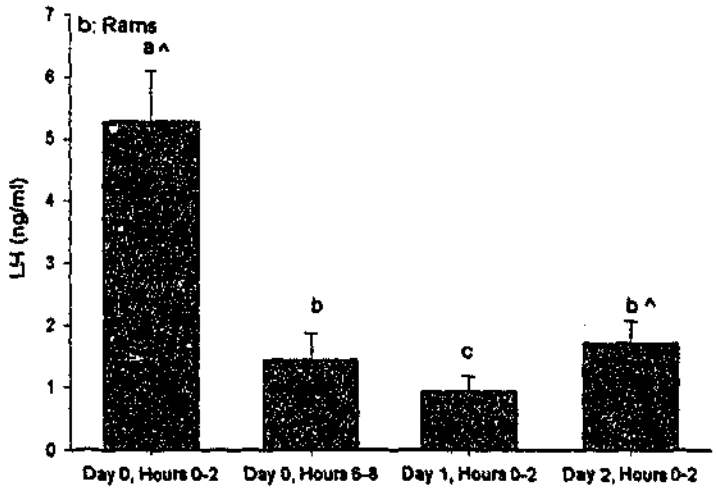


Figure 7.4

Mean (±SEM) plasma concentrations of LH (ng/ml) in ewes (a) and in rams (b) during the first two hours of sampling on Day 0, the last two hours of the infusion of GnRH, the first two hours of sampling on Day 1 and the first two hours of sampling on Day 2, which were both prior to treatment with the GnRH analogue. Significant differences within sexes are indicated by different subscripts ($p<0.05$). Significant differences ($p<0.05$) between sexes are indicated by ^.

7.3.3. The effect of isolation and restraint stress on pulsatile LH secretion induced by a GnRH analogue

Both mean plasma concentrations of LH (Table 7.1) and baseline concentrations of LH (Table 7.2) during the injections with the GnRH analogue were significantly higher ($p<0.05$) on Day 2 than on Day 1, but there were no differences between the sexes in these parameters, so the data from each group were combined. The amplitude of LH pulses detected in response to the GnRH analogue was significantly higher ($p<0.05$) in ewes than in rams (Table 7.3). There were no consistent significant changes in LH pulse amplitude, mean plasma concentrations of LH or baseline concentrations between the control periods and the period of isolation and restraint stress in either sex.

	Period 1 (injections 1-3)	Period 2 (injections 4-6)
Mean (±SEM) plasma concentrations of LH on Day 1 (ng/ml)	2.7±0.3	2.9±0.3*
Mean (±SEM) plasma concentrations of LH on Day 2 (ng/ml)	5.3±0.4	5.9±0.5

Table 7.1

Mean (±SEM) plasma concentrations of LH (ng/ml) during Period 1 and 2 on Day 1 and 2. Mean plasma concentrations of LH were significantly higher ($p<0.05$) on Day 2 than on Day 1. *Measurements taken during the period of isolation and restraint stress.

	GnRH Analogue Injection					
	Period 1			Period 2		
	1	2	3	4	5	6
Mean (±SEM) baseline concentrations of LH (ng/ml) on Day 1	0.9±0.1	1.1±0.1	1.5±0.2	1.5±0.2*	1.3±0.1*	1.6±0.2*
Mean (±SEM) baseline concentrations of LH (ng/ml) on Day 2	1.5±0.2	2.7±0.3	3.1±0.4	3.2±0.5	3.2±0.4	3.5±0.4

Table 7.2

Mean (±SEM) baseline concentrations of LH (ng/ml) prior to each injection of a GnRH analogue given on Day 1 and Day 2. Baseline concentrations of LH were significantly higher ($p<0.05$) on Day 2 than on Day 1. * Measurements taken during the period of isolation and restraint stress.

	GnRH Analogue Injection on Day 1						GnRH Analogue Injection on Day 2					
	Period 1			Period 2			Period 1			Period 2		
	1	2	3	4*	5*	6*	1	2	3	4	5	6
Mean (±SEM) LH pulse amplitude in ewes	2.5±0.6	4.7±0.6	3.1±0.4	2.8±0.3	3.1±0.4	3.9±0.8	7.8±1.1	5.6±0.8	6.4±0.6	4.5±0.5	5.9±0.8	5.7±0.7
Mean (±SEM) LH pulse amplitude in rams	2.3±0.7	2.4±0.4	2.3±0.4	2.1±0.4	2.0±0.4	2.6±0.4	3.8±0.3	4.6±0.5	3.5±0.3	3.7±0.2	3.8±0.4	4.2±0.6

Table 7.3

Mean (±SEM) amplitude of LH pulses (ng/ml) arising from injections of a GnRH analogue given on Day 1 and Day 2. LH pulse amplitude was significantly higher ($p<0.05$) in ewes than in rams on Day 1 and Day 2. * Measurements taken during the period of isolation and restraint stress.

7.3.4. Mean plasma concentrations of cortisol

During the infusion with GnRH antiserum on Day 0 of the experiment, mean plasma concentrations of cortisol significantly increased ($p<0.05$) from basal concentrations (7.8±0.4ng/ml) (Figure 7.5). The elevation in mean (±SEM) plasma concentrations of cortisol was significantly higher ($p<0.05$) from 90 to 210 minutes after the commencement of the infusion in gonadectomised rams than in gonadectomised rams treated with testosterone. There were no significant differences between the mean plasma concentrations of cortisol observed in gonadectomised ewes and gonadectomised ewes treated with oestrogen and no differences between the sexes (Figure 7.5).

On the day of isolation and restraint stress (Day 1), mean (±SEM) plasma concentrations of cortisol in the four hours prior to the imposition of isolation and restraint stress were not significantly different between ewes (15.7±1.1ng/ml) and rams (16.0±1.1ng/ml) (Figure 7.6a and b). In response to isolation and restraint stress, mean plasma concentrations of cortisol were significantly elevated ($p<0.05$). The peak plasma concentrations of cortisol in response to isolation and restraint stress in ewes were significantly higher ($p<0.05$) than those in rams. Cortisol concentrations had returned to prestress concentrations by three hours after the imposition of isolation and restraint stress in ewes, and by one hour after the imposition of isolation and restraint stress in rams. In rams and ewes, plasma concentrations of cortisol were significantly elevated ($p<0.05$) during the first three hours of sampling (0 to 150minutes). During this period, the peak plasma concentrations of cortisol were significantly higher ($p<0.05$) in ewes than that observed in rams. In both rams and ewes, mean plasma concentrations of cortisol were significantly higher on the day of isolation and restraint stress than on the control day. There were no sex differences in the mean plasma concentrations of cortisol measured on the control day (Figure 7.6c and d).

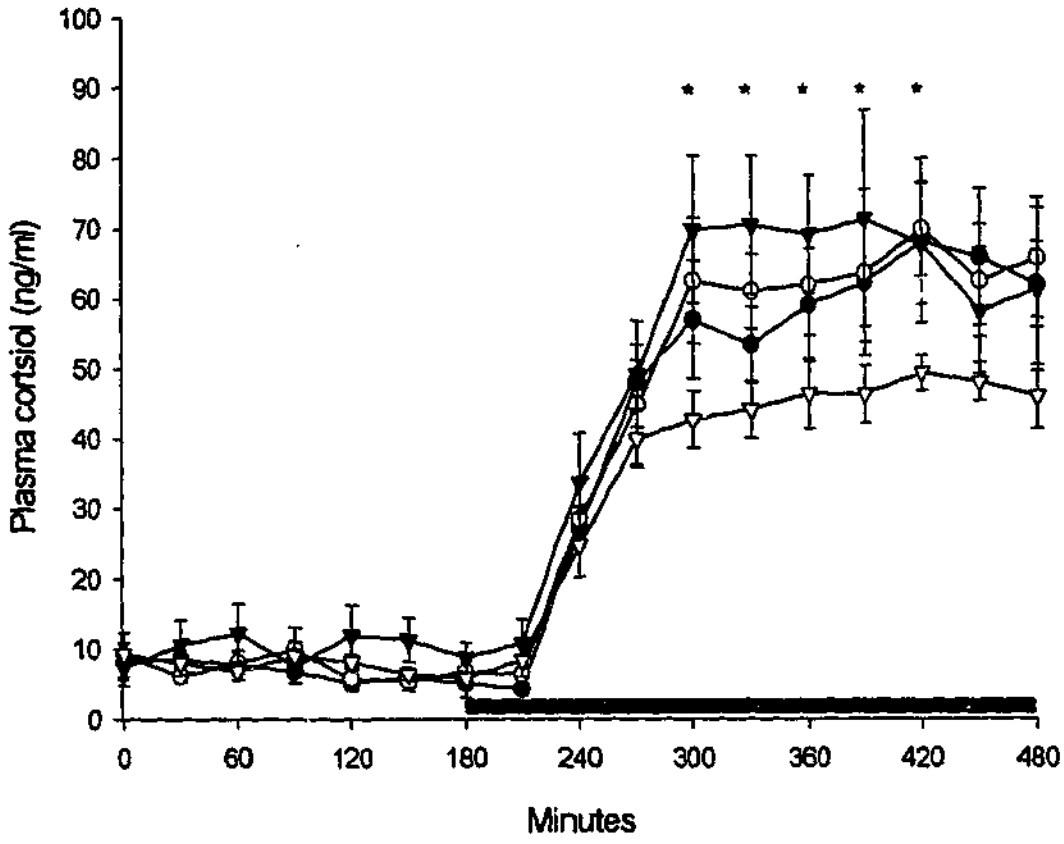


Figure 7.5

Mean (±SEM) plasma concentrations of cortisol (ng/ml) in gonadectomised ewes (●), gonadectomised ewes treated with oestrogen (○), gonadectomised rams (▼) and gonadectomised rams treated with testosterone (▽), before and during the infusion with GnRH antiserum (represented by the black bar). There was a significant increase in plasma concentrations of cortisol from 240 minutes until 480 minutes after the commencement of sampling in all groups ($p<0.05$). The increase in plasma concentrations of cortisol during the infusion was significantly higher (*$p<0.05$) in gonadectomised rams than in gonadectomised rams treated with testosterone from 300 until 420 minutes after the commencement of sampling.

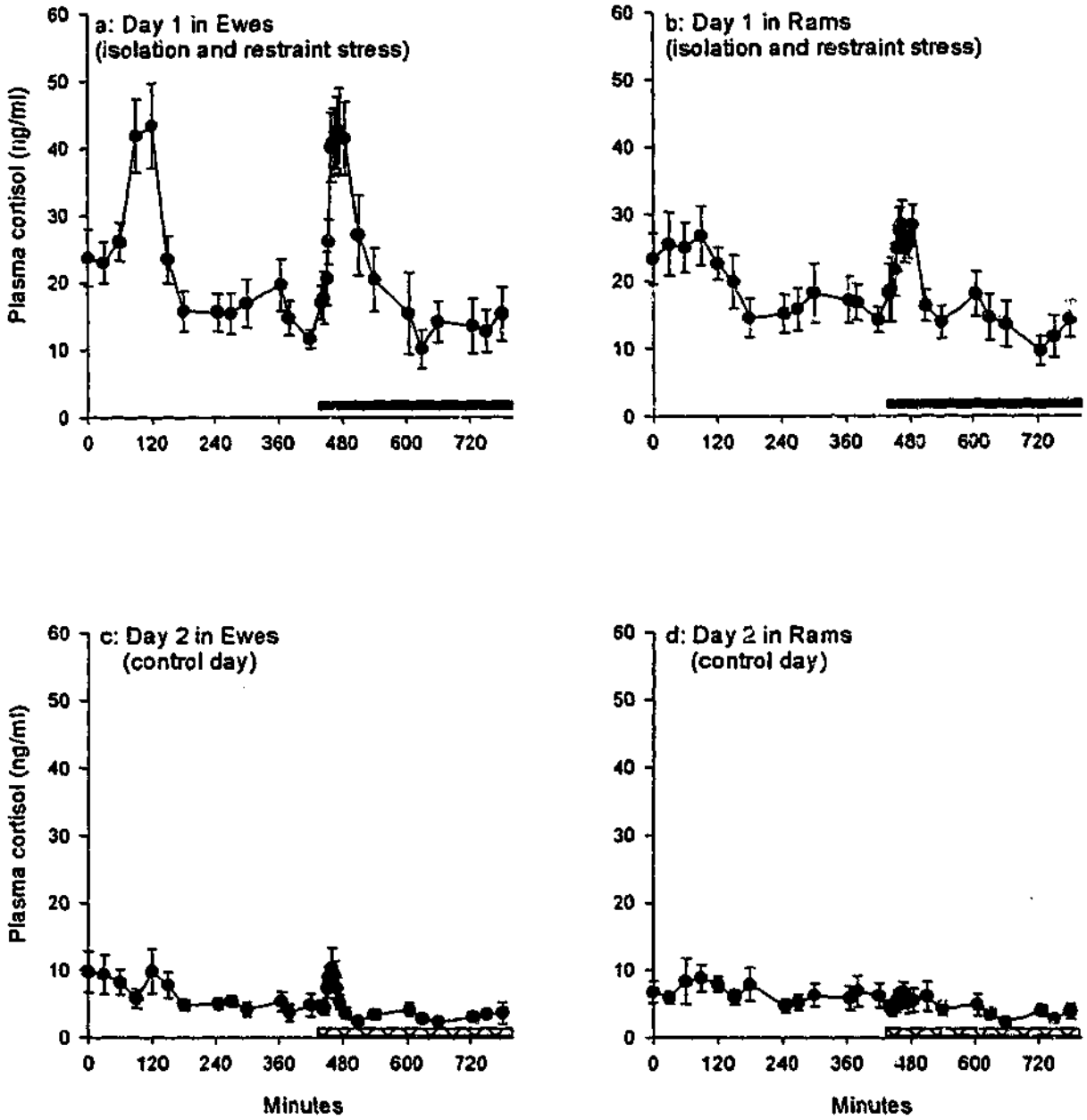


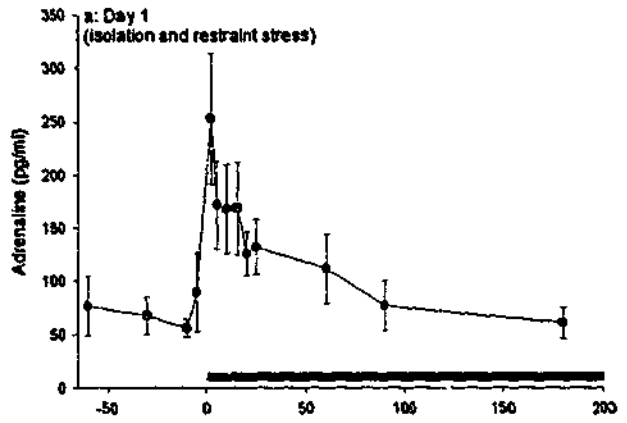
Figure 7.6

Mean (±SEM) plasma concentrations of cortisol (ng/ml) in ewes (a) and rams (b) on Day 1 of the study and in response to isolation and restraint stress (represented by the black bar), and on Day 2 (the control day) in ewes (c) and rams (d). The control period equivalent to isolation and restraint stress is represented by the hatched bar on Day 2. Significant differences are described in the results text.

7.3.5. Mean plasma concentrations of the catecholamines

There were no sex differences and no effect of the sex steroids on the mean plasma concentrations of the catecholamines so data from all groups were combined. Mean plasma concentrations of adrenaline were significantly increased ($p<0.05$) above baseline concentrations at 2, 5, 10, 15, 20 and 25 minutes after the onset of isolation and restraint stress on Day 1 (Figure 7.7a). There were no consistent significant changes in the plasma

concentrations of adrenaline measured on the control day (Day 2) of the experiment (Figure 7.7b). There was also a significant increase ($p<0.05$) in the mean plasma concentrations of DHPG 2, 5, 15, 20 and 25 minutes after the onset of isolation and restraint stress on Day 1 (Figure 7.8a). There were no consistent significant changes in the plasma concentrations of DHPG on the control day of the experiment (Day 2; Figure 7.8b). No consistent significant changes in the plasma concentrations of DOPA or noradrenaline were observed in response to isolation and restraint stress in any group (data not shown).



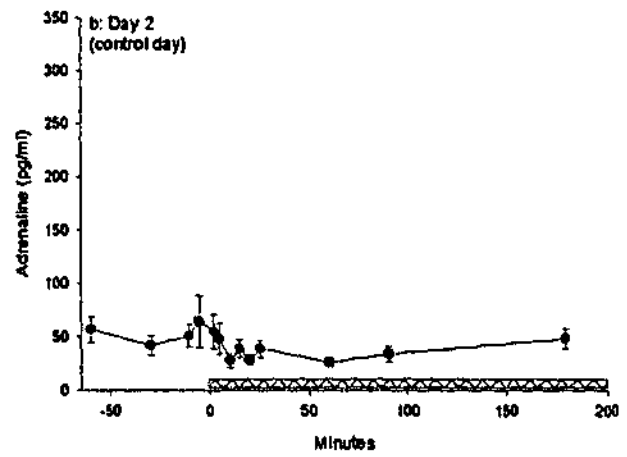


Figure 7.7

Mean (±SEM) plasma concentrations of adrenaline (pg/ml) on Day 1 and Day 2 of the experiment in ewes and rams with and without sex steroids (combined). The period of isolation and restraint stress is indicated by the black bar on Day 1, the equivalent period on the control day is represented by the hatched bar. Mean plasma concentrations of adrenaline were significantly increased ($p<0.05$) above baseline concentrations at 2, 5, 10, 15, 20 and 25 minutes after the onset of isolation and restraint stress on Day 1. There were no consistent significant changes in the plasma concentrations of adrenaline measured on the control day (Day 2) of the experiment.

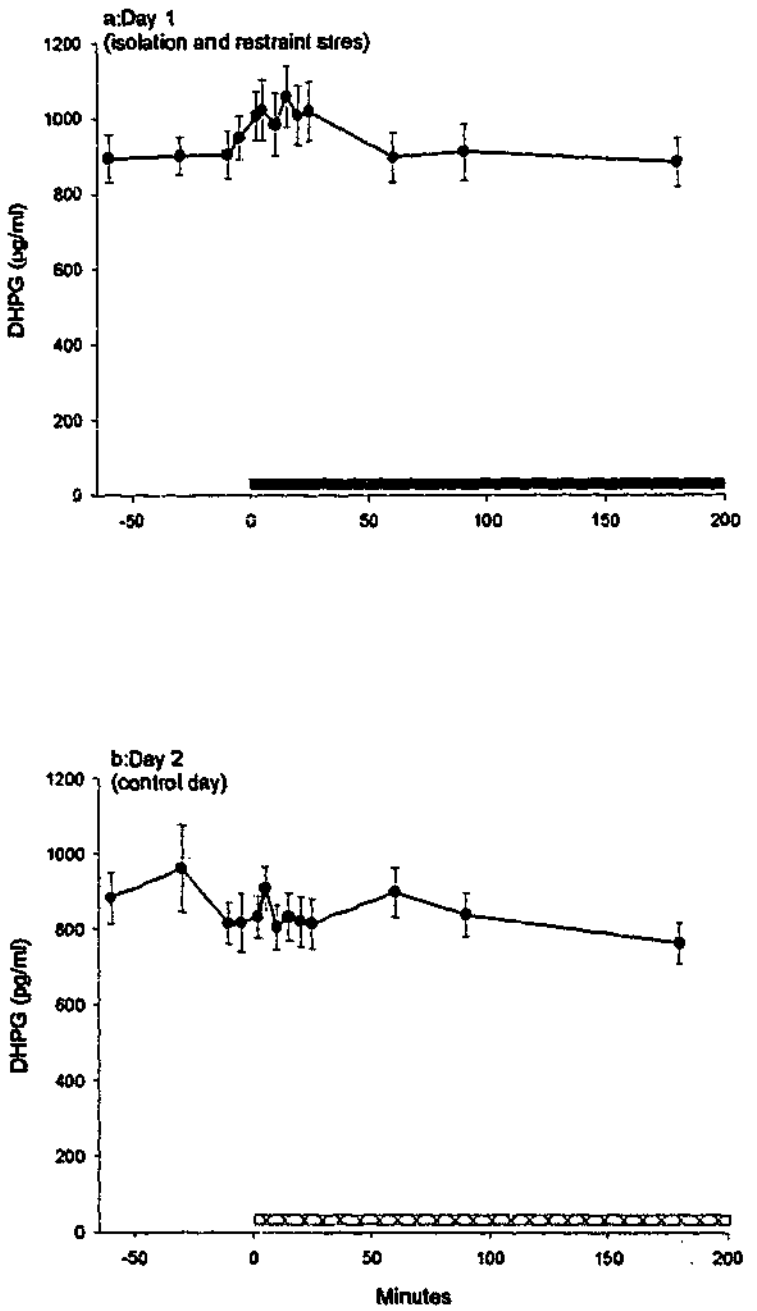


Figure 7.8

Mean (±SEM) plasma concentrations of DHPG (pg/ml) on Day 1 and Day 2 of the experiment in ewes and rams with and without sex steroids (combined). The period of isolation and restraint stress is indicated by the black bar on Day 1, the equivalent period on the control day is represented by the hatched bar. Mean plasma concentrations of DHPG were significantly increased ($p<0.05$) above baseline concentrations at 2, 5, 10, 15, 20 and 25 minutes after the onset of isolation and restraint stress on Day 1. There were no consistent significant changes in the plasma concentrations of DHPG measured on the control day (Day 2) of the experiment.

7.4. Discussion

The results from this study showed that in gonadectomised ewes with or without oestrogen treatment, and in gonadectomised rams with or without testosterone treatment, isolation and restraint stress did not induce a decrease in pituitary responsiveness to the GnRH analogue des-gly^{10}GnRH ethylamide. This suggests that decreases in pituitary responsiveness to GnRH do not contribute to the decrease in LH pulse amplitude previously observed during isolation and restraint stress (Tilbrook *et al.* 1999a). These results do show, however, sex differences in the response of the pituitary to stimulation by the GnRH analogue. They also demonstrate sex differences in the hypothalamo-pituitary adrenal response to isolation and restraint stress and an effect of testosterone on the hypothalamo-pituitary adrenal response to the infusion of protein in rams.

The results of this experiment suggest that previous decreases in LH pulse amplitude during isolation and restraint stress in sheep (Tilbrook *et al.* 1999a) are most likely to be due to a decrease in the hypothalamic secretion of GnRH, rather than a decrease in pituitary responsiveness to GnRH. It is possible that a decrease in GnRH pulse amplitude during isolation and restraint stress results in a decrease in the amplitude of the resulting LH pulses. This can only be verified by direct measurements of GnRH during isolation and restraint stress but this has hitherto not been done. Other studies in sheep have demonstrated a reduction in GnRH-stimulated LH secretion during restraint in rams (Matteri *et al.* 1984), combined isolation and restraint in rams (Tilbrook *et al.* 1999a), or transport stress in ewes (Dobson *et al.* 1999). Unlike in the present study, a hypothalamo-pituitary intact animal model was used. It is possible, therefore, that the results were confounded by a decrease in GnRH secretion from the hypothalamus, thus complicating interpretation of these studies. An advantage of the current model is that any such confounding influence is removed.

This study demonstrates a sex difference in the responsiveness of the pituitary gland to the GnRH analogue. Ewes consistently had larger LH pulse amplitudes than rams. This observation has not been made previously in the hypothalamo-pituitary disconnected model (Chapter 5 and Chapter 6). Sex differences in the response of the pituitary to GnRH

were also not observed in hypothalamo-pituitary intact sheep (Tilbrook *et al.* 1999a). The mechanisms which underlie this sex difference are unknown and may be due to sex differences in the concentration of GnRH receptors in gonadotrophs. It is also possible that these sex differences are only apparent due to the use of the GnRH analogue in the current study, which is more potent than the native GnRH peptide.

Sex differences in the hypothalamo-pituitary adrenal response to stress have been previously reported by Turner *et al.* (2002a), who showed that the response to isolation and restraint stress was higher in gonadectomised ewes than in gonadectomised rams. These results are confirmed in the present study. This sex difference was also observed at the beginning of sampling on Day 1, when plasma concentrations of cortisol were elevated, and was not observed in the basal co: centrations of cortisol. Unlike the results of Chapter 6, there was no sex difference in the duration of the increase in adrenaline secretion in response to isolation and restraint stress. It is possible that there may be sex differences in the interactions between the hypothalamo-pituitary adrenal axis and the sympathoadrenal system. These interactions may be interrupted in the hypothalamo-pituitary disconnected model but remain intact in the model used in the current study. Alternatively, the high degree of variability in the adrenaline data from the current study may mask any existing sex differences.

There was no effect of the sex steroids on the hypothalamo-pituitary adrenal response to isolation and restraint stress, confirming results previously found in this laboratory (Tilbrook *et al.* 1999a). This is unlike the results from Pecins-Thompson & Keller-Wood (1994) and Komesaroff *et al.* (1998) which both demonstrated an effect of oestrogen on the cortisol and ACTH responses to different stressors. Based on studies in males, it may also have been anticipated that the cortisol response to stress would be reduced by the presence of testosterone (Hileman *et al.* 1996). This was not observed in response to isolation and restraint stress in the current study, although it was observed in response to the infusion of GnRH antiserum on Day 0. Being a solution high in protein content, it is likely that the infusion of this solution may have placed temporary stress on the cardiovascular system that resulted in the activation of the hypothalamo-pituitary adrenal axis. It is unlikely that the increase in cortisol was due to an infusion of cortisol or any cortisol-releasing factors in

the serum, as these would have been eliminated by ion-exchange chromatography (in the case of steroids) or filtered through the 100kDa membrane (in the case of proteins). This increase in cortisol, however, did not appear to affect the stress response to isolation and restraint stress on the following day, which was similar to that previously observed in this laboratory (Tilbrook *et al.* 1999a; Turner *et al.* 2002a).

Plasma concentrations of cortisol in the present study were not elevated to the same extent as those observed in the first experiment presented in Chapter 5. They are also lower than the plasma concentrations of cortisol reported by a study from Breen & Karsch (2004), who showed a decrease in LH secretion and no change in GnRH secretion when plasma concentrations of cortisol were elevated to approximately 150ng/ml in gonadectomised ewes. A further study by these authors showed that when plasma concentrations of cortisol were elevated to 150ng/ml in gonadectomised ewes treated with oestradiol to block endogenous GnRH secretion, the amplitude of LH pulses arising from stimulation by exogenous GnRH administration was reduced. It is possible therefore, that there may still be a reduction in pituitary responsiveness due to increased plasma concentrations of cortisol during stress, but that this reduction may only occur within a certain range of plasma concentrations of cortisol. Given that the elevation in plasma concentrations of cortisol due to isolation and restraint stress is routinely approximately 40ng/ml, it is suggested that the decrease in LH pulse amplitude observed in this range is not due to an effect of cortisol at the pituitary, but may be due to an effect of cortisol or some other mediator of stress at the hypothalamus.

The use of the passive immunisation model employed in the current study provides a powerful model for investigating changes in pituitary responsiveness to GnRH, and may also have application for investigating other hypothalamic-hypophysiotrophic factors. Measurement of the plasma concentrations of LH stimulated by endogenous GnRH showed that mean plasma concentrations of LH, and the secretion of LH pulses, were successfully suppressed by treatment with the GnRH antiserum. This suppression can be attributed to the presence of antibodies to the C-terminus of the GnRH peptide, as the GnRH analogue des-gly^{10} GnRH ethylamide was still active in stimulating LH secretion. Furthermore, this suppression was not observed in animals whic were infused with the

control serum which had been processed using the same techniques as the serum from immunised rams.

In conclusion, these results suggest that the decrease in LH pulse amplitude previously observed during isolation and restraint stress can not be attributed to a decrease in pituitary responsiveness, and that a decrease in the hypothalamic secretion of GnRH is more likely to explain the effect of this stressor on LH pulse amplitude.

Chapter 8 ~ General Discussion

Collectively, the experiments presented in this thesis suggest that there are mechanisms by which the responsiveness of the pituitary to GnRH stimulation may be reduced during elevated plasma concentrations of the glucocorticoids, or during the imposition of the psychological stressor, isolation and restraint stress. These studies also suggest that in some circumstances, there may be sex differences in the extent to which pituitary responsiveness is affected by these conditions. Furthermore, other variables, such as the degree to which plasma concentrations of cortisol are elevated and seasonal variability may also be important in determining whether there is an effect of cortisol or stress on pituitary responsiveness to GnRH. Finally, the studies presented in this thesis provide further evidence for sex differences in the response of the hypothalamo-pituitary adrenal axis and the sympathoadrenal system to stress.

The studies presented in Chapter 4 and in Chapter 5 demonstrated that elevation of the plasma concentrations of cortisol, caused by the infusion of cortisol, was successful in reducing both the amplitude of endogenously occurring LH pulses (Chapter 4), and the amplitude of LH pulses stimulated by treatment with exogenously administered GnRH (Chapter 5). Together, these studies suggest that there may be a direct effect of elevated plasma concentrations of cortisol on the pituitary gland to reduce pituitary responsiveness to GnRH. The mechanisms through which cortisol may influence the function of the pituitary are unknown, although glucocorticoid receptors have been identified on gonadotrophs in the rat (Kononen *et al.* 1993). These receptors may provide the framework through which cortisol could act directly on the gonadotroph to reduce its responsiveness to stimulation by GnRH. An effect of cortisol on the oestradiol-stimulated increase in the pituitary concentration of the GnRH receptor has also been shown in prepubertally castrated rams (Adams *et al.* 1999; Daley *et al.* 1999b), suggesting that, in some circumstances, cortisol may affect pituitary responsiveness by decreasing the concentration of GnRH receptors in the pituitary. Further studies are required to investigate whether this occurs under the experimental paradigms used in the experiments presented here.

Sex differences in the extent to which LH secretion was suppressed during cortisol treatment were also demonstrated in Chapter 4 and Chapter 5. In the study presented in Chapter 4, the suppression of the secretion of LH in gonadectomised rams was found to occur at a lower dose of cortisol and over shorter intervals than that which occurred in gonadectomised ewes. In the first study presented in Chapter 5, LH pulse amplitude was reduced during treatment with cortisol in gonadectomised rams, but not in gonadectomised ewes. These studies suggest that there may be sex differences in the effects of cortisol on LH secretion in sheep. In particular, these sex differences may exist, in part, at the pituitary. The sex differences in the mechanisms by which cortisol decreases LH pulse amplitude are yet to be determined, but may involve a difference in the sensitivity of the pituitary gland to cortisol. Furthermore, the physiological significance of these sex differences is unclear. One hypothesis may be that LH secretion in the female has evolved to be more resistant to elevated plasma concentrations of cortisol than LH secretion in the male, as perturbations in hormonal secretion at any stage of the oestrous cycle are more likely to have a greater effect on successful reproduction than in the male, where the production of the gametes is continuous, rather than cyclic. A similar hypothesis was suggested by Moberg (1987). These studies also suggest that there may be an inherent sex difference in the responsiveness of the pituitary gland to treatment with GnRH, as ewes passively immunised against GnRH had a greater response to the GnRH analogue des-gly^{10} GnRH ethylamide than rams. This sex difference has not been observed in the hypothalamo-pituitary disconnected model (Chapter 5 and Chapter 6), or in hypothalamo-pituitary intact sheep (Tilbrook *et al.* 1999a). It is possible that it may arise due to differences in the binding properties of the analogue and the endogenous peptide. Further work is required if this is to be determined.

As a whole, these studies suggest that the extent to which plasma concentrations of cortisol are elevated may be important in determining whether or not pituitary responsiveness to GnRH secretion is affected. This was demonstrated in Chapter 4 and Chapter 5, where different plasma concentrations of cortisol were associated with differences in the changes in the pulsatile secretion of LH, or in the responsiveness of the pituitary gland to GnRH. This has been demonstrated by other studies in sheep, which showed that the parameters of LH secretion affected by cortisol treatment in the ewe were dose dependent (Debus *et al.*

2002). Dose dependent effects of synthetic and natural glucocorticoids have also been demonstrated in rats. Importantly, Brann *et al.* (1990) showed that cortisol suppressed LH secretion in oestrogen primed rats at a range of lower doses, but as the dose increased, this effect of cortisol was no longer observed. The range at which elevated plasma concentrations of cortisol are no longer effective in suppressing LH secretion may differ between males and females. This could explain why there was no effect of cortisol on pituitary responsiveness in hypothalamo-pituitary disconnected ewes, while there was an effect in rams. An effect of cortisol on LH secretion, but not on GnRH secretion has been demonstrated in ewes by contemporaneous sampling from the jugular vein and from the hypophysi ` portal blood vessels (Breen & Karsch 2004). This study, therefore, provides convincing evidence for an effect of cortisol on pituitary responsiveness to GnRH stimulation. Differences in plasma concentrations of cortisol are unlikely to explain the different results, however, as the plasma concentrations of cortisol achieved in both studies were similar. These authors suggest that it is possible that the cortisol-induced suppression of the response of the pituitary to GnRH is mediated by an indirect pathway that is dependent upon an intact hypothalamo-pituitary unit. The existence of such an indirect pathway would fit with the findings presented in this thesis and would explain the differences this data and that of Breen & Karsch (2004). As yet, such a pathway has not been described.

Seasonal factors may also be important in determining the effect of elevated plasma concentrations of cortisol and stress on pituitary responsiveness to GnRH as seasonal differences may explain the different results obtained between the first and second studies of Chapter 5 and Chapter 6. The mechanisms by which season may influence the extent to which cortisol or stress can impact on pituitary responsiveness are, as yet, unknown. Differences in melatonin secretion between seasons may be one possible mechanism which results in the alteration of the function of the pituitary (Brook & Marshall 1996) and melatonin receptors have been found in areas of the pituitary which are rich in gonadotrophs (Helliwell & Williams 1992), supporting the concept that this mechanism may exist. Further to this Sakurai & Adams (1998), showed an interaction between the duration of oestrogen negative feedback and season, in that the suppression of gonadotroph responsiveness by oestradiol was longer during the anoestrous season than

during the breeding season. This effect was reversed during the breeding season by continuous administration of melatonin. In rams, seasonal differences on the feedback effects of inhibin and testosterone on pituitary secretion of the gonadotrophins have also been reported (Tilbrook *et al.* 1999c). Therefore, it appears that season, and possibly the actions of melatonin, may have the ability to alter gonadotroph function. Further investigation is required in this area if such effects are to be elucidated.

A non-hypothalamo-pituitary adrenal mechanism of suppression of pituitary responsiveness may also be suggested from the results described in Chapter 6. These experiments demonstrated that there was a decrease in pituitary responsiveness to GnRH during the imposition of isolation and restraint stress in the non-breeding season in hypothalamo-pituitary disconnected animals. Given that the hypothalamo-pituitary adrenal axis is not activated by stress in this animal model, it is possible that this effect of stress on pituitary responsiveness may have been due to the contemporaneous increase in adrenaline, or to another mechanism not induced by activation of the hypothalamo-pituitary adrenal axis, such as the opioidergic system. Furthermore, a neural mechanism may also be postulated, as synapses between nerve fibres and gonadotrophs have been identified in the rat (Liu *et al.* 1996). Further work is required to investigate whether these neurones are activated in response to stress. This short-term decrease in pituitary responsiveness was not observed in the final study, in which hypothalamo-pituitary intact rams and ewes were exposed to isolation and restraint stress during the breeding season. This may be explained by differences in the animal model used in the two studies. Disruption of the hypothalamo-pituitary adrenal axis may interrupt interactions between this axis and the sympathoadrenal system, therefore altering the response of the sympathoadrenal system to stress. Thus, the response of the sympathoadrenal system may be effective in suppressing gonadotroph responsiveness to GnRH in the hypothalamo-pituitary disconnected animals but not in the hypothalamo-pituitary intact animals. A detailed examination of differences in the sympathoadrenal response to stress between these two animal models may be important before a role for adrenaline in the suppression of pituitary responsiveness to GnRH can be determined.

These studies confirm previous findings that there are sex differences in the response of the hypothalamo-pituitary adrenal axis and sympathoadrenal system to stress. Sex differences in the secretion of cortisol in response to isolation and restraint stress were observed in the final study as plasma concentrations of cortisol during stress were higher in females than in males. This finding is similar to observations reported in a previous study (Turner *et al.* 2002a). No sex differences were observed in basal concentrations of cortisol during any of the studies presented in this thesis, suggesting that the sex differences in cortisol secretion occur in response to stress. It is possible that these sex differences in the response of the hypothalamo-pituitary adrenal axis may be due to sex differences in mechanisms which occur at the hypothalamus and higher brain centres, the pituitary or the adrenal gland. Indeed, evidence exists for sex differences at all three of these sites (Canny *et al.* 1999). Furthermore, testosterone was associated with a reduction in the increase in cortisol induced by the infusion of protein in males. This finding confirms results previously reported in sheep (Hileman *et al.* 1996) and in rats (Handa *et al.* 1994; Patchev & Almeida 1998). There was no effect of oestrogen on cortisol secreted in response to isolation and restraint stress. This was somewhat unexpected as oestrogen treatment was associated with the reduced secretion of ACTH and cortisol in response to the stress of exposure to a barking dog in ovariectomised ewes (Komesaroff *et al.* 1998). Differences in the stressors imposed may explain the different results observed, although both stressors are likely to have a psychological component. The significance of a stress response which is modified by sex or the sex steroids is unknown. It is possible that the suppressive effects of testosterone, and possibly oestrogen, on the hypothalamo-pituitary adrenal response to some stressors may result in the protection of the reproductive system from unnecessary bouts of inhibition during stress. These mechanisms may be particularly relevant in the breeding season when plasma concentrations of testosterone are higher in rams, or during the follicular phase of the oestrous cycle in ewes, when plasma concentrations of oestrogen are elevated. At these times, exposure to stress may have a greater impact on the success of reproduction. Therefore, a suppressive effect of oestrogen and testosterone on the hypothalamo-pituitary adrenal axis response to stress may be of benefit from an evolutionary perspective.

In conclusion, the studies presented in this thesis suggest that there are likely to be mechanisms by which pituitary responsiveness to GnRH stimulation is reduced during stress. Sex differences in the effects of stress or elevated plasma concentrations of cortisol on LH secretion may also exist, although no role for the sex steroids has been suggested by the studies presented in this thesis. It is possible, however, that there may be other factors which determine the extent to which pituitary responsiveness to GnRH is suppressed during stress or elevated plasma concentrations of cortisol, such as season, the elevations in plasma concentrations of cortisol attained by cortisol treatment or the imposition of stress, increases in the plasma concentrations of adrenaline and the interplay between the sympathoadrenal axis and the hypothalamo-pituitary adrenal axis.

Appendix 1

Published papers arising from this thesis

1. Stackpole,C.A.; Turner,A.J.; Clarke,I.J.; Lambert,G.W.; Tilbrook,A.J. (2003) Seasonal differences in the effect of isolation and restraint stress on the luteinizing hormone response to gonadotropin-releasing hormone in hypothalamo-pituitary disconnected, gonadectomized rams and ewes *Biology of Reproduction* **69**: 1158-1164

Conference and Seminar Papers

Stackpole, C.A., Turner, A.I., Clarke, I.J., Tilbrook, A.J. (2001) Isolation and restraint stress does not affect the LH response to GnRH in hypothalamo-pituitary disconnected rams and ewes. *Proceedings of the Australian Society for Reproductive Biology* **32**: 80

Stackpole,C.A.; Turner,A.I.; Clarke,I.J.; Tilbrook,A.J. (2003) Cortisol does not suppress the luteinizing hormone (LH) response to gonadotropin-releasing hormone (GnRH) in hypothalamo-pituitary disconnected rams and ewes. *Biology of Reproduction Supplement 1* **68**: 288

References

Adam CL & Findlay PA 1998 Inhibition of luteinizing hormone secretion and expression of c-*fos* and corticotrophin-releasing factor genes in the paraventricular nucleus during insulin-induced hypoglycaemia in sheep. *Journal of Neuroendocrinology* **10** 777-783.

Adams TE, Sakurai H & Adams BM 1999 Effect of stress-like concentrations of cortisol on estradiol-dependent expression of gonadotropin-releasing hormone receptor in orchidectomized sheep. *Biology of Reproduction* **60** 164-168.

Ahima RS & Harlan RE 1992 Glucocorticoid receptors in LHRH neurons. *Neuroendocrinology* **56** 845-850.

Ajika K, Kalra SP, Fawcett CP, Krulich L & McCann SM 1972 The effect of stress and nembutal on plasma levels of gonadotropins and prolactin in ovariectomized rats. *Endocrinology* **90** 707-715.

Altemus M, Roca C, Galliven E, Romanos C & Deuster P 2001 Increased vasopressin and adrenocorticotropin responses to stress in the midluteal phase of the menstrual cycle. *The Journal of Clinical Endocrinology and Metabolism* **86** 2525-2530.

Ariznavarreta C, Calderon MD, Tresguerres JA & López-Calderón A 1989 Effect of adrenomedullectomy and propanolol treatment on the response of gonadotrophins to chronic stress in male rats. *Journal of Endocrinology* **120** 275-279.

Axelrod J & Reisine TD 1984 Stress Hormones: Their interaction and regulation. *Science* **224** 452-459.

Baldwin DM & Sawyer CH 1974 Effects of dexamethasone on LH release and ovulation in the cyclic rat. *Endocrinology* **94** 1397-1403.

Barnes MA, Kazmer GW, Birrenkott GP & Grimes LW 1983 Induced gonadotropin release in adrenocorticotropin-treated bulls and steers. *Journal of Animal Science* **56** 155-161.

Barry J 1979 Immunohistochemistry of luteinising-hormone-releasing hormone-producing neurons of the vertebrates. *International Review of Cytology* **60** 179-221.

Battaglia DF, Beaver AB, Harris TG, Tanhehco E, Viguié C & Karsch FJ 1999 Endotoxin disrupts the estradiol-induced luteinizing hormone surge: Interference with estradiol signal reading, not surge release. *Endocrinology* **140** 2471-2479.

Battaglia DF, Bowen JM, Krasa HB, Thrun LA, Viguié C & Karsch FJ 1997 Endotoxin inhibits the reproductive neuroendocrine axis while stimulating adrenal steroids: A simultaneous view from hypophyseal portal and peripheral blood. *Endocrinology* **138** 4273-4281.

Battaglia DF, Brown ME, Krasa HB, Thrun LA, Viguié C & Karsch FJ 1998 Systemic challenge with endotoxin stimulates corticotropin-releasing hormone and arginine vasopressin secretion into hypophyseal portal blood: Coincidence with gonadotropin-releasing hormone suppression. *Endocrinology* **139** 4175-4181.

Berga SL 1995 Stress and amenorrhea. *The Endocrinologist* **5** 416-421.

Berga SL, Daniels TL & Giles DE 1997 Women with functional hypothalamic amenorrhea but not other forms of anovulation display amplified cortisol concentrations. *Fertility and Sterility* **67** 1024-1030.

Bertok L 2003 Bacterial endotoxins and their effects. *Orvosi Hetilap* **139** 1947-1953.

Blake CA 1976 Effects of intravenous infusion of catecholamines on rat plasma luteinizing hormone and prolactin concentrations. *Endocrinology* **98** 99-104.

Blank MS, Fabbri A, Catt KJ & Dufau ML 1986 Inhibition of luteinizing hormone release by morphine and endogenous opiates in cultured pituitary cells. *Endocrinology* **118** 2097-2101.

Braden TD, Fry C & Sartin JL 1998 Effects of interleukins on secretion of luteinizing hormone from ovine pituitary cells. *American Journal of Veterinary Research* **59** 1488-1493.

Brann DW, Putnam CD & Mahesh VB 1990 Corticosteroids and regulation of gonadotropin and prolactin secretion in the rat. *Endocrinology* **126** 159-166.

Breen KM, Billings HJ, Debus N & Karsch FJ 2004 Endotoxin inhibits the surge secretion of gonadotropin-releasing hormone via a prostaglandin-independent pathway. *Endocrinology* **145** 221-227.

Breen KM & Karsch FJ 2004 Does cortisol inhibit pulsatile luteinizing hormone secretion at the hypothalamic or pituitary level? *Endocrinology* **145** 692-698.

Breen KM, Macfarlane M, Sakurai H, Adams B & Adams T 1999 Stress-like levels of cortisol increase the negative feedback potency of estradiol in female sheep. *Biology of Reproduction* **60** 276.

Bremner WJ, Cumming IA, Winfield C, de Kretser DM & Galloway D 1984 A study of the reproductive performance of mature romney and merino rams throughout the year. In *Reproduction in Sheep*, pp 16-19. Eds DR Lindsay & DT Pearce. Canberra. Australian Academy of Science and Australian Wool Corporation.

Broadbear JH, Hutton LC & Canny BJ 2004 Sex differences in the pituitary-adrenal response following acute antidepressant treatment in sheep. *Psychopharmacology* **171** 450-457.

Brook C & Marshall N 1996 The Hypothalamopituitary axis. In *Essential Endocrinology*, edn 3, pp 30-53. Eds JLH O'Riordan & PG Malan. Melbourne: Blackwell Science Pty Ltd.

Cagampang FRA, Cates PS, Sandhu S, Strutton PH, McGarvey C, Coen CW & O'Byrne KT 1997 Hypoglycaemia-induced inhibition of pulastile luteinizing hormone secretion in

female rats: Role of oestradiol, endogenous opioids and the adrenal medulla. *Journal of Neuroendocrinology* **9** 867-872.

Canny BJ, Funder JW & Clarke IJ 1989 Glucocorticoids regulate ovine hypophysial portal levels of corticotropin-releasing factor and arginine vasopressin in a stress-specific manner. *Endocrinology* **125** 2532-2539.

Canny BJ, O'Farrell KA, Clarke IJ & Tilbrook AJ 1999 The influence of sex and gonadectomy on the hypothalamo-pituitary-adrenal axis of the sheep. *Journal of Endocrinology* **162** 215-225.

Caraty A, Martin GB & Montgomery G 1984 A new method for studying pituitary responsiveness *in vivo* using pulses of LH-RH analogue in ewes passively immunized against native LH-RH. *Reproduction, Nutrition and Development* **24** 439-448.

Caraty A & Skinner DC 1999a Dynamics of steroid regulation of GnRH secretion during the oestrus cycle of the ewe. *Annales d'Endocrinologie* **60** 68-78.

Caraty A & Skinner DC 1999b Progesterone priming is essential for the full expression of the positive feedback effect of estradiol in inducing the preovulatory gonadotropin-releasing hormone surge in the ewe. *Endocrinology* **140** 165-170.

Caraty A, Miller DW, Delaleu B & Martin GB 1997 Stimulation of LH secretion in sheep by central administration of corticotrophin-releasing hormone. *Journal of Reproduction & Fertility* **111** 249-257.

Carrasco GA & Van de Kar LD 2003 Neuroendocrine pharmacology of stress. *European Journal of Pharmacology* **463** 235-272.

Cates PS, Forsling ML & O'Byrne KT 1999 Stress-induced suppression of pulsatile luteinising hormone release in the female rat: Role of vasopressin. *Journal of Neuroendocrinology* **11** 677-683.

Cates PS & O'Byrne KT 2000 The area postrema mediates insulin hypoglycaemia-induced suppression of pulsatile LH secretion in the female rat. *Brain Research* **853** 151-155.

Chantaraprateep P & Thibier M 1978 Effects of dexamethasone on the responses of luteinizing hormone and testosterone to two injections of luteinizing hormone releasing hormone in young postpubertal bulls. *Journal of Endocrinology* **77** 389-395.

Chen MD, O'Byrne KT, Chiappini SE, Hotchkiss J & Knobil E 1992 Hypoglycemic ' stress' and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey: Role of the ovary. *Neuroendocrinology* **56** 666-673.

Chen MD, Ördög T, O'Byrne KT, Goldsmith JR, Connaughton MA & Knobil E 1996 The insulin hypoglycaemia-induced inhibition of gonadotropin-releasing hormone pulse generator activity in the Rhesus monkey: Roles of vasopressin and corticotropin-releasing factor. *Endocrinology* **137** 2012-2021.

Chrousos GP & Gold PW 1992 The concepts of stress and stress system disorders. *JAMA* **267** 1244-1252.

Clarke IJ 1995a Evidence that the switch from negative to positive feedback at the level of the pituitary gland is an important timing event for the onset of the preovulatory surge in LH in the ewe. *Journal of Endocrinology* **145** 271-282.

Clarke IJ 1995b The preovulatory LH surge: A case of a neuroendocrine switch. *TEM* **6** 241-247.

Clarke IJ 1996 The Hypothalamo-pituitary axis. In *Scientific Essentials of Reproductive Medicine*, pp 120-132. Eds SG Hillet, Kitchener & Neilson. London: Saunders.

Clarke IJ 2001 Sex and season are major determinants of voluntary food intake in sheep. *Reproduction, Fertility and Development* **13** 577-582.

Clarke IJ & Cummins JT 1982 The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinising hormone (LH) secretion in ovariectomised ewes. *Endocrinology* **111** 1737-1739.

Clarke IJ & Cummins JT 1985 Increased gonadotropin-releasing hormone pulse frequency associated with estrogen-induced luteinizing hormone surges in ovariectomized ewes. *Endocrinology* **116** 2376-2833.

Clarke IJ, Cummins JT & de Kretser DM 1983 Pituitary gland function after disconnection from direct hypothalamic influences in the sheep. *Neuroendocrinology* **36** 376-384.

Clarke IJ, Cummins JT, Jenkin M & Phillips DJ 1989 The oestrogen-induced surge of LH requires a 'signal' pattern of gonadotrophin-releasing hormone input to the pituitary gland in the ewe. *Journal of Endocrinology* **122** 127-134.

Clarke IJ, Horton RJE & Doughton BW 1990 Investigation of the mechanism by which insulin-induced hypoglycemia decreases luteinizing hormone secretion in ovariectomized ewes. *Endocrinology* **127** 1470-1476.

Clarke IJ & Tilbrook AJ 1999 Ovulation is timed by the brain. In *Hormones and Women's Health: The Reproductive Years*, Ed LA Salamonson. Melbourne: Gordon and Breach Academic Publishers.

Coleman ES, Elsasser TH, Kemppainen RJ, Coleman DA & Sartin JL 1993 Effect of endotoxin on pituitary hormone secretion in sheep. *Neuroendocrinology* **58** 111-122.

Cook CJ 2002 Glucocorticoid feedback increases the sensitivity of the limbic system to stress. *Physiology and Behavior* **75** 455-464.

Crichton JS, Lishman AW, Hundley M & Amies C 1991 Role of dihydrotestosterone in the control of sexual behaviour on castrated male sheep. *Journal of Reproduction & Fertility* **93** 9-17.

da Costa AP, Ma X, Ingram CD, Lightman SL & Aguilera G 2001 Hypothalamic and amygdaloid corticotropin-releasing hormone (CRH) and CRH receptor-1 mRNA expression in the stress-hyporesponsive late pregnant and early lactating rat. *Molecular Brain Research* **91** 119-130.

Dadoun F, Guillaume V, Sauze N, Farisse J, Velut JG, Orsoni JC, Gaillard R & Oliver C 1998 Effect of endotoxin on the hypothalamic-pituitary-adrenal axis in sheep. *European Journal of Endocrinology* **138** 193-197.

Daley CA, MacFarlane MS, Sakurai H & Adams TE 1999a Effect of stress-like concentrations of cortisol on follicular development and the preovulatory surge of LH in sheep. *Journal of Reproduction & Fertility* **117** 11-16.

Daley CA, Sakuri H, Adams BM & Adams TE 1999b Effect of stress-like concentrations of cortisol on gonadotroph function in orchidectomized sheep. *Biology of Reproduction* **60** 158-163.

Dallman MF, Akana SF, Scribner KA, Bradbury MJ, Walker CD, Strack AM & Cascio CS 1991 Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis. *Journal of Neuroendocrinology* **4** 517-526.

Deaver DR & Dailey RA 1982 Effects of dopamine, norepinephrine and serotonin on plasma concentrations of luteinizing hormone and prolactin in ovariectomized and anestrous ewes. *Biology of Reproduction* **27** 624-632.

Debus N, Breen KM, Barrell GK, Billings HJ, Brown M, Young EA & Karsch FJ 2002 Does cortisol mediate endotoxin-induced inhibition of pulsatile luteinizing hormone and gonadotropin-releasing hormone secretion? *Endocrinology* **143** 3748-3758.

Dees WL, Sorenson Jr AM, Kemp WM & McArthur NH 1981 Immunohistochemical localisation of gonadotropin-releasing hormone (GnRH) in the brain and infundibulum of the sheep. *Cell and Tissue Research* **215** 181-191.

de Kloet ER, Wallach G & McEwen BS 1975 Differences in corticosterone and dexamethasone binding to the rat brain and pituitary. *Endocrinology* **96** 598-609.

Dobson H, Essawy SA & Alam MGS 1988 Suppression of LH response to gonadotrophin-releasing hormone or oestradiol by ACTH (1-24) treatment in anoestrous ewes. *Journal of Endocrinology* **118** 193-197.

Dobson H & Smith RF 1995 Stress and reproduction in farm animals. *Journal of Reproduction & Fertility Supplement* **49** 451-461.

Dobson H & Smith RF 1998 Stress and subfertility. *Reproduction in Domestic Animals* **33** 107-111.

Dobson H & Smith RF 2000 What is stress, and how does it affect reproduction? *Animal Reproduction Science* **60-61** 743-752.

Dobson H, Tebble JE, Phogat JB & Smith RF 1999 Effect of transport on pulsatile and surge secretion of LH in ewes in the breeding season. *Journal of Reproduction & Fertility* **116** 1-8.

Dobson H, Tebble JE, Smith RF & Ward WR 2001 Is stress really all that important? *Theriogenology* **55** 65-73.

Doney JM, Gunn RG & Griffiths JG 1973 The effect of premating stress on the onset of oestrus and on ovulation rate in scottish blackface ewes. *Journal of Reproduction & Fertility* **35** 381-384.

Doney JM, Gunn RG, Smith WF & Carr WR 1976 Effects of pre-mating environment stress, ACTH, cortisone acetate or metyrapone on oestrus and ovulation in sheep. *Journal of Agricultural Science, Cambridge* **87** 127-132.

Driancourt MA, Royere D, Hedon B & Levasseur MC 1993 Oestrous and menstrual Cycles. In *Reproduction in Mammals and Man*, pp 589-603. Eds C Thibault, MC Levasseur & RHF Hunter. Paris: Ellipses.

Du Ruisseau P, Taché Y, Brazeau P & Collu R 1978 Pattern of adenohypophyseal hormone changes induced by various stressors in female and male rats. *Neuroendocrinology* **27** 257-271.

Dubey AK & Plant TM 1985 A suppression of gonadotropin secretion by cortisol in castrated male Rhesus monkeys *(Macaca mulatta)* mediated by the interruption of hypothalamic gonadotropin-releasing hormone release. *Biology of Reproduction* **33** 423-431.

Eisenhofer G, Huynh TT, Hiroi M & Pacak K 2001 Understanding catecholamine metabolism as a guide to the biochemical diagnosis of pheochromocytoma. *Reviews in Endocrine and Metabolic Disorders* **2** 297-311.

Engelmann M, Ebner K, Landgraf R, Holsboer F & Wotjak CT 1999 Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats. *Journal of Neuroendocrinology* **11** 867-872.

Engler D, Pham T, Fullerton MJ, Funder JW & Clarke IJ 1988 Studies of the regulation of the hypothalamic-pituitary-adrenal axis in sheep with hypothalamic-pituitary disconnection. I. Effect of an audiovisual stimulus and insulin-induced hypoglycemia. *Neuroendocrinology* **48** 551-560.

Engler D, Pham T, Fullerton MJ, Ooi G, Funder JW & Clarke IJ 1989 Studies of the secretion of corticotrophin-releasing factor and arginine vasopressin into the hypophysial-portal circulation of the conscious sheep I. Effect of an audiovisual stimulus and insulin-induced hypoglycaemia. *Neuroendocrinology* **49** 367-381.

Euker JS, Meites J & Riegle GD 1975 Effects of acute stress on serum LH and prolactin in intact, castrate and dexamethasone-treated male rats. *Endocrinology* **96** 85-92.

Euker JS & Riegle GD 1973 Effects of stress on pregnancy in the rat. *Journal of Reproduction & Fertility* **34** 343-346.

Fabre-Nys C, Blache D, Hinton MR, Goode JA & Kendrick KM 1994 Microdialysis measurement of neurochemical changes in the mediobasal hypothalamus of ovariectomized ewes during oestrus. *Brain Research* **649** 282-296.

Familiari M, Smith AI, Smith R & Funder JW 1989 Arginine vasopressin is a much more potent stimulus to ACTH release from ovine anterior pituitary cells than ovine corticotropin-releasing factor. 1. In vitro studies. *Neuroendocrinology* **50** 152-157.

Ferin M 1999 Stress and the reproductive cycle. *The Journal of Clinical Endocrinology and Metabolism* **84** 1768-1774.

Freudenberg MA, Ness T, Kumazawa Y & Galanos C 1993 The role of cytokines in endotoxic shock and in endotoxin hypersensitivity. *Immunitat und Infektion* **21** 40-44.

Fulkerson WJ & Tang BY 1979 Ultradian and circadian rhythms in the plasma concentration of cortisol in sheep. *Journal of Endocrinology* **81** 135-141.

Fuquay JW & Moberg GP 1983 Influence of the pituitary-adrenal axis on the induced release of luteinizing hormone in rams. *Journal of Endocrinology* **99** 151-155.

Gambacciani M, Yen SSC & Rasmussen DD 1986 GnRH release from the mediobasal hypothalamus: in vitro inhibition by corticotropin-releasing factor. *Neuroendocrinology* **43** 533-536.

Geschwind II & Dewey R 1968 Dynamics of luteinizing hormone (LH) secretion in the cycling ewe: a radioimmunoassay study. *Proceedings of the Society for Experimental Biology and Medicine* **129** 451-455.

Gindoff PR & Ferin M 1987 Endogenous opioid peptides modulate the effect of corticotropin-releasing factor on gonadotropin release in the primate. *Endocrinology* **121** 837-842.

Goldstein DS 1987 Stress-induced activation of the sympathetic nervous system. *Baillière's Clinical Endocrinology and Metabolism* **1** 253-278.

Gonzaléz-Quijano, MI, Ariznavarreta, C, Martin, A.I, Tresguerres, JA, and Lopez-Calderon, A 1991. Naltrexone does not reverse the inhibitory effect of chronic restraint on gonadotropin secretion in the intact male rat. *Neuroendocrinology* **54**, 447-453.

Goubillon ML & Thalabard JC 1996 Insulin-induced hypoglycemia decreases luteinizing hormone secretion in the castrated male rat: Involvement of opiate peptides. *Neuroendocrinology* **64** 49-56.

Griffiths JG, Gunn RG & Doney JM 1970 Fertility in scottish blackface ewes as influenced by climatic stress. *Journal of Agricultural Science, Cambridge* **75** 485-488.

Hamada T, Nakajima M, Takeuchi Y & Mori Y 1996 Pheromone-induced stimulation of the hypothalamic gonadotropin-releasing hormone pulse generator in ovariectomized, estrogen-primed goats. *Neuroendocrinology* **64** 313-399.

Handa RJ, Burgess LH, Kerr JE & O'Keefe JA 1994 Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Hormones and Behavior* **28** 464-476.

Harbuz MS & Lightman SL 1992 Stress and the hypothalamo-pituitary-adrenal axis: acute, chronic and immunological activation. *Journal of Endocrinology* **134** 327-339.

Hayashi KT & Moberg GP 1987 Influence of acute stress and the adrenal axis on regulation of LH and testosterone in the male Rhesus monkey (*Macaca mulatta*). *American Journal of Primatology* **12** 263-273.

Heisler LE, Pallotta CM, Reid RL & van Vugt DA 1993 Hypoglycemia-induced inhibition of luteinizing hormone secretion in the Rhesus monkey is not mediated by endogenous opioid peptides. *Journal of Clinical Endocrinology and Metabolism* **76** 1280-1285.

Heisler LE, Tumber AJ, Reid RL & van Vugt DA 1994 Vasopressin mediates hypoglycaemia-induced inhibition of luteinizing hormone secretion in the ovariectomized thesus monkey. *Neuroendocrinology* **60** 297-304.

Helliwell RJA & Williams LM 1992 Melatonin binding sites in the ovine brain and pituitary: Characterisation during the oestrous cycle. *Journal of Neuroendocrinology* **4** 287-294.

Herbert J 1987 Neuroendocrine responses to social stress. *Baillière's Clinical Endocrinology and Metabolism* **1** 467-490.

Hileman SM, Lubbers LS, Petersen SL, Kuehl DE, Scott CJ & Jackson GL 1996 Influence of testosterone on LHRH release, LHRH mRNA and proopiomelanocortin mRNA in male sheep. *Journal of Neuroendocrinology* **8** 113-121.

Hinojosa-Laborde C, Chapa I, Lange D & Haywood JR 1999 Gender differences in sympathetic nervous system regulation. *Clinical and Experimental Pharmacology and Physiology* **26** 122-126.

Horton RJE, Li JY, Cummins JT, Smith IA, Shen PJ & Clarke IJ 1990 Morphine decreases LH secretion in ovariectomized ewes only after steroid priming and not by direct pituitary action. *Neuroendocrinology* **52** 612-617.

Howlett TA & Rees LH 1987 Endogenous opioid peptides and human reproduction. In *Oxford Reviews of Reproductive Biology*, edn 9, pp 260-293. Ed JR Clarke. Oxford: Clarendon Press.

Hunter MG, Hindle JE, McLeod BJ & McNeilly AS 1988 Treatment with bovine follicular fluid suppresses follicular development in gonadotrophin-releasing hormone-treated anoestrous ewes. *Journal of Endocrinology* **119** 95-100.

Jackson GL, Kuehl D & Rhim TJ 1991 Testosterone inhibits gonadotrophin-releasing hormone pulse frequency in the male sheep. *Biology of Reproduction* **45** 188-191.

Jackson GL & Kuehl DE 2000 Interactions of photoperiod, testosterone and naloxone on GnRH and LH pulse parameters in the male sheep. *Domestic Animal Endocrinology* **18** 97-110.

Ju G 1999 Evidence for direct neural regulation of the mammalian anterior pituitary. *Clinical and Experimental Pharmacology and Physiology* **26** 757-759.

Karsch FJ, Cummins JT, Thomas GB & Clarke IJ 1987 Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing hormone in the ewe. *Biology of Reproduction* **36** 1207-1218.

Kawate N, Inaba T & Mori J 1993 Effects of cortisol on the amounts of estradiol-17β and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. *Animal Reproduction Science* **32** 15-25.

Kellendonk C, Gass P, Kretz O, Schütz G & Tronche F 2002 Corticosteroid receptors in the brain: Gene targeting studies. *Brain Research Bulletin* **57** 73-83.

Keller-Wood M 1998 ACTH responses to CRF and AVP in pregnant and nonpregnant ewes. *American Journal of Physiology* **274** R1762-R1768.

Komesaroff PA, Esler M, Clarke IJ, Fullerton MJ & Funder JW 1998 Effects of estrogen and estrous cycle on glucocorticoid and catecholamine responses to stress in sheep. *American Journal of Physiology* **275** E671-E678.

Kononen J, Hokaneimi J, Gustafsson Jå & Pelto-huikko M 1993 Glucocorticoid receptor colocalization with pituitary hormones in the rat pituitary gland. *Molecular and Cellular Endocrinology* **93** 97-103.

Krulich L, Hefco E, Illner P & Read CB 1974 The effects of acute stress on the secretion of LH, FSH, prolactin and GH in the normal male rat, with comments on their statistical evaluation. *Neuroendocrinology* **16** 293-311.

Lado-Abeal J, Clapper JA, Chen Zhu B, Hough CM, Syapin PJ & Norman RL 2002 Hypoglycemia-induced suppression of luteinizing hormone (LH) secretion in intact female Rhesus Macaques: Role of vasopressin and endogenous opioids. *Stress* **5** 113-119.

Lado-Abeal J, Rodriguez-Arnao J, Newell-Price JDC, Perry LA, Grossman AB, Besser GM & Trainer PJ 1998 Menstrual abnormalities in women with Cushing's disease are correlated with hypercortisolemia rather than raised circulating androgen levels. *Journal of Clinical Endocrinology and Metabolism* **83** 3083-3088.

Lee S, Miselis R & Rivier C 2002 Anatomical and functional evidence for a neural hypothalamic-testicular pathway that is independent of the pituitary. *Endocrinology* **143** 4447-4454.

Lee VWK, Cumming IA, de Kretser DM, Findlay JK, Hudson B & Keogh EJ 1976 Regulation of gonadotrophin secretion in rams from birth to sexual maturity I. Plasma LH, FSH and testosterone levels. *Journal of Reproduction & Fertility* **46** 1-6.

Lehman MN, Karsch FJ, Robinson JE & Silverman AJ 1988 Ultrastructure and synaptic organization of luteinizing hormone-releasing hormone (LHRH) neurons in the anestrous ewe. *Journal of Comparative Neurology* **273** 447-458.

Lehman MN, Robinson JE, Karsch FJ & Silverman AJ 1986 Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. *Journal of Comparative Neurology* **244** 19-35.

Li PS 1994 Modulation by cortisol of luteinizing hormone secretion from cultured porcine anterior pituitary cells: effects on secretion induced by phospholipase C, phorbol ester and cAMP. *Naunyn-Schmiedeberg's Archives of Pharmacology* **349** 107-112.

Li PS & Wagner WC 1983 In vivo and in vitro studies on the effect of adrenocorticotropic hormone or cortisol on the pituitary response to gonadotropin releasing hormone. *Biology of Reproduction* **29** 25-37.

Li XF, Mitchell JC, Wood S, Coen CW, Lightman SL & O'Byrne KT 2003 The effect of oestradiol and progesterone on hypoglycaemic stress-induced suppression of pulsatile luteinizing hormone release and on corticotropin-releasing hormone mRNA expression in the rat. *Journal of Neuroendocrinology* **15** 468-476.

Lightman SL & Young WSI 1989 Lactation inhibits stress-mediated secretion of corticosterone and oxytocin and hypothalamic accumulation of corticotropin-releasing factor and enkephalin messenger ribonucleic acids. *Endocrinology* **124** 2358-2364.

Lincoln GA, Ebling FJP & Martin GB 1987 Endogenous opioid control of pulsatile LH secretion in rams: modulation by photoperiod and gonadal steroids. *Journal of Endocrinology* **115** 425-438.

Lincoln GA & Short RV 1980 Seasonal breeding: nature's contraceptive. *Recent Progress in Hormone Research* **36** 1-52.

Liptrap RM 1993 Stress and reproduction in domestic animals. *Annals of the New York Academy of Science* **697** 275-284.

Liu YY, Morris JF & Ju G 1996 Synaptic relationship of substance P-like immunoreactive nerve fibres with gland cells of the anterior pituitary in the rat. *Cell and Tissue Research* **285** 227-234.

Long TD, Ellingrod VL, Kathol RG, Christensen LE, Resch DS & Perry PJ 2000 Lack of menstrual cycle effects on hypothalamic-pituitary adrenal axis response to insulin-induced hypoglycaemia. *Clinical Endocrinology* **52** 781-787.

López-Calderón, A, Ariznavarreta, C, Calderon, MD, and Tresguerres, JA 1987 Gonadotropin inhibition during chronic stress: role of the adrenal gland. *Journal of Steroid Biochemistry* **27** 609-614.

López-Calderón A, Gonzaléz-Quijano MI, Tresguerres JAF & Ariznavarreta C 1990 Role of LHRH in the gonadotrophin response to restraint stress in intact male rats. *Journal of Endocrinology* **124** 241-246.

Loriaux L & Nieman L 1990 Stress and reproduction: The role of cortisol. In *Neuroendocrine Regulation of Reproduction*, pp 307-310. Eds SSC Yen & WW Vale. Massachusetts: Serono Symposia.

MacFarlane MS, Breen KM, Sakurai H, Adams BM & Adams TE 2000 Effect of duration of infusion of stress-like concentrations of cortisol on follicular development and the preovulatory surge of LH in sheep. *Animal Reproduction Science* **63** 167-175.

Maddocks S, Kern S & Setchell BP 1995 Investigating local regulation of the testes of ruminants. *Journal of Reproduction & Fertility - Supplement* **49** 309-319.

Martínez de la Escalera G, Choi ALH & Weiner RI 1992 Generation and synchronisation of gonadotropin-releasing hormone (GnRH) pulses: Intrinsic properties of the GT1-1 GnRH neuronal cell line. *Proceedings of the National Academy of Science, USA* **89** 1852-1855.

Mason JW 1968a "Over-all" hormonal balance as a key to endocrine organization. *Psychosomatic Medicine* **30** 791-808.

Mason JW 1968b The scope of psychoendocrine research. *Psychosomatic Medicine* **30** 565-575.

Mason JW 1975 Emotion as reflected in patterns of endocrine integrations. In *Emotions - Their Parameters and Measurements*, pp 143-181. Ed L Levi. New York: Raven.

Matteri RL & Moberg GP 1982a Effect of cortisol or adrenocorticotrophin on release of luteinizing hormone induced by luteinizing hormone releasing hormone in the dairy heifer. *Journal of Endocrinology* **92** 141-146.

Matteri RL & Moberg GP 1982b Effect of cortisol or adrenocorticotrophin on release of luteinizing hormone induced by lutienizing hormone releasing hormone in the dairy heifer. *Journal of Endocrinology* **92** 141-146.

Matteri RL, Watson JG & Moberg GP 1984 Stress or acute adrenocorticotrophin treatment suppresses LHRH-induced LH release in the ram. *Journal of Reproduction & Fertility* **72** 385-393.

McFarlane A, Coghlan J, Tresham J & Wintour EM 1995a Cortisol feedback in adrenalectomized adult sheep. *American Journal of Physiology* **269** E10-E17.

McFarlane A, Coghlan J, Tresham J & Wintour EM 1995b Corticotropin-releasing factor alone, but not arginine vasopressin alone, stimulates the release of adrenocorticotropin in the conscious intact sheep. *Endocrinology* **136** 1821-1827.

McNeilly AS, Picton HM, Campbell BK & Baird DT 1991 Gonadotrophic control of folllicular growth in the ewe. *Journal of Reproduction & Fertility - Supplement.* **43** 177-186.

Meczekalski B, Tonetti A, Monteleone P, Bernardi F, Luisi S, Stomati M, Luisi M, Petraglia F & Genazzani AR 2000 Hypothalamic amenorrhea with normal body weight: ACTH, allopregnanolone and cortisol responses to corticotropin-releasing hormone test. *European Journal of Endocrinology* **142** 280-285.

Medvedev OS, Esler MD, Angus JA, Cox HS & Eisenhofer G 1990 Simultaneous determination of plasma noradrenaline and adrenaline kinetics. Responses to nitroprusside-induced hypotension and 2-deoxyglucose induced glucopenia in the rabbit. *Naunyn-Schmiedeberg's Archives of Pharmacology* **341** 192-199.

Melis GB, Mais V, Gambacciani M, Paoletti AM, Antinori D & Fioretti P 1987 Dexamethasone reduces the postcastration gonadotropin rise in women. *Journal of Clinical Endocrinology and Metabolism* **65** 237-241.

Moberg GP 1985a Biological response to stress: Key to assessment of animal well-being? In *Animal Stress*, pp 27-49. Ed GP Moberg. Baltimore: Williams & Wilkins.

Moberg GP 1985b Influence of stress on reproduction: Measure of well-being. In *Animal Stress*, pp 245-267. Ed GP Moberg. Baltimore: Williams & Wilkins.

Moberg GP 1987 Influence of the adrenal axis upon the gonads. *Oxford Reviews of Reproductive Biology* **9** 456-496.

Moberg GP 1991 How behavioral stress disrupts the endocrine control of reproduction in domestic animals. *Journal of Dairy Science* **74** 304-311.

Moberg GP, Watson JG, Stoebel DP & Cook R 1981 Effect of cortisol and dexamethasone on the oestrogen-induced release of luteinizing hormone in the anoestrous ewe. *Journal of Endocrinology* **90** 221-225.

Moenter SM, Caraty A & Karsch FJ 1990 The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. *Endocrinology* **127** 1375-1384.

Moor RM 1977 Sites of steroid production in ovine graafian follicles in culture. *Journal of Endocrinology* **73** 143-150.

Munck A, Guyre PM & Holbrook NJ 1984 Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Reviews* **5** 25-44.

Nangalama AW & Moberg GP 1991 Interaction between cortisol and arachidonic acid on the secretion of LH from ovine pituitary tissue. *Journal of Endocrinology* **131** 87-94.

Naylor AM, Porter DWF & Lincoln DW 1990 Central administration of corticotrophin-releasing factor in the sheep: effects on secretion of gonadotrophins, prolactin and cortisol. *Journal of Endocrinology* **124** 117-125.

Neumann ID, Wigger A, Torner L, Holsboer F & Landgraf R 2000 Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and

female rats: Partial action within the paraventricular nucleus. *Journal of Neuroendocrinology* **12** 235-243.

Niezgoda J, Bobek S, Wronska-Fortuna D & Wierzchos E 1993 Response of the sympatho-adrenal axis and adrenal cortex to short-term restraint stress in sheep. *Journal of Veterinary Medicine* **40** 631-638.

Nomura M, Saito J, Ueta Y, Muglia LJ, Pfaff DW & Ogawa S 2003 Enhanced up-regulation of corticotropin-releasing hormone gene expression in response to restraint stress in the hypothalamic paraventricular nucleus of oxytocin gene-deficient male mice. *Journal of Neuroendocrinology* **15** 1054-1061.

Norman RL 1994 Corticotropin-releasing hormone effects on luteinizing hormone and cortisol secretion in intact female Rhesus Macaques. *Biology of Reproduction* **50** 949-955.

Norman RL, McGlone J & Smith CJ 1994 Restraint inhibits luteinizing hormone secretion in the follicular phase of the menstrual cycle in Rhesus Macaques. *Biology of Reproduction* **50** 16-26.

Norman RL & Smith CJ 1992 Restraint inhibits luteinizing hormone and testosterone secretion in intact male Rhesus Macaques: Effects of concurrent naloxone administration. *Neuroendocrinology* **55** 405-415.

Olster DH & Ferin M 1987 Corticotropin-releasing hormone inhibits gonadotropin secretion in the ovariectomized Rhesus monkey. *Journal of Clinical Investigation and Metabolism* **65** 262-267.

Ono N, Bedran de Castro J, Khorram O & McCann SM 1985 Role of arginine vasopressin in control of ACTH and LH release during stress. *Life Sciences* **36** 1779-1786.

Pacák K & Palkovits M 2001 Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. *Endocrine Reviews* **22** 502-548.

Padmanabhan V, Keech C & Convey EM 1983 Cortisol inhibits and adrenocorticotropin has no effect on luteinizing hormone-releasing hormone-induced release of luteinizing hormone from bovine pituitary cells *in vitro*. *Endocrinology* **112** 1782-1787.

Parrott RF, Mission BH & de la Riva CF 1994 Differential stressor effects on the concentrations of cortisol, prolactin and catecholamines in the blood of sheep. *Research In Veterinary Science* **56** 234-239.

Patchev VK & Almeida OFX 1998 Gender specificity in the neural regulation of the stress response. *Molecular Neurobiology* **16** 63-77.

Pecins-Thompson M & Keller-Wood M 1994 Prolonged absence of ovarian hormones in the ewe reduces the adrenocorticotropin response to hypotension, but not to hypoglycemia or corticotropin-releasing factors. *Endocrinology* **134** 678-684.

Petraglia F, Sutton S, Vale W & Plotsky P 1987 Corticotropin-releasing factor decreases plasma luteinizing hormone levels in female rats by inhibiting gonadotropin-releasing hormone release into hypophysial-portal circulation. *Endocrinology* **120** 1083-1088.

Peyrin L, Cottet-Emard JM, Gonnet C, Rochas A, le Maho Y & Goffart M 1981 Urinary excretion of catecholamines in the night monkey (Aotus trivirgatus)(Primates, Cebidae). *Archives Internationales de Physiologie et de Biochimie* **89** 345-356.

Phillips DJ & Clarke IJ 1990 Effects of the synthetic glucocorticoid dexamethasone on reproductive function in the ewe. *Journal of Endocrinology* **126** 289-295.

Phogat JB, Smith RF & Dobson H 1999 Effect of transport on pituitary responsiveness to exogenous pulsatile GnRH and oestradiol-induced LH release in intact ewes. *Journal of Reproduction & Fertility* **116** 9-18.

Polkowska J, Dubois MP & Domanski E 1980 Immunocytochemistry of luteinising hormone releasing hormone (LHRH) in the sheep hypothalamus during various reproductive stages. *Cell and Tissue Research* **208** 327-341.

Polkowska J & Przekop F 1983 Immunocytochemical changes in hypothalamic and pituitary hormones after acute and prolonged stressful stimuli in the anoestrous ewe. *Acta Endocrinologica (Copenhagen)* **118** 269-276.

Radovick S, Wondisford FE, Nakayama Y, Yamada M, Cutler Jnr, GB & Weintraub BD 1990 Isolation and characterization of the human gonadotropin-releasing hormone gene in the hypothalamus and placenta. *Molecular Endocrinology* **4** 476-480

Raetz CR, Ulevitch RJ, Wright SD, Sibley CH, Ding A & Nathan CF 1991 Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *The FASEB Journal* **5** 2652-2660.

Rasmussen DD & Malvern PV 1983 Effects of confinement stress on episodic secretion of LH in ovariectomized sheep. *Neuroendocrinology* **36** 392-396.

Reul JM & de Kloet ER 1985 Two receptor systems for corticosterone in rat brain: Micro-distribution and differential occupation. *Endocrinology* **117** 2505.

Richards EM, Hua Y & Keller-Wood M 2003 Pharmacology and physiology of ovine corticosteroid receptors. *Neuroendocrinology* **77** 2-14.

Ringstrom SJ & Schwartz NB 1985 Cortisol suppresses the LH, but not the FSH, response to gonadotropin-releasing hormone after orchidectomy. *Endocrinology* **116** 472-474.

Ringstrom SJ, Suter DE, Hostetler JP & Schwartz NB 1992 Cortisol regulates secretion and pituitary content of the two gonadotropins differentially in female rats: effect of gonadotropin-releasing hormone antagonist. *Endocrinology* **130** 3122-3128.

Rivier C & Rivest S 1991 Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: Peripheral and central mechanisms. *Biology of Reproduction* **45** 523-532.

Roca CA, Schmidt PJ, Altemus M, Deuster P, Danaceau MA, Putnam K & Rubinow DR 2003 Differential menstrual cycle regulation of hypothalamic-pituitary-adrenal axis in women with premenstrual syndrome and controls. *The Journal of Clinical Endocrinology and Metabolism* **88** 3057-3063.

Romero LM 2002 Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology* **128** 1-24.

Rosen H, Jameel ML & Barkan AL 1988 Dexamethasone suppresses gonadotropin-releasing hormone (GnRH) secretion and has direct pituitary effects in male rats: Differential regulation of GnRH receptor and gonadotropin responses to GnRH. *Endocrinology* **122** 2873-2880.

Saketos M, Sharma N & Santoro NF 1993 Suppression of the hypothalamic-pituitary-ovarian axis in normal women by glucocorticoids. *Biology of Reproduction* **49** 1270-1276.

Sakurai H & Adams TE 1998 The effect of season and melatonin on GnRH-induced LH secretion in oestradiol-treated orchidectomized sheep. *Journal of Endocrinology* **158** 277-283.

Salacinski P, McLean C, Sykes JE, Clement-Jones VV & Lowry PJ 1981 Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3α, 6α-diphenyl glycoluril (iodogen). *Analytical Biochemistry* **117** 136-146.

Salisbury RL, Krieg Jr. RJ & Seibel HR 1980 Effects of arginine vasotocin, oxytocin, and arginine vasopressin on steroid-induced surges of luteinizing hormone and prolactin in ovariectomized rats. *Acta Endocrinologica* **94** 166-173.

Samuels MH, Luther M, Henry P & Ridgway EC 1994 Effects of hydrocortisone on pulsatile pituitary glycoprotein secretion. *Journal of Clinical Endocrinology and Metabolism* **78** 211-215.

Sapolsky RM 1985 Stress-induced suppression of testicular function in the wild baboon: Role of glucocorticoids. *Endocrinology* **116** 2273-2278.

Sapolsky RM 1994 *Why Zebras Don't Get Ulcers*. New York: W.H. Freeman and Company.

Sapolsky RM, Romero M & Munck AU 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparatory actions. *Endocrine Reviews* **21** 55-89.

Seeman TE, Singer B, Wilkinson CW & McEwen B 2001 Gender differences in age-related changes in HPA axis reactivity. *Psychoneuroendocrinology* **26** 225-240.

Selvage DJ & Rivier C 2003 Importance of the paraventricular nucleus of the hypothalamus as a component of a neural pathway between the brain and the testes that modulates testosterone secretion independently of the pituitary. *Endocrinology* **144** 594-598.

Selye H 1946 The general adaptation syndrome and the diseases of adaptation. *Journal of Clinical Endocrinology and Metabolism* **6** 117-230.

Shalts E, Xia L, Xiao E & Ferin M 1994 Inhibitory effect of arginine-vasopressin on LH secretion in the ovariectomized Rhesus monkey. *Neuroendocrinology* **59** 336-342.

Skinner DC & Herbison AE 1997 Effects of photoperiod on estrogen receptor, tyrosine hydroxylase, neuropeptide Y, and β-endorphin immunoreactivity in the ewe hypothalamus. *Endocrinology* **138** 2585-2595.

Smart D, Forhead AJ, Smith RF & Dobson H 1994 Transport stress delays the oestradiol-induced LH surge by a non-opioidergic mechanism in the early postpartum ewe. *Journal of Endocrinology* **142** 447-451.

Smith R.F. & Dobson H 2002 Hormonal interactions within the hypothalamus and pituitary with respect to stress and reproduction in sheep. *Domestic Animal Endocrinology* **23** 75-85.

Smith RF & Dobson H 2002 Hormonal interactions within the hypothalamus and pituitary with respect to stress and reproduction in sheep. *Domestic Animal Endocrinology* **23** 75-85.

Stoebel DP & Moberg GP 1982a Effect of adrenocorticotropin and cortisol on luteinizing hormone surges and estrous behavior of cows. *Journal of Dairy Science* **65** 1016-1024.

Stoebel DP & Moberg GP 1982b Repeated acute stress during the follicular phase and luteinizing hormone surge of dairy heifers. *Journal of Dairy Science* **65** 92-96.

Sundberg DK 1995 Chemical Messenger Systems. In *Neuroscience in Medicine*, pp 403-426. Ed PM Conn. Philadelphia: J.B. Lippincott Company.

Suter DE & Schwartz NB 1985a Effects of glucocorticoids on responsiveness of luteinizing hormone and follicle-stimulating hormone to gonadotropin-releasing hormone by male rat pituitary cells *in vitro*. *Endocrinology* **117** 855-859.

Suter DE & Schwartz NB 1985b Effects of glucocorticoids on secretion of luteinizing hormone and follicle-stimulating hormone by female rat pituitary cells *in vitro*. *Endocrinology* **117** 849-854.

Suter DE, Schwartz NB & Ringstrom SJ 1988 Dual role of glucocorticoids in regulation of pituitary content and secretion of gonadotropins. *American Journal of Physiology* **254** E595-E600.

Swartz SR & Moberg GP 1986 Effects of epinephrine, norepinephrine, and dopamine on gonadotropin-releasing hormone-induced secretion of luteinizing hormone *in vitro*. *Endocrinology* **118** 2425-2431.

Taché Y, Du Ruisseau P, Ducharme JR & Collu R 1978 Pattern of adenohypophyseal hormone changes in male rats following chronic stress. *Neuroendocrinology* **26** 208-219.

Taché Y, Ducharme JR, Charpenet G, Haour F, Saez J & Collu R 1980 Effect of chronic intermittent immobilization stress on hypophyso-gonadal function of rats. *Acta Endocrinologica* **93** 168-174.

Terasawa E, Keen KL, Mogi K & Claude P 1999 Pulsatile release of luteinizing hormone-releasing hormone (LHRH) in cultured LHRH neurons derived from the embryonic olfactory placode of the rhesus monkey. *Endocrinology* **140** 1432-1441.

Thibier M & Rolland O 1976 The effect of dexamethasone (DXM) on circulating testosterone (T) and luteinizing hormone (LH) in young postpubertal bulls. *Theriogenology* **5** 53-60.

Tilbrook AJ, Canny BJ, Serapiglia MD, Ambrose TJ & Clarke IJ 1999a Suppression of the secretion of luteinizing hormone due to isolation/restraint stress in gonadectomised rams and ewes is influenced by sex steroids. *Journal of Endocrinology* **160** 469-481.

Tilbrook AJ, Canny BJ, Stewart BJ, Serapiglia MD & Clarke IJ 1999b Central administration of corticotrophin releasing hormone but not arginine vasopressin stimulates the secretion of luteinizing hormone in rams in the presence and in the absence of testosterone. *Journal of Endocrinology* **162** 301-311.

Tilbrook AJ & Clarke IJ 1995 Negative feedback regulation of the secretion and actions of GnRH in male ruminants. *Journal of Reproduction & Fertility - Supplement* **49** 297-306.

Tilbrook AJ & Clarke IJ 2001 Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males. *Biology of Reproduction* **64** 735-742.

Tilbrook AJ, de Kretser DM & Clarke IJ 1993 Human recombinant inhibin A and testosterone act directly at the pituitary to suppress plasma concentrations of FSH in castrated rams. *Journal of Endocrinology* **138** 181-189.

Tilbrook AJ, de Kretser DM & Clarke IJ 1999c Seasonal changes in the negative feedback regulation of the secretion of the gonadotrophins by testosterone and inhibin in rams. *Journal of Endocrinology* **160** 155-167.

Tilbrook AJ, de Kretser DM, Cummins JT & Clarke IJ 1991 The negative feedback effects of testicular steroids are predominantly at the hypothalamus in the ram. *Endocrinology* **129** 3080-3092.

Tilbrook AJ, Turner AI & Clarke IJ 2000 Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction* **5** 105-113.

Tilbrook AJ, Turner AI & Clarke IJ 2002a Stress and reproduction: Central mechanisms and sex differences in non-rodent species. *Stress* **5** 83-100.

Tilbrook AJ, Turner AI, Lambert GW & Clarke IJ The bodyweight of gonadectomized ewes influences the cortisol response to isolation/restraint stress.

Torpy DJ & Chrousos GP 1996 The three-way interactions between the hypothalamic-pituitary-adrenal and gonadal axes and the immune system. *Baillière's Clinical Rheumatology* **10** 181-198.

Turner, A. I. Repeated acute elevation of cortisol did not affect reproduction in female pigs. 1998.
Ref Type: Thesis/Dissertation

Turner AI, Canny BJ, Hobbs RJ, Bond JD, Clarke IJ & Tilbrook AJ 2002a Influence of sex and gonadal status of sheep on cortisol secretion in response to ACTH and on cortisol and LH secretion in response to stress: Importance of different stressors. *Journal of Endocrinology* **173** 113-122.

Turner AI, Hemsworth PH & Tilbrook AJ 2002b Susceptibility of reproduction in female pigs to impairment by stress and the role of the hypothalamo-pituitary-adrenal axis. *Reproduction, Fertility and Development* **14** 377-391.

Turner AI, Rivalland ETA, Clarke IJ, Lambert GW, Morris MJ & Tilbrook AJ 2002c Noradrenaline, but not neuropeptide Y, is elevated in cerebrospinal fluid from the third cerebral ventricle following audiovisual stress in gonadectomized rams and ewes. *Neuroendocrinology* **76** 373-380.

Udelsman R & Chrousos GP 1988 Hormonal responses to surgical stress. *Advances in Experimental Medicine and Biology* **245** 265-272.

Van de Kar LD & Blair ML 1999 Forebrain pathways mediating stress-induced hormone secretion. *Frontiers in Neuroendocrinology* **20** 1-48.

van Vugt DA, Piercy J, Farley AE, Reid RL & Rivest S 1997 Luteinizing hormone secretion and corticotropin-releasing factor gene expression in the paraventricular nucleus of Rhesus monkeys following cortisol synthesis inhibition. *Endocrinology* **138** 2249-2258.

Vellucci SV & Parrott RF 1994 Expression of c-fos in the ovine brain following different types of stress, or central administration of corticotrophin-releasing hormone. *Experimental Physiology* **79** 241.

Watanobe H & Habu S 2003 Adrenal glucocorticoids do not mediate impaired reproductive function induced by lipopolysaccharide in rats. *Neuroendocrinology* **78** 23-28.

Weinstock M, Razin M, Schorer-Apelbaum D, Men D & McCarty R 1998 Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. *International Journal of Developmental Neuroscience* **16** 289-295.

Welsh Jnr TH, Bambino TH & Hseuh AJW 1982 Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis in vitro. *Biology of Reproduction* **27** 1138-1146.

Welsh Jnr TH & Johnson BH 1981 Stress-induced alterations in secretion of corticosteroids, progesterone, luteinizing hormone, and testosterone in bulls. *Endocrinology* **109** 185-190.

Wetsel WC, Valença MM, Merchenthaler I, Liposits Z, López FJ, Weiner RI, Mellon PL & Negro-Vilar A 1992 Intrinsic pulsatile secretory activity of immortalized luteinizing

hormone-releasing hormone-secreting neurons. *Proceedings of the National Academy of Science, USA* **89** 4149-4153.

Williams CY, Harris TG, Battaglia DF, Viguié C & Karsch FJ 2001 Endotoxin inhibits pituitary responsiveness to gonadotropin-releasing hormone. *Endocrinology* **142** 1915-1922.

Williams CY, Nishihara M, Thalabard JC, Grosser PM, Hotchkiss J & Knobil E 1990 Corticotropin-releasing factor and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey. *Neuroendocrinology* **52** 133-137.

Wingfield JC & Sapolsky RM 2003 Reproduction and resistance to stress: When and how. *Journal of Neuroendocrinology* **15** 711-724.

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ & Mathieson JC 1990 CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249** 1431-1433.

Xiao E & Ferin M 1988 The inhibitory action of corticotropin-releasing hormone on gonadotropin secretion in the ovariectomized Rhesus monkey is not mediated by adrenocorticotropic hormone. *Biology of Reproduction* **38** 763-767.

Xiao E, Luckhaus J, Niemann W & Ferin M 1989 Acute inhibition of gonadotropin secretion by corticotropin-releasing hormone in the primate: Are the adrenal glands involved? *Endocrinology* **124** 1632-1637.

Xiao E, Xia-Zhang L & Ferin M 2000 Inhibitory effects of endotoxin on LH secretion in the ovariectomized monkey are prevented by naloxone but not by an interleukin-1 receptor antagonist. *Neuroimmunomodulation* **7** 6-15.

Xiao E, Xia-Zhang L & Ferin M 2002 Inadequate luteal function is the initial clinical cyclic defect in a 12-day stress model that includes a psychogenic component in the Rhesus monkey. *The Journal of Clinical Endocrinology and Metabolism* **87** 2232-2237.

Young EA 1995 The role of gonadal steroids in hypothalamic-pituitary-adrenal axis regulation. *Critical Reviews in Neurobiology* **9** 371-381.