Sex differences in response to adrenocorticotropin (ACTH) administration in sheep

Elize van Lier
Department of Clinical Chemistry
Swedish University of Agricultural Sciences
Uppsala
Sweden

Animal and Forage Sciences Department
Faculty of Agriculture
Universidad de la República
Montevideo
Uruguay

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Abstract

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This thesis summarises and discusses results of two separate studies on the effects of adrenocorticotropin (ACTH) on the secretion of steroid hormones in sheep of different sex and gonadal status. Furthermore, it includes a study on the effects of ACTH and progesterone on luteinising hormone (LH) secretion in castrated rams as well as a study on sex differences in oestrogen receptor (ER) content of adrenal glands of sheep.

Testosterone concentrations of castrated males before and after ACTH administration were below the detection limit of the assay used. After ACTH administration cortisol, progesterone and 17α-hydroxy pregnenolone (17OHP5) were elevated for several hours and differed from pre-ACTH levels. Cortisol after ACTH administration was higher in females than in males in both the breeding and the non-breeding season. Gonadectomy eliminated the differences between the ewes and the rams. Rams showed a lower response in the breeding season than in the non-breeding season, which was not observed in the ewes.

After ACTH administration the number of LH pulses in the first half of the experimental period was significantly lower than it was in the second half. No effect was seen on mean LH secretion. Progesterone treatment had no effect on either the number of LH pulses or LH secretion.

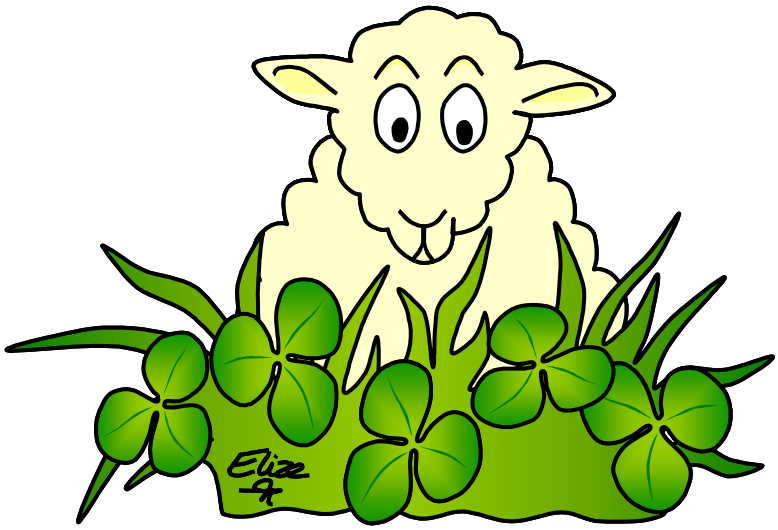
Ewes had higher levels of cytosolic ERs in the adrenals than did the rams and gonadectomy increased ER levels. No differences were observed in messenger ribonucleic acid (mRNA) levels for ER sub-type α (ERα) and insulin-like growth factor I (IGF-I). Progesterone receptor (PR) mRNA levels were reduced in ovariectomised ewes and enhanced in castrated rams. All of the animals had positive nuclear staining for ERα in the adrenal cortex, and no differences were observed between the groups.

The studies revealed progesterone and 17OHP5 secretion after ACTH administration, with the adrenal gland being the most likely source. Sex differences were observed in ACTH-induced cortisol secretion in intact sheep. Adrenocorticotropin decreased LH pulsatility in recently castrated rams. We demonstrated the existence of ER in the adrenal gland of sheep and found varying sensitivity to oestrogens as the ER levels differed among gender and gonadal status. Oestrogens may affect steroidogenesis directly at the adrenal cortex, which suggests that oestrogens are partly responsible for the sex differences in cortisol secretion in sheep.

Key words: hypothalamus-pituitary-adrenal (HPA) axis, *Ovis aries*, reproduction, sex differences, steroids, stress response

Author's address: Elize van Lier, Department of Clinical Chemistry, SLU, SE-750 07 UPPSALA, Sweden. On leave from the Animal and Forage Sciences Department, Faculty of Agriculture, Avda. E. Garzón 780, 12900 MONTEVIDEO, Uruguay. Email: vanlier@adinet.com.uy

To Alejandro Castillo



Contents

Appendix

Papers I–IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I–IV):

I. Van Lier, E., Andersson, H., Pérez-Clariget, R. & Forsberg, M. 1998. Effects of administration of adrenocorticotrophic hormone (ACTH) on extragonadal progesterone levels in sheep. *Reproduction in domestic animals 33,* 55–59.

II. Van Lier, E., Regueiro, M., Pérez-Clariget, R., Andersson, H., Kindahl, H. & Forsberg, M. 1999. Effects of adrenocorticotrophin (ACTH) and progesterone on luteinising hormone (LH) secretion in recently castrated rams. *Animal reproduction science 55,* 115–126.

III. Van Lier, E., Pérez-Clariget, R. & Forsberg, M. 2003. Sex differences in cortisol secretion after administration of an ACTH analogue in sheep during the breeding and non-breeding season. *Animal reproduction science,* in press.

IV. Van Lier, E., Meikle, A., Bielli, A., Åkerberg, S., Forsberg, M. & Sahlin, L. Sex differences in oestrogen receptor levels in adrenal glands of sheep during the breeding season. *Submitted.*

Abbreviations

17OHP4: 17α-hydroxy progesterone
17OHP5: 17α-hydroxy pregnenolone
3βHSD: 3β-hydroxy steroid dehydrogenase/Δ4–Δ5 isomerase
A4: androstenedione
ACTH: adrenocorticotropin
AR: androgen receptor
AVP: arginine vasopressin
cAMP: cyclic adenosine monophosphate
cDNA: complementary deoxyribonucleic acid
CRH: corticotropin-releasing hormone
DHEA: dehydro-epiandrosterone
ENR/h: estimated net response per hour
ER: oestrogen receptor
ERα: oestrogen receptor sub-type α
ERβ: oestrogen receptor sub-type β
FGF: fibroblast growth factor
GC: glucocorticoid
GH: growth hormone
GnRH: gonadotropin-releasing hormone
HPA: hypothalamus-pituitary-adrenal
HPG: hypothalamus-pituitary-gonadal
IGF-I: insulin-like growth factor I
i.m.: intramuscular/intramuscularly
i.v.: intravenous/intravenously
LBA: ligand-binding assay
LH: luteinising hormone
LW: live weight
mRNA: messenger ribonucleic acid
NT: neurotransmitter
oERα: ovine oestrogen receptor sub-type α
oPR: ovine progesterone receptor
P4: progesterone
P450: cytochrome P450 family
P450aldo: P450 aldosterone synthetase
P450c11: P450 11β-hydroxylase
P450c17: P450 17α-hydroxylase/17–20 lyase
P450c21: P450 21-hydroxylase
P450scc: P450 cholesterol side-chain cleavage
PR: progesterone receptor
RIA: radio-immunoassay
s.c.: subcutaneous/subcutaneously
StAR: steroidogenic acute regulatory (protein)
S-Tferase: sulphotransferase
TGF: transforming growth factor

Introduction

Stress and livestock production

Stress can be considered to be any threat, perceived or real, to homeostasis (Munck, Guyre & Holbrook, 1984; Rivier & Rivest, 1991). When expectations are not met by real or anticipated perceptions of the internal or external environment the animal is stressed. The biological response to a threat to homeostasis is variable, depending on the type, duration and frequency of the stressor. In general terms stress activates the sympatho-adrenal system and the hypothalamus-pituitary-adrenal (HPA) axis as well as the immune system, and alters behaviour (Moberg, 2000).

In animal husbandry we can find many stress factors – or stressors – derived from interactions of animals with humans, animals or their environment. The most easily understood stressors are those in which humans impose 'unnatural' conditions on the animals with productive purposes, such as transport, change of housing, shearing, treatments, *etc*. The impact of these stressors is not always the same as animals have great adaptive capacity and can learn to recognise situations as 'non-threatening'. Conversely they can also easily forget when situations are not repeated with certain frequency. This adaptive ability varies from animal to animal, which makes prediction of the response to a given stressor very difficult. Fear of humans is an important element of these stressors. Between-animal interactions include instability of social hierarchy, group forming and over-population. The establishment of a hierarchical order when new members are introduced to a group is a stressful event. It takes time to establish dominance and this interferes with other behaviours such as feeding behaviour. Similar stress occurs when the group size is so large that individuals have difficulties recognising each other and need to establish a pecking order at each encounter. Among the environmental stress factors are climatic factors, lack of stimulation (*e.g.* in animals housed in a poor environment) and excessive stimulation (*e.g.* noise) (for a review, see Hemsworth & Barnett, 2000).

Stress facilitates four categories of problems which can be of economic importance in animal husbandry: 1) interference in livestock production and reproduction; 2) increased sensitivity to infectious diseases and psychosomatic symptoms; 3) alterations of behaviour; and 4) shock. A full description of the effects of stress in animal husbandry is beyond the scope of this thesis. Nevertheless it is important to keep in mind that human interactions with animals can have profound effects on the physiological and psychological status of these animals. In the end stress may be translated into economic loss in livestock production. In this thesis the focus is on the interactions between stress and reproduction.

Endocrine response to stress

In response to stress the sympatho-adrenal axis as well as the HPA axis is activated, resulting in a cascade of hormonal events (Fig. 1). Catecholamines

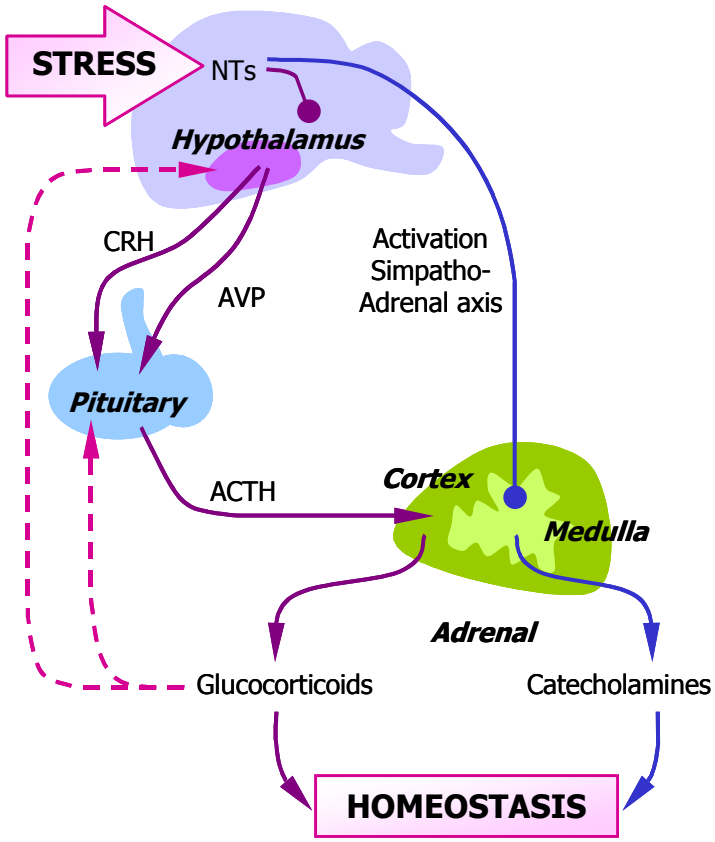


Fig. 1. Schematic representation of the endocrine response to stress. ACTH: adrenocorticotropin; AVP: arginine vasopressin; CRH: corticotropin-releasing hormone and NTs: neurotransmitters. Solid lines: stimulation; broken lines: inhibition. Adapted from Matteri, Carroll & Dyer, 2000.

are secreted in a matter of seconds from the adrenal medulla and corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the hypothalamus. Together CRH and AVP stimulate the pituitary to secrete ACTH, which in turn stimulates the secretion of glucocorticosteroids by the adrenal cortex. The glucocorticoids (GCs) have permissive, stimulatory, suppressive and preparative effects on the different systems of the organism, all of which are aimed at maintaining homeostasis (for a review, see Sapolsky, Romero & Munck, 2000). Through classic negative feedback regulation by the GCs the secretion of CRH, AVP and ACTH is suppressed, and thus the activity of the system is reset to basal levels.

The responses to stress play an integrative role in maintaining homeostasis. In general the endocrine responses are directed towards inhibition of functions such as growth and reproduction, and towards maintenance and survival. The changes in biological function during stress result in a shift of biological resources away from activities occurring before the stressor and are the 'biological costs of stress' (Moberg, 2000). For most stressors, the biological cost is negligible because the

stressors are short-lived. During prolonged or severe stress the biological cost becomes a significant burden to the body. During stress growth hormone (GH) is increased for energy mobilisation and insulin-like growth factor I (IGF-I) is decreased to reduce growth; prolactin is increased; and thyroid hormones are inhibited by sub-nutrition and stimulated by cold stress (for a review, see Matteri, Carroll & Dyer, 2000).

Adrenal glands

The adrenal glands are bilateral structures located craniomedially to the kidneys. They consist of two endocrine tissues, the primarily steroid-producing adrenal cortex and the catecholamine-producing chromaffin cells in the medulla (Naaman Répérant & Durand, 1997). The medulla, which is innervated by the splanchnic nerve and connected to the sympathetic nervous system, secretes catecholamines (adrenaline and noradrenaline). The cortex is of importance for the maintenance of homeostasis as it regulates sodium balance (via mineralocorticoids) as well as glucose levels (via GCs). During foetal life the adrenal cortex regulates intra-uterine homeostasis, the maturation of foetal organ systems and, in species such as the sheep, also the timing of parturition (Messiano & Jaffe, 1997).

The adrenal cortex is organised into three zones (Fig. 2) (Feige *et al.*, 1998). Underneath the capsule lies the zona glomerulosa which secretes aldosterone (mineralocorticoid) when stimulated by angiotensin II. The zona fasciculata secretes mainly cortisol in mammals and corticosterone in rodents (GCs). The third layer, the zona reticularis, is very pronounced in primates, smaller in other mammals such as cattle and sheep, and absent in lower vertebrates and some rodents. This zone produces androgens, mainly dehydro-epiandrosterone (DHEA). The functional architecture is based on specific expression of key enzymes within the different zones (Conley & Bird, 1997). The adrenal cortex undergoes continuous regeneration, and stem cells are believed to be located directly below the capsule and between the glomerulosa and the fasciculata zones (Feige *et al.*, 1998). These cells proliferate and are then displaced centripetally until they reach the junction to the medulla. No clear separation between cortical and medullar cells exists and the two endocrine tissues are intimately related (Ehrhart-Bornstein *et al.*, 1998).

The regulation of adrenocortical steroidogenesis is complex. Recent studies have demonstrated that the adrenals produce a wide variety of hormones, neuropeptides, neurotransmitters and cytokines, which interact within the gland (for a review, see Ehrhart-Bornstein *et al.*, 1998). Steroidogenesis is induced by the binding of ACTH to its membrane receptor on the adrenocortical cell. This binding activates adenylate cyclase, resulting in increased intracellular levels of cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase. The activated protein kinase has several functions, including activation of cholesterol esterase, stimulation of cholesterol transport into the mitochondrion and activation of the cholesterol C27-side-chain cleavage (scc) P450 complex. Adrenocorticotropin also induces gene transcription and translation of the enzymes which are involved in the biosynthesis of steroids. But apart from ACTH,

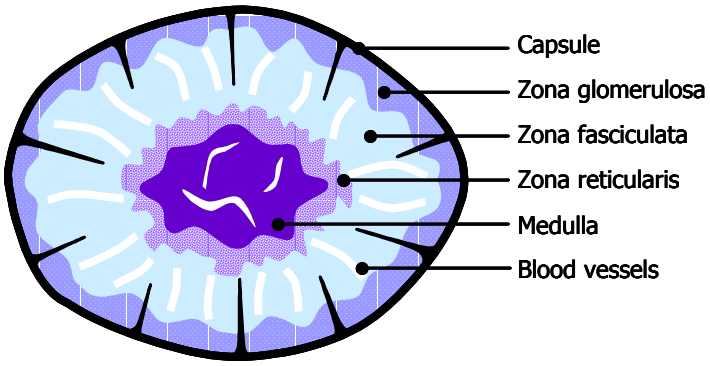


Fig. 2. Schematic representation of the adrenal gland.

a number of other regulatory factors such as insulin-like growth factors (IGFs), fibroblast growth factor (FGF) and transforming growth factor (TGF) have been shown to participate in the control of adrenal cortex homeostasis (for a review, see Feige *et al.*, 1998). Furthermore, ACTH receptors are up-regulated by their ligand, ACTH, and also, by locally produced IGF-I (Penhoat, Jaillard & Saez, 1989; Picard-Hagen *et al*., 1997).

Steroid hormones are derived from cholesterol, a 27-carbon molecule composed of three six-carbon rings and one five-carbon ring, with an eight-carbon side-chain (see, *e.g.*, Van Lier, 1998). The first step in steroid synthesis taking place in the mitochondria is the transformation of cholesterol into pregnenolone by cleavage of six carbons from the side-chain. Pregnenolone is then transported to the endoplasmic reticulum for further processing (Henricks, 1991). There are several enzyme complexes involved in steroidogenesis (Fig. 3). Pregnenolone can be metabolised by either 3β-hydroxy steroid dehydrogenase/Δ4–Δ5 isomerase (3βHSD) or P450 17α-hydroxylase/17–20 lyase (P450c17). These two enzyme complexes have the ability to act on more than one substrate. The one, 3βHSD, acts upon pregnenolone, 17α-hydroxy pregnenolone (17OHP5) and DHEA isomerising the Δ5 bond and introducing a ketone at C3, while P450c17 acts upon pregnenolone, progesterone and 17OHP5, oxidising the molecule at C17 and cutting off the remaining side-chain. Therefore, pregnenolone can be metabolised either to progesterone (Δ4 pathway) or to 17OHP5 (Δ5 pathway). Which of the metabolic pathways will be taken depends on the competition between 3βHSD and P450c17 for pregnenolone. These reactions occur in the zona fasciculata which expresses both these enzyme complexes. Progesterone as well as 17OHP5 are metabolised to 17α-hydroxy progesterone (17OHP4), then to deoxycortisol and finally, to cortisol (for a review, see Conley & Bird, 1997).

Sex differences in the response to stress

The HPA axis responds differently in males and females. In rats a sex difference exists in the response to stress, with a greater overall response in females than in males (Handa *et al.*, 1994). These sex differences in the HPA function

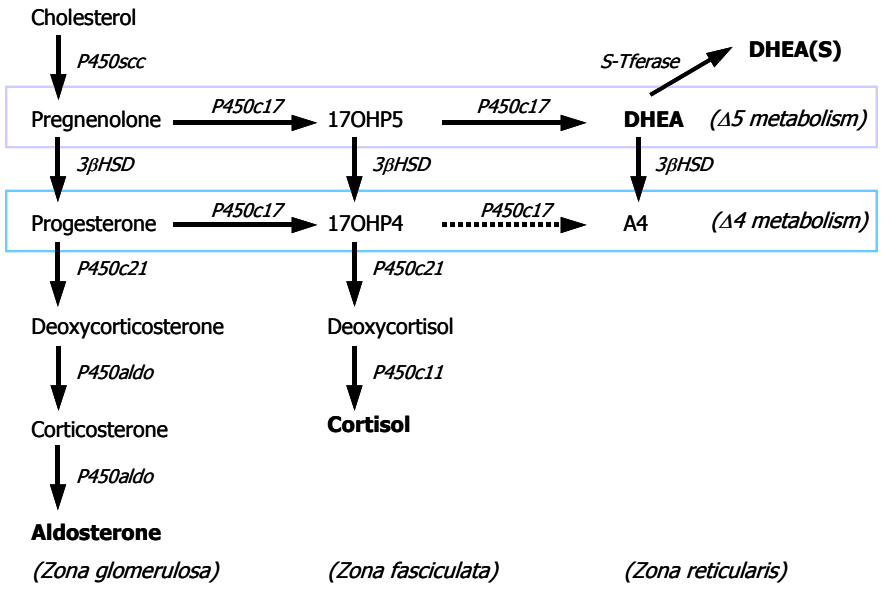


Fig. 3. Adrenocortical steroid biosynthesis in higher mammals (not rodents). The broken arrow indicates an enzymatic reaction available in pigs, but not in humans or ruminants. P450: cytochrome P450 family; P450scc: cholesterol side-chain cleavage; 3βHSD: 3β-hydroxy steroid dehydrogenase/Δ4–Δ5 isomerase; P450c17: P450 17α-hydroxylase/17–20 lyase; P450c21: P450 21-hydroxylase; P450c11: P450 11β-hydroxylase; P450aldo: P450 aldosterone synthetase; S-Tferase: Sulphotransferase. Adapted from Conley & Bird, 1997.

are in part due to differences in the circulating gonadal steroid milieu (Handa *et al.*, 1994). Gonadal steroids have been shown to affect the HPA function and activity under both basal and stressful conditions in rats (Kitay, 1961; Viau & Meaney, 1991) and primates (Xiao *et al.*, 1994). Nevertheless the regulatory pathways of gonadal steroid modulation of the activity of the HPA axis are unknown (Yukhananov & Handa, 1997).

Sex steroids have organisational effects on the brain during foetal and neonatal development. Present during the perinatal period, they can organise neuronal substrates, resulting in life-long alterations in endocrine function, including the HPA axis (Patchev & Almeida, 1998). Furthermore, sex steroids also have effects on the HPA axis in the adult, modulating its response (Ogilvie & Rivier, 1997; McCormick *et al.*, 1998). These steroid effects can be reversed by gonadectomy or mimicked by steroid replacement regimes (Ogilvie & Rivier, 1997).

Most studies have focused on centrally located gender differences in the HPA axis (specifically in the hypothalamus), and it has been argued that the differences downstream in the cascade of events generated by activation of this axis are mediated by differences in ACTH secretion (Burgess & Handa, 1992; Handa *et al.*, 1994). In rats gonadal steroids alter HPA activity. Oestradiol enhances ACTH secretion and testicular androgens suppress adrenal secretion of corticosterone (Ogilvie & Rivier, 1997). How these steroids bring about these effects and the exact location of their site of action are still matters under investigation.

In male rats castration has been reported to increase ACTH and corticosterone secretion as compared with intact males, and treatment with androgens after castration resulted in the same stress response as shown by intact males (Handa *et al.*, 1994). Castration of the male rat increased hypothalamic CRH content and the number of CRH-immunoreactive cells in the paraventricular nucleus by removal of an androgen-dependent repression (Bingaman *et al.*, 1994). In the rat the HPA responses to stress seem to be sensitive to variations in circulating testosterone levels since ACTH and corticosterone levels have been observed to be negatively correlated to testosterone levels in intact and castrated testosterone-replaced males in response to stress (Viau & Meaney, 1996). Independent and interactive effects of testosterone and corticosterone on the regulation of the rat HPA axis have been demonstrated by Viau *et al.* (1999), and these probably occur upstream of the paraventricular nucleus.

Oestrogen modulation of the rat HPA axis involves a reduction in feedback sensitivity to corticosterone (especially by interfering with GC receptor function), with an increase in pituitary secretion of ACTH, at least under stressful conditions (Burgess & Handa, 1992, 1993; Young, 1995).

From the studies in the rat it follows that the action of oestrogens on the HPA axis is one of stimulation, which partly arises from impaired GC-negative feedback at central sites, resulting in increased ACTH and GC secretion. However, direct actions at the adrenal gland cannot be ruled out. Androgens have inhibitory effects on the HPA axis, which are probably mediated centrally, though there also seem to be direct effects on the adrenals, which reduce ACTH-stimulated GC production. On the whole the HPA and hypothalamus-pituitary-gonadal (HPG) axes interact at multiple levels, which makes the physiological interpretation difficult, especially because conflicting results have been reported between and within species. In recent years, however, it has become evident that the response to stress as well as the effect of the sex of the animal varies according to the type and duration of the stressor (Rivier, 1999; Turner *et al.*, 2002).

Over the past decades sheep have been used to investigate stress and the response of these animals to stress. Nevertheless comparisons between the sexes have only recently gained attention. Publications on the subject come mainly from a group in Australia (Canny *et al.*, 1999; Tilbrook *et al.*, 1999; Tilbrook, Turner & Clarke, 2000, 2002; Turner *et al.*, 2002). Their findings will be reviewed in the next section and in the General discussion of this thesis.

Effects of stress on reproduction

It is still not completely understood how stress affects reproduction. Stress-related hormones can influence the HPG axis at the hypothalamus (to affect gonadotropin-releasing hormone (GnRH) secretion) and the pituitary (to affect gonadotropin secretion), with direct effects on the gonads being of less importance (Tilbrook, Turner & Clarke, 2000). The effect of stress on reproductive functions and the mechanisms mediating these effects depend on the type, duration and frequency of the stimulus (Rivier & Rivest, 1991).

Possible stress target sites

There is evidence that the immediate changes in luteinising hormone (LH) secretion in response to stress are mediated at the level of GnRH-secreting neurons, while long-term effects also involve peripheral mechanisms such as alteration in pituitary and gonadal responsiveness (Rivier & Rivest, 1991). In the hypothalamus neurotransmitters such as catecholamines (adrenaline, noradrenaline and dopamine) and endogenous opiates (*e.g.* β-endorphin) can affect the GnRH-secreting neurons. These neurotransmitters can affect the rhythm of the GnRH pulse generator. Hormones such as CRH, ACTH and steroids can affect the GnRH neurons. In the pituitary gland the effects can be either direct (through ACTH, CRH, endogenous opiates, cortisol and other steroids) or indirect by central interference of GnRH secretion, reducing the stimulus on the gonadotropes.

The effects of stress on the gonads have only been observed in primates and rodents. In rats high levels of GCs reduce the sensitivity to LH and LH receptor concentration of the testes. Tilbrook, Turner & Clarke (2002) have emphasised the importance of understanding how the different pathways that are activated during stress affect the secretion and actions of GnRH and how stress influences the feedback actions of gonadal steroids and inhibin. They also propose that the mechanisms by which stress affects reproduction are likely to differ between males and females.

Stress and reproduction in the female

Female reproduction is especially vulnerable to stress since it relies on a series of endocrine events whose temporal relationship is so critical that any disruption in the sequence will jeopardise reproductive success. The most clear example is interference with the correct timing of the pre-ovulatory LH surge (Dobson & Smith, 2000), leading ultimately to reproductive failure due to problems related to ovulation or behavioural oestrus (Parr, Davis & Tilbrook, 1989). Although stressors can cause foetal loss in mid- to late pregnancy, the highest stress-induced reproductive losses occur as a result of the interference with hypothalamus-pituitary function; early embryonic losses result from inappropriate exposure of the oocyte to gonadotropins within the follicle (Staigmiller & Moor, 1984; Dobson & Smith, 1995). Transport delays the onset of the LH surge in both cows and sheep (Nanda, Ward & Dobson, 1989; Smart *et al.*, 1994), when the stressor is initiated within hours of the onset of the LH surge. Repeated exposure to GnRH during transport of ewes during the breeding season has been shown to result in lower LH responses to the second and third injection of GnRH as compared with non-transported ewes (Phogat, Smith & Dobson, 1999). Stress can inhibit or delay ovulation or decrease ovulation rate; as a result of stress the oestrous cycles can be longer or shorter and dissociation of oestrus and ovulation can occur. These effects do not occur in all stressful situations, and therefore it is difficult to predict the results. Nevertheless they do increase the risk of reproductive failure.

Stress and reproduction in the male

Acute stress inhibits erection as the sympathetic system over-rules the parasympathetic system and local heat stress on the testes severely affects spermatogenesis. As in the female, stress in the male interferes with LH secretion (Tilbrook *et al.*, 1999). Different ACTH treatments in rams reduce the LH response to exogenous GnRH suggesting reduced pituitary sensitivity to GnRH (Matteri, Watson & Moberg, 1984). In the male, studies on the effects of stress have mainly focused on LH secretion and its mechanisms of regulation. However, apart from heat stress, it is not clear whether the effects of stress on LH secretion affect male fertility.

Outline and aims of the study

As mentioned in the Introduction, several stress conditions alter or depress reproductive performance in mammals (Rivier & Rivest, 1991; Moberg, 1991; Dobson & Smith, 1995, 1998; Dobson *et al.*, 2001). The interaction between the HPA axis and the HPG axis is not directed in one way. The gonads also affect the way the HPA axis responds to stress. The general aim of this thesis was to gain further information about the interactions between the HPA and HPG axes. This thesis is composed of several studies which address different aspects of these interactions:

Paper I

The mammalian adrenal cortex is known to secrete sex steroids besides the mineralocorticoids and GCs (Conley & Bird, 1997). We wanted to know whether the sheep adrenal gland is capable of secreting progesterone and testosterone when stimulated by its tropic hormone. Therefore the effect of exogenous ACTH on serum progesterone in wethers and ovariectomised ewes and on serum testosterone concentrations in wethers was evaluated (**Paper I**).

Paper II

In wethers and rams (both during and outside the breeding season) exogenous ACTH has been found to suppress plasma LH concentrations and depress the LH response to exogenous GnRH challenge (Cox & Mohamed, 1988; Mohamed & Cox, 1988; Mohamed, Cox & Moonan, 1988). Matteri, Watson & Moberg (1984) propose that in the ram, environmental stress may inhibit the response of the pituitary gland to GnRH through a hormonal component of the HPA axis other than cortisol. Since we had found increases in progesterone levels in both wethers and ovariectomised ewes after exogenous ACTH, with an especially large increase in wethers (**Paper I**), we hypothesised that progesterone could be involved in suppressing LH secretion in rams. Therefore, the effects of ACTH and progesterone on LH concentration and pulse frequency in recently castrated rams were evaluated (**Paper II**).

Paper III

Although we did not perform a formal comparison between the wethers and the ovariectomised ewes in **Paper I**, the observed patterns of cortisol and progesterone secretion made us suspect that there are gender-related differences in steroid secretion from the adrenal glands. Sex differences in cortisol secretion have been observed in primates and rodents (Kitay, 1961; Viau & Meaney, 1991; Xiao *et al.*, 1994) and in sheep differences have been observed *in vitro* (Canny *et al.*, 1999). Therefore a comparison was made of the cortisol concentrations in sheep of different sex and gonadal status after adrenal cortex stimulation by an ACTH analogue in the breeding and non-breeding season (**Paper III**).

Paper IV

In **Paper III** we found that also in sheep the response of the adrenal gland to exogenous ACTH administration – in terms of cortisol secretion – was affected by sex and gonadal status. This direct stimulation of the adrenal cortex by exogenous ACTH suggested that sex differences are localised in the adrenal cortex and are probably brought about by the sex steroids. As the effects of steroid hormones on the target tissues are exerted through their binding to a specific receptor, the first step in understanding the mechanisms by which steroid hormones affect adrenal steroidogenesis is to demonstrate the presence of their receptors. Oestrogens seem to be involved in the regulation of the rat adrenal cortex (Kitay, 1963) and oestrogen receptors (ERs) have been found in the adrenal gland of rodents and primates (Cutler *et al.*, 1978; Calandra *et al.*, 1980; Hirst *et al.*, 1992). Hence the hypothesis that the differences in cortisol secretion in sheep of different sex are partly due to a different adrenal sensitivity to oestrogen. Thus ER was characterised in adrenal glands of sheep of different sex and gonadal status in the breeding season (**Paper IV**). Markers of oestrogen action such as progesterone receptor (PR) mRNA and IGF-I mRNA were also measured (**Paper IV**). To make a comparison the uterus was included in this study as a classic oestrogen target tissue.

Materials and methods

Experimental designs

The studies were conducted at an experimental station (**Papers I–III**) and at the Faculty of Agriculture (**Paper IV**) both in Uruguay, located at 35º latitude south. All animals were from the Corriedale breed and were accustomed to handling. Before and between the trials, the animals were kept on pasture with free access to water. During the experiments the animals always had free access to water and feed (**Papers II–IV**). Before the experiment in **Paper IV**, the animals were kept on pasture during the day and were housed overnight with extra feeding and water. All animal experimentation was performed in compliance with regulations set by the National Board for Laboratory Animals in Sweden (Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden).

Paper I

Eight adult ewes and eight 30-month-old wethers (castrated at 1–2 months of age), all from the same flock, were included. The ewes were ovariectomised by paramedial laparotomy a month before the experiment began. Wethers and ewes were treated as two independent groups. All of the animals were used in two different trials, with a resting period of 1 week between samplings in early autumn (late March – early April). In the first trial each animal received 2 ml of sterile saline (0.9%) intravenously (i.v.). In the second trial animals were given 30 IU of porcine ACTH (Acton Prolongatum®, Ferring, Sweden) i.v. Blood samples were taken by venipuncture from the jugular vein for 8 h. The sampling interval was 30 min during the first 4 h and 60 min during the last 4 h. Sampling began at 09:30 h. Injections of saline or ACTH were given after 2 h of sampling.

Paper II

Six two-year-old Corriedale rams from the same flock were used during the winter (June – July). The rams were castrated and given a recovery period of 15 days. The day before castration, samples were taken in order to establish the pre-castration LH levels. The rams were divided into two groups, an untreated group (group U: n = 2) and a treated group (group T: n = 4). During the sampling periods, blood samples were taken every 15 min for 8 h beginning at 09:30 h. Sampling periods 0, 1 and 3 were all control samplings. In period 2 the animals were treated with 0.5 mg of an ACTH analogue (Tetracosactid, Synacthen Depot®, 1 mg/mL; Ciba Geigy, Basle, Switzerland) i.v. immediately after the first sample was taken. At the same time group U received 2 mL of sterile saline (0.9%) i.v. Before sampling in period 4 the animals were treated with subcutaneous (s.c.) progesterone implants for a total of 5 days (part of a Controlled Internal Drug Release (CIDR) dispenser, Eazi Breed®, InterAg, Hamilton, New Zealand). Sampling was done on the last day of the treatment. Group U had empty implants (silicone tubes) put in place during the same period.

The same protocol as in **Paper II** was performed with intact rams in the breeding season (March). Sampling period 0 was eliminated and the number of treated rams included was five. The results have not been published elsewhere.

Paper III

Two trials were conducted, the first during late spring (*i.e.* in the non-breeding season) and the second during the late summer (*i.e.* in the breeding season). Twelve female and twelve male adult Corriedale sheep were used in the non-breeding season. Three weeks prior to the trial six of the rams and six of the ewes were gonadectomised. In the breeding season ten female and nine male adult Corriedale sheep were used, all of which had been included in the non-breeding season trial, except for one intact ewe that had to be replaced because of a problem not related to the experiment. Animals were divided in four different groups according to sex and status: intact rams, castrated rams, intact ewes and ovariectomised ewes. On the day of frequent blood sampling, samples were taken every 15 min for 9 h, and each animal received 0.5 mL of an ACTH analogue (Synacthen Depot®, as in **Paper II**) intramuscularly (i.m.) immediately after the sample at 1:30 h from the beginning of the trial. Previous to the trials periodic samples were taken from the intact ewes for progesterone determination in order to assess luteal activity in the non-breeding season and to establish the stage of the oestrous cycle in the breeding season. Live weight (LW) was recorded before and after the samplings.

Paper IV

Thirteen female and nine male adult sheep were used during the autumn (May, breeding season). These animals were the same as in **Paper III** in the breeding season, apart from the addition of three extra intact ewes. Castration had been performed 5.5 months prior to the experiment. Since ER regulation in the uterus is well understood and the concentrations of ER vary across the oestrous cycle (Clark, Schrader & O'Malley, 1992), ewes in follicular and luteal phase were included in this study. Oestrus was synchronised with intravaginal sponges impregnated with medroxyprogesterone acetate for 12 days. After sponge withdrawal oestrus was checked twice daily with a ram. The animals were divided into five different groups according to sex and gonadal status: intact rams, castrated rams, intact ewes in the follicular phase, intact ewes in the luteal phase and ovariectomised ewes. Animals of the same group were sacrificed on the same day: intact rams and ewes in the follicular phase on the day after detected oestrus, gonadectomised sheep 4 days later, and ewes in the luteal phase 11 days after detection of oestrus. Blood samples were obtained every morning, from sponge withdrawal until the day of sacrifice. Live weight was recorded on the day of sacrifice.

Methods

Sampling procedures

The frequent blood sampling was done in small indoor pens, while daily samples were sometimes taken outdoors (**Paper III**). The day before a frequent blood sampling period, each animal's neck was shorn and fitted with an indwelling jugular catheter for collection of blood samples. Only in **Paper I** was frequent sampling done by venipuncture of the jugular vein, as was the case with all of the daily samplings (**Papers III** & **IV**). The blood samples were processed within 2 h of collection and either serum (**Papers I** & **II**) or plasma (heparinised tubes: **Papers III** & **IV**) was obtained after centrifugation. Then the serum or plasma was immediately frozen and stored at –20 ºC until analysed.

In **Paper IV** tissue samples were obtained at sacrifice: the adrenal glands and the uteri were removed and dissected on ice. The fresh weights of the left and right adrenals and the uteri were recorded. The tissues were packed and immediately frozen in liquid nitrogen and then stored at –80 °C. Half of each adrenal gland was fixed in paraformaldehyde (10%) and thereafter embedded in paraffin for immunohistochemical staining. For ER determination and mRNA studies in the uterus, a portion of the horn next to the uterine-tubular junction (upper uterus) was used.

Hormone determinations and definitions

All hormone determinations were done by radio-immunoassay (RIA) (**Papers I–IV**). Dilutions of sheep sera or plasma containing high concentrations of cortisol, progesterone and testosterone produced displacement curves parallel to standards provided in the Coat-A-Count, solid-phase RIA kits (Diagnostic Products Corporation, Los Angeles, CA, USA) (**Papers I–IV**). Oestradiol was analysed by a liquid-phase RIA previously validated for ovine plasma (Meikle *et al.,* 1997) (**Paper IV**), as was LH (Forsberg *et al.*, 1993) (**Paper II**). 17α-hydroxy pregnenolone was analysed with an ICN tritium kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). In all assays quality control samples containing endogenous hormone were assayed in duplicate at the beginning and end of each assay for the calculation of the intra- and inter-assay coefficients of variation. The intra-assay coefficients of variation for two control samples in the 17OHP5 assay (low: 282 nmol/L and high: 496 nmol/L) were 11.7% and 9.2%, respectively. The analytical detection limit of the assay was 108 nmol/L.

Basal levels of cortisol and progesterone were calculated individually using samples taken before treatments, not taking into account the first sample (**Papers I–III**). In **Paper I** for the estimation per hour and per animal of the hormonal responses to the saline and ACTH challenges, samples taken at hourly intervals after injection were used to calculate the mean hormone production per hour. Individual basal levels were subtracted in order to obtain the net response per hour. Since the hormone levels did not reach basal levels before the sampling finished, the results are expressed as *estimated* net response/hour (ENR/h).

The LH pulse (**Paper II**) was defined using the following criteria: 1) a peak had to occur within three samples of the previous nadir with a rise of >1 μg/L, or, if the peak occurred after the third sample from the nadir, the rise from the previous sample had to be ≥2 μg/L; and 2) the subsequent decline to the next nadir had to be >1 μg/L. These criteria were adapted from Goodman & Karsch (1980). Luteinising hormone pulses were counted for each animal during each period. Mean LH levels were calculated on all samples for each animal during each period.

Testosterone pulses (**Paper III**) were defined as increases within three samples from the previous nadir, resulting in a maximum of at least four times the concentration of the previous nadir, which was followed by a progressive decline. Pulse amplitude was defined as the difference between the maximum pulse concentration and the previous nadir concentration. Pulse frequency was taken as pulses per hour.

Since three of the five intact ewes were in the luteal phase of the oestrous cycle transformation was performed on the progesterone results of the intact ewes in the breeding season (unpublished results, study III) in order to limit the effect of progesterone secretion by the ovary. This was done as follows: progesterone base levels were calculated for the castrated ewes in the breeding season (0.4 nmol/L) and then subtracted from the progesterone concentrations at time 0 of each intact ewe. The resulting values were individually subtracted from every progesterone concentration. Values <0.4 nmol/L were replaced by 0.4 so that no value would be below the base level of the castrated ewes in the breeding season.

Oestrogen receptor determination

Oestrogen receptor determination was performed in cytosolic fractions by a ligand-binding assay (LBA) measuring both ERα and ERβ (**Paper IV**). The term 'cytosolic receptors' is used to indicate receptors found in the supernatant fraction of a tissue homogenate after high-speed centrifugation (Perrot-Applanat, Guichon-Mantel & Milgrom, 1992). Oestrogen receptors are nuclear receptors, but owing to the homogenisation procedure the receptors are found in the cytosol after centrifugation. Nuclear receptors (representing receptors that are more tightly bound to the nuclei) could be extracted in a 0.4 M KCl buffer from the nuclear pellet, but for the adrenal gland, the levels of extracted nuclear receptors were below the LBA detection limit. Therefore the cytosolic receptors from the LBA determinations are considered to represent the total number of ERs in the adrenal gland. The receptor-binding activities were analysed by the linear regression test of Scatchard's inverse model, allowing a statistical estimation of receptor parameters (Scatchard, 1949).

ERα, PR and IGF-I mRNA

The determination of mRNA was performed by a solution hybridisation method (**Paper IV**), a quantitative technique with a high sensitivity and capacity to analyse many samples in the same assay (every set of tissue samples was run in one assay). The hybridisation probes used for ovine ERα (oERα) and ovine PR (oPR) mRNA determinations were derived from plasmids containing 360 or 314

bp complementary deoxyribonucleic acids (cDNAs) from the oER and oPR, respectively (Ing, Spencer & Bazer, 1996). The hybridisation probe used for IGF-I mRNA determinations was derived from a 775 bp RsaI-EcoRI fragment cDNA of the human IGF-I and was kindly supplied by Dr Peter Rotwein of Washington University School of Medicine, St. Louis, MO, USA.

Immunolocalisation of ERα

A standard immunohistochemical technique (avidin-biotin-peroxidase, Vectastain Elite; Vector Laboratories, Burlingame, CA, USA) (**IV**) was used to visualise ERα immunostaining intensity and distribution (Wang *et al.*, 1999). The monoclonal antibody used to detect ERα was ER C-311 (cat# sc-787, Santa Cruz, CA, USA). After a general inspection of each slide, a quantitative image analysis (Leica Imaging Systems Ltd., Cambridge, UK) was performed to estimate the expression of ERα in the adrenal cortex. The main objective of immunostaining was to analyse the zona fasciculata, which is involved in the secretion of cortisol (Conley & Bird, 1997). Consequently, no formal analysis was performed of the zona glomerulosa, zona reticularis and medulla.

Statistical analyses

The SAS statistical package (Statistical Analysis Systems, 1996, 1999–2000) was used to perform the statistical analyses. At all times an alpha level of $P < 0.05$ was used to determine significance and P-values between 0.05 and 0.10 were considered as a tendency. The response of cortisol and progesterone to ACTH was compared with the corresponding basal level (**Paper I**). The results of the saline trial were compared with those obtained in the ACTH trial. The differences were analysed using the Student's *t*-test (**Paper I**). The ovariectomised ewes and wethers were considered as two independent groups, and therefore no comparison was made between sexes. Analysis of variance was performed on the data in **Papers II**–**IV**. Different SAS procedures were used. The General Linear Model (GLM) procedure along with the Ryan-Einot-Gabriel-Welsch multiple range test was used to analyse LH pulse data and mean LH levels (**Paper II**). The MIXED procedure along with the Tukey-Kramer test was used in **Papers III** & **IV**. In study III a repeated measurements analysis of variance of the cortisol (**Paper III**) and progesterone data was performed. The covariance structure used in the analysis was autoregressive order 1. The main effects studied were *sex* (male v. female), *status* (intact v. gonadectomised) and *season* (non-breeding v. breeding). The effect of *time* was considered as the repeated measures factor. *Season* was taken as a between-animal effect and animal was in the experimental error. Analysis of variance was also performed on the variable of LW taking into account sex, status and season. In **Paper IV**, the two main effects studied were *sex* and *status*, each with two levels, male v. female and intact v. gonadectomised, respectively. The variables analysed were ER (adrenals and uteri), ratio of nuclear area staining positively for ERα (adrenals), ERα mRNA (adrenals and uteri), PR mRNA (adrenals and uteri), IGF-I mRNA (adrenals and uteri), oestradiol, LW and the tissue weight of the adrenal glands and the uteri.

Results

In all of the studies the treatments were successful in eliciting the effects they were designed for. Administration of ACTH effectively induced cortisol secretion whenever applied, while saline did not (**Papers I–III**). In **Paper I** we used natural ACTH and in **Papers II** and **III** a synthetic analogue was used because the porcine ACTH was no longer available. No differences were observed in the effectiveness of either product. Progesterone implants (**Paper II**) successfully increased progesterone serum concentrations (mean ± SEM: 2.2 ± 0.1 nmol/L). Gonadectomy was successful in all cases; testosterone concentrations in the wethers (**Paper I**) and in the castrated rams were non-detectable (**Papers II–IV**) and ovariectomy in females resulted in progesterone levels near the detection limit of the assay (**Papers I**, **III** & **IV**). In **Paper IV** the intact females were synchronised and after sponge withdrawal oestrous signs were observed in all intact ewes except for one of the ewes in the luteal phase group. At the time of sacrifice a large follicle was present in the ovaries of ewes in the follicular phase and a corpus luteum in the ovaries of all of the ewes in the luteal phase, confirming synchronisation.

*Effects of administration of ACTH on extra-gonadal progesterone (**Paper I**) and 17α-hydroxy pregnenolone levels in sheep*

The testosterone concentrations determined in the samples from the wethers in the ACTH trial were below the detection limit of the assay in all cases. Administration of saline solution did not affect cortisol or progesterone levels in either sex. One male maintained elevated levels of cortisol and progesterone for 1 h after a sample had been taken with some difficulty. On that occasion, the animal did not stand still and only after 8 min of struggling with it could a sample be obtained. It is important to note that the actual puncture of the jugular vein and the collection of the sample was always swift, taking between 30 and 60 sec. No significant increases in cortisol or progesterone were observed before ACTH administration. After ACTH administration the concentrations of both hormones remained significantly elevated for 2–3 h.

Although a formal comparison of the cortisol and progesterone levels between the ovariectomised ewes and wethers in **Paper I** was not made, the progesterone levels after ACTH were surprisingly high in the wethers. At the time we hypothesised that for cortisol synthesis, the Δ4 pathway (via progesterone) would be preferred over the Δ5 pathway (via 17OHP5) in wethers. In some of the samples 17OHP5 was evaluated in order to gain more insight into steroid synthesis. Considerable amounts of 17OHP5 were released into the blood stream upon ACTH administration both in ovariectomised ewes and in wethers (Fig. 4), but no inverse relationship between progesterone and 17OHP5 could be seen (unpublished results).

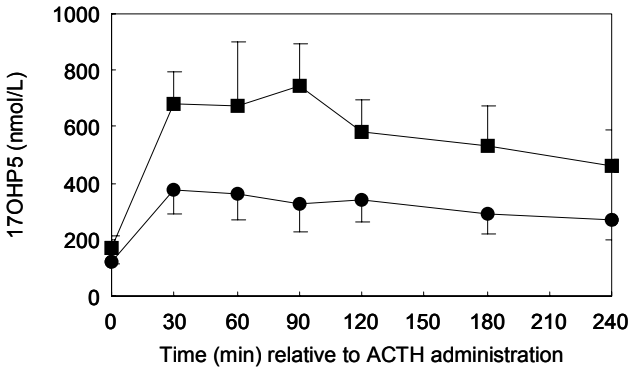


Fig. 4. Mean (± SEM) concentrations of 17OHP5 (nmol/L) after ACTH administration in ovariectomised ewes (dot, n = 5) and wethers (square, n = 5) (unpublished results).

*Effects of ACTH and progesterone on LH secretion in recently castrated rams (**Paper II**) and intact rams*

Before castration testosterone levels were low, while after castration they were below the detection limit of the assay. Cortisol and progesterone concentrations were basal before castration in all animals. No significant differences were found either in the untreated group or in the control samplings of the treated group when compared with pre-castration levels. The ACTH treatment resulted in significant increases in both cortisol and progesterone levels for 3 h when compared with levels in the control animals. When progesterone implants were used, the treated group exhibited increased serum progesterone levels, which were different from both the pre-castration basal level and from the levels in the untreated animals during the same period. Cortisol levels in this period were not different from basal levels.

All of the animals exhibited low LH levels and showed hardly any pulse activity before castration. Following castration both the number of LH pulses and the mean LH production increased significantly in all of the animals, while the differences during the post-castration periods were not significant. In order to obtain a more detailed description of the LH patterns during the ACTH trial, the sampling period was split into two equal parts and the number of pulses and the mean LH levels in both parts were compared. This division was based on the subjective observation that fewer pulses occurred in the first half of the ACTH trial. The number of pulses found for the treated group during the first half of this period was significantly different from that seen during the second half; no such differences had been seen in the previous control period. No effect on mean LH secretion was seen during ACTH treatment since the LH levels were not different from those in the previous control period and levels did not differ when the first half of the ACTH treatment period was compared with the second half. Progesterone treatment had no effect either on the number of LH pulses or on LH secretion.

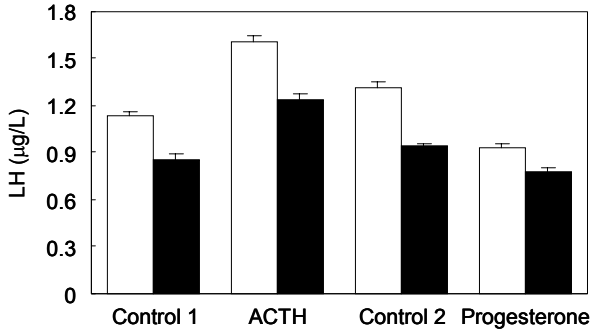


Fig. 5. Mean (± SEM) concentrations of LH (µg/L) in intact rams in the breeding season. Open bars: control rams (n = 2); solid bars: treated rams (n = 5) (unpublished results).

The effects of ACTH and progesterone were also evaluated in intact rams during the breeding season (unpublished results). After ACTH treatment in intact rams cortisol and progesterone were significantly higher than during the control periods. The rams treated with progesterone implants had elevated progesterone concentrations but no increased cortisol levels. The LH concentrations in intact rams in the breeding season were low and it was not possible to identify pulses. For this reason only the mean concentrations were analysed. The two untreated rams had higher LH concentrations than the treated group during all of the periods even in the control periods, when no treatments were applied (Fig. 5). Furthermore, all of the periods differed, in the treated rams as well as in the untreated rams. The changes in LH concentration over the periods were of the same magnitude for the treated and the untreated rams. When merging the LH data of the two control periods and expressing the LH concentrations of the other periods as a percentage of the merged controls, we found no differences between treated and untreated rams. However, the differences between the periods were maintained.

*Sex differences in cortisol and progesterone secretion after administration of an ACTH analogue in sheep during the breeding and non-breeding season (**Paper III**)*

The ewes had a lower LW than the rams, regardless of season. Both the intact and the castrated rams lost weight during the summer, resulting in a significant difference between seasons that was not seen in the ewes. The mean testosterone concentrations, the pulse number and the frequency differed from season to season, but not the pulse amplitude. During the breeding season the rams had either one or two pulses of testosterone during the 9-h sampling period. In the non-breeding season three of the six rams had testosterone levels that were low and presented no pulses. The other three rams, however, each showed one pulse. In both seasons the females showed higher cortisol levels after ACTH administration than males, though the difference appeared less marked in the non-breeding season. The cortisol response in the ewes was not affected by season.

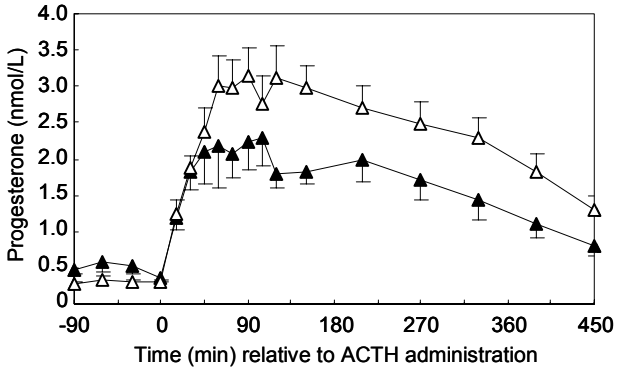


Fig. 6. Mean (± SEM) levels of progesterone (nmol/L) in sheep. Time 0 indicates time of ACTH administration. Solid triangles: breeding season; open triangles: non-breeding season (unpublished results).

The rams, however, showed a lower response in the breeding season. Gonadectomy reduced the response in the ewes but had no effect in the rams. Gonadectomy also eliminated the differences between the ewes and the rams, inasmuch as the intact rams had lower levels of cortisol compared with the intact females, with the cortisol levels of the gonadectomised animals of both sexes being between those of the gonad-intact groups. The rams lost weight during the summer, but this did not affect the cortisol results, as a regression analysis between LW and cortisol showed no relation between these parameters in either the rams or the ewes. The cortisol profiles of the intact rams showing testosterone pulses in the non-breeding season were in the lower range, while the profiles of the rams not showing these pulses were higher, except for one animal. In the breeding season the intact ewes were in different phases of the oestrous cycle. This difference in cycle phase did not lead to any distinction between the cortisol profiles of these ewes.

All animals showed significant increases in progesterone secretion after ACTH administration (Fig. 6, unpublished results). The overall progesterone secretion was higher in the non-breeding season than in the breeding season. However, neither sex nor gonadectomy affected this response.

Oestradiol was detected in the samples of two animals from each group before and after ACTH administration. Owing to the high assay variation at low concentrations (Table 1) no statistical analysis was performed. Nevertheless gonadectomised sheep had detectable concentrations of oestradiol.

*Sex differences in oestrogen receptor levels in adrenal glands of sheep in the breeding season (**Paper IV**)*

Progesterone on the day of sacrifice was high in ewes in the luteal phase and was near the detection limit of the assay in all of the other groups. On the day of sacrifice oestradiol was low in all of the animals, including ewes in the follicular phase (Table 2) although in this group oestradiol levels were high on the 2 days

Table 1. *Mean (± SEM) oestradiol concentrations (pmol/L) in sheep*

	Females		**Males**		**Intact**		**Gonadectomised**	
Pre-ACTH	8.5	(±1.4)	9.5	(±2.2)	11.8	(±1.3)	6.3	(±0.8)
Post-ACTH	10.5	(±1.6)	11.0	(±2.1)	13.8	(±0.9)	7.8	(±0.5)
Mean	9.5	(±1.1)	10.3	(±1.5)	12.8	(±0.8)	7.0	(±0.5)

The analytical detection limit of the assay was 2 pmol/L. The coefficient of variation for the control sample (6.6 pmol/L) was 20.7%.

preceding sacrifice. Testosterone was detectable only in the intact rams, with two rams showing high and the other two rams having low concentrations.

Ewes had higher levels of cytosolic ER in the adrenals than the rams did and gonadectomy increased the ER concentration in both sexes. The ER content of the adrenals of the ovariectomised ewes differed from that in intact rams and ewes in the luteal phase. The cytosolic ER levels measured in uterine tissue from the ewes in follicular and luteal phase differed significantly. No differences were observed in mRNA levels of ERα and IGF-I in the adrenal glands. Progesterone receptor mRNA levels were reduced in ovariectomised ewes and enhanced in castrated rams. Progesterone receptor mRNA levels tended to be higher in ewes in the follicular phase than in ovariectomised ewes and intact rams. Uterine mRNA levels of ERα and PR were highest in ewes in the follicular phase, and IGF-I mRNA was lowest in ovariectomised ewes. All of the animals had positive nuclear staining for ERα in the zona fasciculata of the adrenal cortex but no differences were observed between the groups.

Table 2. *Mean (± SEM) oestradiol concentrations in sheep*

	Females		**Males**		**Intact**		**Gonadectomised**	
Mean (pmol/L)	6.5	(±0.7)	5.6	(±1.1)	8.0	(±0.7)	3.9	(±0.4)

The analytical detection limit of the assay was 3 pmol/L. The coefficient of variation for the control sample (7.1 pmol/L) was 15.1%.

General discussion

Sampling by venipuncture did not induce stress in the animals (**Paper I**). When comparing the cortisol concentrations obtained from both the saline trial and samples collected before the ACTH injection (**Paper I**), with the basal levels from **Papers II** and **III** (in which indwelling jugular vein catheters were used), and with values described in other studies (McNatty, Cashmore & Young, 1972; Fulkerson & Tang, 1979; Przekop *et al.,* 1985; Jones *et al.*, 1989), the concentrations can all be considered to be normal basal levels.

Adrenal steroid secretion

Cortisol

Exogenous ACTH administration in sheep caused an increase in circulating cortisol concentrations (**Papers I–III**). In all of the studies the cortisol response after the ACTH injection (**Papers I–III**) was similar in magnitude to that observed by Benhaj & Cooke (1985) who used long-acting ACTH. Such cortisol concentrations can be considered to be similar to peak levels that are induced by natural stress, as indicated by the level that was reached by one of the wethers in the saline trial (**Paper I**). The resistance to restraint in this case could have been the stressor rather than venipuncture since cortisol secretion had already occurred.

Progesterone

After ACTH administration (**Papers I** & **II**, and unpublished results from study III) progesterone concentrations showed an increase in all the animals, with the most likely source being the adrenal glands. This increase was especially large in wethers in the first experiment (**Paper I**). We have demonstrated that upon adrenal cortex stimulation by ACTH, progesterone is secreted in substantial amounts, irrespective of sex and the presence or absence of the gonads. The physiological significance of this secretion is not clear but in most of the animals substantial levels were reached (**Paper I**).

In the female rat, progesterone of adrenal origin seems to be important for a normal pre-ovulatory LH surge, and corticosteroids do not appear to be involved. While the role of the ovaries as triggering the pre-ovulatory LH surge is unquestioned there is increasing evidence, in rats as well as primates, suggesting that adrenal pre-ovulatory secretion of progesterone may be important for synchronisation and potentiation of the pre-ovulatory gonadotropin surge (for a review, see Brann & Mahesh, 1991). Acute stress in an oestrogen-primed rat would be able to induce a pre-ovulatory LH surge and ovulation, whereas chronic stress or an ill-timed acute stress would have inhibitory effects on the pre-ovulatory gonadotropin surge (Putnam, Brann & Mahesh, 1991). This may account for the contradictory reports on the effect of stress on LH secretion.

Testosterone

We did not detect any increase in testosterone concentrations in wethers or in castrated rams after injection of ACTH, although the assay used was highly specific for testosterone (**Papers I–III**). However, testosterone is not the predominant androgen secreted by the adrenal cortex. In some species, especially in humans, the zona reticularis secretes substantial amounts of DHEA and also, androstenedione (A4), which can be converted peripherally to androgens with potent effects (Conley & Bird, 1997). Androgens other than testosterone have not been tested in our samples, and therefore their secretion by the ovine adrenal glands cannot be ruled out.

Oestradiol

Even if the intra-assay coefficient of variation was high for low concentrations, oestradiol was detectable in unstressed gonadectomised sheep (**Paper IV**). This could be an indication of an extra-gonadal source of oestradiol or of its androgen precursors. Whether or not the source is the adrenal cortex cannot be assessed by our results. Nevertheless Adams *et al.* (1990) report the existence of non-ovarian sources of oestrogen since in their study the uterine weight in ovariectomised ewes immunised against oestrogens was lower than it was in non-immunised ovariectomised ewes. Furthermore, they observed increased adrenal weight and decreased oestrogen content in uterine tissue in these immunised ewes. It is probable that these oestrogens (and/or their precursors) came from the adrenal gland as adrenalectomy has been shown to decrease the concentration of oestradiol in the uterus of ovariectomised ewes (Atkinson & Adams, 1988). Aromatase activity has been reported to occur in foetal and newborn porcine adrenal glands, but not in primates or ruminants (Conley *et al.*, 1996). These findings raise the question about the physiological role of such extra-gonadal oestradiol. Oestradiol concentrations in intact and gonadectomised sheep need to be evaluated with a more sensitive assay in order to detect a possible effect of gonadectomy.

17α-hydroxy pregnenolone

From the results it can be seen that this steroid precursor can be found in the blood of unstimulated gonadectomised sheep, but there was no indication of a difference in the steroidogenic pathway between ewes and wethers. In human, primate and ruminant adrenal glands Δ4 is the dominant pathway as 3βHDS activity predominates over P450c17. Furthermore, in these species 17OHP4 is not a substrate for A4 synthesis in the adrenal due to poor Δ4 lyase activity of P450c17. The predominance of the Δ4 pathway and the lack of Δ4 lyase activity facilitate efficient cortisol synthesis (for a review, see Conley & Bird, 1997).

On a molar basis, 17OHP5 concentrations were over a 100-fold higher than progesterone concentrations in both groups. However, 17OHP5, which is a direct precursor of DHEA (synthesised by the zona reticularis), was not measured in the samples. In sheep, adrenal androgen secretion appears to be <3.5 nmol/L (Conley & Bird, 1997). The physiological significance of these high serum concentrations of 17OHP5 is not clear.

The HPA axis and LH secretion in rams

Before castration of the rams the LH levels were low, which is typical for the non-breeding season (winter) (Pérez-Clariget, Forsberg & Rodriguez-Martinez, 1998) (**Paper II**). In the untreated rams after castration no changes in pulse were seen, but there was a slight decrease in the mean LH levels towards the end of the experiment. Although it was not statistically significant, this decrease may indicate that the hypothalamus was regaining control over LH secretion. If so, this could be due to the steroid-independent effect of season, which directly influences the hypothalamic GnRH secretion (Lincoln, 1984; Xu *et al.,* 1992).

Adrenocorticotropin hormone was found to suppress LH pulsatility during the first 4 h after administration, which was when cortisol and progesterone levels were elevated, although it did not affect the mean LH levels. Juniewicz, Johnson & Bolt (1987) effectively reduced LH levels for 2.5 h by using an i.v. bolus injection of ACTH when studying the effect of adrenal steroids on testosterone and LH secretion in the ram. Although they did not analyse pulse activity, a similar pattern of delay in pulsatility could be seen in their results. Other authors (Fuquay & Moberg, 1983; Matteri, Watson & Moberg, 1984; Mohamed, Cox & Moonan, 1988; Mohamed & Cox, 1988; Cox & Mohamed, 1988) have reported suppression of LH secretion in intact and castrated rams treated with ACTH, with or without a GnRH challenge. Analysis of LH pulsatility was not performed in any of these studies. Their findings, together with our results, confirm that a hormonal component(s) of the HPA axis is (are) involved in stress-induced interference of LH secretion.

Administration of ACTH has been seen to result in increased levels of both cortisol and progesterone in wethers and intact and castrated rams (**Papers I–III**). In **Paper II** we used s.c. implants to evaluate the effect of progesterone alone. Progesterone did not affect either LH secretion or the pulsatility. Tonic LH secretion of the pituitary gland is the result of an interplay between a stimulatory input from the brain and an inhibitory feedback from the gonads. Reports which discuss the effect of progesterone on the HP axis are contradictory. In the bull Welsh & Johnson (1981) have shown that stress causes an increase in the secretion of corticosteroids and progesterone and results in a decrease in the secretion of LH and testosterone, which indicates a negative influence of endogenous corticosteroids upon testosterone production by bovine testes. The authors suggested that progesterone may be a component of this negative relationship.

Goodman & Karsch (1980) propose that progesterone acts on the hypothalamus, since in their study long-term progesterone treatment in ovariectomised ewes decreased the frequency of LH pulses and basal LH secretion, but did not reduce the pituitary gland response to an exogenous GnRH pulse. While this is true for the female model, Sakurai, Adams & Adams (1997) found no suppression of LH secretion in wethers implanted with progesterone. However, they found that progesterone does reduce GnRH receptors and GnRH receptor-mRNA in gonadotrophs, suggesting that progesterone acts directly at pituitary gland loci to

affect gonadotroph function. This reduction in GnRH receptor-mRNA is also reported in *in vitro* studies on ovine pituitary gland tissue by Wu, Sealfon & Miller (1994). It appears that the negative feedback potency of progesterone differs between gonadectomised male and female sheep. This difference in sensitivity to progesterone may reflect differences in tissue distribution of PRs between the sexes (Sakurai, Adams & Adams, 1997).

One possible reason for not finding any effect of progesterone on LH secretion and pulsatility is that the steroid feedback mechanisms seem to be different in the breeding and non-breeding season. In their review, Tilbrook & Clarke (1995) mention that opioids are involved in the inhibitory effects of steroids on the secretion of GnRH, but that the effects of these opioids may be confined to the breeding season in the ram. An illustrative example is that naloxone (an opioid antagonist) when administered in the non-breeding season did not result in an increase in LH secretion. However, our results do not allow us to confirm participation of progesterone in the mechanism by which stress interferes with LH secretion. We did not find any effect of progesterone on pulse frequency, which is one of the important physiological cues that regulate reproduction (**Paper II**). Nevertheless the progesterone treatment may have lasted long enough to have induced PR down-regulation in the pituitary gland and therefore it may have masked any effect of progesterone treatment.

No statistical analysis of LH pulsatility was performed on the data on the intact rams in the breeding season (study II, unpublished results) as the LH levels were too low to be able to distinguish pulses. The mean LH levels differed among treatment periods as well as between treated and non-treated rams. However, control rams had higher mean LH levels than did the rams that received ACTH and progesterone, even in the periods when no treatments were applied. The absence of effects of the treatments may have been due to lack of sensitivity of the assay used. No conclusion on the effect of the treatments could be drawn from these results.

Adrenocorticotropin-induced cortisol secretion

Sex differences in cortisol secretion have previously been observed in primates and rodents (Kitay, 1961; Viau & Meaney, 1991; Xiao *et al.*, 1994). This observation can now also be extended to sheep: in our study female sheep secreted more cortisol after ACTH administration than male sheep did (**Paper III**). By directly stimulating the adrenal cortex with exogenous ACTH, the stress activation of the HPA axis was circumvented, indicating that sex differences also operate at the level of the adrenal cortex. There was no difference in cortisol responses after ACTH administration in gonadectomised sheep, and their response was between that of the groups of intact sheep. Gonadectomy possibly reduced the sex differences, rendering the gonadectomised animals more alike, which suggests that the sex differences in secretory activity of the adrenal cortex were due, at least in part, to the circulating sex hormones in the intact animals. Several reports have mentioned successful elimination of sexual differences by gonadectomy at the

adrenal level in rodents (rat: El-Migdadi, Gallant & Brownie, 1995; mouse: Perry & Stalvey, 1992).

Studies in rats have shown that the sex-linked differences in the secretory activity of the adrenal glands are maintained by circulating hormones, acting not only at the hypothalamus-pituitary level but also directly on adrenocortical cells (Nowak *et al.*, 1995). Corticosterone production in adrenocortical cells from ovariectomised rats has been reported to increase after steroid replacement by oestradiol benzoate (Lo, Chang & Wang, 2000). Another indication suggesting a direct effect of oestradiol on the adrenal cortex comes from a study in rats by Atkinson & Waddell (1997), who found differences in basal corticosterone levels (not accompanied by any differences in basal ACTH levels) in males and in females at different stages of the oestrous cycle.

In sheep, ACTH-induced cortisol production was found to be greater in adrenal gland cultures derived from females than from males (Canny *et al.*, 1999), but no effect of gonadectomy was seen. Tilbrook *et al.* (1999) did not find differences in cortisol secretion between gonadectomised male and female sheep *in vivo*, which is in accordance with our findings. Nevertheless in their study sex steroid replacement in gonadectomised sheep did not reveal any sex differences in cortisol secretion. Their study, however, did not include intact males and females. Even so, it can be argued that sex steroid replacement mimicking the hormonal milieu of intact animals should suffice to elicit the supposed sex differences, although such hormone replacement therapies in general do not follow the naturally occurring patterns of secretion. This may lead to non-physiological concentrations of a particular hormone, which could possibly interfere with the receptor dynamics of the different hormones and thus mask their effects. Researchers from the same group, recognising the importance of including intact sheep, later investigated the effect of sex and gonadal status in sheep on ACTH-induced cortisol secretion and did not find any differences among the groups of animals (Turner *et al.*, 2002). This is in conflict with our findings since we clearly observed sex differences after ACTH administration in this species, both in the breeding season and in the non-breeding season. Our study and that by Turner *et al.* (2002) are not entirely comparable as a different type and dose of ACTH and route of administration (0.5 mg Synacthen Depot® i.m. in our study v. 0.2 μg/kg LW Synacthen i.v. in their study) were used, and also, the sheep breeds varied (Corriedale v. Romney Marsh). However, a possible explanation for this discrepancy may be that these investigators studied the animals during the transition from breeding to non-breeding season.

In our study the differences between sexes seemed to be reduced in the non-breeding season, which is a period of relative gonadal endocrine quiescence, when the levels of gonadal steroids in both sexes are reduced (ewes: Bartlewski, Beard & Rawlings, 1999; rams: Pérez *et al.*, 1997; Mandiki *et al.*, 1998; Gerlach & Aurich, 2000). Season affected cortisol secretion in the males but not in the females. It is interesting to note that rams in the breeding season as well as rams showing testosterone pulses in the non-breeding season had lower cortisol profiles after ACTH administration, which would be an indication of androgen effects on the HPA axis other than the central sites. Administration of ACTH in bulls

following the onset of puberty resulted in decreased cortisol secretion as compared with steers, suggesting direct modulation of steroidogenesis by androgens at the adrenal cortex (Verkerk & Macmillan, 1997). As three of the six intact rams in the non-breeding season were coming into season, they might have had higher cortisol profiles if we had performed the experiment somewhat earlier in spring. Even so, difference between seasons still existed for the rams. The seasonal difference in LW was not responsible for the seasonal difference in cortisol in the rams. Only the intact rams seemed to have lower cortisol levels after ACTH administration in the breeding season compared with the non-breeding season, which was not seen in the castrated rams in spite of having lost weight during summer as well. Though the physiological importance of the sex difference in seasonal effect on cortisol secretion is not clear at this point, it does show that season has to be taken into account when evaluating sex differences in the HPA axis in sheep.

The lack of seasonal differences in the ewes is not easily explained. If oestrogens in the breeding season were partly responsible for the higher cortisol levels in ewes after ACTH administration compared with rams, then how did the ewes maintain a high cortisol response in the non-breeding season? This may be explained by a sensitivity compensation mechanism, with an inverse relationship between sex steroid receptor abundance and circulating hormone concentrations (Young & Crews, 1995). The adrenal gland may be highly sensitive to oestrogen in the non-breeding season and may therefore be able to maintain a high cortisol response despite low oestrogen levels.

Molecular studies on the adrenal gland

How do the sex steroids modulate cortisol secretion from the adrenal glands? If we hypothesise that the sex steroids act directly upon the adrenal glands, we first have to determine the presence of their receptors in adrenal tissue. Both androgen receptors (ARs) (Calandra *et al.*, 1980) and ERs (Cutler *et al.*, 1978; Calandra *et al.*, 1980; Hirst *et al.*, 1992) have been found in the adrenal gland of rodents. In our study we focused on the ERs since at the time a LBA for AR was not available in our laboratory. To our knowledge this was the first time that ER had been determined in ovine adrenal tissue. We demonstrated oestrogen-binding capacity in the adrenal gland by LBA, and ERα mRNA levels by solution hybridisation, and also showed the presence of the ERα protein by immunostaining (**Paper IV**).

Even though the ER concentrations in the adrenal glands were low compared with those in the uterus, we were able to find differences among the groups (**Paper IV**). Female sheep had higher levels than male sheep did and gonadectomy resulted in an increase in ER in both sexes. This increase in adrenal ER observed in gonadectomised sheep compared with their intact counterparts suggests that the regulation of ER in the adrenal gland is under control of the sex steroids. As oestradiol was lowest in plasma of gonadectomised sheep, the high levels of ER in the adrenals from these sheep may be explained by the above-mentioned sensitivity compensation mechanism (*i.e.* an inverse relationship between sex steroid receptor abundance and circulating hormone concentrations) (Young & Crews, 1995). In line with this Atkinson & Adams (1988) using ovariectomised

ewes report that in their study withdrawal of an extra-gonadal source of oestrogen precursors by adrenalectomy led to increased uterine ER concentration while the oestradiol content in the uterine tissue was extremely low. In our study the ER concentrations in the intact ewes seemed to follow the same pattern as previously described for the ovine uterus, with up-regulation by oestradiol (follicular phase) and down-regulation by progesterone (luteal phase) (Rexroad, 1981a, 1981b). The immunohistochemical study of the adrenal cortex did not reveal any of the sex differences found by the LBA as the overall low staining intensity made a semi-quantitative study impossible (**Paper IV**). However, it did indicate that all of the steroid-secreting cells in the cortex (zona glomerulosa: mineralocorticoids; zona fasciculata: GCs; and zona reticularis: androgens) would be sensitive to oestrogen action as they all presented nuclear ERα staining.

The uterine content of ER in the intact ewes in the follicular phase was several times that of the luteal phase (**Paper IV**), as could be expected (Rexroad, 1981b). This is in agreement with the high levels of uterine ERα mRNA found in the follicular phase, indicative of a high transcription rate as compared with the luteal phase. In the adrenals the ERα mRNA levels were the same for all of the groups. The differences in adrenal ER content must therefore have been due to post-transcriptional factors affecting receptor inactivation and/or receptor turnover (**Paper IV**). The ERα mRNA levels could not explain the differences in adrenal ER. Although the regulation of ER in the adrenal gland appears to be dependent on sex steroids as in the uterus, the discrepancy between transcription rate and actual ER protein content in the adrenal suggests that ER regulation is tissue-specific.

In order to evaluate oestrogen activity in the adrenal gland a uterine oestrogen activity marker, PR mRNA (**Paper IV**), was measured (Clark & Peck, 1979). Progesterone receptor mRNA levels in the uterus were highest in the follicular phase, which is consistent with increased oestrogen activity as described by Spencer & Bazer (1995). Although the difference was not as marked as in the uterus, adrenal PR mRNA tended to be higher in ewes in the follicular phase suggesting a direct oestrogen induction of PR in the adrenal. The lowest levels of adrenal PR mRNA were observed in the ovariectomised ewes in spite of their high adrenal ER levels. This was consistent with the low oestradiol concentrations in plasma in these ewes: the high adrenal ER content was not coupled to oestrogen activity, and resulted in poor induction of PR mRNA. Castration in the rams seemed to enhance the adrenal levels of PR mRNA, which may indicate a suppressive effect of testosterone on PR concentration in the intact male. We can only speculate on this as we have no data on the ARs in sheep adrenal glands. Nevertheless the physiological relevance of this increase in PR mRNA levels in the castrated ram is not clear.

Another uterine oestrogen activity marker is IGF-I mRNA. In the liver, IGF-I expression is principally regulated by GH, whereas in the uterus oestradiol and progesterone induce IGF-I expression and subsequent uterine growth (Sahlin, Norstedt & Eriksson, 1994; Simmen *et al.*, 1990). Indeed, in our study the uterine weight was highest in ewes in the follicular and luteal phase and lowest in the ovariectomised ewes, and this was reflected in the uterine IGF-I mRNA (**Paper**

IV). Our finding that in the adrenal glands IGF-I mRNA levels did not differ among the groups suggests that IGF-I mRNA is probably not an indicator of oestrogen activity in the adrenal gland and may be regulated mainly by other hormones (e.g. GH). The relative adrenal weight observed in this study was almost identical to that recorded in sheep by Canny *et al.* (1999) and was higher in females than in males. In the adult adrenal gland, IGF-I has a regulatory function and is not involved in growth as it is in the foetal gland (Feige *et al.*, 1998). Therefore other growth factors and possibly oestrogens may be responsible for the observed gender difference in relative weight of these glands.

The differences in adrenal sensitivity to oestrogens according to gender (**Paper IV**) were consistent with the gender differences in adrenal cortisol response to ACTH administration (**Paper III**). In general the ewe would have a greater capacity to respond to oestrogen by increasing cortisol secretion. With our results it is not possible to disclose the exact mechanisms by which oestrogens participate in the regulation of adrenal cortex functions. However, some mechanisms by which oestrogens may possibly affect adrenal steroidogenesis have been suggested. They include 1) influencing adrenocortical sensitivity to ACTH (Atkinson & Waddell, 1997; Lo, Chang & Wang, 2000); 2) increasing availability of the steroid precursor (cholesterol) by affecting the steroidogenic acute regulatory (StAR) protein (Townson *et al.*, 1996; Stocco, 2001); and 3) a stimulatory effect on the induction of enzymes in the synthesis of GCs (Perry & Stalvey, 1992).

How does it all make sense?

On the whole, it is clear that the HPA and HPG axes interact at multiple levels, which makes the physiological interpretation difficult, especially because there are conflicting results in the literature, both between and within species. Furthermore, different stressors result in different responses from the HPA axis (Tilbrook, Turner & Clarke, 2002). Handa *et al.* (1994) suggest that androgen suppression of the HPA axis may have evolved to reduce acute hormonal stress responses and consequently maintain reproductive competence.

Reproduction in the male is essentially different from reproduction in the female in that gamete production is a continuous process once puberty is reached. In the female the gametes are all present at birth and after puberty they are shed cyclicly during ovulation, which is a very vulnerable event. Any alteration of the pre-ovulatory LH surge can compromise conception. In the ram, spermatogenesis rarely ever stops even though sheep are seasonal breeders (Lincoln & Short, 1980). Sperm quality and quantity are affected by season (Amir & Volcani, 1965; Bielli *et al.*, 1997) but the ram retains its fertilising capacity throughout the non-breeding season. This may reflect an energy-saving mechanism as the likelihood of finding cyclic ewes in the non-breeding season is very low. During the breeding season in the rams the androgen suppression of the HPA axis is probably maximum allowing the animal to escape from any detrimental effect of stress on its reproductive status.

It is reasonable to think that each sex has its own strategy for dealing with the effects of stress. In the female the costs of reproduction are extremely high when all the resources spent on gestation, lactation and survival of the offspring are taken into account. Ways to avoid these costs in a hostile or unstable environment would be to prevent conception or induce early embryonic loss. Stress has been consistently reported to interfere with the timing of the pre-ovulatory LH surge in many species, jeopardising conception (Parr, Davis & Tilbrook, 1989; Moberg, 1991; Dobson & Smith, 2000). The male, on the other hand, has to successfully cope with stress during mating, as competition between males is a very important aspect of reproduction in herd animals. Each male competes to have its own genes perpetuated within the population. In this line of thinking one could expect the male strategy to be one of energy conservation during winter or stress without completely shutting down the process in order to be able to breed whenever an opportunity presents itself. These sex differences in reproductive strategies ('opportunistic' v. 'strategic') may offer an explanation as to why the HPA and HPG axes interact differently in each sex (Fig. 7).

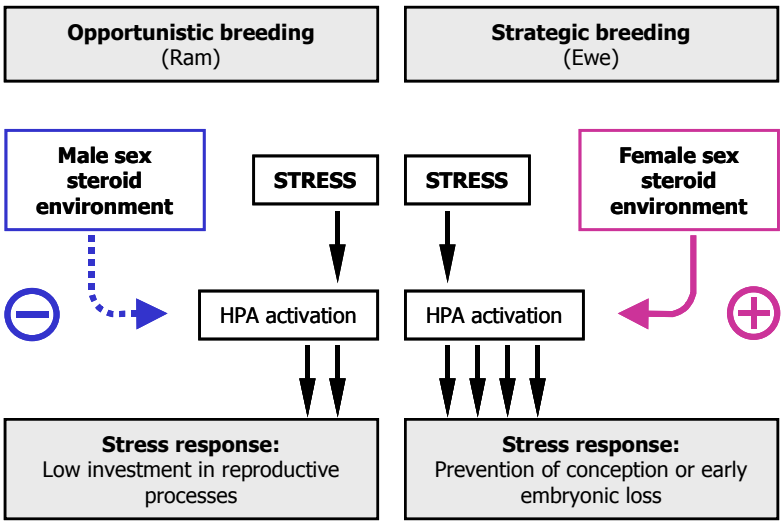


Fig. 7. Model for possible sex-related interactions between stress and reproduction. In the male the prevailing sex steroids dampen the stress response, ensuring sperm availability under extreme circumstances, while in the female the prevailing sex steroids enhance the stress response and thus increase the risk of reproductive failure.

Conclusions

Based on the results of the present studies the following conclusions can be drawn:

- Venipuncture in animals accustomed to handling does not induce stress in terms of cortisol secretion, while restraint does (**Paper I**).
- Testosterone is not among the androgens secreted by sheep adrenal glands upon ACTH stimulation (**Papers I–III**).
- Administration of ACTH induces secretion of progesterone in intact and castrated male and female sheep (**Papers I–III**).
- There is no difference in the preferred pathway for cortisol secretion between ovariectomised ewes and wethers (**Paper I**).
- Ewes secrete more cortisol after ACTH administration than rams do, regardless of season (**Paper III**).
- Gonadectomy of sheep eliminates the gender differences in ACTH-induced cortisol secretion (**Paper III**).
- Season affects ACTH-induced cortisol secretion in intact rams but not in intact ewes (**Paper III**).
- Sex steroids participate in the regulation of steroidogenesis in the adrenal glands (**Papers III** and **IV**).
- Oestrogen receptors are present in the adrenal glands of sheep (**Paper IV**).
- Treatment with ACTH decreases LH pulsatility in recently castrated rams in the winter (**Paper II**).
- Prolonged progesterone treatment in recently castrated rams does not affect either mean LH levels or LH pulsatility (**Paper II**).

Future perspectives

Several questions arose during the course of these studies. Adrenocorticotropin stimulation results in different adrenal responses according to sex, and the sex steroids are supposed to be at least partly responsible for that. Firstly, we must therefore ask how the sex steroids bring about these effects. It would be interesting to try to directly manipulate cortisol secretion in gonadectomised sheep with oestradiol and testosterone and their antagonists to establish whether their effects can be reproduced and whether they are mediated through their receptors.

Also, what is the role of seasonal differences in cortisol secretion in rams and why are they not seen in the ewe? The sensitivity of the adrenal glands to sex steroids throughout the seasons needs to be assessed. And what is the significance, if any, of the low levels of oestradiol in plasma of unstressed gonadectomised sheep? Could the adrenal gland itself provide precursors for oestradiol synthesis? If so, taking this together with higher adrenal ER levels in the ewe, a positive feedback loop may be suspected which would keep the ewe highly sensitive to stress, regardless of season.

It will be interesting to see the progress in adrenal physiology. Besides the interactions of the sex steroids with the adrenal glands, many other substances (interleukins, IGFs, prolactin, GH and other growth factors) interact with them. The integrative role of the adrenal glands in homeostasis is probably far more extensive than is known today.

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