Suppression of the secretion of luteinizing hormone due to isolation/restraint stress in gonadectomised rams and ewes is influenced by sex steroids

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Abstract

In this study we used an isolation/restraint stress to test the hypothesis that stress will affect the secretion of LH differently in gonadectomised rams and ewes treated with different combinations of sex steroids. Romney Marsh sheep were gonadectomised two weeks prior to these experiments. In the first experiment male and female sheep were treated with vehicle or different sex steroids for 7 days prior to the application of the isolation/restraint stress. Male sheep received either i.m. oil (control rams) or 6 mg testosterone propionate injections every 12 h. Female sheep were given empty s.c. implants (control ewes), or 2×1 cm s.c. implants containing oestradiol, or an intravaginal controlled internal drug release device containing $0 \cdot 3$ g progesterone, or the combination of oestradiol and progesterone. There were four animals in each group. On the day of application of the isolation/restraint stress, blood samples were collected every 10 min for 16 h for the subsequent measurement of plasma LH and cortisol concentrations. After 8 h the stress was applied for 4 h. Two weeks later, blood samples were collected for a further 16 h from the control rams and ewes, but on this day no stress was imposed. In the second experiment, separate control gonadectomised rams and ewes ($n=4$/group) were studied for 7 h on 3 consecutive days, when separate treatments were applied. On day 1, the animals received no treatment; on day 2, isolation/restraint stress was applied after 3 h; and on day 3, an i.v. injection of $2 \mu g/kg$ ACTH$_{1-24}$ was given after 3 h. On each day, blood samples were collected every 10 min and the LH response to the i.v. injection of 500 ng GnRH administered after 5 h of sampling was measured. In Experiment 1, the secretion of LH was suppressed during isolation/

restraint in all groups but the parameters of LH secretion (LH pulse frequency and amplitude) that were affected varied between groups. In control rams, LH pulse amplitude, and not frequency, was decreased during isolation/restraint whereas in rams treated with testosterone propionate the stressor reduced pulse frequency and not amplitude. In control ewes, isolation/restraint decreased LH pulse frequency but not amplitude. Isolation/restraint reduced both LH pulse frequency and amplitude in ewes treated with oestradiol, LH pulse frequency in ewes treated with progesterone and only LH pulse amplitude in ewes treated with both oestradiol and progesterone. There was no change in LH secretion during the day of no stress. Plasma concentrations of cortisol were higher during isolation/restraint than on the day of no stress. On the day of isolation/restraint maximal concentrations of cortisol were observed during the application of the stressor but there were no differences between groups in the magnitude of this response. In Experiment 2, isolation/restraint reduced the LH response to GnRH in rams but not ewes and ACTH reduced the LH response to GnRH both in rams and ewes. Our results show that the mechanism(s) by which isolation/restraint stress suppresses LH secretion in sheep is influenced by sex steroids. The predominance of particular sex steroids in the circulation may affect the extent to which stress inhibits the secretion of GnRH from the hypothalamus and/or the responsiveness of the pituitary gland to the actions of GnRH. There are also differences between the sexes in the effects of stress on LH secretion that are independent of the sex steroids.

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Introduction

Stress can have a negative effect on reproduction (for reviews see Moberg 1987, Rivier & Rivest 1991, Dobson

& Smith 1995) but the mechanisms involved are poorly understood. Although different stressful stimuli can activate a number of pathways, the activation of the hypothalamo–pituitary–adrenal axis is one of the most

common responses to stress (Rivier & Rivest 1991, Handa *et al.* 1994). There is evidence in a number of species that there are sex differences in the response of the hypothalamo–pituitary–adrenal axis to a variety of stressors and it has been proposed that these differences are due principally to the influence of the sex steroids (Handa *et al.* 1994, Da Silva 1995, Young 1995, Patchev & Almeida 1998). Although there are sex differences in the function of stress pathways, such as the hypothalamo–pituitary–adrenal axis, it is unknown if there are also differences between the sexes in the extent to which stress may deleteriously affect the reproductive axis and whether the effect of stress on reproduction is influenced by the sex steroids. There have not been, to our knowledge, any comparisons of the effects of a standard stressor on the gonadotrophic axis in males and females of the same species. Furthermore, the effects of sex steroids on the impact of stress on reproduction has never been investigated in males although there is evidence to suggest that ovarian steroids may influence the impact of stress on luteinizing hormone (LH) secretion in females. In female rhesus monkeys that were restrained in a chair for sampling, LH pulses (Dierschke *et al.* 1970) and hypothalamic multiunit electrical activity (Wilson *et al.* 1984) were detected in ovariectomised but not intact animals. Insulin-induced hypoglycaemia inhibited hypothalamic multiunit electrical activity to a greater extent in intact than in ovariectomised animals and this reduced responsiveness in the ovariectomised females was reversed when they were treated with oestradiol (Chen *et al.* 1992). Moreover, when female rhesus monkeys were stressed by 6 h of chair restraint the levels of LH were suppressed in the follicular phase but not in the luteal phase of the menstrual cycle (Norman *et al.* 1994). In ewes, habituation to the effects of confinement on LH secretion varied with the stage of the oestrous cycle (Rawlings & Cook 1991).

To establish if there are sex differences in the effects of stress on reproduction, and to determine if the sex steroids influence these effects, it is important to compare the effects of a stressor that is easily quantified and reproducible, and has been shown to be effective in suppressing reproduction. Psychological stressors, such as isolation, and physical stressors, such as restraint, are potent activators of the hypothalamo–pituitary–adrenal axis in both male and female sheep (for reviews see Moberg 1985, 1987, Dobson & Smith 1995) and have been shown to inhibit the secretion of LH. For instance, confinement of ovariectomised ewes for 3–4 h suppressed the frequency and amplitude of LH pulses (Rasmussen & Malven 1983) and restraint of intact rams for 3 h resulted in a reduced LH response to an injection of gonadotrophin releasing hormone (GnRH) (Matteri *et al.* 1984). To test the hypothesis that sex steroids influence the effects of stress on LH secretion in male and female sheep, we have compared the effect of isolation/restraint stress on the secretion of LH in

gonadectomised male and female sheep treated with different combinations of sex steroids.

Materials and Methods

Animals

Adult Romney Marsh rams and ewes were studied in 2 experiments at the Victorian Institute of Animal Science, Werribee, Australia (38 ° latitude) during the breeding season for this breed (Bremner *et al.* 1984). The animals were from the same lambing, had been reared at the Victorian Institute of Animal Science and were 3 years old at the time of the experiments. Eight rams and sixteen ewes were used in Experiment 1 and five rams and five ewes were used in Experiment 2. Two weeks prior to these experiments, the animals were gonadectomised and housed individually in pens in an animal house. A maintenance ration and water were available *ad libitum* during this time. On the day of treatment and sampling, the animals were fed after the sampling was completed but water was always available. On the day prior to treatment and sampling, each animal was fitted with an indwelling jugular catheter (Dwellcath, Tuta Laboratories, Lane Cove, Australia) for the collection of blood samples.

The care and use of the animals in this experiment conformed with 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.

Experimental procedure

Experiment 1: effect of isolation and restraint on the secretion of LH in gonadectomised rams and ewes treated with combinations of sex steroids The gonadectomised rams and ewes underwent 4 h of isolation and restraint after 7 days of treatment with sex steroids or the appropriate controls. There were 6 groups of 4 animals: (1) castrated rams injected (i.m.) with oil every 12 h for 7 days (control rams), (2) castrated rams injected (i.m.) with 6 mg testosterone propionate (Sigma Chemical Company, St Louis, MO, USA) every 12 h for 7 days, (3) ovariectomised ewes that received 2 empty s.c. implants for 7 days (control ewes), (4) ewes treated for 7 days with two 1 cm s.c. implants containing oestradiol, (5) ewes treated for 7 days with progesterone by means of an intravaginal controlled internal drug release device (Riverina Artificial Breeders Pty Ltd, Albury, NSW, Australia) containing 0·3 g progesterone and (6) ewes treated for 7 days with both oestradiol and progesterone as in treatments 4 and 5.

On the day of sampling and treatment, blood samples (5 ml) were collected every 10 min for 16 h. The stress treatment of isolation/restraint was then imposed on each

animal for 4 h between 8–12 h of sampling. The samples were assayed for LH and cortisol.

To ensure that there were no weight related effects on the volume of blood collected that may affect the results, the liveweights of the animals were measured and the packed cell volume (%) was estimated on blood samples at regular intervals throughout the sampling period. There were no significant differences between the mean (± S.E.M.) liveweights of the control rams (50·3 ± 2·1 kg), castrated rams treated with testosterone propionate (53·8 ± 3·4 kg), control ewes (48·6 ± 2·8 kg), or ovariectomised ewes treated with oestradiol (53·4 ± 1·0 kg), progesterone (49·0 ± 3·1 kg) or oestradiol and progesterone (48·3 ± 2·1 kg). There was a significant ($P<0.05$) effect of time on packed cell volume but there was no consistent trend to this effect and there was no time × group interaction. Importantly, there were no differences between groups in packed cell volume.

At the time of isolation/restraint, each animal was removed from the pen (1·38 m long × 0·42 m wide × 0·9 m high) in which it had been for 7 days and was placed for the remainder of the sampling period in a novel pen of the same size that was completely enclosed on all sides and the top by hessian (RN & G Lowin, Fitzroy, Australia) so that visual contact with other animals was not possible. Furthermore, each animal was fitted with a harness (Radford *et al.* 1960) that was used to restrain the animal to the side of the pen. The movement of the animal was restricted so that only the head could be moved. The animals were able to drink water freely during this time. Each pen in which the isolation/restraint treatment was imposed was separated by a pen of identical dimensions that did not contain an animal. After 4 h, the hessian was removed from the pen and the harness was removed so that the animal was able to move freely within the pen and to see other animals in the animal house.

Two weeks after the isolation/restraint treatment, blood samples (5 ml) were collected every 10 min for 16 h (no stress) from the 4 control rams (group 1 above) and the 4 control ewes (group 3 above). These samples were also assayed for LH and cortisol.

Experiment 2: effect of isolation and restraint and adrenocorticotrophin (ACTH) on the release of LH in response to gonadotrophin–releasing hormone (GnRH) in gonadectomised rams and ewes The release of LH following an i.v. injection of 500 ng GnRH (Auspep, South Melbourne, Australia) was determined in gonadectomised rams ($n=5$) and ewes ($n=5$) on a day when no stress was imposed, during isolation/restraint stress and following an i.v. injection of 2 µg/kg ACTH (Synacthen, Ciba-Geigy Australia Ltd, Pendle Hill, Australia). On the first day (no stress), blood samples (5 ml) were collected every 10 min for 5 h and GnRH was injected. Samples were then taken every 5 min for 30 min then every 10 min for a further 90 min (i.e. up to 2 h after

injection of GnRH). On days 2 and 3 an identical sampling and GnRH treatment regimen was used. After 3 h of sampling on day 2, isolation/restraint stress was imposed on each animal for 4 h as described for Experiment 1. Thus, on this day, the injection of GnRH was given after 2 h of isolation/restraint. On day 3, ACTH was given after 3 h of sampling, 2 h before the GnRH injection. The samples were assayed for LH.

Radioimmunoassays

The plasma concentrations of LH were measured in a radioimmunoassay as described by Lee *et al.* (1976) using NIH LH S18 as the standard. The mean (± S.E.M.) sensitivity of the 15 LH assays conducted in Experiment 1 was 0·18 ± 0·03 ng/ml with a range in sensitivities from 0·1 to 0·4 ng/ml. The values of LH were always above the sensitivity of the particular assay used to measure LH in each animal. For these assays, the intra–assay coefficients of variation were 3·9% at 2·3 ng/ml and 7·3% at 3·6 ng/ml and the interassay coefficients of variation were 9·7% and 12·3% respectively at these concentrations. In Experiment 2, 7 assays were conducted with a mean (± S.E.M.) sensitivity of 0·17 ± 0·05 ng/ml, intra–assay coefficients of variation of 7·8% at 2·3 ng/ml and 10·2% at 4·3 ng/ml and interassay coefficients of variation of 8·2% at 1·9 ng/ml and 11·1% at 4·2 ng/ml.

The plasma concentrations of cortisol were measured in Experiment 1 by radioimmunoassay after extraction with dichloromethane using the method described by Bocking *et al.* (1986). An aliquot of 200 µl was taken from each sample within each hour of the experiment and was pooled. An aliquot of 100 µl of each of these pools was assayed for cortisol. Furthermore, each sample during the first hour of the isolation/restraint treatment, and the corresponding hour from the control sampling, was assayed for cortisol for each animal. A total of 8 assays was conducted with a mean (± S.E.M.) sensitivity of 0·08 ± 0·02 ng/ml and a range in sensitivities of 0·06 to 1·0 ng/ml. The values of cortisol were always above the sensitivity of the particular assay used to measure cortisol in each animal. The intra-assay coefficients of variation were 8·2% at 29 ng/ml and 14·0% at 68 ng/ml and the interassay coefficients of variation were 15·8% and 14·2% respectively at these concentrations.

Statistical analyses

In Experiment 1, a factorial repeated measures analysis of variance was used to analyse the plasma concentrations of LH, the number of pulses of LH/h and the amplitude of LH pulses. The factors were groups (gonadectomised rams and ewes given different steroid treatments) and the repeat variables were the periods before, during and after the isolation/restraint treatment. Comparisons were also made between groups. To assess if the effects of isolation/restraint on the secretion of LH varied between groups,

each of the parameters of LH secretion during the period of isolation/restraint were expressed as a ratio of pre-treatment values and compared using analysis of variance. The frequency and amplitude of LH were defined according to Karsch *et al.* (1987). The same comparisons were made for the control rams and ewes for the corresponding periods on the day when no stress was imposed. Each of the parameters of LH secretion was also compared between the day of isolation/restraint and the day when no stress was imposed for the control rams and ewes.

Repeated measures analysis of variance was also used to analyse the plasma concentrations of cortisol in Experiment 1. Furthermore, the plasma concentrations of cortisol during the first hour of isolation/restraint treatment were analysed within groups and comparisons were also made between groups. The same comparisons were made for control rams and ewes for the corresponding periods of sampling on the day when no stress was imposed. For control rams and ewes, the plasma concentrations of cortisol before, during and after isolation/restraint, for each hour on the day of isolation/restraint treatment, and during the first hour of isolation/restraint were also compared with the corresponding periods of sampling on the day of no stress.

For each parameter of LH and cortisol in these analyses, homogeneity of variance was checked and transformation of data was not necessary.

Analysis of variance was used to compare the live-weights of rams and ewes in each group in Experiment 1 and the packed cell volume of blood samples collected from animals in each group in Experiment 1. These data did not require transformation.

In Experiment 2, the area (ng/ml/h) under the LH response curve and the peak plasma concentrations (ng/ml) of LH following an injection of GnRH were compared between the days of no stress, isolation/restraint and treatment with ACTH using repeated measures analysis of variance. The between subject factor was sex (male or female) and the repeat variable was day (no stress, isolation/restraint stress and ACTH treatment). The peak concentrations of LH were determined by subtracting the basal concentrations of LH over the 2 h before injection of GnRH from the highest concentration of LH following injection of GnRH. Homogeneity of variance was checked and, for the area under the LH curve, data required log transformation.

For both experiments, paired comparisons were made, where appropriate, using least significant differences.

Results

Experiment 1: plasma LH

Analysis of the plasma concentrations of LH showed that, within animals, there were significant ($P<0.01$) effects of stage and a significant ($P<0.01$) stage × group interaction

and there was a significant ($P<0.01$) between groups effect. Similarly, for the number of LH pulses/h, there was a significant ($P<0.01$) effect of stage, a significant ($P<0.05$) stage × group interaction and a significant ($P<0.01$) between groups effect. For the amplitude of LH pulses, there was a significant ($P<0.01$) effect of stage, no stage × group interaction and a significant ($P<0.01$) effect of group.

Isolation/restraint stress reduced the secretion of LH in all animals. In contrast, for the control rams and control ewes there were no changes in the plasma concentrations of LH, number of LH pulses/h or the amplitude of LH pulses during sampling on the day when no stress was imposed (Fig. 1).

As expected, treatment with sex steroids reduced the secretion of LH. There were differences between groups of animals in the plasma concentrations of LH, number of LH pulses/h and the amplitude of LH pulses due to the different steroid treatments. Nonetheless, there were no differences between groups in any of the parameters of LH during the period of isolation/restraint expressed as a ratio of the pre-treatment values, indicating that the relative differences between groups did not change when the isolation/restraint treatment was imposed.

Effect of isolation/restraint on LH in rams

Mean plasma concentrations of LH In control rams the plasma concentrations of LH were significantly lower during ($P<0.01$) and after ($P<0.05$) the period of isolation/restraint than before imposition of the stressor (Table 1). Furthermore, the concentrations during isolation/restraint were lower ($P<0.05$) than after treatment. In rams treated with testosterone propionate the concentrations of LH did not change significantly across the experiment (Table 1). Nevertheless, due to negative feedback by testosterone, these concentrations were extremely low and were significantly ($P<0.01$) less than the concentrations in control rams, making it difficult to demonstrate changes due to the isolation/restraint treatment.

Number of LH pulses/h There was no effect of isolation/restraint on the number of LH pulses/h in control rams. In rams treated with testosterone propionate, however, the number of LH pulses/h was significantly ($P<0.05$) fewer during the isolation/restraint period than before or after treatment but there were no differences between the pre- and post-treatment periods (Table 1).

Amplitude of LH pulses The mean amplitude of LH pulses was significantly ($P<0.05$) reduced during the period of isolation/restraint in control rams but was not affected in rams treated with testosterone propionate (Table 1).

Effect of isolation/restraint on LH in ewes

Mean plasma concentrations of LH In control ewes, the plasma concentrations of LH were significantly ($P<0.01$)
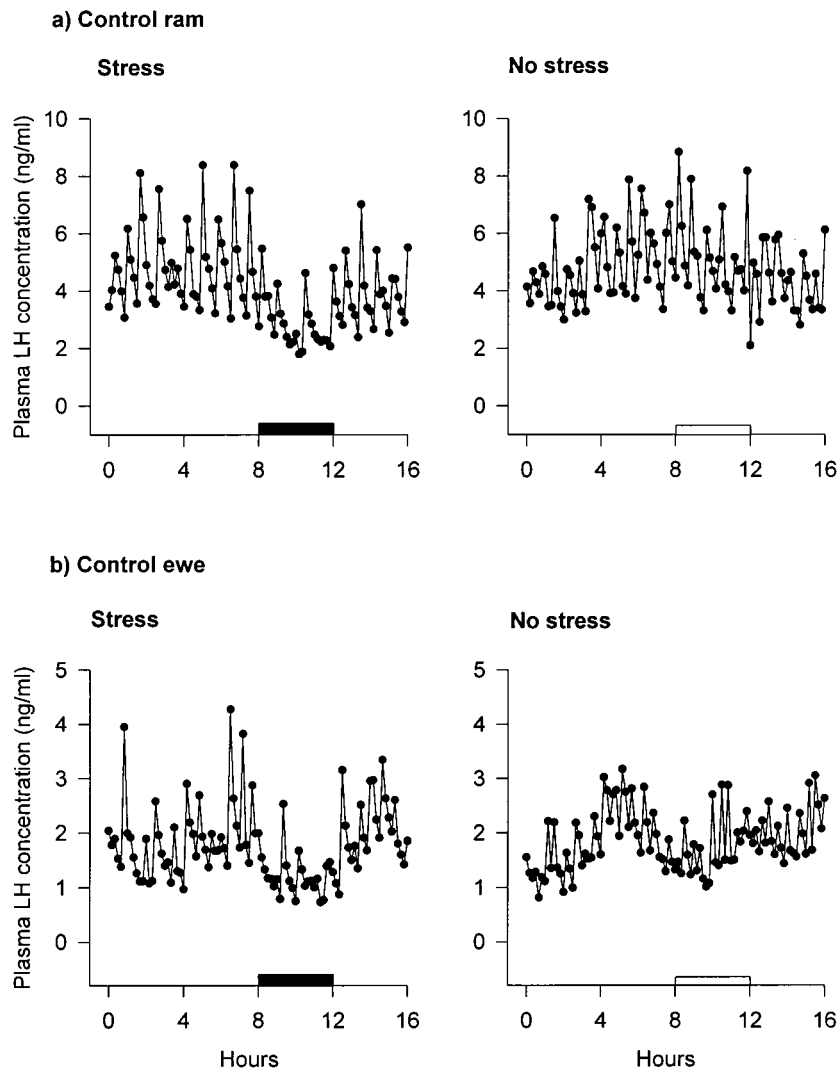
Figure 1 Profiles of plasma LH (ng/ml) from a representative control ram (a) and a control ewe (b) over the 16 h of sampling on the day of isolation/restraint (Stress) and on the day when no stress was imposed (No stress) in Experiment 1. The 4 h of isolation/restraint stress are indicated by the closed bar and the open bar coincides with the corresponding period of sampling on the day of no stress.

lower during the period of isolation/restraint than before treatment but the concentrations after treatment were not different from the pre-treatment concentrations (Table 1). The effects of isolation/restraint on LH concentrations in ewes treated with oestradiol were similar to those seen in control ewes, being lower ($P<0.05$) during treatment than before or after treatment (Table 1). The plasma concentrations of LH in ewes treated with progesterone were significantly ($P<0.05$) reduced during isolation/restraint and remained low for the remainder of the sampling time. In ewes treated with the combination of oestradiol and progesterone the concentrations of LH did not change significantly across the experiment (Table 1) but, as in

castrated rams treated with testosterone propionate, these values were low due to the negative feedback effects of the steroids and this may have masked the effects of stress on this parameter.

Number of LH pulses/h In control ewes, there were significantly ($P<0.05$) fewer LH pulses/h during the period of isolation/restraint than before or after treatment and there were less ($P<0.05$) LH pulses during the post-treatment period than the pre-treatment period. There was no effect of isolation/restraint on the number of LH pulses/h in ewes treated with oestradiol and progesterone (Table 1). In ewes treated with oestradiol

Table 1 Mean ($\pm$ S.E.M.) plasma concentration of LH (ng/ml), number of LH pulses per hour and amplitude of LH pulses (ng/ml) in gonadectomised rams and ewes treated with combinations of sex steroids before, during and after isolation/restraint stress. All animals were sampled every 10 min for 8 h before the isolation/restraint stress was imposed (Pre-treatment), throughout the 4 h of isolation/restraint (During treatment) and for 4 h after the treatment (Post-treatment). Rams received i.m. injections of oil (Control rams) or 6 mg testosterone propionate/12 h for 7 days (TP) while ewes received an empty s.c. implant for 7 days (Control ewes), 2 × 1 cm implants containing oestradiol for 7 days (E), an intravaginal controlled internal drug release device containing 0·3 g progesterone for 7 days (P) or both oestradiol and progesterone (E+P)

	LH concentration (ng/ml)			LH pulses per hour			LH pulse amplitude (ng/ml)		
	Pre-treatment	During treatment	Post-treatment	Pre-treatment	During treatment	Post-treatment	Pre-treatment	During treatment	Post-treatment
Stress treatment groups									
Control rams	3·6 ± 0·6[a]	2·0 ± 0·4[b]	3·1 ± 0·3[c]	1·1 ± 0·0	1·1 ± 0·2	1·4 ± 0·1	2·7 ± 0·8[a]	1·3 ± 0·4[b]	2·1 ± 0·3[a]
TP	0·2 ± 0·0	0·2 ± 0·0	0·2 ± 0·0	0·8 ± 0·2[a]	0·2 ± 0·1[b]	0·4 ± 0·1[b]	0·1 ± 0·0	0·1 ± 0·0	0·1 ± 0·0
Control ewes	2·3 ± 0·4[a]	1·6 ± 0·2[b]	2·7 ± 0·2[a]	1·9 ± 0·3[a]	1·0 ± 0·2[b]	1·4 ± 0·2[c]	1·9 ± 0·9	1·3 ± 0·2	2·0 ± 0·5
E	1·7 ± 0·4[a]	0·9 ± 0·2[b]	1·6 ± 0·3[a]	2·5 ± 0·3[a]	1·6 ± 0·2[b]	1·6 ± 0·1[b]	0·9 ± 0·1[a]	0·4 ± 0·1[b]	0·8 ± 0·1[a]
P	2·9 ± 0·9[a]	1·4 ± 0·4[b]	1·2 ± 0·4[b]	0·7 ± 0·1[a]	0·3 ± 0·1[b]	0·3 ± 0·1[b]	5·2 ± 1·9	3·1 ± 0·8	3·5 ± 0·8
E+P	0·3 ± 0·0	0·2 ± 0·0	0·2 ± 0·0	0·4 ± 0·1	0·2 ± 0·1	0·4 ± 0·2	0·3 ± 0·1[a]	0·1 ± 0·1[b]	0·2 ± 0·1[b]

For each parameter, different superscripts within rows (i.e. between pre-treatment, during treatment and post-treatment) represent significant differences between means [a,b,c]$P<0·05$.

or progesterone the number of LH pulses/h was also significantly less during the isolation/restraint period than before or after treatment but there were no differences between the pre- and post-treatment periods (Table 1).

Amplitude of LH pulses Isolation/restraint did not affect the amplitude of LH pulses in control ewes or ewes treated with progesterone (Table 1). In contrast, the mean amplitude of LH pulses was significantly ($P<0·05$) reduced during the period of isolation/restraint in ewes treated with oestradiol and ewes treated with both oestradiol and progesterone (Table 1). In ewes treated with oestradiol and progesterone the LH pulse amplitude remained depressed for the remainder of the experiment (Table 1).

Experiment 1: plasma cortisol

For plasma concentrations of cortisol, there was a significant ($P<0·01$) effect of stage and a significant ($P<0·05$) stage × group interaction within animals. There was also a significant ($P<0·01$) between group effect.

In control rams and control ewes the plasma concentrations of cortisol during the period of isolation/restraint were significantly ($P<0·05$) higher than during the corresponding period on the day when there was no stress (Table 2). In control rams, the plasma concentrations of cortisol during the first 3 h of sampling on the day of no stress were significantly ($P<0·05$) higher than the concentrations for this period on the day of isolation/restraint. Thereafter, the concentrations of cortisol did not differ significantly between the sampling days until the isolation/ restraint treatment was imposed, when the concentrations of cortisol were higher ($P<0·05$) than during the day of no stress (Fig. 2). In control ewes, the plasma concentrations

of cortisol were significantly ($P<0·05$) higher throughout the day that isolation/restraint was imposed than on the day of no stress (Fig. 2). There were no differences between rams and ewes in the plasma concentrations of cortisol at any stage of the experiment.

There were no statistically significant differences in plasma concentrations of cortisol between groups on the day of isolation/restraint. In all groups the mean plasma concentrations of cortisol increased significantly ($P<0·05$) during the first 3–4 h of sampling (Fig. 2). Maximal concentrations of cortisol occurred during the first hour of isolation/restraint (data not shown). In control rams and ewes the plasma concentrations of cortisol did not change significantly over the first hour of isolation/restraint or during the corresponding hour of sampling on the day of no stress but the concentrations of cortisol were significantly ($P<0·01$) higher during isolation/restraint than during the day of no stress. Similarly to control rams and ewes, the plasma concentrations of cortisol did not vary significantly across the first hour of isolation/restraint in the other treatment groups and there were no differences

Table 2 Mean ($\pm$ S.E.M.) plasma concentrations of cortisol (ng/ml) in gonadectomised rams treated with i.m. injections of oil/12 h for 7 days (Control rams) and ewes treated with an empty s.c. implant for 7 days (Control ewes) during the 4 h of isolation/restraint (Stress) and the corresponding period of sampling when no stress was imposed (No stress)

	No stress	Stress
Control rams	10·9 ± 2·5[a]	28·8 ± 3·5[b]
Control ewes	7·9 ± 1·6[a]	22·3 ± 3·3[b]

Within rows, different superscripts represent significant ($P<0·05$) differences between means.
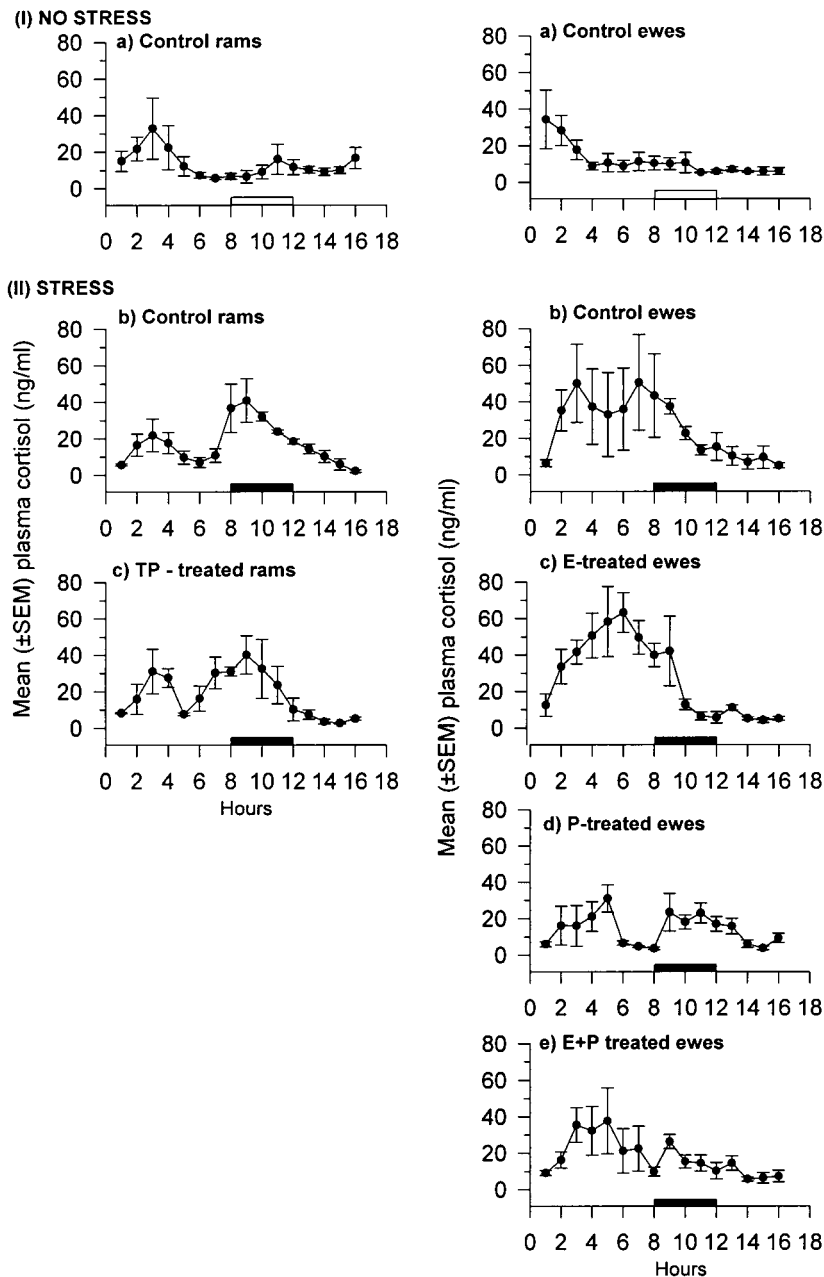
Figure 2 Mean (± S.E.M.) plasma concentrations of cortisol (ng/ml) in control rams and control ewes during (I) the day of no stress and (II) the day of isolation/restraint (stress) in Experiment 1. The mean (± S.E.M.) plasma concentrations of cortisol are also shown for rams treated with testosterone propionate (TP-treated rams), ewes treated with oestradiol (E-treated ewes), ewes treated with progesterone (P-treated ewes) and ewes treated with oestradiol and progesterone (E+P-treated ewes) for the day that isolation/restraint (stress) was imposed in Experiment 1. The closed bar represents the 4 h of isolation/restraint (stress) and the open bar the corresponding period during the day of no stress.

Table 3 Mean (± S.E.M.) plasma concentrations of cortisol in gonadectomised rams and ewes treated with different combinations of sex steroids during the first hour of isolation/restraint (stress) and the corresponding hour during the day when no stress was imposed (No stress). Rams received i.m. injections of oil (Controls) or 6 mg testosterone propionate/12 h for 7 days (TP) while ewes received an empty s.c. implant for 7 days (Control), 2 × 1 cm implants containing oestradiol for 7 days (E), an intravaginal controlled internal drug release device containing 0·3 g progesterone for 7 days (P) or both oestradiol and progesterone (E+P). Only Control rams and ewes were sampled on both the day of isolation/restraint stess and the day of no stess

	No stress	Stress
Rams		
Control	$9·5 \pm 2·1^a$	$47·0 \pm 5·4^b$
TP	—	$32·2 \pm 5·0^b$
Ewes		
Control	$8·8 \pm 1·3^a$	$40·6 \pm 3·8^b$
E	—	$31·6 \pm 3·3^b$
P	—	$37·3 \pm 4·1^b$
E+P	—	$32·5 \pm 2·5^b$

Within rows, different superscripts indicate significant differences between means [a,b]$P<0·05$.

between any groups in these concentrations (Table 3). After the application of the isolation/restraint stress, plasma concentrations of cortisol fell, and the lowest concentrations of cortisol were observed in the 4 h following the treatment (Fig. 2).

Experiment 2

For both the area under the LH curve and the peak concentrations of LH achieved following injection of GnRH, the analysis showed that, within animal, there was a significant ($P<0·01$) effect of day but no interaction between day and sex. There were no significant between sex effects.

Rams The release of LH in gonadectomised rams following an injection of GnRH was reduced ($P<0·05$) during isolation/restraint and after treatment with ACTH (Fig. 3). The area under the LH response curve (Fig. 4) and peak concentrations of LH achieved after injection of GnRH (Fig. 4) were significantly lower ($P<0·05$) after 2 h isolation/restraint and 2 h after injection of ACTH than during no stress. The area under the LH curve after injection of ACTH was significantly ($P<0·05$) lower after injection of ACTH than during isolation/restraint but the peak concentrations of LH achieved did not differ between the period of isolation/restraint and after injection of ACTH (Fig. 4).

Ewes In ovariectomised ewes, the release of LH after injection of GnRH was affected only after treatment with ACTH (Fig. 3). The area under the LH curve following

injection of GnRH did not differ significantly between the no stress and isolation/restraint but was lower ($P<0·05$) 2 h after injection of ACTH than during the no stress or isolation/restraint periods (Fig. 4). There were no differences between any stages of the experiment in the peak plasma concentrations of LH achieved following injection of GnRH (Fig. 4).

Discussion

The results of Experiment 1 show that the secretion of LH in sheep was suppressed by 4 h of isolation and restraint and suggest that the mechanism by which this stress treatment inhibited the secretion of LH may be influenced by sex steroids. Furthermore, our results indicate that there is a sex difference, at least with gonadectomised sheep, in the way that isolation/restraint stress inhibits the secretion of LH. Although the secretion of LH was suppressed in all groups of gonadectomised sheep by the isolation/restraint treatment, the parameters of LH secretion that were affected differed between gonadectomised rams and ewes and between sheep given different treatments of sex steroids. Specifically, the effect of this stressor on the frequency and the amplitude of LH pulses differed between groups. Decreases in the frequency of LH pulses during isolation/restraint indicate that the activity of GnRH neurones in the hypothalamus was inhibited by the stressor because, both in rams (Caraty & Locatelli 1988, Jackson *et al.* 1991, Tilbrook *et al.* 1991) and ewes (Clarke & Cummins 1982, Clarke *et al.* 1987), the secretion of GnRH is pulsatile and there is a high degree of concordance between pulses of GnRH and LH. Changes in the amplitude of LH pulses, however, could indicate actions of stress on the hypothalamus, to decrease the amplitude of GnRH pulses, and/or on the pituitary gland to reduce the responsiveness of the pituitary to the actions of GnRH. Experiment 2 focussed on the responsiveness of the pituitary gland to GnRH and showed that there were differences between gonadectomised rams and ewes in the effects of isolation/restraint on the LH response to GnRH. Therefore, our results indicate that androgens in male sheep and oestradiol and progesterone in female sheep, as well as the sex of the gonadectomised sheep, influenced the extent to which the stress of isolation/restraint inhibited the activity of GnRH neurones in the hypothalamus and/or the actions of GnRH on the pituitary gland to stimulate the release of LH.

It appears that androgens mediate the effects of isolation/restraint stress on the hypothalamic synthesis and/or secretion of GnRH in rams since, in Experiment 1, the stressor decreased the frequency but not the amplitude of LH pulses when castrated rams were treated with testosterone propionate. In contrast, in the absence of testosterone the stressor caused an inhibition of the
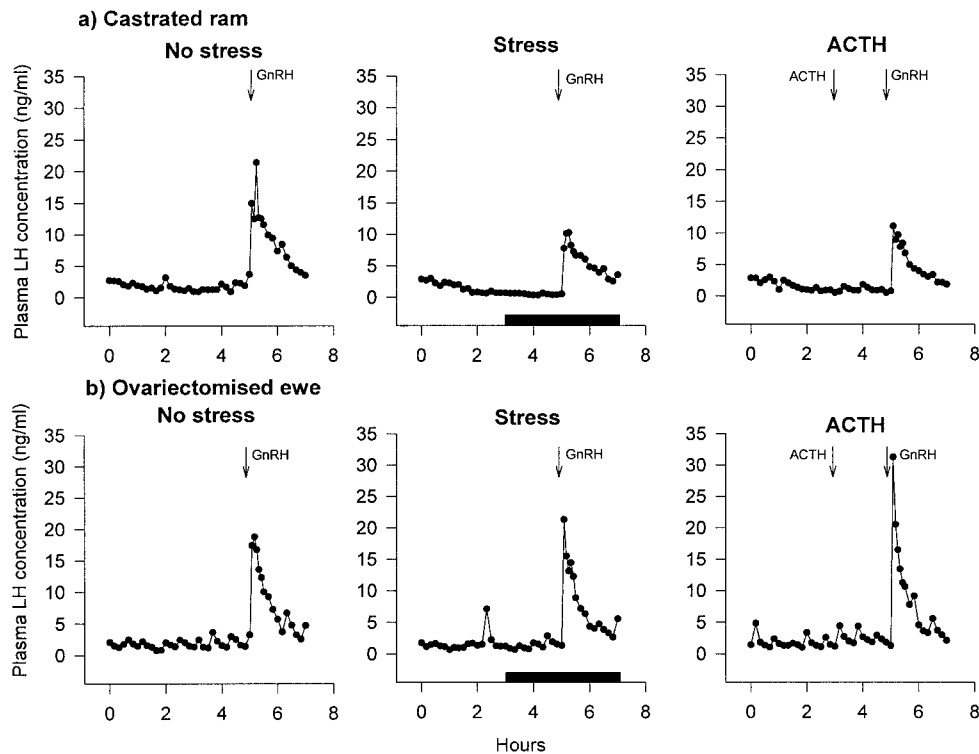
Figure 3 Plasma concentrations of LH (ng/ml) in a representative castrated ram (a) and an ovariectomised ewe (b) over the 8 h of sampling in Experiment 2 for the day when no stress was imposed (No stress), the day of treatment with isolation/restraint (Stress) and the day of injection with ACTH (ACTH). The bar indicates the 4 h of isolation/restraint. Injections of GnRH and ACTH are indicated by arrows.

pituitary to respond to the actions of GnRH. In the control rams in Experiment 1, the amplitude but not the frequency of LH pulses was decreased by isolation/ restraint and, in Experiment 2, isolation/restraint and treatment with ACTH reduced the LH response to GnRH in castrated rams. Although there have been reports of a reduced LH response to exogenous GnRH in rams following 3 h of restraint stress (Matteri *et al.* 1984) or treatment with ACTH (Fuquay & Moberg 1983, Matteri *et al.* 1984) these studies differed from ours in that intact rams of mixed breeds and ages were used and the stress treatment did not involve isolation. Moreover, it is not possible to determine from the experimental protocols used in these studies if the secretion and/or release of GnRH was also affected. In intact male rhesus monkeys that were stressed by immobilisation it was found that there was no difference between controls and immobilised animals in the LH response to an injection of GnRH (Goncharov *et al.* 1984) which suggests that the responsiveness of the pituitary to GnRH was not affected by the stressor. This may be because endogenous androgens mediated the effects of stress at the hypothalamus, similar to our findings with rams, although comparisons with castrated male monkeys were not made. Chair restraint of male rhesus monkeys for 6 h suppressed levels of LH

(Norman & Smith 1992) but the authors did not present a pulse analysis to establish which parameters of LH secretion were affected by the stressor. Our results clearly indicate that, in Romney Marsh rams, androgens influence the extent to which isolation/restraint stress invokes mechanisms that act on the hypothalamus or pituitary to inhibit the secretion of LH.

In ovariectomised ewes not treated with steroids the frequency but not the amplitude of LH pulses was inhibited by the isolation/restraint stress in Experiment 1 suggesting that the predominant effects of this stress in these animals was at the level of the GnRH neurones. It is unlikely that isolation/restraint stress inhibits the ability of the pituitary to respond to GnRH in ovariectomised ewes because there was no effect of isolation/restraint on the LH response to GnRH in Experiment 2. Rasmussen and Malven (1983) found a decrease in the frequency of LH pulses in ovariectomised ewes that were confined for 4–5 h but they also reported a decrease in the amplitude of LH pulses. The reason for this discrepancy between studies is not known but our isolation/restraint treatment differed from, and was probably more severe than, the confinement treatment of Rasmussen & Malven (1983) in which the movement of the ewe was only moderately restricted. Also, different breeds of sheep were used in each study and
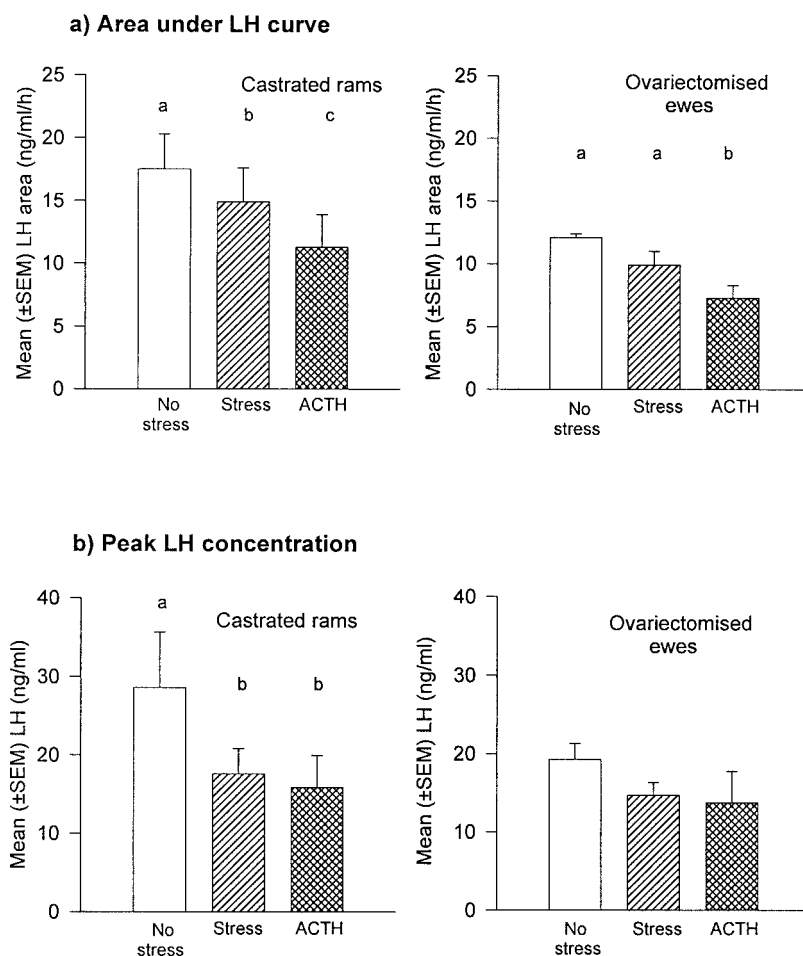
a) Area under LH curve

b) Peak LH concentration

Figure 4 Mean (± S.E.M.) area under the LH response curve (ng/ml/h) (a) and peak plasma concentrations of LH (ng/ml) (b) in castrated rams and ovariectomised ewes after an injection of GnRH for the day when no stress was imposed (No stress), the day of isolation/restraint (Stress) and the day of injection with ACTH (ACTH) in Experiment 2. Within a panel, significant differences between means are indicated by different superscripts, [a,b,c]P<0·05.

the sheep may have been ovariectomised for different periods before the stress treatments were imposed. In ewes, oestradiol is clearly able to influence the actions of stress on the activity of GnRH neurones and may also play a role in affecting the pituitary responsiveness to the actions of GnRH because, in gonadectomised ewes treated with oestradiol, isolation/restraint stress reduced both the frequency and amplitude of LH pulses. Progesterone, on the other hand, appears to mediate the effects of stress on LH secretion through actions directly on the brain since only the frequency, and not the amplitude, of LH pulses was reduced during stress. The combined treatment with oestradiol and progesterone resulted in a reduction in LH pulse amplitude but not frequency during isolation/restraint stress. Nonetheless, the frequency of LH pulses was low in these animals due to the negative feedback

actions of the steroid treatment and the mean number of pulses of LH per hour were halved in these ewes when the isolation/restraint treatment was applied, even though the result was not statistically significant. The decreases in LH pulse amplitude in the ovariectomised ewes treated with oestradiol or oestradiol and progesterone in Experiment 1 may represent changes in the responsiveness of the pituitary to GnRH although it has been reported that isolation and restraint did not affect the LH response to GnRH in oestradiol-treated anoestrous ewes (Dobson & Smith 1995). It is possible that these decreases in LH pulse amplitude during isolation/restraint may have been due to hypothalamic effects of stress because it has recently been found that stress in ewes can have different effects on the pattern of secretion of GnRH and LH. The systemic immune challenge of ovariectomised ewes with endotoxin

resulted in suppression of the amplitude of GnRH pulses with no significant effect on GnRH pulse frequency while both the amplitude and frequency of LH pulses were inhibited (Battaglia *et al.* 1997). There is clearly a need for a systematic approach to determine the extent to which a particular sex-steroid milieu influences the effects of isolation/restraint stress on the brain to inhibit the synthesis and secretion of GnRH, and on the pituitary gland to suppress the LH-releasing actions of GnRH.

Although sex steroids clearly influenced the effect of isolation/restraint stress on the frequency and amplitude of LH pulses in Experiment 1, there was a sex difference in the impact of stress on LH secretion that was independent of the actions of the sex steroids. In the gonadectomised rams that were not treated with steroids (control rams) there was a decrease in the amplitude but not the frequency of LH pulses during stress, whereas in the control ewes, the frequency and not the amplitude of LH pulses was inhibited. Further, in Experiment 2 it was shown that isolation/restraint stress decreased the pituitary responsiveness to GnRH in gonadectomised rams but not in gonadectomised ewes. The reasons for these sex differences are unknown. Collectively, the results of this study suggest that stress will inhibit the secretion of LH by different mechanisms in males and females and these mechanisms involve both steroid-dependent and steroid-independent components.

The plasma concentrations of cortisol were significantly increased in all groups during the isolation/restraint treat-ment in Experiment 1 indicating that this treatment stimulated the hypothalamo–pituitary–adrenal axis. This finding is in keeping with other studies that have shown that isolation and restraint treatments stimulate the hypothalamo–pituitary–adrenal axis in sheep (see Intro-duction). In this experiment, however, there were no differences between gonadectomised rams and ewes, or between gonadectomised sheep given different sex-steroid treatments, in either the basal or stress-induced concen-trations of cortisol. Although there is some evidence that sex and gonadal factors may play a role in modulating the response of the hypothalamo–pituitary–adrenal axis to a variety of stimuli in sheep *in vivo* (Cook 1997, 1998) and *in vitro* (Canny *et al.* 1999), the current study specifically compared the effects of physiologically relevant concen-trations of androgens, oestrogens and progesterone on the activity of the hypothalamo–pituitary–adrenal axis in gonadectomised sheep. The lack of a sex difference and the lack of effect of the sex steroids on the activity of the hypothalamo–pituitary–adrenal axis in gonadectomised sheep differs from observations in rats where there are clear sex differences and where it has been proposed that oestrogens enhance while androgens depress the activity of the hypothalamo–pituitary–adrenal axis (see Handa *et al.* 1994, Da Silva 1995, Young 1995, Patchev & Almeida 1998 for reviews). The discrepancies between our findings

and those reported for rats probably represent differences between species and/or different stressors. Indeed, Dobson and Smith (1995) drew attention to the importance of species differences when studying stress and reproduction, particularly when comparing findings in the rat to those in domestic species. Furthermore, it has been observed that sex differences in the function of the hypothalamo–pituitary–adrenal axis are severely reduced or absent when the gonads have been removed. For example, in rhesus monkeys, ovariectomy of females (Smith & Norman 1987*b*) and castration of males (Smith & Norman 1987*a*) reduced the circadian patterns of cortisol secretion and the amplitude changes in cortisol concentrations were similar in males and females. Our study was conducted with gonadectomised sheep and there is a need to undertake a study in which both the basal and stress-induced function of the hypothalamo–pituitary–adrenal axis is compared in intact male and female sheep. If sex differences exist, our data suggest that these are likely to be due to factors other than the sex steroids.

The plasma concentrations of cortisol increased at the beginning of the sampling period both on the day that no stress was imposed and the day of isolation/restraint treatment in Experiment 1. Whereas the animals in the current study were normally fed in the morning, on the days of experimentation food was not offered until the night when sampling had been completed. Furthermore, on these days, there were more people in close proximity to the animals than on non-experimentation days. Both of these factors may have contributed to the acute stimulation of the hypothalamo–pituitary–adrenal axis. There is evi-dence in sheep that the changes in plasma cortisol con-centrations throughout the day can be influenced by the presentation of food or the expectation that food will be presented. For example, primary or secondary peaks in plasma cortisol have been observed in sheep at, or just prior to, the time of feeding (Keller-Wood *et al.* 1988). More-over, there was a diurnal rhythm in plasma concentrations of cortisol in pregnant ewes fed once daily, with the levels being highest just before food presentation, whereas there was no such rhythm in ewes that were fed at frequent intervals throughout the day (Simonetta *et al.* 1991). Furthermore, peaks in the plasma concentrations of cortisol have been observed in sheep in the morning when human activity may have been increased (McNatty *et al.* 1972). Irrespective of the reason for the initial increase in cortisol secretion on the days of sampling in Experiment 1, the concentrations decreased and remained low during the day of no stress. On the day of isolation/restraint, however, cortisol secretion tended to remain elevated prior to the application of the stressor, and in all groups the lowest concentrations of cortisol were observed after removal of the isolation/restraint stress.

The extent to which cortisol may have been responsible for the suppression in the secretion of LH in Experiment 1 cannot be established from the current data. The secretion

of LH was reduced only when the isolation/restraint treatment was imposed and was not affected when plasma concentrations of cortisol were high early in the sampling period. In addition, cortisol concentrations did not remain elevated during the period of isolation/restraint when LH secretion was invariably suppressed. This suggests that either the pattern of secretion of cortisol was not adequate to inhibit the secretion of LH during this period, or cortisol does not have major effects on suppressing the secretion of LH in gonadectomised sheep. Indeed, there is no convincing evidence that cortisol plays a major role in the stress-induced suppression of LH secretion in sheep. While infusion of cortisol for 19 h into ovariectomised ewes reduced tonic LH secretion and the LH response to GnRH in one study (Porter *et al.* 1990), another study showed that treatment of ovariectomised ewes with dexamethasone had no effect on LH secretion with or without overlying oestrogen treatment in either the breeding or non-breeding seasons and had no effect on the LH response to GnRH (Phillips & Clarke 1990). Neither cortisol nor dexamethasone treatments affected an oestradiol-induced LH surge in ewes (Moberg *et al.* 1981, Phillips & Clarke 1990) and treatment of intact or adrenalectomised rams with cortisol did not alter the LH responses to 100 μg GnRH (Fuquay & Moberg 1983).

Since the hypothalamo–pituitary–adrenal axis was stimulated by isolation/restraint when compared with no stress, and the secretion of LH was depressed, it remains possible that component(s) of this axis other than cortisol may act to suppress the secretion of LH. Nevertheless, it is unlikely that these effects would have been due to either corticotrophin-releasing factor (CRF) or arginine vasopressin (AVP). The central administration of CRF to sheep has been to found to have no effect (Clarke *et al.* 1990) or to increase LH secretion (Naylor *et al.* 1990, Caraty *et al.* 1997) and central i.c.v treatment of ewes with AVP did not affect LH secretion (Clarke *et al.* 1990). In contrast, ACTH is able to suppress LH secretion in sheep. In Experiment 2, treatment with ACTH inhibited the LH response to GnRH in both gonadectomised rams and ewes and this is similar to findings previously reported (Fuquay & Moberg 1983, Matteri *et al.* 1984, Dobson *et al.* 1988). Furthermore, these effects of ACTH are likely to occur via a mechanism that does not involve the activation of the adrenal glands (Fuquay & Moberg 1983, Matteri *et al.* 1984, Dobson *et al.* 1988). Despite the ability of ACTH to inhibit the LH response to GnRH in sheep, this hormone is not necessarily a significant mediator of the effects of isolation/restraint on LH secretion. Presumably, the secretion of ACTH would have increased with the activation of the hypothalamo–pituitary–adrenal axis early in the sampling period in Experiment 1 but LH secretion did not decrease during this time. Indeed, the results of Experiment 2 provide evidence that ACTH that may have been released during isolation/restraint in gonadectomised ewes does not act directly on the pituitary to inhibit the

actions of GnRH. Whereas ACTH inhibited the actions of GnRH in both gonadectomised rams and ewes, isolation/restraint caused this effect only in the rams.

In summary, our results show that sex steroids influence the mechanisms by which isolation/restraint stress suppresses the secretion of LH in gonadectomised sheep. The predominance of particular sex steroids in the circulation influenced the extent to which the stress reduced the frequency and/or amplitude of LH pulses. The mechanisms for these various effects of stress could include suppression of the synthesis and/or secretion of GnRH and/or reduction in the responsiveness of the pituitary gland to the actions of GnRH. Furthermore, there are also steroid-independent differences between gonadectomised rams and ewes in the way that isolation/restraint stress inhibits LH secretion. In gonadectomised rams, but not ewes, isolation/restraint inhibited the actions of GnRH on the pituitary to release LH. In contrast, there were no effects of sex steroids, or of sex, on the concentrations of cortisol in gonadectomised sheep.

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