Plasma and ovarian oestradiol and the variability in the LH surge induced in ewes by the ram effect

Claude Fabre-Nys¹, Audrey Chanvallon^{1,†}, Nathalie Debus², Dominique François³, Frédéric Bouvier⁴, Joelle Dupont¹, Lionel Lardic¹, Didier Lomet¹, Christelle Ramé¹ and Rex J Scaramuzzi^{1,5}

¹UMR 7247 Physiologie de la Reproduction et des Comportements, CNRS, Institut Français du Cheval et de l'Équitation, Université de Tours, PRC Centre INRA Val de Loire, 37380 Nouzilly, France, ²UMR 868 'Élevage des Ruminants en Régions Chaudes', 2 Place Viala, 34060 Montpellier Cedex 1, France, ³INRA SAGA, Chemin de Borde-Rouge Auzeville, BP 52627, 31326 Castanet Tolosan, France, ⁴INRA Bourges, Domaine de la Sapinière, 18390 Osmoy, France and ⁵Department of Comparative Biological Sciences, Royal Veterinary College, Hawkshead Lane, South Mimms, Hertfordshire AL9 7TA, UK

Correspondence should be addressed to C Fabre-Nys; Email: claud.fabre@tours.inra.fr

[†]A Chanvallon is now at Institut de l'Élevage, Oniris, BP 40706, 44307 Nantes, France

Abstract

The proportion of anoestrous ewes ovulating after exposure to a sexually active ram is variable mainly due to whether an LH surge is induced. The aim of this study was to determine the role of oestradiol (E₂) in the ram-induced LH surge. In one study, we measured the plasma concentrations of E₂ in ewes of different breeds before and after the 'ram effect' and related these patterns to the presence and latency of the LH surge, while another compared ovarian responses with the 'ram effect' following exposure to rams for 2 or 12 h. In all ewes, the concentration of E₂ increased 2–4 h after rams were introduced and remained elevated for 14.5 ± 0.86 h. The quantity of E₂ secreted before the LH surge varied among breeds as did the mean concentration of E₂. The granulosa cells of IF ewes collected after 12 h exposure to rams secreted more E₂ and progesterone and had higher levels of STAR than the 2 h group but in MV ewes there was no differences between these groups for any of these parameters. These results demonstrate that the LH surge induced by the rams is a result of increased E₂ secretion associated with increased levels of STAR in granulosa cells and that these responses varied among breeds. The results suggest that the variable occurrence of a LH surge and ovulation may be the result of variable ovarian responses to the 'ram effect' and insensitivity of the hypothalamus to the E₂-positive feedback signal.

Free French abstract: A French translation of this abstract is freely available at <http://www.reproduction-online.org/content/149/5/511/suppl/DC1>.

Reproduction (2015) **149** 511–521

Introduction

In sheep, goats and many wild ungulates, reproduction is seasonal and the females give birth when the environmental conditions are most favourable for the survival of their young. Ewes have oestrous cycles when day length is decreasing (the breeding season), which stop as day length increases (anoestrus). During anoestrus, their ovaries release very little oestradiol (E₂), there are no spontaneous pre-ovulatory luteinising hormone (LH) surges and the ewes do not ovulate. However, the introduction of a sexually active male into a group of seasonally anoestrous ewes will induce an immediate increase in the pulsatile secretion of LH (short-term LH response). This socio-sexual stimulation of ovulation often referred to as the 'ram effect', initiates a sequence of

physiological events that in some of ewes culminates in a LH surge and ovulation (Martin *et al.* 1986, Ungerfeld 2007). A similar phenomenon occurs in goats and in many wild ungulates in which shortening and concentrating the period of sexual activity reduce the risks of predation. In farm animals, this is a simple technique for inducing fertile mating outside of the breeding season in a way that is compatible with sustainable, green and ethical agriculture (Martin *et al.* 2004). However, the response to the 'ram effect' is variable both among breeds and within breeds and this variability is a serious practical limitation to the more wide-spread use of this technique. The origins of this variability are poorly understood. In a previous study, we reported that 93% of ewes had 'short-term' LH responses to the 'ram effect'. We also reported that the

frequency of subsequent LH surges was highly variable ranging from 0 to 100%, depending on breeds and period of stimulation (Oldham *et al.* 1978, Chanvallon *et al.* 2011). Together, these results pointed to the induction of the LH surge as the major cause of variability of response to the 'ram effect'.

In all mammalian species, the LH surge is induced by an increase in secretion of the hypothalamic neuropeptide, gonadotrophin-releasing hormone (GNRH). In species that ovulate spontaneously, such as the sheep, the rat, the rhesus monkey or the human, secretion of GNRH is tightly controlled by ovarian steroids: progesterone secreted by corpora lutea and E_2 secreted predominately by large oestrogenic follicles. The secretion of E_2 increases during the follicular phase and this increase is essential for the LH surge (positive feedback). The LH surge occurs only after a period of prolonged exposure to the above threshold concentrations of E_2 (Corker *et al.* 1969, Karsch *et al.* 1973, 1979, Smith *et al.* 1975, Hauger *et al.* 1977). In sheep, the characteristics of the relationship between the pre-ovulatory rise in systemic concentrations of E_2 and the onset of the LH surge, and presumably of GNRH, varies among breeds (Land *et al.* 1976, Cahill *et al.* 1981, Ben Saïd *et al.* 2007).

It is widely assumed that the LH surge occurring in anoestrous ewes after introduction of rams is induced by mechanisms similar to those occurring during the breeding season, that is, the increased pulsatile secretion of LH induced by exposure to rams increases the secretion of ovarian E_2 that by positive feedback induces the pre-ovulatory surges of GNRH and LH (Martin *et al.* 1986). However, there is very little published data describing the precise pattern of E_2 concentrations either before or after the 'ram effect'. In fact, one study from 1978 concluded that there was no change in the concentrations of plasma E_2 in ewes stimulated by the 'ram effect' (Knight *et al.* 1978). Recent results from our laboratory have indicated that there was a close relationship between the induction of a LH surge after introduction of rams and increased systemic concentrations of E_2 at the time of the LH surge (Johnson *et al.* 2011).

Our hypothesis is that the plasma concentration of E_2 in anoestrous ewes can predict the likelihood and timing of the LH surge following the 'ram effect'. More specifically, we propose that among breed, variability in the LH response to the 'ram effect' is due to variability in the threshold concentration of E_2 necessary to trigger a LH surge (i.e. hypothalamo-hypophyseal sensitivity) and/or a failure of the ovaries to respond to the male-induced pulsatile secretion of LH thus, producing insufficient E_2 to induce a LH surge (ovarian sensitivity). To test these hypotheses, we carried out two experiments. In the first, we measured the systemic concentrations of E_2 before and after the introduction of rams in four breeds of anoestrous sheep that have different patterns of response to the 'ram effect' and then related these concentrations to the occurrence and

timing of the LH surge. In a second experiment, we compared the ovarian follicular response under conditions known to differentially affect LH secretion and ovulatory response to the 'ram effect' in two of the breeds studied in the first experiment.

Materials and methods

The experiments were carried out in accordance with French and European regulations on the care and welfare of animals in research and with authorisation of the Ministry of Agriculture and with their ethical approval (no. 006259 and 2012-01-2).

For our first study (the four breeds study), the plasma samples to be analysed were selected from an existing set of samples that had been previously collected for another experiment. The data from this earlier experiment have been published (Chanvallon *et al.* 2011). In this experiment, adults anoestrous ewes from four breeds (Mouton Vendéen (MV), Ile de France (IF), Romane (R) and M d'Arles (M)) were exposed to sexually active males at different periods of anoestrus and their responses recorded and analysed. In the published experiment, we reported the concentrations of LH from -1.5 h before to 56 h after the 'ram effect' (samples every 15 min up to $+4$ h and then every 4 to $+56$ h) and these data were used as the basis for the selection of ewes for the present experiment. We selected samples from ewes that had LH surges 2–56 h after ram introduction (IF, $n=18$; M, $n=17$; R, $n=15$ and MV, $n=7$) as well as ten ewes that had not had a LH surge by 56 h (three ewes from IF, R and MV; one M). To avoid bias, we controlled for the possible effects of time of anoestrus by randomisation within anoestrous periods. E_2 concentration was assayed in samples taken 1.5 h before and a few minutes before the introduction of the rams (0 h) and in those taken at 2, 4, 8 and 12 h after the introduction of the rams and then every 4 h until the LH surge or 56 h in ewes without a LH surge. Details of the blood sampling and processing procedures have been published (Chanvallon *et al.* 2011).

Anoestrous ewes from MV and IF breeds, known to differ in the occurrence of ovulation after exposure to rams (Chanvallon *et al.* 2011), were used in the second study (the ovarian response study). The ewes had been isolated from all contact with rams for more than 2 months before the start of the experiment. The treatments were i) exposure to sexually active rams for 2 h (male 2 h: IF, $n=6$ and MV, $n=4$), a condition that stimulates LH pulsatile secretion; ii) exposure to a sexually active ram for 12 h (male 12 h: IF, $n=9$ and MV, $n=4$), a condition that induces a LH surge in some but not all ewes and iii) exposure to unfamiliar anoestrous ewes for 2 h (controls: IF, $n=6$ and MV, $n=4$), a condition that has no effect on LH secretion. The ovaries of each ewe were collected at slaughter after 2 h ('control 2 h' and 'male 2 h' ewes) or 12 h ('male 12 h' ewes) by a licensed butcher in the licensed abattoir at the INRA laboratory.

Isolation and culture of granulosa cells

Immediately after slaughter, the ovaries were removed and immersed for 15 min in isotonic saline containing Fungizone (Gibco BRL) and antibiotics (penicillin and streptomycin).

For each ewe, granulosa cells from the right ovary were collected from different follicles as described below and stored at -80°C before total RNA extraction. Those obtained from the left ovary were cultured. More precisely, the ovaries were transferred to culture plates containing McCoy's medium and the small follicle (SF; 1.0–2.0 mm) medium follicle (MF; >2.0–3.5 mm) and antral follicle (LF; >3.5 mm) were dissected free. Each follicle was then slit open in McCoy's medium, and granulosa cells were removed by gently scraping the interior surface of the follicle with a platinum loop. The cell suspensions were centrifuged at 300 *g* for 7 min. The granulosa cells were pooled by follicle class within ovaries. Given the low number of granulosa cells for the MF and LF groups, only granulosa cells from the SF class were analysed for both E_2 and progesterone production and mRNA expression.

For the culture experiments, pellets were re-suspended in culture medium (McCoy's 5A medium (Sigma) containing bicarbonate supplemented with 20 mmol/l HEPES, 100 kIU/l penicillin, 0.1 g/l streptomycin, 3 mmol/l L-glutamine, 0.1% BSA (w/v), 0.1 $\mu\text{mol/l}$ androstenedione, 5 mg/l transferrin and 20 $\mu\text{g/l}$ selenium). The granulosa cells were then cultured in serum-free conditions. Briefly, cells were counted and their viability was determined (ranged between 70 and 85%) by Trypan blue exclusion. The cultures were performed in 48-well plates for the production of progesterone and E_2 or in six-well plates for the RNA measurements. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in a serum-free culture medium containing either no exogenous factors, ovine follicle-stimulating hormone (oFSH) alone (10^{-8} M), insulin-like growth factor 1 (IGF1) alone (10^{-8} M) or their combination (oFSH+IGF1, 10^{-8} M) for 48 h. For each ewe, each combination of treatment was tested in quadruplicate. After 48 h of stimulation, the collected medium was stored at -20°C before progesterone and E_2 assay.

RNA analysis

Total RNA was extracted from SF granulosa cells obtained from right ovary of each group of ewe (exposure for 2-h to an unfamiliar ewe (control) or to a sexually active ram for either 2 or 12 h) using RNeasy kits according to the manufacturer's procedure (Qiagen). The levels of RNA for STAR protein (STAR), 3β -hydroxy steroid dehydrogenase (HSD3B) and P450 side chain cleavage enzyme (CYP11A1) was quantified by measuring the absorbance at 260 nm. The samples were stored at -80°C until use. Reverse transcribed total RNA (1 μg) was denatured and retro-transcribed with the reverse transcriptase Moloney murine leukaemia virus (MMLV; 15 U) in a 20 μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 200 μM of each dNTP (Amersham),

50 pmol of oligo(dT)15 and 5 U of ribonuclease inhibitor. All were incubated at 37°C for 1 h. Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix (Bio-Rad) and 250 nm of specific primers as given in Table 1, in a total volume of 20 μl using a MyiQ Cycle device (Bio-Rad). The samples were tested in duplicate on the same plate and PCR amplification with water, instead of cDNA, was used systematically as a negative control. After incubation for 2 min at 50°C and a denaturation step of 10 min at 95°C , samples were subjected to 40 cycles (30 s at 95°C , 30 s at 60°C and 30 s at 72°C), followed by acquisition of the melting curve. Primers' efficiencies (*E*) were performed from serial dilutions of a pool of all cDNA analysed and ranged from 1.8 to 2. Three reference genes: *PPIA*, *RPL19* and *ACTB* were used (the description of these primers is given in Table 1). For each gene, expression was calculated according to primer efficiency and *C_q*: $\text{expression} = E^{-C_q}$. Then, the relative expression of *STAR*, *CYP11A1* or *HSD3B*/geometric mean of the three reference genes was analysed.

Assay of LH in plasma

The concentration of LH in plasma was measured by EIA as described previously (Faure *et al.* 2005). The sensitivity of the assay was 0.10 ng/ml and the intra-assay and inter-assay coefficients of variation (CV) for a reference sample (0.5 ng/ml) were 9.3 and 5.2% respectively.

Assay of progesterone and E_2 in culture medium

The concentration of progesterone in the culture medium was measured by an EIA protocol as described previously (Canepa *et al.* 2008). The intra-assay CV averaged <10%. The results are expressed as the amount of steroid (ng) secreted per 48 h per 100 mg of protein. The results are expressed as mean $\pm$ S.E.M. For each ewe, each combination of treatment was tested in quadruplicate. The E_2 concentration in the culture medium was determined by using by EIA using the E_2 Assay Kit from Cayman Chemical (distributed by Interchim, Montluçon, France). The intra-assay CV averaged <10%. The results are expressed as mean $\pm$ S.E.M. For each ewe, each combination of treatment was tested in quadruplicate.

Assay of E_2 in plasma

The concentrations of E_2 in jugular venous plasma were determined using the HRP- E_2 DIASource Immunoassay ELISA Kit (E2-EASIA/KAP0621; DIASource immunoassay SA, Louvain la Neuve, Belgium), adapted for the detection of E_2 in ovine

Table 1 Oligonucleotide primers sequences.

Gene	Protein	Forward (5'–3')	Reverse (5'–3')
<i>PPIA</i>	Cyclophilin A	GCATACAGGTCCTGGCATCT	TGTCCACAGTCAGCAATGGT
<i>RPL19</i>	Ribosomal protein L19	AATCGCCAATGCCAATCT	CCCTTTCGCTTACCTATACC
<i>ACTB</i>	Beta actin	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCACTATACC
<i>CYP11A1</i>	P450 side-chain cleavage enzyme	AGAGAATCCACTTTCGCCACATC	GGTCTTTCTCCAGGTTCTGAC
<i>HSD3B1</i>	3β -hydroxy steroid dehydrogenase	GTGAGCTTCTGCTCAGTCC	CTCCTTGGTTTCTGCTTGG
<i>STAR</i>	STAR protein	CCCATGGAGAGGCTTTATGA	CCCATGGAGAGGCTTTATGA

plasma. Aliquots (300 µl) of plasma, or standards in steroid-free plasma, in glass tubes were extracted with 3 ml of ethyl acetate/cyclohexane (Carlo Erba RPE, 99.8% min, for analysis-ACS reagent V/V) by mixing for 5 min. After resting for 2 h, tubes were centrifuged for 15 min and then frozen in liquid nitrogen. The solvent layer was decanted, transferred into new glass tubes and evaporated to dryness under nitrogen. The standards and samples were reconstituted in 160 µl of 0.1 M Tris (a mixture of Trisma base and Trisma hydrochloride, Sigma), 1 mM EDTA (Titriplex III, Merck) and 150 µl was incubated in an ELISA plate, previously coated with anti-rabbit mouse IgG from the DIASource Kit, with the antibody to E₂ also from the DIASource Kit but, diluted at 50% of the concentration recommended, for 24 h at 22 °C on a horizontal shaker set at 700 ± 100 revolutions/min. The HRP coupled E₂ (E₂-HRP) from the DIASource Kit (50 µl) was added to each well at the concentration recommended for the kit, shaken for 1 h at 22 °C on a horizontal shaker set at 700 ± 100 revolutions/min and then for 23 h at 4 °C without shaking. The plate was washed three times with the washing solution supplied with the kit and dried on absorbent paper. Then, 200 µl of 3,3',5,5'-tetramethylbenzidine substrate were added to each well and incubated for 30 min at 22 °C on a horizontal shaker set at 700 ± 100 revolutions/min. Finally, 50 µl of the STOP reagent also part of the kit (0.9 N solution of sulphuric acid) was dispensed into each well. The absorbance of each well was read at a wavelength of 450 nm (with a reference wavelength at 620 nm) within 1 h of the addition of the STOP reagent. The concentrations of E₂ were calculated using the RIA SMART

programme (Canberra Packard-Packard instrument Co., Saint-Quentin-en-Yvelines, France) with 4PL logistic regression.

Validation of E₂ assay

Cross-reactivities were <2% for oestrone, oestriol, E₂-3-glucuronide and E₂-17-glucuronide, and <0.1% for E₂-17-valerate, cortisol, progesterone, DHEA-S, testosterone, androstenediol, norgestrel, premarin and equilin. The assay sensitivity was 0.39 pg/ml. The recovery of E₂ was tested by adding 0.5, 1.0, 2.0 and 5.0 pg/ml of E₂ to steroid-free plasma. The percentage recoveries of added E₂ were 80, 109, 123 and 99% for 0.5, 1, 2 and 5 pg/ml respectively. The fitted regression line had a slope of 1.03 and the correlation between added and recovered E₂ was 0.98. The recovery of E₂ was similarly tested using demineralised water instead of steroid-free plasma. The percentage recoveries were respectively, 106, 83, 80 and 74% at 0.5, 1.0, 2.0 and 5.0 pg/ml, and the regression line has a slope of 0.73 and the correlation was 0.99. The 'intra-assay' and 'inter assay' CV determined at three concentrations of E₂ were as follows: 16.6 and 15.3% (at 0.46 pg/ml), 11.9 and 11.7% (at 0.80 pg/ml), 4.6 and 4.7% (at 5.13 pg/ml).

Statistical analyses

E₂ data from the first study were analysed after grouping the data around time '0' relative to the introduction of rams. The pattern of E₂ concentrations was considered as 'discontinuous' if the concentrations of E₂ fluctuated more than 25% between

Figure 1 The pattern of concentrations of oestradiol (E₂; open circles, dotted lines) and LH (closed circles and solid lines) in anoestrous ewes after the introduction of sexually active rams at time 0. Examples are shown of ewes that did not have a LH surge within the 56 h sampling window (Mouton Vendéen no. 1b and R no. 2b) and of ewes with a LH surge and a continuous pattern of secretion of E₂ (Ile de France no. 14, R no. 1, M nos 33 and 7), or a discontinuous pattern of secretion of E₂ (Ile de France no. 1 and Mouton Vendéen no. 4).

Figure 2 The pattern of concentrations of E_2 in four breeds of anoestrous ewes (Mouton Vendéen, $n=10$; Ile de France, $n=21$; R, $n=18$ and M, $n=18$) following exposure to a sexually active ram at time 0. Data have been centred about time '0' (the introduction of rams) and are expressed as mean $\pm$ S.E.M. Concentrations in Mouton Vendéen are lower than those in all other breeds 8, 12 h after ram introduction (indicated by asterisks; $P<0.008$) and lower than Ile de France and R at 16 and 20 h after male introduction (indicated by triangle; $P<0.004$). Statistical analyses were made using mixed model ANOVA under general linear model followed by paired comparisons between breeds using Bonferroni's correction.

the time of introduction of rams and the onset of the LH surge; otherwise E_2 concentration patterns were considered as 'continuous'. The data were then re-analysed after grouping them around the time of the peak of the LH surge. The quantity of E_2 secreted after the 'ram effect' was estimated from a plot of E_2 concentrations in plasma between the time of ram introduction and the maximum concentration of the LH surge as the area under this curve in mm^2 using a triangulation procedure and from a baseline of 0. An LH surge was defined as an increase in LH, lasting at least 4 h and with a minimum of one value above 10 ng/ml. The onset of the LH surge was defined as the first point preceding the sustained increase and when the LH concentration was at least three times the S.D. of the baseline (Moenter *et al.* 1990).

Statistical tests were carried out using SPSS version 20 (SPSS, Inc., IBM Company Headquarters). The data were tested for normality and when the data were not normally distributed they were either transformed to logarithms or analysed using non-parametric tests. The data involving measures over time were analysed using a mixed model ANOVA run under the general linear model with time as a repeated measure. Data from cell culture, RNA analyses and for the latency of the

LH surge were analysed using a univariate ANOVA run under the general linear model. For both types of ANOVA, *post hoc* paired comparisons within treatments were performed using the Bonferroni's correction if the ANOVA analysis showed an overall treatment effect that was significant. Overall and within breed correlations were tested using the non-parametric Spearman's rank test. The proportions were tested using the χ^2 test. Differences were considered statistically significant when $P<0.05$ and as a trend toward significance when the P value was between 0.05 and 0.10.

Results

The 'four breeds study'

Eight examples (two from each breed) of the different patterns for the plasma concentration of E_2 and LH after introduction of rams are illustrated in Fig. 1. In all ewes (67/67), the concentration of E_2 increased significantly following the introduction of rams, although the pattern and the magnitude of the increases were highly variable (Fig. 1). The statistical analysis showed that the concentrations increased after the introduction of rams ($P<0.001$). The increase in E_2 concentrations was detected 2 h after ram introduction ($P<0.0001$) and was greater in ewes that went on to have an LH surge than in ewes that did not (1.98 ± 0.25 and 0.86 ± 0.36 pg/ml respectively; $P<0.012$). In 65% of the ewes with an LH surge, there was a continuous pattern of increased E_2 (Fig. 1: IF, no. 14; R, no. 1 and M, nos 33 and 7) whereas in the other 35% had a discontinuous pattern of increased E_2 (Fig. 1: IF, no. 1 and MV, no. 4). In ewes with a surge starting between 8 and 56 h after introduction of rams, the mean plasma concentrations of E_2 were above 2 pg/ml for 14.5 ± 0.86 h (min 6 h and max 36 h). There was no difference in the concentrations of LH before the onset of the LH surge (averaged for each ewe from the time of male introduction and the onset of the LH surge) between the ewes with a continuous (1.23 ± 0.10 ng/ml) and a discontinuous (1.39 ± 0.23 ng/ml) pattern of secretion of E_2 .

The concentrations of E_2 varied among breeds ($P<0.012$, Fig. 2) and the interaction between time and breed was significant ($P<0.001$). Paired comparisons showed that the concentrations of E_2 in the MV ewes were significantly lower than those in IF

Table 2 The quantity of oestradiol (mean $\pm$ S.E.M.) secreted after the 'ram effect' estimated as the area (mm^2) under the response curve between the time rams were introduced and the time of maximum concentration of LH and latency of the onset of the LH surge (h) in ewes with a surge or between the time rams were introduced and 56 h in ewes without a LH surge.

	Breed of ewe			
	Ile de France	M	Mouton Vendéen	R
Area under the curve (mm^2) in ewes with a LH surge	$68\pm9.7^{a,b}$ (18)	54 ± 6.1^a (17)	$86\pm17.1^{a,b}$ (7)	102 ± 12.8^b (15)
Mean latency of the surge (h)	$20.8\pm3.39^{a,c}$	16.9 ± 2.04^a	$40.6\pm4.22^{b,c}$	31.2 ± 4.217^c
Area under the curve (mm^2) in ewes without LH surge	$99\pm18.4^{a,b}$ (3)	120 (1)	32 ± 11.4^a (3)	$73\pm10.3^{a,b}$ (3)

The value in brackets indicates the number of ewe per group. Values with a different superscript are significantly different at a P value of 0.05.

Figure 3 The pattern of concentrations of oestradiol in four breeds of anoestrous ewes (Mouton Vendéen, $n=7$; Ile de France, $n=18$; R, $n=15$ and M, $n=17$) following exposure to a sexually active ram. Data have been centred around the time when the maximum concentration of LH was observed (time 0) and are expressed as mean $\pm$ S.E.M. Statistical analysis were made using mixed model ANOVA under general linear model and showed an effect of time ($P<0.037$) but no effect of breed and no interaction of time and breed.

($P<0.051$), R ($P<0.011$) and M ewes ($P<0.011$). The concentrations in R, IF and M ewes did not differ. The proportion of MV ewes (0/7) with a 'continuous' pattern of E_2 concentrations was lower than that in IF (14/18; $P<0.001$), M (13/17; $P<0.001$) and R (10/15; $P=0.003$) ewes. There were no differences in proportions among IF, M and R ewes with 'continuous' pattern of E_2 .

The quantity of E_2 secreted after the introduction of rams, estimated from the area under the curve, varied with breed ($P<0.001$, Table 2). R ewes with a LH surge secreted significantly more E_2 ($P<0.001$) than M with a LH surge. IF, M and MV did not differ.

The latency to the LH surge was longer in MV than in IF ewes ($P<0.01$) and M ($P<0.001$), but R only differed significantly from M ewes ($P<0.02$, Table 2). The area under the E_2 curve was correlated with the latency to the LH surge both when pooled among breeds ($r=0.779$; $P<0.0001$) and by breed (IF: $r=0.932$; $P<0.001$; M: $r=0.6293$; $P<0.01$; MV: $r=0.7456$; $P<0.05$ and R:

$r=0.8304$; $P<0.001$). In ewes that did not have an LH surge within the 56 h sampling period, the area under the E_2 curve did not differ significantly from those with an LH surge, although for the MV breed it tended to be lower ($P=0.08$).

Analysis of the concentrations of E_2 grouped around the maximum concentration of LH (Fig. 3) showed a significant effect of time ($P=0.037$), but no effect of breed and no interaction of time with breed. In all ewes that had a surge later than 8 h after the introduction of rams ($n=52$), the concentration of E_2 in plasma 4 h before the LH surge exceeded 2.5 pg/ml (IF, 5.11 ± 0.40 pg/ml; M, 4.47 ± 0.42 pg/ml; MV, 3.73 ± 0.82 pg/ml and R, 4.73 ± 0.56 pg/ml) and in most ewes (41/57) the mean concentration at 0 h was lower ($P<0.0001$) than at -4 h (3.73 ± 0.20 pg/ml vs 4.60 ± 0.27 pg/ml).

The ovarian response study

The effects of exposure to either unfamiliar ewes (controls) or sexually active rams on the number of ovarian follicles are presented in Table 3. Exposure to a ram 2 or 12 h had no effect on the number of follicles in any size category present on the ovaries compared with those on ovaries of ewes exposed to unfamiliar ewes for 2 h in either the MV or IF ewes. But in IF ewes, the number of SFs tended to differ ($P=0.062$) and paired comparisons showed that the 12 h group was significantly different from the 2 h group ($P=0.021$). This tendency was not observed in MV ewes.

The *in vitro* production of E_2 by cultured granulosa cells was higher ($P<0.001$) in IF ewes exposed to rams for 12 h (Fig. 4, top panel) compared with those exposed to rams for 2 h. But *in vitro* concentrations of E_2 did not differ between IF ewes exposed to rams for 2 h compared with those exposed for 2 h. The conditions of culture also affected E_2 output by the cultured granulosa cells from IF ewes and all culture treatments were significantly different from the others (all paired comparison $P<0.001$). The order from the lowest to highest production of E_2 was controls < IGF1 < FSH < (FSH + IGF1) (Fig. 4, top panel).

Table 3 The effect of exposure to a ram for 2 h (male 2 h) or 12 h (male 12 h) or to an unfamiliar ewe (control) on the total number of ovarian follicle >1.0 mm in diameter (mean $\pm$ S.E.M.) and the number of small (diameter: 1.0–2.0 mm), medium (diameter: <2.0–3.5 mm) and large (diameter: <3.5 mm) follicles in anoestrus Ile de France and Mouton Vendéen ewes (mean $\pm$ S.E.M.).

Treatment	Classification of follicles			
	Total	Small	Medium	Large
Ile de France				
Control ($n=6$)	24.0 $\pm$ 2.9	22.2 $\pm$ 2.9	0.5 $\pm$ 0.4	1.3 $\pm$ 0.5
Male 2 h ($n=6$)	29.8 $\pm$ 2.9	27.5 $\pm$ 4.7	1 $\pm$ 0.3	1.3 $\pm$ 0.5
Male 12 h ($n=9$)	19.0 $\pm$ 2.7	16.2 $\pm$ 2.9*	1 $\pm$ 0.4	1.8 $\pm$ 0.2
Mouton Vendéen				
Control ($n=4$)	32.5 $\pm$ 1.1	28.8 $\pm$ 5.4	2.8 $\pm$ 1.2	1.0 $\pm$ 0.5
Male 2 h ($n=4$)	29.8 $\pm$ 9.2	27.0 $\pm$ 9.4	1 $\pm$ 0.7	1.0 $\pm$ 0.8
Male 12 h ($n=3$)	41.0 $\pm$ 3.1	39.3 $\pm$ 14.2	0.0	1.3 $\pm$ 0.6

In both breeds, there was no effect of treatment on the number of follicles. However, there was a trend towards significance in Ile de France ewes ($P=0.062$) and paired comparison showed a lower number of small follicles in the male 12 h group than in the male 2 h group (* $P<0.021$).

Figure 4 The effect of exposure for 2 h to an unfamiliar ewe (control; light grey columns) or to a sexually active ram for either 2 h (medium grey columns) or 12 h (dark grey columns) on oestradiol secretion *in vitro* by granulosa cells from anoestrous Ile de France (top panel, $n=6$) or Mouton Vendéen (bottom panel, $n=4$) ewes after 48 h culture in enriched McCoy's 5A medium (without FCS) and in the absence (control) or presence of FSH (10^{-8} M), IGF1 (10^{-8} M) or IGF1 + FSH (both 10^{-8} M). Steroid secretion was normalised against the protein concentration of each well. Results are presented as mean $\pm$ S.E.M. Within the *in vitro* treatment groups, different letters indicate significant differences ($P<0.05$ univariate ANOVA under general linear model). There were no significant differences within the *in vitro* treatment groups for the Mouton Vendéen ewes.

In MV ewes, there were no significant differences among the three groups (Fig. 4, bottom panel) either among the control cultures or the cells cultured with FSH, IGF1 or both (Fig. 4, bottom panel).

The *in vitro* production of progesterone by cultured granulosa cells was higher in IF ewes exposed to rams for 12 h (Fig. 5, top panel) compared with those exposed to rams for 2 h ($P<0.001$), but progesterone concentrations were not different in ewes exposed to rams vs ewes for 2 h. Similar to E_2 concentrations, the conditions of culture also affected progesterone output by the cultured granulosa cells from IF ewes. And all culture treatments were significantly different from the others (all paired comparison $P<0.001$). The order from the lowest to highest production of progesterone was controls < IGF1 < FSH < (FSH+IGF1) (Fig. 5, top panel). In MV ewes, there were no significant differences among the three groups (Fig. 5, bottom panel) either among

the control cultures or the cells cultured with FSH, IGF1 or both (Fig. 5, bottom panel).

The level of mRNA for StAR was higher in IF ewes exposed to rams for 12 h compared with those in IF ewes exposed to either other ewes ($P<0.001$) or to rams ($P<0.001$) for 2 h (Fig. 6, top panel). However, in the MV ewes, the level of the *STAR* mRNA expression was similar in ewes exposed to ewes for 2 h or exposed to rams for 2 or 12 h (Fig. 6, bottom panel). In both IF and MV ewes, the mRNA expression of *HSD3B* and *CYP11A1* was not affected by exposure to ewes for 2 h or exposure to rams for 2 or 12 h (Fig. 6).

Discussion

Our results show that in most anoestrous ewes, the LH surge induced by the socio-sexual stimulus of introduction of rams is preceded by an increase in circulating concentrations of E_2 for between 8 and 56 h after the introduction of rams. The duration and the pattern of

Figure 5 The effect of exposure for 2 h to an unfamiliar ewe (control; light grey columns) or to a sexually active ram for either 2 h (medium grey columns) or 12 h (dark grey columns) on progesterone secretion *in vitro* by granulosa cells from anoestrous Ile de France (top panel, $n=6$) or Mouton Vendéen (bottom panel, $n=4$) ewes after 48 h culture in enriched McCoy's 5A medium (without FCS) and in the absence (control) or presence of FSH (10^{-8} M), IGF1 (10^{-8} M) or IGF1 + FSH (both 10^{-8} M). Steroid secretion was normalised against the protein concentration of each well. Results are presented as mean $\pm$ S.E.M. Within the *in vitro* treatment groups, different letters indicate significant differences ($P<0.05$ univariate ANOVA under general linear model). There were no significant differences within the *in vitro* treatment groups for the Mouton Vendéen ewes.

Figure 6 The expression of mRNA in ovine granulosa cells from anoestrous Ile de France (top panel) or Mouton Vendéen (bottom panel) ewes exposed for 2 h to an unfamiliar ewe (control; light grey columns: IF, $n=6$ and MV, $n=4$) or to a sexually active ram for either 2 h (medium grey columns: IF, $n=6$ and MV, $n=4$) or 12 h (dark grey columns: IF, $n=6$ and MV, $n=3$). Total mRNA was extracted from granulosa cells and reverse transcribed and real-time RT-PCR was performed. The geometric mean of three housekeeping genes (*PPIA*, *RPL19* and *ACTB*) was used to normalise gene expression. Results are presented as mean $\pm$ S.E.M. Different letters indicate significant differences ($P < 0.05$ univariate ANOVA under general linear model).

these increases were variable and some of the variability appears to be associated with the breed of ewe. In our previous experiment, we showed that ram-induced pulsatile secretion of LH did not differ among breeds (Chanvallon *et al.* 2011). In the MV breed, the variability in the response to the 'ram effect' seemed to be caused primarily by a reduced ovarian response to LH resulting in lower concentrations of E_2 in plasma and presence of LH surges at a lower frequency and/or a longer latency. However, in R ewes, the plasma concentrations of E_2 after the introduction of rams were not higher than that in IF or M ewes; However, the quantity of E_2 secreted (estimated as the area under the curve) before the LH surge was higher in the R breed, suggesting that the variability in this breed was due to a lower sensitivity of the hypothalamo-hypophyseal complex to the positive feedback effect of E_2 .

In all ewes, the concentration of E_2 increased within 4 h of the 'ram effect'. Large follicles are the predominant source of E_2 in ewes (Baird & Scaramuzzi 1976). These follicles secrete E_2 following each pulse of LH both in the breeding season (Baird *et al.* 1976) and in anoestrus (Scaramuzzi & Baird 1977). There were large follicles (>3.5 mm diameter) present in most ewes (Table 3). The increased concentration of E_2 following the 'ram effect' is undoubtedly the result of increased pulsatile

secretion of LH that in itself is a response to the socio-sexual stimulation that always follows exposure to an unfamiliar sexual partner (Hawken *et al.* 2007, Chanvallon *et al.* 2011, Jorre de St Jorre *et al.* 2012). Sheep have particularly low concentrations of blood E_2 , especially during anoestrus, and present considerable technical difficulties when attempting to assay the circulating concentrations of E_2 . Consequently, until recently there has been little detailed analysis of the concentrations of circulating E_2 in the anoestrous ewes ovulating in response to the introduction of rams. One report from our group (Johnson *et al.* 2011) showed that the plasma concentrations of E_2 increased 8 h after the introduction of rams and that the occurrence of the LH surge in response to socio-sexual stimulation was strongly related to the concentration of E_2 during the 48 h following the introduction of ram. In addition, there have been a number of reports in the males of several species, where exposure to females increased LH secretion and that this was followed by increased testosterone secretion (Katongole *et al.* 1971, Rose *et al.* 1972, Kamel *et al.* 1975, Gonzalez *et al.* 1988). Thus, a neuro-endocrine response to socio-sexual stimulation seems to be a common physiological mechanism allowing animals to adapt to socio-sexual conditions. Interestingly, this was also the case in ewes that did not go on to have a LH surge, suggesting that the immediate increase in the pulsatile secretion of LH following socio-sexual stimulation and the following LH surge, although closely associated, are distinct phenomena. In an earlier study (Chanvallon *et al.* 2011), we were able to show that a short-term increase in the pulsatile secretion of LH following the introduction of a ram was an almost universal response occurring in 104 out of 112 ewes (93%). In this study, the short-term response was not always followed by a LH surge and only 71 (68%) went on to have a LH surge. This study extends these observations and shows that the short-term increase in pulsatile LH induced by the 'ram effect' is consistently followed by an increase in follicular E_2 secretion but that the temporal pattern and duration of the increase in the concentration of E_2 is variable.

In about two thirds of the ewes with a LH surge, the concentrations of E_2 remained elevated from the time of the 'ram effect' until the LH surge (continuous responses), an interval of between 8 and 56 h. In the other third, the concentrations of E_2 were more variable (discontinuous responses), with several episodes of increased concentrations of E_2 before the LH surge. These patterns are very similar to those seen before the LH surge in cyclic ewes (Baird *et al.* 1976) and in other spontaneous ovulators such as the rat (Smith *et al.* 1975) and the rhesus monkey (Hotchkiss *et al.* 1971), and suggests that the ram-induced LH surge is a consequence of E_2 -positive feedback (see review by Goodman (1994)). We did not find any significant difference in the concentrations of LH between ewes

with continuous or discontinuous patterns of secretion of E_2 . The discontinuous patterns of secretion of E_2 differed among breeds and this pattern was seen in all the ewes of the MV breed and ewes of this breed also had lower concentrations of E_2 suggesting reduced ovarian responses to the introduction of rams.

Cultured granulosa cells secreted both E_2 and progesterone. Those from IF ewes responded to both socio-sexual stimulation for 12 h and also to the addition of FSH and IGF1, known stimulants of granulosa cells steroidogenesis (Campbell *et al.* 1996). There is an extensive literature on the effects of IGF1 and FSH both alone and in combination on the secretion of E_2 by cultured granulosa cells from various species (Campbell *et al.* 1996, 2003, Spicer *et al.* 2002) and our data show close agreement with this literature. The ability of cultured granulosa cells from the IF breed to respond to FSH and IGF1 was associated with increased levels of *STAR* mRNA, suggesting a key role of this step in the response to the 'ram effect'. Cultured granulosa cells from the MV breed behaved very differently, they were completely non-responsive to either socio-sexual stimulation or to stimulation by FSH, IGF1 or a combination of both. The low levels of *STAR* of MV anoestrous ewes may compromise their ability to secrete E_2 in response to the 'ram effect' and could be responsible for the generally poorer responses to the 'ram effect' than other breeds (Chanvallon *et al.* 2011).

When the data for E_2 were centred around the time of the LH surge rather than the time when the rams were introduced, the concentrations of E_2 in the 20 h before the LH surge were still higher than baseline, but the breed difference were no longer significant. This apparent contradiction can be explained first by the longer latency between the introduction of the rams and the start of the LH surge in some breed (R and MV). It can also be explained by the fact that in all animals with LH surges, the concentration of E_2 exceeded the threshold concentration for positive feedback. In cyclic ewes, the amount of E_2 required for induction and maintenance of the LH surge has been defined by studies in ovariectomised, E_2 -treated ewes, and is much lower than that normally present in intact cyclic ewes during the follicular phase of the oestrous cycle (Evans *et al.* 1997).

Similarly to intact ewes (Karsch *et al.* 1979), the mean concentrations of E_2 in most ewes were lower 4 h after the maximum concentrations of the LH surge than at the peak of the LH surge. This suggests that the feedback regulations of the ovary during anoestrus are identical to those during the breeding season.

The total amount of E_2 present before the LH surge was positively correlated with the latency of the LH surge and differed among breeds; the R breed required more E_2 before the LH surge than the M breed. Similar breed differences in the concentration of E_2 before the surge have been observed during the breeding season

and have often been linked with the prolificity of the breed (Land *et al.* 1976, Cahill *et al.* 1981). In one study using both intact and ovariectomised ewes, Ben Saïd *et al.* (2007) showed that Romanov ewes, a breed that has multiple ovulations, require higher concentrations of E_2 and for a longer duration for the induction of a LH surge than IF ewes. The R is a breed developed from a cross between Romanov and Berrichon du Cher breeds. The R had a higher quantity of E_2 before the LH surge compared with M ewes, but the concentrations of E_2 after the introduction of rams were not different compared with other breeds. This suggests a higher threshold concentration and longer duration of exposure to E_2 are required to induce an LH surge as observed previously in Romanov breed (Ben Saïd *et al.* 2007). This probably explains why R sheep that are less seasonal (Chanvallon *et al.* 2011) have more variable responses to the introduction of rams compared with the equally less seasonal M sheep.

In MV ewes the time required to reach the threshold concentration of E_2 for the induction of the LH surge was longer than for the other breeds, thus explaining the longer latency in this breed. The levels of *STAR* mRNA expression were higher in IF ewes exposed to the male for 12 h, but this was not the case for MV. This finding is consistent with the findings from the culture experiments and the blood concentrations of E_2 because *STAR* is an essential step and rate-limiting step in steroidogenesis (Clark & Stocco 1996). In an earlier study, we have shown that *STAR* mRNA in the granulosa cells of follicles from anoestrous ewes was increased by pre-treatment with progesterone (Brown *et al.* 2014) a treatment known to improve the response to the 'ram effect'. The synthesis of *STAR* in the ovary is stimulated rapidly by LH (Selvaraj *et al.* 1996, Light & Hammes 2013) and following the 'ram effect' there is a rapid increase in the secretion of pulsatile LH. Taken together these data suggest that the inability of follicles in the ovaries of anoestrous ewes to synthesise *STAR* is a major reason for the variability of the response to the 'ram effect'. This result could explain the poor response of the MV.

In conclusion, our study shows that the presence or absence of an LH surge and therefore an ovulation after the socio-sexual stimulus of the 'ram effect' are linked with the ability of the ovary to synthesise *STAR* and thus secrete sufficient E_2 in response to the male-induced increase in LH pulsatility and in the ability to cause a preovulatory surge in response to this increase in E_2 .

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The research was supported by grants from the Région Centre (DURAREP; 2008 00030333 and DURAREP 2; 2011 00064290) and the European Union Framework 6 funding program (MEXC-CT-2006-042499). R J Scaramuzzi was the recipient of an EU Marie Curie Chair of Excellence (MEXC-CT-2006-042499). A Chanvallon was the recipient of a PhD studentship jointly funded by the 'Convention Industrielle de Formation par la Recherche', the 'Centre Interrégional d'Information et de Recherche en Production Ovine' and the 'Association Nationale de la Recherche et de la Technologie'.

Acknowledgements

The authors wish to thank the staff from INRA experimental stations in Nouzilly, Bourges and Frejorgues for the care of the animals and for their help during the experiments. They would also like to thank several MSc students, S Bertaux, C Buron, M David, A Durand, J Gauthier, C Journault, A Pelletier and O Robineau, who participated in various aspects of these experiments.

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Received 7 November 2014

First decision 2 January 2015

Revised manuscript received 20 January 2015

Accepted 18 February 2015