

## Research article

## Light-induced sexually active rams provoke LH preovulatory surges and enhances LH concentrations in ewes after progestagen treatment

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## ABSTRACT

The effect of the introduction of sexually active rams in spring on LH secretion in ewes after progestagen treatment has been studied. Two rams were induced into a sexually active state by exposure to 2 months of long days (16 h of light/d) from 1 January (SAR), and another 2 rams were exposed to the natural photoperiod, so that they were not sexually activated in spring (control; C). At the end of the long-day period, rams were returned to natural photoperiod conditions. Fifteen ewes synchronized in estrus by intravaginal sponges were assigned to three groups at sponge withdrawal (hour 0): SAR (n = 5), exposed to SAR rams; C (n = 5), exposed to C rams, and ISO (n = 5), kept isolated from rams. Twenty-four hours after pessary removal (hour 0), rams were introduced into the SAR and C groups. Three SAR ewes presented preovulatory LH surges; the proportion was significantly ( $P < 0.05$ ) higher in this group (3/5) than in the others (C: 0/5, ISO: 0/5). SAR introduction induced a more marked ( $P < 0.05$ ) increase in mean LH plasma concentrations (before rams:  $1.34 \pm 0.19$ ; after rams:  $6.94 \pm 2.66$  ng/ml) than C (before:  $0.96 \pm 0.29$ ; after:  $3.60 \pm 1.44$ ) or ISO (before:  $1.26 \pm 0.42$ ; after:  $2.14 \pm 1.36$ ) groups, and significantly ( $P < 0.05$ ) higher plasma LH levels after ram introduction. In conclusion, only light-treated sexually activated rams induced LH preovulatory surges in ewes in the seasonal anestrus, when ewes are synchronized with progestagen treatment in the absence of eCG.

## 1. Introduction

Photoperiod is one of the most significant highlights of sheep reproductive physiology, which presents a clear seasonal pattern [1]. Its annual variation induces changes in the activity of the hypothalamic-pituitary axis, altering pulsatile gonadotrophin releasing hormone (GnRH) discharges, and in turn, luteinizing hormone (LH) secretion, so that seasonal changes in ovine reproductive condition are detected; those changes reflect differences in sensitivity to the negative feedback of circulating estradiol [2]. Thus, the negative feedback of estradiol increases when day length increases, reducing dramatically the secretion of GnRH and LH, and avoiding ovulations. In contrast, the estradiol negative feedback decreases when day length decreases, so that the secretion of GnRH and LH increase and ovulations to occur.

Biostimulation, defined as the presence of a male to stimulate the reproductive characteristics of females, such as onset of puberty, estrous signs, ovulation induction, etc, has been used in the last few years as a useful tool to reduce the use of exogenous hormones and drugs to control and improve the productivity of sheep and goats [3]. With that in mind,

we have developed management protocols for sheep flocks that raised the possibility of sustainable systems for the reproductive management of sheep. We have shown that the presence of rams sexually activated in spring by exposure to long days for two months, with or without melatonin implants, lengthen ovarian and estrous activity in Rasa Aragonesa ewes in spring, practically eliminating their seasonal anestrus [4], moves forward the reactivation of sexual activity in ewes in the middle of the seasonal anestrus, after lambing at the end of the reproductive season [5], and produces early puberty of ewe-lambs born in September [6]. In addition, those photoperiod-melatonin-treated, sexually activated rams, when used in a ram effect protocol, significantly increase the number of ewes that become pregnant and the number of lambs born per ewe in May on conventional [7] and organic farms [8].

Recently, we demonstrated that the continuous presence of sexually active rams in spring avoids the seasonal decrease in plasma LH concentrations, probably, by preventing the seasonal negative feedback of estradiol on LH secretion [9]. Thus, the hypothesis of the present study was that the presence of sexually active rams -maintained by photoperiod therapy-prevents a seasonal drop in LH plasma concentrations in females,

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and that the annual photoperiodic inhibition of LH central activity can be overridden by sociosexual stimuli in sheep. To investigate that possibility, anestrus ewes were synchronized in estrus in March and were abruptly exposed to control or light-treated rams.

## 2. Material and methods

The experiment was conducted at the experimental farm of the University of Zaragoza, Spain (41° 40'N 0° 53' W). The Ethics Committee for Animal Experiments at the University of Zaragoza approved all of the procedures performed in the study. The care and use of animals were in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

### 2.1. Rams

Four sexually experienced adult Rasa Aragonesa rams (5–7 years of age, LW: 102 ± 5 kg, BCS: 3.22 ± 0.10) were kept permanently in a shaded open pen under natural photoperiod before the photoperiodic treatments. The photoperiodic-treated rams were induced into a sexually active state by exposure to two months of long days (16 h of light/d) in an open pen between 1 Jan and 2 Mar (Sexually Activated rams, SAR, n = 2). Control rams (n = 2) were kept in a shaded, open pen and exposed to natural photoperiod (15 h and 12 min, and 9 h and 10 min of light at the summer and winter solstices, respectively) so that they were not sexually activated in spring (control rams, C). Lighting was controlled by an electronic timer, using artificial light in the morning (06:00 to 09:00) and at night (16:00 to 22:00), and light intensity was at least 300 lx at the level of the eyes of the animals [10]. At the end of the long-day period, rams were returned to natural photoperiod conditions.

Rams were offered a diet formulated to fulfil their maintenance requirements. The diet comprised 0.75 kg of pellets and 1 kg of barley straw per day, providing 8.5 MJ of metabolizable energy per ram. The pelleted diet consisted of barley (85%) and soy bean (15%). The animals had unrestricted access to water and mineral supplement.

### 2.2. Ewes

Anovulatory Rasa Aragonesa ewes (n = 15, two years of age, LW: 55 ± 4 kg, BCS: 3.42 ± 0.25), which had been isolated from rams for at least 4 mo, were synchronized in estrus using intravaginal sponges that contained 30 mg fluorogestone (FGA) (Syncro-Part, CEVA Salud Animal, Barcelona, Spain) for 12 d. At sponge withdrawal (20 Mar), ewes were assigned to one of three groups that were housed in different barns: SAR group (n = 5), exposed to SAR rams, C group (n = 5), exposed to C rams, and ISO group (n = 5), which were kept isolated from rams throughout the experiment. Twenty-four hours after pessary removal (hour 0), rams were introduced into the SAR and C groups.

Ewes were chosen from the experimental flock at the University of Zaragoza after their ovarian state had been confirmed based on two weekly transrectal scans, before sponge insertions. A ewe was considered anovulatory if the corpus luteum was absent in the two ultrasonographs. They were fed 0.42 kg of pellets and 0.70 kg of barley straw per day, providing 7.8 MJ of metabolizable energy per ewe. The pelleted diet was the same than that offered to rams.

### 2.3. LH surge determination

Blood samples were obtained at 6-hour intervals after sponge withdrawal and until ram introduction, 24 h later. Thereafter, samples were collected at 4-hour intervals until 60 h after ram introduction. The onset of a preovulatory LH surge was indicated by the first of three consecutive samples that had a plasma LH concentration of ≥5 ng/ml. The end of a preovulatory LH surge was indicated by the first of three consecutive samples that had a plasma LH concentration of ≤5 ng/ml. The duration of

a preovulatory LH surge was the number of hours between the onset and the end of the preovulatory surge [11].

### 2.4. LH determination

Samples were collected by jugular venipuncture into 5-ml heparinized tubes. Immediately after collection, the samples were centrifuged at 3000 x g for 20 min, and the resultant plasma was stored at -20 °C until LH analysis, which was assayed by RIA [12]. The sensitivity of the LH assay was 0.1 ng/ml and the intra-assay coefficient of variation was 5.5%. All samples were run in a single assay.

### 2.5. Statistical analyses

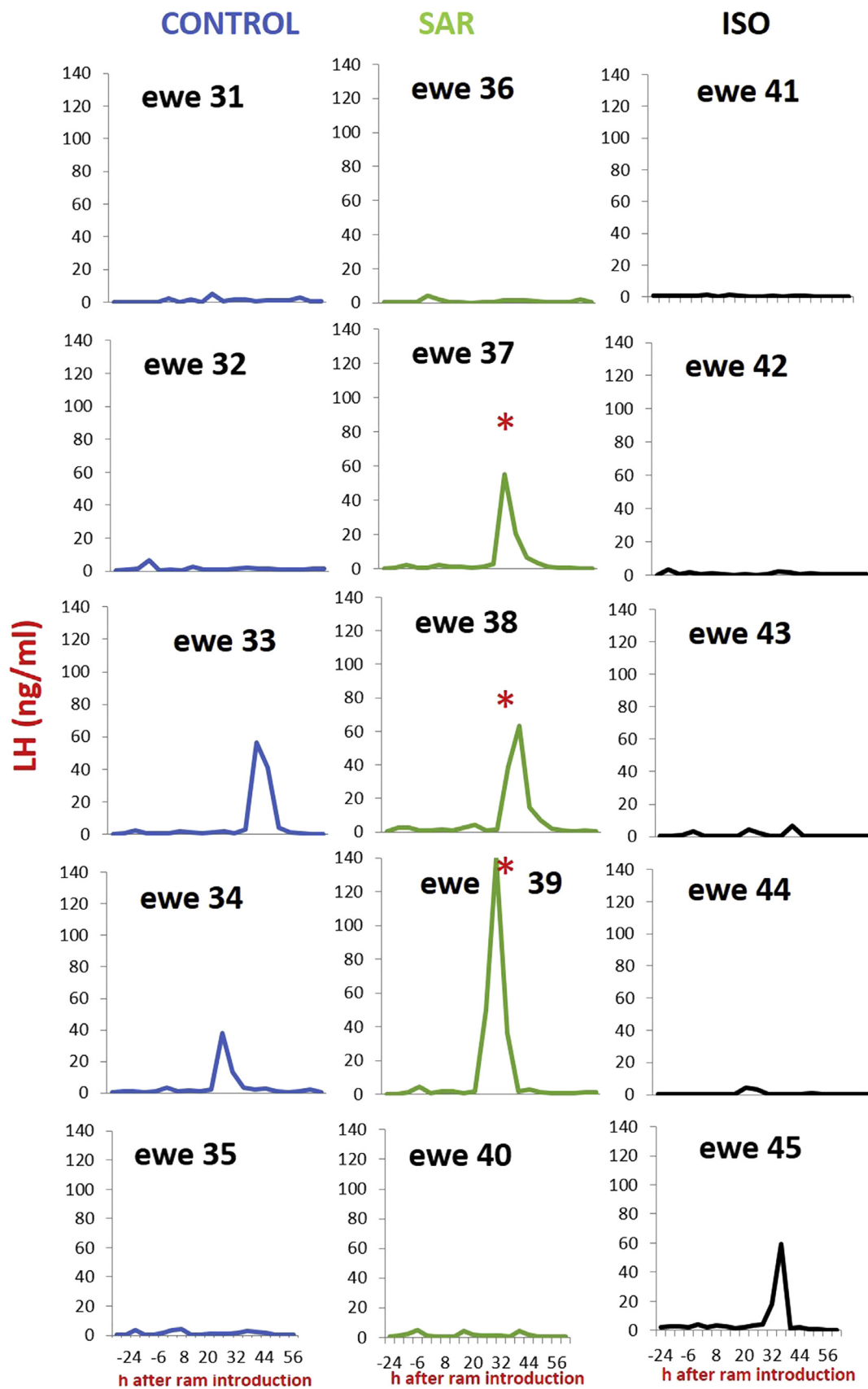
Differences in the proportion of the ewes in each group that displayed estrus or an LH surge within the period of observation were evaluated statistically by chi-square tests. Differences in mean LH concentrations, LH peak amplitude, the area under the curve, the onset of LH surge and its duration were compared by ANOVA with the presence of SAR or C rams, or isolation as the main effect. Differences in the LH levels of each group before and after ram introduction were compared by paired T-test for related samples.

## 3. Results and discussion

Four of five ewes in the SAR and in the C group presented estrus signs. Three SAR ewes presented preovulatory LH surges and the proportion of ewes that presented an LH surge was significantly ( $P < 0.05$ ) different among groups (SAR: 3/5, C: 0/5, ISO: 0/5) (Figure 1). Before ram introduction, plasma LH levels were similar among groups; however, after ram introduction, SAR ewes presented significantly ( $P < 0.05$ ) higher LH levels than did the ewes in the other groups (Table 1). Furthermore, the SAR group, only, experienced a significant ( $P < 0.05$ ) increase in LH levels after ram introduction. None of the ewes in the C and ISO groups presented an LH preovulatory surge; therefore, differences among groups in LH peak amplitude, the area under LH curve, and the onset of the LH surge were not compared.

This experiment has confirmed the results of previous experiments with the same breed at the same latitude [9], in which either the continuous presence of light-treated sexually activated rams in spring, or their sudden introduction into a group of anestrus ewes of this work, counterbalance and prevents the seasonal decrease in plasma LH concentrations, probably, by altering the seasonal negative feedback of estradiol on LH secretion. In addition, the results of this experiment are similar to those involving goats, in which the permanent presence of sexually active bucks or their introduction prevented a decrease in plasma LH concentrations in OVX + E2 goats in the seasonal anestrus, or increased their plasma LH concentrations, respectively [13].

Although sexual-performance tests were not performed in this experiment to avoid the effect of previous contact with females on sexual activity, especially in the control rams, our previous studies have confirmed that light-treated rams exhibit higher sexual behavioral expression [14] than do rams kept under the natural seasonal photoperiod, and have high plasma testosterone concentrations in spring [6, 7, 14]. It has been found [15] that the proportion of ewes that ovulated was highest in groups that had been exposed to rams that exhibited intense, rather than moderate, sexual behavior, and concluded that, in addition to a pheromone signal, the sexual intensity of the ram is important in initiating ovarian cycles. In goats, bucks subjected to photoperiodic treatments that were similar to those used in the present experiment to stimulate their sexual activity in spring were more efficient in stimulating LH pulsatility and ovulations in intact goats than were untreated males that displayed low levels of sexual behavior [16, 17]. In addition, the sexual behavior of males contributes to the maintenance of high LH pulsatility up to 24 h after introduction into a group of anovulatory goats [18], and the expression of intense sexual behavior by male goats is



**Figure 1.** Plasma LH concentrations of Rasa Aragonesa ewes that had been either exposed in late March to rams that had been induced into a sexually active state by exposure to 2 months of long days from 1 Jan (SAR), or rams exposed to natural photoperiod variations (C), or kept isolated from rams (ISO). \* indicates an pre-ovulatory LH surge, which was indicated by the first of three consecutive samples that had a plasma LH concentration of  $\geq 5$  ng/ml [11].

**Table 1.** Mean ( $\pm$ S.E.) plasma LH concentrations before (from sponge withdrawal until 6 h before ram introduction, 24 h later) and after ram introduction (4–60 h after ram introduction), and LH peak value, area under the LH curve, onset of the LH surge and its duration, and the onset of estrus for SAR, C, and ISO Rasa Aragonesa ewes. They had been exposed to rams that were induced into a sexually active state by exposure to 2 months of long days from 1 Jan (SAR), rams exposed to natural photoperiod variations (C), or kept isolated from rams (ISO). A preovulatory LH surge was defined as the first of three consecutive samples that had a plasma LH concentration of  $\geq 5$  ng/ml. The end of a preovulatory LH surge was defined as the first of three consecutive samples that had a plasma LH concentration of  $\leq 5$  ng/ml. The duration of a preovulatory LH surge was the number of hours between the onset and the end of the preovulatory surge [11]. x, y Differences before vs. after ram introduction,  $P < 0.05$ ; a,b indicate significant differences among groups at  $P < 0.05$ .

	LH before rams (ng/ml)	LH after rams (ng/ml)	LH Peak (ng/ml)	Area under LH curve	Onset LH surge (h after ram introduction)	Duration LH surge (h)	Onset estrus (h after ram Intr.)
SAR	1.34 $\pm$ 0.19 <sup>x</sup>	6.94 $\pm$ 2.66 <sup>ya</sup>	86.08 $\pm$ 26.98	848 $\pm$ 321	28 $\pm$ 2	13 $\pm$ 1	45 $\pm$ 4
C	0.96 $\pm$ 0.29	3.60 $\pm$ 1.44 <sup>b</sup>	n/a	n/a	n/a	n/a	45 $\pm$ 4
ISO	1.26 $\pm$ 0.42	2.14 $\pm$ 1.36 <sup>b</sup>	n/a	n/a	n/a	n/a	n/a

necessary to induce a LH preovulatory surge and ovulation in seasonally anovulatory goats [19].

Some animals of the SAR and C groups presented estrus signs with no LH peak; in fact, the levels of estradiol required to induce the GnRH/LH surge are higher than that needed to induce estrous behavior [20]. In our experiment, two control ewes responded to the introduction of non-treated rams with a slight increase in their LH levels, although they were not LH surges. Elsewhere, we showed that ewes housed with control rams exhibit a high frequency of silent ovulations in the seasonal anestrus [4] or halfway LH concentrations compared to OVX + E2 ewes that had been exposed to either treated rams or kept isolated from them [9], which suggests that the rams of Mediterranean breeds that are exposed to the natural photoperiod exhibit a 'residual' sexual activity that can influence ewes in the control groups, but are not sufficiently sexually active to induce complete GnRH/LH activity in the ewes.

#### 4. Conclusion

In conclusion, only light-treated sexually activated rams induced LH preovulatory surges in ewes in the seasonal anestrus, when ewes are synchronized with progestagen treatment in the absence of eCG.

#### Declarations

##### Author contribution statement

J. Abecia: Conceived and designed the experiments; performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

P. Chemineau and J. Delgado: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Keller: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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##### Competing interest statement

The authors declare no conflict of interest.

##### Additional information

No additional information is available for this paper.

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