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Exposure of rams in sexual rest to sexually activated males in spring increases plasma LH and testosterone concentrations

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ABSTRACT

Eight stimulating rams, and twelve stimulated rams, were used to determine whether a similar endocrine response to the introduction of sexually active males in spring in a flock of ewes is observed in a flock of rams. The stimulating rams (n = 4) were induced into a sexually active state by exposure to 2 months of long days (16 h light/d) (15 December-15 February). At the end of the long-day period, rams were returned to the natural photoperiod. Control-stimulating rams (n = 4) were kept under the natural photoperiod. On April 20, stimulated rams were divided into 2 groups, and joined with activated (ACT; n = 6) or control stimulating rams (C; n = 6). On the day of ram introduction, stimulated rams were blood sampled for 8 h at 20-min intervals, from 4 h before to 4 h after ram introduction, and next day from 24 to 28 h after ram introduction, and analyzed for plasma LH concentrations, and 10, 20 and 30 days after ram introduction to measure plasma testosterone levels. Mean (±SEM) plasma LH concentrations (ng/ml) of stimulated rams were similar during the 4 h before stimulating-ram introduction (ACT: 0.59 ± 0.03; C: 0.53 ± 0.04; P > 0.05). The introduction of the photoperiod-treated stimulating rams increased LH concentrations of stimulated rams during the 4 h after their introduction (1.14 ± 0.37) compared with the C group (0.51 ± 0.03; P < 0.05), especially during the first hour (ACT: 0.93 ± 0.16; C: 0.49 ± 0.03; P < 0.05), and during the blood sampling period 24–28 h after ram introduction (0.75 ± 0.07 vs. 0.58 ± 0.04; P < 0.05). Before the introduction of stimulating rams, the LH pulse frequencies and amplitudes did not differ between groups; however, LH pulsatility was higher at 4 h (0.58 ± 0.11 pulses/h; P < 0.05), and had trend to be higher 24 h (0.50 ± 0.06) (P = 0.10) after the introduction of the photoperiod-treated stimulating rams compared with the control-stimulating rams (0.29 ± 0.08 and 0.29 ± 0.10, respectively). As for LH pulses, there was an effect of group (P < 0.05) on LH amplitude, which presented a trend to be higher in ACT rams 4 h after ram introduction (1.68 ± 0.30; P < 0.10) and higher 24 h (1.07 ± 0.08; P < 0.05) after ram introduction, compared with LH amplitudes of C rams (0.71 ± 0.06 and 0.82 ± 0.07, respectively). Plasma testosterone concentrations of rams exposed to photoperiod-treated activated rams were higher than those of rams exposed to control-stimulating rams, at 4 h, 20 and 30 days after ram introduction (P < 0.05). In conclusion, sexually active rams in spring are able to stimulate LH and testosterone secretion of other rams in sexual rest, a phenomenon we called "ram-to-ram effect".

1. Introduction

Changes in the negative feedback of testosterone (rams) and estradiol (ewes) on LH secretion are the key factors influencing the seasonality of reproduction in sheep [1]. The well-known "ram effect," which is generated by rams housed with anovulatory ewes previously isolated from rams [2], induces a proportion of the flock to ovulate, displaying estrus, and getting pregnant, can profoundly alter the timing of the sexual season. The use of 'biostimulation' (i.e., the stimulatory effects on reproductive characteristics of

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females, such as the onset of puberty, estrous expression, and ovulation induction, that are induced by the presence of a male) in place of exogenous hormones to control and improve the productivity of sheep and goats, could be useful to consider in the future [3].

We found that rams rendered sexually active by exposure to long days for two months in the spring, which have higher plasma testosterone concentrations than untreated-rams, and a sexual activity similar to the breeding season, extend the ovarian and estrous activity of Rasa Aragonesa ewes, effectively inhibiting their seasonal anestrus [4]. Since sexually active rams induced LH preovulatory surges in ewes in the seasonal anestrus [5], the continuous presence of these rams prevented the seasonal decrease in plasma LH concentrations in OVX + E ewes, preventing the seasonal negative feedback of estradiol on LH secretion [6].

The possibility that these mechanisms can act in the opposite direction has been investigated, and a “ewe to ram” effect has been reported, in which the continuous presence of ewes in estrus in spring increased rams' testicular volume, some testicular echogenic characteristics were modified, and they had higher testosterone levels, with no changes in LH pulsatility [7]. We recently discovered for the first time that bucks rendered sexually active in spring by a photoperiodic treatment, stimulated the LH and testosterone secretion in bucks in seasonal sexual rest, a phenomenon known as the “buck-to-buck effect” [8]. We expected that the abrupt introduction of sexually active rams would promote LH and testosterone secretion in rams during their seasonal sexual rest, because sexually active rams are particularly effective at stimulating endocrine and ovulatory activity in seasonally anestrous ewes. The goal of this study was to determine how rams in sexual rest responded to photoperiod-treated, sexually active rams in the short term, a phenomenon that we called the “ram-to-ram effect".

2. Materials and methods

2.1. Ethical note

The research was carried out at the experimental farm of the University of Zaragoza (latitude: 41° 63′ N, longitude: 0° 53′ W). All of the procedures used in the study were authorized by the University of Zaragoza's Animal Experiments Ethics Committee. The Spanish Policy for Animal Protection (RD 53/2013), which complies with the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific reasons, was in charge of animal care and usage.

2.2. General conditions of the study

Males of the Rasa Aragonesa breed, which is emblematic of the “Mediterranean” sheep breeds, were used in this study. Ewes in the northern part of Spain have a three-month anestrous season from April to July, despite the fact that 10–45% of ewes ovulate in the spring [9], and their LH secretion is sensitive to photoperiod changes [10,11]. The sexual activity of Rasa Aragonesa rams can be promoted by melatonin treatments, and show a strong seasonal fluctuation in semen characteristics [12–14]. Rams were fed to satisfy their LW requirements and allowed full access to water and mineral salts [15].

2.3. Experimental design

2.3.1. Stimulating rams

Before the photoperiodic treatments, eight rams were maintained in an open enclosure (6 × 12 m) under natural photoperiodic settings and divided into two groups [4]. One group was exposed to 2 months of long days (16 h of light/day) in a closed pen (5 × 7 m; 8.75 m^2/ram) between 15 December and 15 February, to stimulate their endocrine and sexual activities (photoperiod-treated activated stimulating rams, n = 4). An electrical timer controlled the lighting, and the light intensity at the level of the animals' eyes was at least 300 lx. Rams were returned to natural photoperiod circumstances at the conclusion of the long days (daylength: 10 h and 35 min). The remaining rams were kept under natural photoperiod conditions (15 h 12 min, and 9 h 10 min of light at the summer and winter solstices, respectively; control-stimulating rams, n = 4) (Fig. 1).

The effects of photoperiod on rams' sexual state were measured using plasma testosterone concentrations. Blood samples were taken monthly from both groups of stimulating rams from the start of the photoperiod therapy until two months after the natural photoperiod was resumed.

2.3.2. Stimulated rams

On April 20, a total of twelve stimulated rams were randomly assigned to one of two groups, which were joined with photoperiod-treated activated stimulating rams (ACT; n = 6) or with control-stimulating rams (C; n = 6). The distances between the two groups of males were more than 300 m, and there was no opportunity for them to see each other, therefore there was no risk of interfering. For 30 days, stimulated rams were housed with stimulating rams.

2.3.3. Measurements

Plasma LH concentrations in stimulated rams were measured every 20 min from 4 h before (7:30–11:30) to 4 h after (11:45–15:10) the introduction of stimulating rams (11:30, hour 0), and again the next day during 4 h (11:30–15:10) to see if any effect observed immediately after the introduction of rams would last longer. The concentrations of testosterone in each group of stimulated males were measured before (−4 h) and after (4 h) the introduction of stimulating males. Following that, testosterone levels were measured on days 10, 20, and 30 following the introduction of males (Fig. 1).

Jugular venipuncture was used to collect all blood samples, which were then placed in heparinized tubes. The samples were immediately centrifuged at 3000×*g* for 10 min after collection, and the plasma samples obtained were kept at −20 °C until RIA analysis. Luteinizing hormone was measured according to Faure et al. [16] in a single assay. The intra-assay CV was 7.2% and the sensitivity was 0.1 ng/mL. In a single assay, testosterone was measured according to Garnier et al. [17]. The intra-assay CV was 8.2% and the sensitivity was 0.1 ng/mL.

2.4. Statistical analysis

To detect differences between treatments, data on plasma LH and testosterone concentrations, as well as LH pulse frequency and amplitude, were examined using a two-way repeated-measures ANOVA. The treatment (group), the sampling period (hours or days), and the interaction between these parameters were all included in the model. When there were significant interactions, the Tukey test was applied for two-by-two individual point comparisons within sampling periods (4 h before the introduction of rams, 4 h after the introduction of rams, and 24–28 h after the introduction of rams). When there were significant interactions, the Student's *t*-test was utilized for two-by-two individual point comparisons. We also estimated the pooled mean of plasma LH concentrations using 4-h time windows (4 h before the introduction of rams, 4 h after introduction of rams, and 24–28 h after introduction of rams). The Tukey test was used for post-hoc

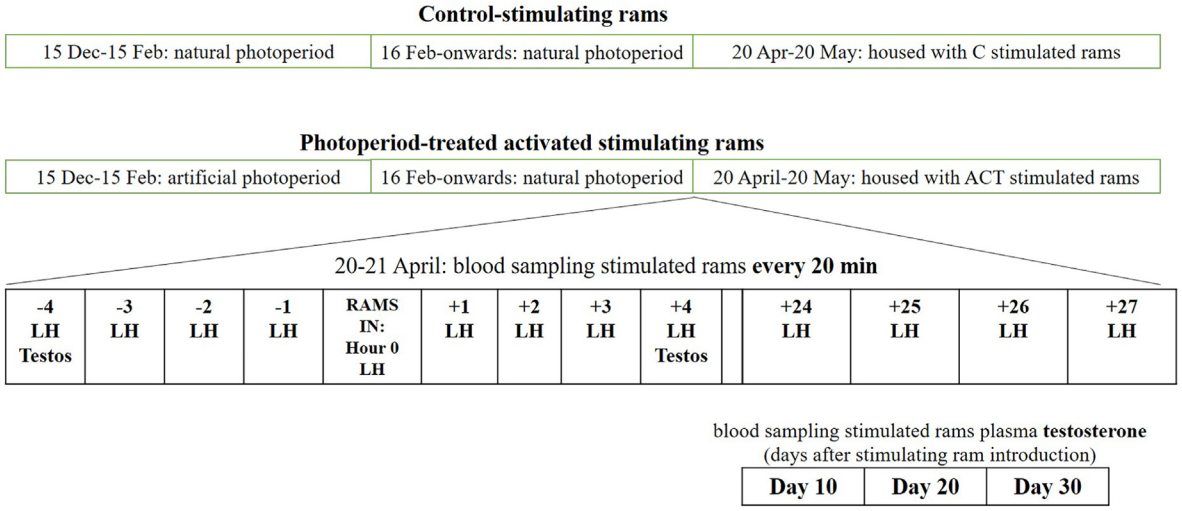

Fig. 1. Experimental design: Between 15 December and 15 February photoperiod-treated activated stimulating rams (n = 4) were exposed to 2 months of long days (16 h of light/day), and were returned to natural photoperiod at the conclusion of the long days. The control-stimulating rams (n = 4) were kept under natural photoperiod conditions. On April 20, 12 stimulated rams were joined either with photoperiod-treated activated stimulating rams (ACT; n = 6) or with control-stimulating rams (C; n = 6), for 30 days. Plasma LH concentrations in stimulated rams were measured every 20 min from 4 h before to 4 h after the introduction of stimulating rams (hour 0), and again the next day during 4 h. The concentrations of testosterone in stimulated males were measured before (−4 h) and after (4 h) the introduction of stimulating males, and on days 10, 20, and 30 following the introduction of males.

comparisons after the two-way ANOVA (group and time of sample) was used to evaluate the means of LH concentrations. When two consecutive values were greater than the two preceding values, and the highest (pulse amplitude) value exceeded the mean basal value by at least four fold the assay's coefficient of variation, an LH pulse was declared to have occurred. The differences were considered significant at the threshold of $P \leq 0.05$, and the data were reported as mean ± SEM.

3. Results

3.1. Plasma testosterone concentrations of stimulating rams

The two groups of rams exhibited equal plasma testosterone levels at the start of the light treatment (15 December) (Fig. 2). Control-stimulating rams had raised their concentrations after 30 days of the photoperiod therapy of the photoperiod-treated activated rams, however there were no statistical differences. Both groups had similar testosterone concentrations at the end of the light treatment of the photoperiod-treated activated rams (15 February), but the activated rams had higher plasma testosterone concentrations than the control rams two months after the photoperiodic treatment (15 April), just before joining the stimulated rams ($P < 0.05$) (Fig. 2).

3.2. Plasma LH concentrations and pulsatility

Plasma LH concentrations were similar during the 4 h before the

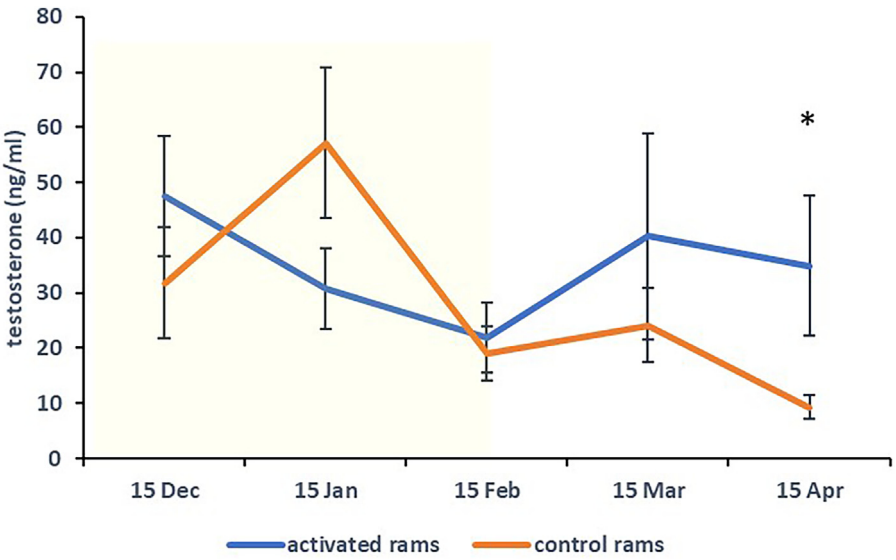

Fig. 2. Plasma testosterone concentrations (mean ± SEM) of rams housed under the natural photoperiod at 41°N (control-stimulating rams) or exposed to two months of long days (16 h of light/d) (300 lx) −photoperiod-treated activated stimulating rams-; at the end of the long-day period, activated rams were returned to the natural photoperiod. Yellow area indicates light treatment. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stimulating-ram introduction, although there was a group effect ($P < 0.05$) on plasma LH concentrations (Table 1). The introduction of the ACT rams led to a dramatic increase in LH plasma concentrations, which remained elevated till the next day (Fig. 3). Photoperiod-treated activated rams increased LH concentrations in stimulated rams over the 4 h after their introduction ($P < 0.05$), especially during the first h (ACT: 0.93 ± 0.16; C: 0.49 ± 0.03 ng/mL; $P < 0.05$), and during the blood collection period 24–28 h after ram introduction ($P < 0.05$) (Table 1).

For LH pulse frequencies, there was a group effect ($P < 0.05$) and an interaction between time and groups ($P = 0.095$). The LH pulse frequencies and amplitudes did not differ across groups prior to the introduction of stimulating rams (Table 1). When compared to C rams, LH pulsatility was higher at 4 ($P < 0.05$) and 24 h ($P = 0.10$) after the introduction of the photoperiod-treated activated rams. There was also a group effect ($P < 0.05$) on LH amplitude, with a trend to be larger in ACT rams 4 h after ram introduction ($P = 0.10$) and larger 24 h ($P < 0.05$) after ram introduction than in C rams (Table 1).

3.3. Plasma testosterone concentrations of stimulated rams

There was an effect of time ($P < 0.05$) and group ($P < 0.01$), and an interaction between time and group ($P < 0.05$) for plasma testosterone concentrations of the stimulated rams. Prior to the introduction of rams, they were low and did not differ between groups (Fig. 4). After that, 4 h after the stimulating rams were introduced, plasma testosterone concentrations in males joined with the photoperiod-treated activated rams were higher than those in males joined with the control-stimulating rams ($P < 0.05$). Similarly, testosterone concentrations in ACT rams were higher than those in C rams 20 and 30 d after the rams were introduced ($P < 0.05$; Fig. 3).

4. Discussion

The findings of this experiment support our hypothesis that sexually active rams in the spring can promote the release of LH and testosterone in other rams who are in a seasonal sexual pause. Thus, exposure to the control, non-active rams had no effect on LH and testosterone secretion of stimulated rams, but the photoperiod-treated activated rams, via the phenomenon that we called the ram-to-ram effect, boosted their LH concentration, amplitude, and pulsatility, as well as plasma testosterone concentrations. These findings are the first to show that rams made sexually active by exposure to a photoperiodic treatment can promote endocrine activity during the seasonal sexual rest, and they confirm our prior results in goats, where the buck-to-buck effect was documented [8]. Overall, our findings show that, when combined with sexually active males, socio-sexual interactions can be employed to enhance male sexual activity during sexual rest in small ruminant males.

We have reported that only light-treated, sexually activated rams induced LH preovulatory surges in ewes in the seasonal anestrus, when ewes are synchronized with progestagen treatment, while control, non-activated rams did not [5]. Furthermore, the current results are comparable to those obtained in goats, in which the permanent presence of sexually active bucks or their introduction prevented or enhanced plasma LH concentrations in OVX + E2 goats, respectively, during seasonal anestrus [18]. Furthermore, a continual presence of sexually active rams or goats was found to suppress the natural seasonal decline in plasma LH concentrations in anestrous ewes, most likely by blocking the seasonal negative feedback of estradiol on LH production [6,19,20]. The key neuroendocrine activity in charge of reproductive seasonality in this species is photoperiod, which is responsible for fluctuations in testosterone negative feedback on LH secretion in rams [21]. Increased gonadal activity during the breeding season is achieved by greatly elevating gonadal responsiveness, with relatively little or no increase in GnRH and LH secretion, as a result of a higher efficiency of negative feedback on the hypothalamus caused by relatively low concentrations of gonadal steroids. In sheep and goats, this seasonal mechanism is able to cause sexual rest [22–24]. The enhanced LH secretion of stimulated rams generated by photoperiod-treated activated rams in our study is most likely due to a reduction or adjustment of the testosterone negative feedback on LH.

Rams joined with the photoperiod-treated activated rams presented a rise in their plasma testosterone concentrations 4 h after the introduction of the rams, and was maintained until 20–30 days later. It is likely that the photoperiod-treated activated rams acted at the kisspeptin neuron level of the arcuate nucleus in charge of the estradiol negative feedback on GnRH secretion, which in turn controls LH secretion [25]. Moreover, in rams, seasonality also induces changes in the testosterone negative feedback on LH secretion, establishing the principal neuroendocrine mechanism in control of for reproductive seasonality. This negative feedback of testosterone enlarges during long days, lessen the secretion of LH and then evoking sexual rest [22]. However, testosterone concentration of light-treated rams did not increase until mid-April, 2 months after the end of the long-day treatment, as we have reported previously, when rams stimulated with long days during the winter do not present high levels of testosterone until 6–7 weeks of the return to the natural photoperiod [26,27]. Again, these results indicate that the sexual-activated rams immediately stimulated the hypothalamus-pituitary-gonads axis of the rams in sexual rest, as observed in ewes joined with similar rams [5]. In an experiment designed to determine the effect of the presence of sexually-activated rams by photoperiod treatment on the onset of puberty of ram-lambs born in autumn [6], we observed that ram-lambs that were reared in the company of sexual-activated adult rams exhibited higher levels of testosterone in the prepuberal period in spring than did ram-lambs that had been kept with non-activated adult rams, although similar to those reared in isolation.

Overall, the findings of this study show that sexually active rams boost the sexual endocrine activity of other rams during the

Table 1
Mean (±SEM) plasma LH concentrations (ng/ml), pulsatility (pulses/h) and amplitude (ng/ml), 4 h before (PRE), 4 h after (POST), and 24 h after the introduction of activated (ACT) rams, rendered sexually active by exposure to 2 months of long days (16 h of light per day) from 15 December, followed by natural photoperiod conditions, or joined with control (C), non-activated rams. a,b $P < 0.05$; c,d $P \leq 0.10$.

	PRE		POST		24 h	
	ACT	C	ACT	C	ACT	C
Plasma concentration (ng/ml)	0.59 ± 0.03	0.53 ± 0.04	1.14 ± 0.37^{a}	0.51 ± 0.03^{b}	0.73 ± 0.07^{a}	0.58 ± 0.04^{b}
Pulsatility (pulses/h)	0.25 ± 0.09	0.29 ± 0.10	0.58 ± 0.11^{c}	0.29 ± 0.08^{d}	0.50 ± 0.06^{a}	0.29 ± 0.10^{b}
Amplitude (ng/ml)	0.94 ± 0.02	0.84 ± 0.05	1.68 ± 0.30^{a}	0.71 ± 0.06^{b}	1.07 ± 0.08^{c}	0.82 ± 0.07^{d}

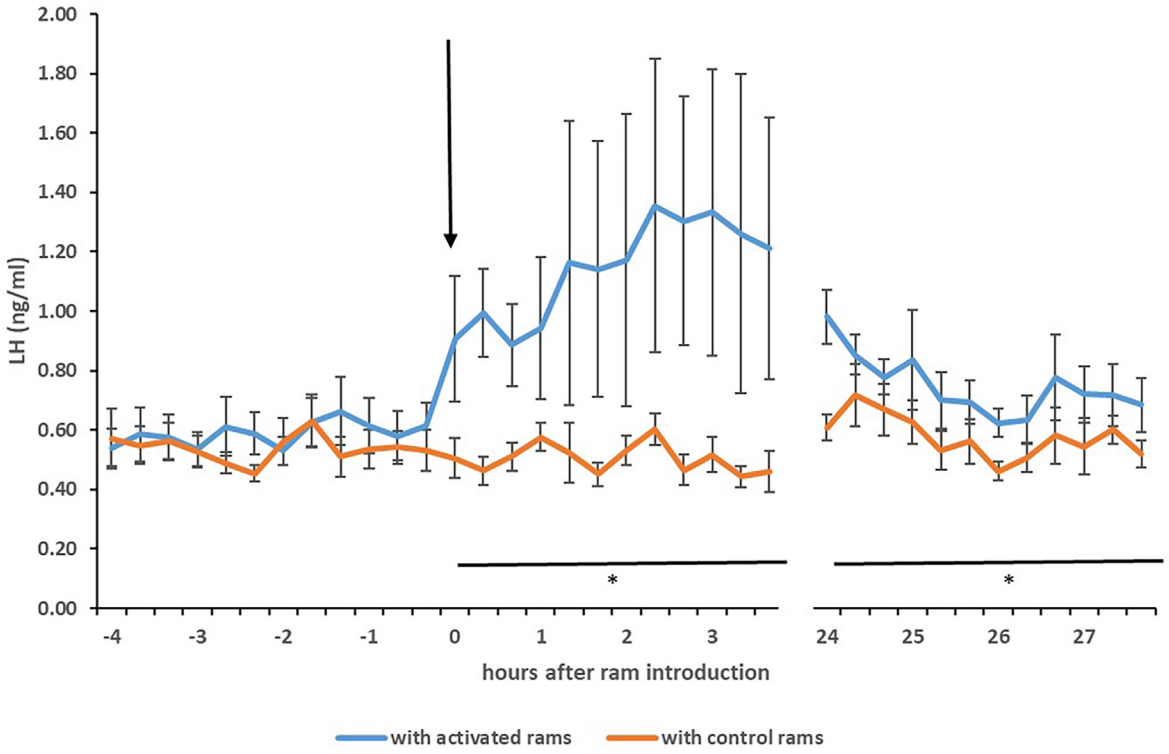

Fig. 3. Plasma LH concentrations (mean ± SEM) of rams housed with photoperiod-treated activated stimulating rams, rendered sexually active by exposure to 2 months of long days (16 h of light per day) from 15 December, followed by natural photoperiod conditions, or housed with control-stimulating, non-activated rams. *P < 0.05. ↓ Indicates the moment of introduction of the rams in each group.

seasonal sexual rest in the spring. These stimulations took place at the pituitary-goads axis and lasted at least a month.

5. Conclusion

In conclusion, the so-called by us "ram-to-ram effect" can enhance the endocrine activity of rams in sexual rest if they are joined with rams who have been made sexually active by a photoperiodic treatment. These findings, together with similar ones reported by our laboratories in male goats [8], suggest that socio-sexual interactions could be used to restrict out-of-season reproduction in these species.

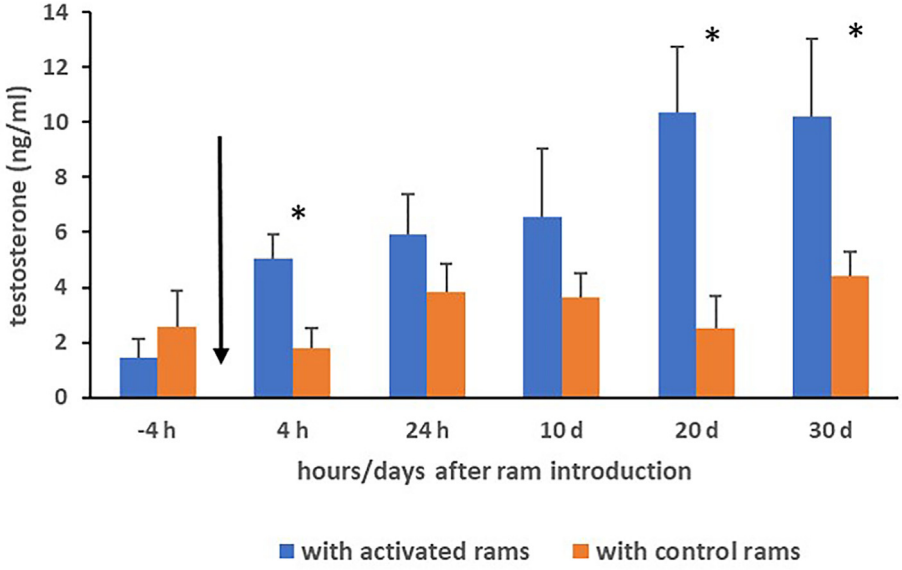

Fig. 4. Plasma testosterone concentrations (mean ± SEM) of rams housed with photoperiod-treated activated stimulating rams, rendered sexually active by exposure to 2 months of long days (16 h of light per day) from 15 December, followed by natural photoperiod conditions, or housed with control-stimulating, non-activated rams. *P < 0.05. ↓ Indicates the moment of introduction of rams in each group.

CRediT authorship contribution statement

José Alfonso Abecia: Conceptualization, Investigation, Formal analysis, Writing - original draft, Funding acquisition, Supervision. **Francisco Canto:** Investigation, Writing-review & editing. **Matthieu Keller:** Conceptualization, Writing - review & editing. **Carlos Palacios:** Investigation, Writing – review & editing. **Philippe Chemineau:** Conceptualization, Writing – review & editing. **José Alberto Delgadillo:** Conceptualization, Writing – review & editing.

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