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TOWARDS A SUSTAINABLE SHEEP REPRODUCTIVE MANAGEMENT: THE PRESENCE OF SEXUALLY ACTIVE RAMS AS A TOOL

Influence of the environment on the sheep milk production
Photoperiod-melatonin-induced, sexually-activated rams increase pregnancy rate and number of lambs per ewe in a ram effect

J.A. ABECIAA, J. ARAYAA, P. CHEMINEAUC, C. PALACIOSD, M. KELLERC, J.A. DELGADILLOB

A IUCA. Departamento de Producción Animal y Ciencia de los Alimentos. Universidad de Zaragoza, Miguel Servet, 177, 50013 Zaragoza, Spain
B Centro de Investigación en Reproducción Caprina, Universidad Autónoma Agraria Antonio Narro, Periférico Raúl López Sánchez y Carretera a Sante Fe, C.P. 27054, Torreón, Coahuila, Mexico
C Physiologie de la Reproduction et des Comportements, UMR INRA, CNRS, Université de Tours, IFCE, Agreenium, 37380 Nouzilly, France
D Departamento de Construcción y Agronomía. Facultad de Ciencias Agrarias y Ambientales. Filiberto Villalobos, 37007, Salamanca, Spain

SUMMARY

Introduction - The use of sexually-activated rams to induce a ram effect in ewes is a practical tool to induce out-of-season sexual activity in ewes.

Aim - To determine whether the introduction of sexually activated rams to a flock of anestrous ewes, to induce a ram effect, would improve the ovulatory response and reproductive performance of Rasa Aragonesa ewes.

Materials and methods - Rams exposed to two months of either long days (16 h of light/d) between 1 Feb and 31 Mar (Treated group, n=4), or the natural photoperiod (Control group, n=4). At the end of the long-day period, rams were returned to the natural photoperiod and received three melatonin implants. Fifty-eight anestrous ewes were assigned to one of three groups: i) mated with Treated rams (TR group, n=17), ii) mated with Control rams (CR group, n=18), or iii) kept isolated from rams during the experiment (Isolated group, n=23). Mating occurred between 11 May and 20 Jun.

Results and discussion - Treated rams performed 81% of nudging, 75% of anogenital sniffing, and 90% of self-urination (P<0.001). Those rams had higher plasma testosterone concentrations than the rams in the Control group at 15 and 30 d (P<0.05) after exposure to the natural photoperiod. All ewes ovulated and presented estrus within the mating period, and 39% of the Isolated ewes began ovulatory activity (P<0.001). TR ewes had a higher proportion of ewes that lambed (100%) and higher fecundity (1.44±0.51 lambs/ewe) than CR ewes (78% and 1.00±0.69 lambs/ewe) (P<0.05).

Conclusions - More ewes that were exposed to sexual-activated rams became pregnant and produced more lambs per ewe than ewes that were exposed to non-treated rams. All ewes in the two groups ovulated; therefore, an increase in the ovulatory response after the ram effect did not cause the improvement in reproductive performance.

KEY WORDS

Sheep, ram effect, extra-light, melatonin, sexual activation.

INTRODUCTION

Reproductive seasonality is the most important factor limiting productivity in sheep farms because lambing occurs at a specific time of year (late winter and spring) which allows lambs to be born at the time the availability of natural food and survival is high. Photoperiod controls reproductive seasonality through circadian secretion of melatonin by the pineal gland, which translates day and night length to the neuroendocrine system. If social or nutritional factors are handled appropriately, seasonal anestrus can be reduced, especially in Mediterranean breeds, which have a shorter seasonal anestrus season than do northern European breeds. The “ram effect”, which involves the reintroduction of males to a flock of ewes in anestrus that had been isolated from rams, is the most well-studied socio-sexual factor for inducing ovulation in sheep during the seasonal anestrus. The practice has been used to advance the breeding season in commercial sheep flocks. After ram introduction, a proportion of the ewes ovulates, present estrus, and become pregnant in the middle of the seasonal anestrus. The introduction of ram induces an increase in the tonic secretion of LH and one or more corpora lutea that have either a normal or short life span associated or not to estrous behavior. For that reason, two peaks of estrus occur at 17-20 d and 21-25 d after ram introduction. Although the ram’s pheromones are the main stimulus in the ram effect, the intensity of the sexual behavior displayed by the ram influences the quality of the response. For example, males categorized as “high sexual performing” stimulated a higher proportion of ewes to ovulate (95%) than did “low sexual performing” rams (78%).
active during the sexual rest by exposure to two months of long days (16 h light) starting between December and February, plus melatonin implants at the end of the artificial photoperiod. This photoperiodic treatment increases plasma testosterone concentrations and improved sexual activity of rams assessed by a serving capacity test\textsuperscript{12,13}. Therefore, the presence of sexually activated rams caused ovarian activity to persist in spring and increased estrous expression\textsuperscript{14}, advanced puberty in autumn-born ewe lambs\textsuperscript{15}, and advanced the resumption of estrous activity in postpartum ewes in spring\textsuperscript{16}. In seasonally anovulatory goats, bucks rendered sexually active by exposure to long days are more efficient than the control ones displaying low sexual behavior to induce LH preovulatory surge and ovulation\textsuperscript{16,17}. Therefore, we hypothesized that the introduction of sexually activated rams to a flock of anestrous ewes to induce a ram effect would improve the ovulatory response and reproductive performance of Rasa Aragonesa ewes. To test this hypothesis, anestrous ewes were exposed to either photoperiodic-treated or control rams in spring.

**MATERIALS 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 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.

**Rams**

The experiment included eight sexually experienced adult Rasa Aragonesa rams (6-9 years of age), which had a mean (± S.E.M.) live weight (LW) of 95 ± 5 kg, and a mean body condition score (BCS) of 3.55 ± 0.10 (on a scale of 0-5 in which 0 = emaciated and 5 = obese\textsuperscript{18}). Before the photoperiodic treatments, the males were kept permanently in a shaded, open pen under natural photoperiod. The photoperiodic-melatonin-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 February and 31 March (Treated group, n=4). Artificial light was provided in the morning (06:00 to 09:00) and the evening (16:00 to 22:00), which was controlled by an electronic timer, and light intensity was >300 lx at the eye-level of the animals\textsuperscript{19}. At the end of the long-day period, rams were returned to the natural photoperiod, and each received three subcutaneous melatonin implants (Melovine, CEV A Salud Animal, Barcelona, Spain). The combination of artificial photoperiod and melatonin treatment increases significantly testosterone secretion in Rasa Aragonesa rams in spring\textsuperscript{21}. Control rams (n=4) were kept in a shaded, open pen and exposed to the natural photoperiod (15 h and 12 min, and 9 h and 10 min of light at the summer and winter solstices, respectively), therefore, they were not sexually activated in spring.

**Ewes and the ram effect**

Fifty-eight anestrous adult Rasa Aragonesa ewes (2-5 years old), which had a mean LW of 58 ± 6 kg, a mean BCS of 3.25 ± 0.07, and been isolated from rams four months before ram introduction, were assigned to one of three groups balanced for LW and BCS: i) ewes exposed to Treated rams (TR group, n=17) between 11 May (ram introduction=day 0) and 20 June (day 40), ii) ewes exposed to Control rams (CR group, n=18) between 11 May (ram introduction=day 0) and 20 June (day 40), and iii) ewes kept isolated from rams during the experiment (Isolated group, n=23) Ewes were selected from the main experimental flock of the University of Zaragoza based on plasma progesterone (P\textsubscript{4}) concentrations in blood samples collected 21, 14, and 1 d before ram introduction. Ewes that had plasma P\textsubscript{4} levels <0.5 ng/ml in the three samples; i.e., anovulatory, were included in the experiment. The three groups of ewes were housed in different barns to prevent any effect on each other through the whole experiment.

Rasa Aragonesa is a local Spanish genotype that has a short (<100 d) anestrous period between May and July, although some (20-40%) ewes might ovulate at other times of the year\textsuperscript{2}. Rams used in this experiment were of the same breed, which exhibits a pronounced seasonal variation in sperm characteristics\textsuperscript{20}.

**Measurements**

Ewes in estrus were identified by the marking harnesses wore by the rams, which left colored marks on the rumps of the ewes. Estrus was confirmed by direct visual observation at 08:00 and 18:00. A female was considered in estrus if she stood immobile when mounted by the ram.

After ram introduction, blood samples were collected once per week to assess ovarian status based on plasma P\textsubscript{4} concentrations. Samples were collected by jugular venipuncture, placed into heparinized tubes, and centrifuged at 3500xg for 30 min. The plasma fraction was stored at −20 °C until P\textsubscript{4} and testosterone concentrations were measured. Based on the analysis, concentrations of plasma P\textsubscript{4} >0.5 ng/ml of plasma were indicative of a previous ovulation. On day 10, the sexual behavior of the rams in each group and their interactions with females were recorded for 30 min. Each of four trained observers visually followed each ram and recorded their instance of nudging, anogenital sniffing, flehmen, self-marking with urine, mounting attempts, and serves\textsuperscript{21}. On day 15, ram activity was recorded by wearable activity monitors designed for dogs (Whistle Lab Inc., San Francisco), which were adapted to suit the ram’s anatomy by adding a neck collar. The monitors were connected to a mobile phone through a Bluetooth connection, and transmitted information about time of activity and rest (h). To assess the sexual status of rams, plasma testosterone concentrations were measured in blood samples that were collected every two weeks from the onset of the photoperiodic treatments (1 February) to one month after the return to the natural photoperiod of the TR group (30 April).

**Pregnancy rates, fertility and prolificacy**

The pregnancy rates (number of pregnant ewes/number of ewes exposed to rams) were based on transrectal ultrasound 30 d after the introduction of the males, which were confirmed after lambing. Fertility (number of ewes lambing/number of ewes exposed to rams), prolificacy (number of lambs born/number of ewes giving birth), and fecundity (number of lambs born/ewes with rams) were calculated at parturition.
Hormonal assays
Progesterone was assayed using an ELISA kit designed for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK), following the manufacturer’s instructions. The sensitivity was 0.27 ng/ml. Intra- and inter-assay coefficients of variation for sample pools of 0.5 and 1 ng/ml were 8.5%, 9.9%, and 12% and 15%, respectively. Plasma testosterone concentrations were measured by radioimmunoassay. Sensitivity was 0.3 ng/ml. Samples were run in a single assay (intra-assay CV = 6%).

Statistical Analyses
Differences in the proportions of ewes that ovulated were evaluated statistically by a chi-squared test. Differences in the proportions of ewes that exhibited estrus and ovulations, ewes that displayed either a short or a normal luteal phase, and the proportions of pregnant ewes were compared by a Fisher’s exact probability test. Differences in plasma hormonal concentrations, prolificacy, and fecundity were tested by one-way ANOVA. Between-group differences in the sexual behavior of rams were analyzed by comparing the frequencies of the behaviors observed in each group with a random repartition, and tested statistically using a Fisher’s exact probability test.

RESULTS
Ovulations and estrus
All ewes exposed to Treated and Control rams ovulated and presented estrus, but only nine of 23 (39%) of the isolated ewes ovulated during the experiment (P<0.001). The time elapsed between the introduction of rams and the first estrus differed significantly (P<0.001) between TR (21.52±1.12 d) and CR ewes (15.89±1.02 d).

Reproductive parameters
The proportion of ewes that lambed was significantly (P<0.05) higher in ewes exposed to Treated (100%) than in those exposed to Control rams (78%). Litter size did not differ significantly between TR (1.44±0.51 lambs/lambing ewe) or CR ewes (1.29±0.47 lambs/lambing ewe). In contrast, fecundity was significantly (P<0.05) higher in ewes exposed to Treated (1.44±0.51 lambs/ewe) than in those exposed to Control rams (1.00±0.69 lambs/ewe).

Sexual behavior and activity of rams
The sexual behavior observed on day 10 differed significantly between the two groups of four rams that were exposed to ewes (Fig. 1). The photoperiodic-melatonin-treated rams were more active than were the Control rams: Treated rams performed 81% (n = 106) of the instances of nudging and 75% (n = 76) of the anogenital sniffing (P<0.001). Nine of 10 (90%) instances of self-urination were performed by the photoperiodic-melatonin-treated rams (P<0.001). The two groups did not differ significantly in the number of flehmen, mounting attempts, and serves. The activity monitors revealed that Treated rams (18.4±3.2 h) were twice as active as were Control rams (9.0±4.0 h) (P<0.01).

Testosterone profile
During the two months of light treatment in the Treated group, rams in the two groups had similar plasma testosterone concentrations (P>0.05; Fig 2); however, after the artificial photoperiod and melatonin treatment plasma testosterone concentrations were higher in the photoperiodic-melatonin-treated rams than they were in the control group at 15 d and 30 d (P<0.05) after exposure to the natural photoperiod.

DISCUSSION
Results of this study show that all TR and CR ewes ovulated, although fertility and fecundity were higher in ewes exposed to Treated than in those exposed to Control rams. This reproductive difference between groups of ewes could be related to the physiological response of rams to the photoperiodic treatments. In fact, long days associated with melatonin improved endocrine and sexual behavior of rams compared with control ones. Altogether, these results show that the exposition to rams rendered sexually active by exposure to long
days and melatonin, improves the reproductive response of seasonally Aragonesa ewes.
In this study, all TR and CR ewes and about 40% of isolated ewes ovulated. These results confirm the weak ovulatory seasonality of Rasa Aragonesa ewes previously described by our group. In addition, the high proportion of Rasa Aragonesa ewes that ovulated when exposed to Control rams agrees with several studies performed with Merino ewes or Creole goats, two breeds displaying also a weak ovulatory seasonality. In the contrary, our results differed from those described in strong seasonal breeds of sheep and goats, in which the proportion of females that ovulated is low when they are isolated or exposed to control rams or bucks, displaying a weak sexual behavior and odor. Therefore, our results indicate that the Rasa Aragonesa breed can be exposed to control rams to induce most females to ovulate during the seasonal anestrous. However, exposure to photoperiod-treated sexually-activated rams is necessary to improve the reproductive performance of these ewes. Indeed, in our study, fertility and fecundity were higher in ewes exposed to Treated than in those exposed to Control rams. This difference is not related to the proportions of females that ovulated when exposed to Treated or Control rams, but it is likely to be related to the endocrine and sperm production of Treated compared with Control males. Therefore, the mean outcome of this study is that the use of photoperiodic-treated rams improves the reproductive performances of ewes, improving the productivity of ewes exposed to Treated rams. The differences acted at the production level because a significantly higher proportion of the ewes lambed and the number of lambs per ewe were higher in the TR group than it was in the CR group of ewes. Differences in libido activity and or semen quality induced by the melatonin treatment probably contributed to the differences in the number of pregnant ewes. In a study that quantified the effects of melatonin implants in Rasa Aragonesa rams in the anestrous season, melatonin increased the proportion of progressive motile spermatozoa, particularly, 46-75 d after melatonin implantation (P<0.01), and the number of spermatozoa attached per oocyte was significantly (P<0.01) higher among ovine oocytes that had been incubated with sperm from implanted rams than it was among those incubated with sperm from non-implanted rams. In what and this experiment, semen from melatonin-implanted rams increased fertility and fecundity in ewes that had been inseminated with spermatozoa obtained 46-60 d after implantation. From the earlier experiment, we concluded that melatonin treatment in rams in the non-breeding season modifies sperm motility and appears to improve fertilization. In the current experiment, however, the increases in fertility and fecundity cannot be attributed to the melatonin implants, entirely, because, on an organic farm, where no hormonal treatments are allowed, the sexual activation of rams in spring by long days, without melatonin, resulted in reproductive performances that were similar to those in the present experiment (ewes that mated with treated rams produced 22% more pregnant ewes, and 0.34 extra lambs per than did ewes that were exposed to control rams).
In the present experiment, the association of artificial photoperiod and exogenous melatonin increased plasma testosterone concentrations and improved the sexual activity of rams. Indeed, plasma testosterone concentrations were higher in Treated than in Control rams after the end of the photoperiodic treatment. In addition, these photoperiodic treated rams displayed more nuzzling, anal-genital sniffing and self-urination events that Control rams. These results confirm our previous finding, and indicate that this photoperiodic treatment allow stimulate the endocrine and sexual behavior of Rasa Aragonesa rams, even if the belong to a weak seasonal breed. This phenomenon also occurs in goat bucks, and is like the differences observed in serving capacity tests that classify rams as either “high performing” or “low performing”. High-performing rams have a better capacity for inducing high lambing percentage, more lambs born, and more live lambs born per ewe than do low-performing rams, as in our study. Undoubtedly, the combined treatment applied in this experiment (melatonin + artificial photoperiod) is not allowed in
organic farming, where the use of exogenous hormones is not allowed. In a couple of works previously reported by our group, we have verified that the elimination of melatonin implants does not modify either testosterone secretion or sexual behavior of rams\(^1\), and that the use of rams exposed only to long days to be used in organic farms in spring is possible, since these rams increased fertility and fecundity of ewes compared with control rams avoiding the use of exogenous hormones\(^2\). However, if the use of exogenous hormones is feasible, melatonin implants will increase the mating efficiency of rams\(^3\) and will produce a higher number of lambs per ewe, as we have demonstrated in the present experiment.

CONCLUSIONS

In conclusion, treatment of Rasa Aragonesa rams with two months of long days and melatonin implants increased their sexual activity and plasma testosterone levels, and more of the ewes that mated with those rams became pregnant and produced more lambs per ewe than did the ewes that mated with non-treated rams. The improvement in reproductive parameters was not caused by a better ovulatory response after the ram effect because all of the ewes in both groups ovulated. More experiments, including a study of the effects of the photoperiodic treatment on semen quality and stimulus signals are needed.

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