Effects of melatonin and undernutrition on the viability of ovine embryos during anestrus and the breeding season

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Abstract

This study examined the effects of melatonin and level of nutrition on embryo yield during anestrous and breeding season. Adult Rasa Aragonesa ewes were assigned randomly to one of the four treatment groups in two experiments using a $2 \times 2 \times 2$ factorial design. Individuals were treated (+MEL) or not treated (−MEL) with a subcutaneous implant of melatonin for 42 d (Melovine®, CEVA) and fed 1.5 (control, C) or 0.5 (low, L) times the daily maintenance requirements for 20 d. Ewes were mated at oestrus (Day = 0) and embryos were recovered on Day 5. Level of nutrition and melatonin supplements did not have a significant effect on ovulation rate or the number of recovered ova per ewe in the Reproductive Season (RS) and the Anestrous Season (AS). During the RS, undernutrition reduced the number of viable embryos per ewe (C: 1.1 ± 0.2; L: 0.6 ± 0.2; $P<0.05$); however, the number of viable embryos per ewe in the L+MEL group (0.2 ± 0.15) was significantly lower than it was in the L, C+MEL and C groups (0.9 ± 0.3, 1.2 ± 0.3, 1.0 ± 0.4, respectively; $P<0.05$). In the AS, nutrition did not have a significant effect on the number of viable embryos per ewe, although melatonin supplements might have improved rates slightly. Embryo viability rate (% viable embryos/embryos recovered) was unaffected by melatonin supplements or level of nutrition in the RS and the AS. Season had a strong effect on the number of viable embryos per functional corpus luteum among ewes in the L+MEL group, only (RS: 0.2 ± 0.1; AS: 0.6 ± 0.2; $P<0.05$). In conclusion, undernutrition impaired the viability of sheep embryos in the RS, particularly among ewes that were given melatonin supplements subcutaneously, but melatonin appeared to improve embryo quality in the AS, which suggests

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that the mechanisms involved in the interactive effects of melatonin and nutrition on embryo development are influenced by season.

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1. Introduction

Embryo viability in sheep can be affected by several factors, such as nutrition, seasonality, melatonin treatment, among others. It is well documented that undernutrition affects reproductive function at multiple levels of the hypothalamus–pituitary–gonadal axis in ruminants (for a review, see Robinson, 1996; Boland et al., 2001; Forcada and Abecia, 2006b). In sheep, subnutrition increases embryo mortality and reduces pregnancy rates (Rhind et al., 1989; Abecia et al., 1995, 1999; Lozano et al., 2003) because of an inadequate oocyte quality and embryo development (for a review, see Abecia et al., 2006). Low dietary intake alters oocyte morphology in cattle (Yaakub et al., 1999) and reduces the number of high quality oocytes and embryos in superovulated ewes (Lozano et al., 2003).

On the other hand, season can also exert a significant effect on embryo viability of sheep. Mitchell et al. (2002) observed that some of the characteristics of the embryos, such as the number of fertilized ova, the number of embryos with <16 cells, and embryo quality were higher in the reproductive season than it was in the anestrous season. Season did not influence the number of embryos recovered after natural estrus (Mitchell et al., 1999) or superovulatory treatment (López-Sebastián et al., 1990; González-Bulnes et al., 2003).

Seasonality in sheep is mediated by photoperiod, which is conveyed to the reproductive-neuroendocrine axis by melatonin (Bittman et al., 1983). Melatonin is released at night and acts in the mediobasal hypothalamus to modulate the pulsatile secretion of GnRH (Karsch et al., 1984; Robinson et al., 1985). Subcutaneous implants of melatonin are used widely to advance the breeding season and to improve reproductive performance during anestrus both in highly seasonal (Haresign et al., 1990) and in Mediterranean ewes (Chemineau et al., 1996; Zuñiga et al., 2002). Subcutaneous implants cause a short-day length-like response without suppressing endogenous secretion (O’Callagan et al., 1991; Malpaux et al., 1997). Melatonin treatment is an effective method for inducing estrous cycles, increasing ovulation and lambing rates during anestrus (Haresign et al., 1990; Robinson et al., 1991; Haresign, 1992; Bister et al., 1999; Luther et al., 2005). In general, melatonin treatment increases fertility and prolificacy in ewes (Forcada et al., 1995; Chemineau et al., 1996; Zuñiga et al., 2002; Abecia et al., 2007). Melatonin treatments can improve the viability of embryos in ewes after superovulation in the anestrous period (Forcada et al., 2006a); however, McEvoy et al. (1998) did not observe significant differences in the number and quality of embryos from superovulated melatonin-treated and untreated donor ewes in the AS. For the reasons above mentioned, melatonin appears to be beneficial to embryo survival (Durotoye et al., 1997; Abecia et al., 2002; Forcada et al., 2006a); thus we hypothesize that melatonin can override the detrimental effects of undernutrition on the same parameter. Furthermore, the effects of undernutrition and melatonin treatments on oocyte and embryo quality might vary seasonally because the endogenous secretion of melatonin and the voluntary intake vary significantly throughout the year (Chilliard et al., 1998). In this study, we investigated the effects of melatonin and level of nutrition on embryo viability in the reproductive and anestrous seasons.
2. Materials and methods

2.1. Animals

The experiment was conducted using 42 adult, cycling, non-pregnant Rasa Aragonesa ewes at the Experimental Farm of the University of Zaragoza, Spain (41°41’N) and in compliance with the requirements of the European Union for Scientific Procedure Establishments and the Ethics Committee of the University of Zaragoza, Spain.

2.2. Reproductive season (RS)

In the RS, at the beginning of the experiment, the 42 adults, cycling, and non-pregnant Rasa Aragonesa ewes had a mean (± S.E.M.) live weight (LW) of 64.1 ± 1.7 kg and a mean body condition (BC) score (Russell et al., 1969) of 3.1 ± 0.1. To confirm ovarian cyclicity, at 7 and 14 d before the application of the melatonin implants, blood samples were collected and plasma progesterone concentrations were measured. All of the ewes had a progesterone concentration >1 ng/ml in at least one of the samples. On 8 December, ewes were randomly assigned to either a group that would receive a subcutaneous melatonin implant (18 mg melatonin, Melovine®, CEVA Salud Animal, Barcelona, Spain) at the base of the ear (n = 21) or to a group that would not receive an implant (n = 21). Although date of implantation could be considered late in the breeding season, it should be noted that Forcada et al. (2002) have previously reported the efficacy of melatonin implants on reproductive parameters of Rasa Aragonesa ewes implanted as late as in mid January. Therefore, the photorefractoriness to short days in this Mediterranean breed would appear not earlier than in mid February.

In mid-January, 42 d after the addition of the melatonin implants, individuals in the two groups were synchronized using a 14-d treatment with intravaginal progestagen pessaries (30 mg Fluorogestone Acetate; Sincropart®, Ceva Salud Animal S.A., Barcelona, Spain). When pessaries were removed, the ewes were injected i.v. with 400 IU of equine chorionic gonadotrophin (eCG) (Sincropart® PMSG, Ceva Salud Animal S.A.). Fig. 1 summarizes the experimental design.

2.3. Anestrus (AS)

At 7 and 14 d before the start of the experiment, blood samples were collected from 42 ewes and their plasma progesterone concentrations were measured. To create a group of anestrous ewes, those that had progesterone levels >1 ng/ml in at least one of their samples were considered cyclic, and were excluded from the study. Consequently, in the AS, the experiment included 31 adults, non-cycling, non-pregnant Rasa Aragonesa ewes that had a mean (± S.E.M.) LW of 57.2 ± 1.2 kg and a BC of 2.9 ± 0.04. On 26 March, 16 of those ewes received a subcutaneous implant of melatonin (18 mg melatonin, Melovine®, CEVA Salud Animal, Barcelona, Spain) at the base of the ear and 15 ewes were not treated and served as a control group. On 6 May, 42 d after the melatonin implants were inserted, the ewes were synchronized using a 14-d treatment with intravaginal progestagen pessaries (30 mg Fluorogestone Acetate; Sincropart®, Ceva Salud Animal S.A., Barcelona, Spain). Ewes were injected i.v. with 480 IU eCG (Sincropart® PMSG, Ceva Salud Animal S.A.) at pessary removal.

In both of the experiments (Fig. 1), after the insertion of the pessaries and until slaughter (Day 5 post-estrus), the ewes were offered diets that differed in the level of nutrition they provided. The control (C) and low (L) groups were fed diets that provided 1.5 and 0.5 times the daily maintenance
requirements, respectively (Agricultural and Food Research Council, 1993), and had unlimited access to water. The 1.5 M diet ensures the maintenance of LW and BC, whereas the 0.5 M diet leads to a 12% reduction in LW and BC after 20 d (Abecia et al., 1995, 1997; Lozano et al., 1998; Sosa et al., 2004; Sosa et al., 2006).

Thus, four groups were considered: (1) ewes that were offered the C diet and did not receive a melatonin implant (C), (2) ewes that were offered the C diet and received a melatonin (C+MEL), (3) ewes that were offered the L diet and did not receive a melatonin implant (L), and (4) ewes that were offered the L diet and received a melatonin implant (L+MEL). Ewes were randomly assigned to each group in both seasons. These groups were replicated in each season.

Ewe fed the C diet received 0.60 kg of pellets and 1 kg of barley straw per day, which provided 12.4 MJ of metabolizable energy (ME) and 9.3% crude protein (CP). The L diet comprised 0.20 kg of pellets and 0.35 kg of barley straw per ewe per day (4.1 MJ ME and 9.1% CP). The pelleted diet consisted of barley (73%), soybean (22%), and a mineral supplement (5%). Live weight and BC were measured at the time of pessary insertion, pessary withdrawal, and at slaughter.

Every 8 h from 24 h after pessary withdrawal, estrus (Day 0) was monitored using intact rams wearing harnesses with marking crayons. To quantify plasma progesterone concentrations, at Days 0, 3, and 5 (slaughter), jugular blood samples were collected in evacuated heparins tubes. To test the effectiveness of the implants, at 45 d after implantation one daytime blood sample was collected from the melatonin-implanted ewes. The samples were centrifuged within 15 min (1000 g for 10 min) and the plasma was stored at $-20^\circ C$.

On Day 5, 20 d after the start of the experimental diets, embryos were collected by mid-ventral laparotomy. Ewes were anesthetized using an i.m. injection of 0.4 ml 2% xylazine (Xilagesic 2%, Calier, Barcelona, Spain), and 10 ml of sodium thiopental (20 mg/ml) (Thiobarbital Braun Medical, Jaén, Spain) administered by i.v. injection 5 min later. The ovarian response was quantified using the number of corpora lutea that were morphologically sound and congruent with an active luteal phase. Uterine horns were exposed and flushed using a Foley catheter and pre-warmed (36°C) phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and antibiotics (penicillin and streptomycin). Ewes were euthanized using an i.v. injection of sodium thiopental (T-61®, Intervet, Salamanca Spain). Ova and embryos were examined under a stereomicroscope (20–40× magnification) and classified based on their stage of development and morphology (Winterberger-Torres and Sevellec, 1987). Morulae and compacted morulae were considered viable embryos, according to the day of pregnancy on which the embryos were recovered. The in vitro viability of embryos was assessed based on the proportion of embryos that were capable of developing into expanded or hatched blastocysts after

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**Fig. 1.** Experimental design for both experiments.
48 h of culture at 39 °C with 5% CO₂. The culture medium consisted of synthetic oviductal fluid (SOF) without glucose, BME essential amino acids, MEM non-essential amino acids (Walker et al., 1996), 10% (v/v) of foetal bovine serum, 1 mM of l-glutamine, 100 UI/ml of penicillin-G, and 100 μg/ml of streptomycin sulphate.

For each ewe, the following variables were recorded: number of corpora lutea, number of recovered ova (oocytes + embryos), recovery rate (number of ova recovered/number of corpora lutea), number of fertilized embryos, number of viable embryos (as compacted morulae), and number of viable embryos after 48 h in culture (as expanded and hatched blastocysts). Fertilization rate was the number of embryos divided by the number of ova recovered. Viability rate was the number of viable embryos divided by the number of ova recovered. Ewes that did not provide recovered ova were excluded from the calculations of fertilization and viability rates.

2.4. Hormone assays

Plasma melatonin concentrations were measured by a direct, solid-phase radioimmunoassay (RIA) using a commercially available kit (Bühlmann RK-MDI; Bühlmann Lab; Switzerland), within a single assay. The sensitivity of the assay was 1.3 pg/mL and the intra-assay CV was 10.9% for low control concentrations (3.3 pg/mL) and 4.9% for high control concentrations (20.6 pg/mL).

Plasma progesterone concentrations were measured by a direct, solid-phase RIA using commercially available kits (Count-A-Count TKPG; DPC) (Meikle et al., 1997). The RIA had a sensitivity of 0.02 ng/mL. The intra-assay CV was 14% for low control concentrations (3 ng/mL), 8.5% for medium concentrations (15 ng/mL), and 7.5% for high concentrations (30 ng/mL). The interassay CV was <15% for all of the standard concentrations.

2.5. Statistical analyses

The experimental was based on a 2 × 2 × 2 factorial design, with nutritional level, melatonin treatment, and season as fixed effects. The effects of the treatments on the development and quality of oocytes and embryos were evaluated using the PROC GEN MOD (Statistical Analysis System; SAS Institute, Cary, NC, USA) with the Poisson distribution specified in a model that included Season (reproductive or anestrus), Nutrition Level (low or control), and Melatonin Treatment (with or without melatonin implant) and their interactions. The values expressed as percentages were arcsine-transformed before being subjected to statistical analyses. The probability level for statistical significance was set to \( P < 0.05 \) and the results are expressed as mean ± S.E.M.

3. Results

3.1. Live weight and body condition

In the RS, the LW of the ewes in the L and L+MEL groups dropped significantly (from 61.8 ± 3.5 to 54.3 ± 3.2 kg and 64.1 ± 2.8 to 54.8 ± 2.5 kg, respectively; \( P < 0.001 \)), but not those in the C and C+MEL groups (from 63.8 ± 3.7 to 64.6 ± 3.6 kg and 64.7 ± 3.7 to 64.4 ± 3.5 kg, respectively). Ewes in the L and L+MEL groups experienced a significant reduction in BC (from 2.8 ± 0.1 to 2.5 ± 0.1 and from 3.0 ± 0.1 to 2.5 ± 0.1, respectively; \( P < 0.001 \)), but ewes in the C and C+MEL groups did not (2.9 ± 0.1 to 3.0 ± 0.1 and 2.9 ± 0.2 to 2.8 ± 0.1, respectively).

In the AS, ewes in the C and C+MEL groups maintained their LW (from 62.5 ± 2.4 to 63.5 ± 2.2 kg and 54.6 ± 0.9 to 53.3 ± 0.9 kg, respectively) and BC (from 3.0 ± 0.6 to 3.0 ± 0.7
and from 2.9 ± 0.1 to 2.9 ± 0.1, respectively); but those in the L and L+MEL groups experienced a significant reduction in LW (from 54.6 ± 3.2 to 52.8 ± 3.2 kg and 56.6 ± 2.2 to 51.8 ± 1.9 kg, respectively; *P* < 0.001) and BC (from 2.9 ± 0.1 to 3.0 ± 0.1 and from 2.75 ± 0.06 to 2.8 ± 0.1, respectively). In the RS and AS, after pessary withdrawal (14 d after the onset of nutritional treatments), the LW and BC of ewes in the L group were significantly lower than were those of the control ewes (*P* < 0.01).

### 3.2. Circulating hormones

In both seasons, when measured during daylight on Day 45, the concentrations of plasma melatonin in all of the ewes that received a melatonin implant were high, which confirmed that the implants functioned properly (mean ± S.E.M.): 53.6 ± 4.2 and 62.6 ± 4.9 pg/ml in January and May, respectively.

In both seasons and all of the groups, progesterone concentrations increased gradually after estrus. In the RS, nutrition level and supplemental melatonin did not have a significant effect on plasma progesterone concentrations at estrus, at Day 3, and at slaughter (Day 5). Plasma progesterone concentrations on Day 5 were 3.7 ± 0.5 ng/mL (C), 3.8 ± 0.5 ng/mL (C+MEL), 4.7 ± 0.7 ng/mL (L), and 4.0 ± 0.8 ng/mL (L+MEL). In the AS, the interaction effect of melatonin × nutrition on plasma progesterone concentrations at estrus presented a trend to significance (C: 0.30 ± 0.09 ng/mL, C+MEL: 0.11 ± 0.09 ng/mL, L: 0.19 ± 0.11 ng/mL, L+MEL: 0.35 ± 0.11 ng/mL; *P* < 0.09), but nutrition or melatonin treatments had no significant effects on plasma concentrations on Day 3 and at slaughter (Day 5). On Day 5, mean plasma progesterone concentrations were 3.6 ± 0.5 ng/mL (C), 3.8 ± 0.5 ng/mL (C+MEL), 2.6 ± 0.6 ng/mL (L), and 3.4 ± 0.6 ng/mL (L+MEL).

### 3.3. Ovulation rate

Ovulation rates did not differ among groups or between seasons (Tables 1 and 2).

### 3.4. Embryo recovery

#### 3.4.1. Reproductive season

Nutrition level had a significant effect on the number of viable embryos (1.1 ± 0.2 vs. 0.6 ± 0.2 in C and L ewes, respectively; *P* < 0.05), and undernutrition reduced the number of viable embryos in ewes treated with melatonin (*P* < 0.05) (Table 1). Melatonin treatment had a detrimental effect on fertilization rate (*P* < 0.05) and, consequently, the numbers of fertilized and viable embryos were consistently lower in the undernourished, melatonin-implanted group ewes than they were in the other groups. Ewes in the L+MEL group exhibited the lowest in vitro viability (40.0%), and differed significantly from the ewes in the C and C+MEL groups (77.8% and 80.0%, respectively; *P* < 0.05) and, to a lesser extent, from the ewes in the L group (66.7%; *P* = 0.07).

#### 3.4.2. Anestrous season

Only one ewe in the L+MEL group did not exhibit estrous behaviour after pessary withdrawal. Two of the ewes in group L (*n* = 5) and two in group L+MEL (*n* = 6) were excluded from the analysis of the AS because they did not exhibit normal corpora lutea at slaughter. On Day 5 after estrus, the groups did not differ significantly in the number of fertilized and viable embryos,
Table 1
Ovarian response and embryo production in the reproductive season by Rasa Aragonesa ewes fed either 1.5 (C) or 0.5 (L) times the maintenance requirements and treated (MEL) or not treated with melatonin 62 d before embryo recovery

<table>
<thead>
<tr>
<th>GROUP</th>
<th>C</th>
<th>C+MEL</th>
<th>L</th>
<th>L+MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ewes</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>No. of ewes in estrus</td>
<td>8/10</td>
<td>9/11</td>
<td>9/11</td>
<td>9/10</td>
</tr>
<tr>
<td>Ovulation rate</td>
<td>1.9 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>No. of recovered ova</td>
<td>1.1 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Recovery rate (%)</td>
<td>60</td>
<td>75</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>No. of fertilized embryos</td>
<td>1.1 ± 0.3c</td>
<td>1.3 ± 0.3c</td>
<td>1.1 ± 0.3c</td>
<td>0.5 ± 0.3d</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>100a</td>
<td>87.5 ± 9.5b</td>
<td>100b</td>
<td>70 ± 12b</td>
</tr>
<tr>
<td>No. of viable embryos</td>
<td>1 ± 0.3a</td>
<td>1.2 ± 0.3a</td>
<td>0.9 ± 0.3a</td>
<td>0.2 ± 0.3 b</td>
</tr>
<tr>
<td>Viability rate (%)</td>
<td>83.3 ± 17c</td>
<td>81.3 ± 15.6c</td>
<td>75 ± 15.6c</td>
<td>40 ± 19.8d</td>
</tr>
<tr>
<td>Pregnancy rate (%)a</td>
<td>75 (6/8)</td>
<td>77.7 (7/9)</td>
<td>88.8 (8/9)</td>
<td>44.4 (4/9)</td>
</tr>
</tbody>
</table>

Different superscript letters (a, b) in the same row indicate significant differences (P<0.05). Different superscript letters (c, d) in the same row indicate differences of P<0.1. Ova = oocytes + embryos.

which indicates that undernutrition and supplemental melatonin did not have a significant effect on these parameters in the AS (Table 2). Supplemental melatonin and undernutrition did not have a significant effect on the in vitro viability of recovered embryos in the AS; thus, the percentages of embryos that were capable of developing into expanded or hatched blastocysts were 87.5% (C), 83.3% (C+MEL and L), and 88.9% (L+MEL).

The interaction between season × melatonin treatments had a significant effect on fertilization rate (P<0.05), and tended to have an effect on embryo viability (P<0.08). In general, unlike in the RS, melatonin had a positive effect on fertilization rate and embryo viability in the AS (Table 2). In the RS, the effect of this interaction was particularly pronounced among the undernourished ewes, in which supplemental melatonin significantly (P<0.05) impaired the number of viable

Table 2
Ovarian response and embryo production in the anestrous season by Rasa Aragonesa ewes fed either 1.5 (C) or 0.5 (L) times the maintenance requirements and treated (MEL) or not treated with melatonin 62 d before embryo recovery

<table>
<thead>
<tr>
<th>GROUP</th>
<th>C</th>
<th>C+MEL</th>
<th>L</th>
<th>L+MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ewes</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>No. of ewes in estrus</td>
<td>8/8</td>
<td>7/7</td>
<td>5/5</td>
<td>5/6</td>
</tr>
<tr>
<td>Ovulation rate</td>
<td>2.4 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>2.0 ± 0.6</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>No. of recovered ova</td>
<td>1.0 ± 0.3</td>
<td>1.6 ± 0.3</td>
<td>1.2 ± 0.4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Recovery rate (%)</td>
<td>42a</td>
<td>73.3b</td>
<td>60b</td>
<td>78b</td>
</tr>
<tr>
<td>No. of fertilized embryos</td>
<td>0.6 ± 0.3c</td>
<td>1.3 ± 0.3d</td>
<td>0.8 ± 0.4c</td>
<td>1.2 ± 0.4d</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>66.7 ± 18</td>
<td>83.3 ± 18</td>
<td>75 ± 22</td>
<td>87.5 ± 22</td>
</tr>
<tr>
<td>No. of viable embryos</td>
<td>0.6 ± 0.3c</td>
<td>1.1 ± 0.3d</td>
<td>0.8 ± 0.4c</td>
<td>1.2 ± 0.4d</td>
</tr>
<tr>
<td>Viability rate (%)</td>
<td>66.7 ± 18.1</td>
<td>75 ± 18.1</td>
<td>75 ± 22.2</td>
<td>87.5 ± 22.2</td>
</tr>
<tr>
<td>Pregnancy rate (%)a</td>
<td>50 (4/8)</td>
<td>71.4 (5/7)</td>
<td>60 (3/5)</td>
<td>80 (4/5)</td>
</tr>
</tbody>
</table>

Different superscript letters (a, b) in the same row indicate significant differences (P<0.05). Different superscripts letters (c, d) in the same row indicate differences of P<0.1. Ova = oocytes + embryos.

a Percentage of ewes with viable embryos on Day 5.
Fig. 2. Number of viable embryos per corpus luteum (CL) in the reproductive season (left panel) and the anestrous season (right panel) in Rasa Aragonesa ewes fed either 1.5 (C) or 0.5 (L) times the LW maintenance requirements and treated (MEL) or not treated with melatonin 62 d before embryo recovery. Mean ± S.E.M. Within the same panel, bars that have different superscripts are significantly different ($P \leq 0.05$).

embryos, whereas, in the AS, embryo viability tended ($P < 0.1$) to improve. The mean number of viable embryos per functional corpus luteum in each group is shown in Fig. 2.

4. Discussion

This study investigated the effects of exogenous melatonin and level of nutrition on embryo viability in Rasa Aragonesa ewes in the anestrous and the reproductive seasons in northern Spain. We hypothesized that the known beneficial effects of exogenous melatonin on embryo survival would override the negative effects of undernutrition at any time of year; however, in this study, the effects of melatonin implants on embryo development differed between the reproductive or anestrous seasons.

In our experiments, nutritional restrictions reduced significantly the mean live weights and body condition of ewes. Although the rate of live weight decrease of undernourished ewes were not similar in both seasons, the effectiveness of the nutritional treatments can be assumed because of the significant differences observed between C and L live weight at slaughter time ($P < 0.01$). Several factors could explain this situation, such as nutritional status of the ewes before the onset of the experiment, metabolic and hormonal factors interacting with season, nutrition and reproduction, environment factors and others. In addition, Clarke (2001) observed a seasonal regulation of food intake in sheep, observing an increment of voluntary food intake under long-day conditions, which in turn, could provoke live weight differences. Our previous studies (Abecia et al., 1997, 1999; Sosa et al., 2006) demonstrated that mature ewes subjected to a degree of undernutrition similar to that of the present experiment for 3–4 weeks exhibited a significant reduction in LW and BC. In addition, underfed ewes exhibited an increase in lipolytic activity (Sosa et al., 2006). However, such short-term undernutrition associated with a progestagen-synchronized estrus does not appear to impair ovulation rate in the reproductive season (Abecia et al., 1997, 1999; Borowczyk et al., 2006) or the anestrous season (Sosa et al., 2006).

It was unexpected that undernutrition impaired embryo viability on Day 5 after the onset of estrus in melatonin-implanted ewes in the reproductive season, but not in the anestrous season, when the melatonin treatment seemed to improve embryo fertilization and viability. Season did not have a significant effect on the number of viable embryos or viability rate. Regardless of the level of nutrition, seasonal anestrus seemed to reduce the fertilization rate and viability rate of the ewes that did not receive melatonin implants. Seasonal differences might be due in part to differences in semen quality; however, low seasonal variations in the volume and quality of
the ejaculates from Rasa Aragonesa rams (Martí et al., 2007) suggest that the higher fertilization rate in the reproductive season might be mainly due to higher oocyte quality at the time of ovulation. Mitchell et al. (2002) reported a higher proportion of unfertilized or degenerated ova in the anestrous period following superovulatory treatments and artificial insemination, and a larger number of small follicles were induced to ovulate in April than in October, and most of them contained immature oocytes at the time of ovulation. In vitro fertilization rates of oocytes recovered from superovulating ewes can be lower in the anestrous season than in the reproductive season (Stenbak et al., 2001). The presence of a corpus luteum before gonadotrophin treatment is beneficial to embryo development (Gonzalez-Bulnes et al., 2002; González-Bulnes et al., 2003), which might be due to the inability of progestagen protocols to suppress LH to the level achieved in the luteal phase (Kojima et al., 1992), thereby inducing inadequate follicular development that can lead to abnormalities in fertilization and embryo development. In our experiment, all of the ewes that were induced to ovulate in anestrus were non-cyclic at the time of sponge insertion.

In our study, four undernourished ewes failed to exhibit functional corpora lutea after ovulation in the anestrous season. Undernutrition is associated with a low uterine expression of progesterone receptors on Day 5 after estrus in ewes (Sosa et al., 2004; Sosa et al., 2006). In addition, progesterone can modulate the endometrial PGF$_{2\alpha}$ secretion by down-regulating the concentration of oxytocin receptor and delaying the time of luteolysis (McCracken et al., 1999). Given that undernutrition during early embryo development in sheep increases uterine in vitro production of PGF$_{2\alpha}$ after an induced estrus (Abecia et al., 1999) or following superovulation (Lozano et al., 2003), undernutrition might lead to a poor uterine environment that compromises the development of embryos. Furthermore, undernutrition can influence earlier embryo development, by reducing oviductal sensitivity to ovarian steroids (Sosa et al., 2008) or by altering oocyte quality (Borowczyk et al., 2006).

In the present experiments, melatonin implants had a significant detrimental effect on fertilization rate in the reproductive season, but not in the anestrous season. Elsewhere, studies have shown that supplemental melatonin in anestrous does not improve fertilization rate in terms of embryos recovered from superovulated ewes (McEvoy et al., 1998; Forcada et al., 2006a) or after IVF of oocytes recovered from superovulatory treatment (Luther et al., 2005). Although McEvoy et al. (1998) found that melatonin implants did not have a significant effect on embryo production and survival after superovulation during anestrous. Our recent studies have shown that supplemental melatonin can reduce significantly the number and rate of non-viable (degenerated and retarded) embryos in superovulated ewes during anestrous (Forcada et al., 2006a). The luteotrophic effect of the pineal hormone observed in vivo or in vitro (Durotoye et al., 1997; Abecia et al., 2002) and the effects of melatonin at the hypothalamic–hypophyseal level (Malpaux et al., 1997) might be involved in the melatonin-induced improvement in embryo viability during anestrous. In our study, it is unclear why melatonin implants had a detrimental effect on fertilization and viability rates in Rasa Aragonesa ewes in the reproductive season. In humans and rats, granulosa cells express specific melatonin binding sites (Yie et al., 1995; Clemmens et al., 2001). In addition, in rats, chronic exposure to estrogen can down-regulate melatonin receptors in the ovaries and, therefore, reduce their expression (Clemmens et al., 2001). Thus, it is possible that over exposure to melatonin during the reproductive season in sheep might be detrimental at the ovarian level and associated with high concentrations of estradiol, particularly around the estrus.

In conclusion, undernutrition impaired the viability of embryos in the RS, particularly in the ewes that were given melatonin implants. In the AS, supplemental melatonin appeared to improve embryo quality. Those results suggest that the mechanisms underlying the interaction between
exogenous melatonin and level of nutrition on embryo development are seasonally regulated, but the nutrition–melatonin interactions and their mechanisms require further study.

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References


