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Changes in melatonin concentrations in seminal plasma are not correlated with testosterone or antioxidant enzyme activity when rams are located in areas with an equatorial photoperiod

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ABSTRACT

In temperate climates, photoperiod and melatonin regulate ram reproduction, modulating hormonal secretions, sperm quality, and seminal plasma composition. Information on the effect of an equatorial photoperiod (12L:12D) on ram reproduction, however, is scarce, and no data on hormonal concentrations and antioxidant enzyme activity in seminal plasma have been reported. Thus, the variation was investigated of melatonin and its relationship with testosterone and antioxidant enzyme activity in the seminal plasma of three sheep breeds in Colombia, when there was a consistent photoperiod during two dry and two rainy seasons per year. Semen was collected once a week from 12 mature rams (four of each breed: Colombian Creole, Hampshire, and Romney Marsh). Seminal plasma was obtained by centrifugation. The concentration of melatonin and testosterone were quantified along with the enzymatic activity of glutathione peroxidase (GPx), glutathione reductase (GRD), and catalase (CAT). Correlation analyses between melatonin and testosterone concentrations or enzymatic activity were also performed. Melatonin concentration was affected by season ($P < 0.05$) but not breed, with lesser concentrations in the first rainy season. Testosterone concentration, however, was affected by breed and season, with greater concentrations ($P < 0.01$) in the Hampshire and Romney Marsh rams during the second dry season. Regarding antioxidant enzyme activity, there was only seasonal variation in GPx activity ($P < 0.05$). When correlation analyses were used for data assessments, there was a negative correlation between melatonin and testosterone concentrations in Hampshire rams. In conclusion, melatonin concentrations in seminal plasma of rams that were located in an area with an equatorial photoperiod was affected by the climatological season but there was no positive correlation with testosterone concentration or antioxidant enzyme activity.

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1. Introduction

In temperate climates, photoperiod through modulation of melatonin secretion regulates reproductive activity in sheep. Photoperiodic signals reach the pineal gland and there is modulation of nocturnal melatonin secretion (Arendt, 1998; Bittman et al., 1983) and subsequent modulation of gonadotropin secretion (Malpaux et al., 1996), gonadal functions, and sexual behavior (Rosa and Bryant, 2003).

Seasonality effects in sheep decrease with the extent of domestication (Lincoln et al., 1990), and also depends on latitude at which the animals are located. Sheep breeds originating from England are highly seasonal (Tempest and Boaz, 1973), whereas those from intermediate latitudes such as Mediterranean breeds have a short anestrus (Forcada et al., 1992). Tropical and equatorial breeds are aseasonal or only have short periods of seasonally induced anestrus (Mahieu et al., 1989; Arroyo et al., 2016) and thus the reproductive pattern is regulated by food availability in relation to the annual rainfall cycle (Rosa and Bryant, 2003). Furthermore, ewes from temperate regions subjected to an equatorial photoperiod (12L:12D) soon afterwards begin to have non-seasonal reproductive patterns (Jackson et al., 1990).

The response of rams to seasonality is less marked than in ewes. In temperate regions ($> 30^{\circ}$ and $< 45^{\circ}$ north or south latitude), there are variations in hormonal concentrations (D'Occhio et al., 1984), sperm quality (Karagiannidis et al., 2000) and testicular volume (Avdi et al., 2004) during different seasons of the year; however, there is no complete cessation of spermatogenesis and sexual activity. This variation in hormonal concentrations in rams during different seasons of the year is reflected in the seminal plasma composition, where concentrations of melatonin and testosterone also have a seasonal pattern. The melatonin concentration in this fluid correlates with that of testosterone and antioxidant enzyme activity (Casao et al., 2010). In tropical (below 23.5° north or south) and subtropical (between 23.5° and 35.0° north or south) regions, photoperiodic signals also regulate seasonality, although to a lesser extent. Nevertheless, variations in testicular size, testosterone concentrations (Milczewski et al., 2015) and several sperm parameters (Aguirre et al., 2007; Cardenas-Gallegos et al., 2012) have been reported. Information on ram reproduction in the equatorial zone (between 10 °N and 10 °S) with a 12L:12D light regimen, however, is scarce. Furthermore, there is no information on the effect of a tropical or equatorial photoperiod on variation in ram seminal plasma hormonal concentrations and antioxidant enzyme activity.

Colombia is an equatorial region where the differences in photoperiod throughout the year are minimal and as a consequence there are no short and long days. The Andean region, the most highly populated in the country, has a bimodal annual rain cycle, with two rainy and two dry seasons. This mountain area has cold and humid weather (Narváez-Bravo and León Aristizábal, 2001). Sheep currently represent 11% of the country's livestock, although in a climate change scenario, the choice of sheep as the primary livestock species is likely to increase (Seo et al., 2010). The Colombian Creole sheep is a native breed which is the result of indiscriminate breeding between European and African sheep since the Spanish conquest in the early 16th century, and thus animals of this breed are highly adapted to the Colombian climate (Ocampo et al., 2017). Additionally, imported wool sheep breeds, such as Hampshire, Romney Marsh and others, have been introduced in the country since 1963, when the first importation occurred. These breeds are characterized by a superior productive performance, but there is relatively little adaptation to the local climatic conditions in terms of fertility (Beaty, 1971).

The aim of this research was to elucidate the effect of an equatorial photoperiod on ram reproduction by investigating the yearly variation of melatonin concentration and its relationship with testosterone concentrations and antioxidant enzyme activity in the seminal plasma of a native (Colombian Creole) and two imported (Hampshire and Romney Marsh) sheep breeds, reared in Colombia when there was a 12L:12D light regimen during two dry and rainy seasons of the year.

2. Materials and methods

2.1. Animals and seminal plasma extraction

All animals used in this study were handled in strict accordance with Colombian Animal Protection Regulations (Law 84/1989, modified by Law 1774/2016). Rams were housed together with uniform nutritional conditions at the Center for Ovine Research, Technological Development and Extension (Centro de investigación, desarrollo tecnológico y extensión ovino –CIDTEO) National University of Colombia, located in Mosquera (4°40′57″ N 74°12′50″ W) at 2510 m above sea level. The ram's diet was based on pasture (*Pennisetum clandestinum*, *Lolium perenne*), supplemented with concentrate (400 g), corn silage (300 g) and mineralized salt (100 g). Local amplitude of the photo-phase throughout the year varies from 12 h 21 min (11 h 39′ of dark) in the summer solstice to 11 h 49 min (12 h 11 min of dark) in the winter solstice, i.e., 32 min of difference between the longest and the shortest day of the year (Fig. 1). The climate of the region is classified as Cfb following the Köppen Climate Classification System. The average temperature is 13.6 °C whereas the annual variation of temperature between coldest and hottest months is 0.7 °C. The mean relative air humidity ranges from 92% in the morning to 70% in the evening, and the mean annual rainfall is 960 mm, with a mean of 205 days with precipitation per year (Fig. 1).

Semen was obtained using an artificial vagina (AV) following the protocol approved by the Bioethics Committee of the National University of Colombia (CB-074-2014). Raw semen was obtained from 12 mature rams (2–5 years old) of three breeds (four Creole; four Romney Marsh and four Hampshire sires). Two successive ejaculates were collected once a week from each ram throughout the year and mixed together for seminal plasma extraction.

Seminal plasma from each mixed semen sample was obtained by centrifugation at 7500 × *g* for 5 min in a microfuge (HERMLE Labortechnik GmbH, Siemensstr 25, D-78564 Wehingen, Germany) at 4 °C. The supernatant was collected and centrifuged again, and the recovered seminal plasma was filtered through a 0.22 mm Millipore membrane (Merck KGaA, Darmstadt, Germany). The seminal

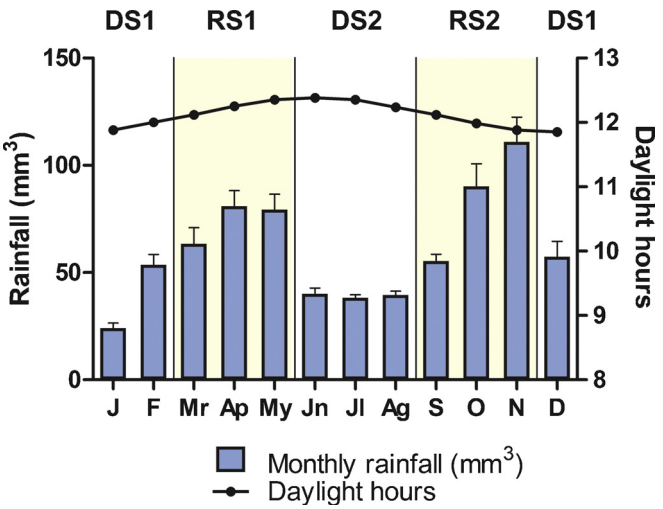

Fig. 1. Monthly rainfall (mm^3, bars) and daylight hours (line) in Mosquera, Colombia (4°40′57″ N 74°12′50″ W); Monthly rainfall is shown as mean ± S.E.M. of five consecutive years, whereas the daylight hours were recorded the 21st of each month.

plasma was stored at −20 °C after adding 10% protease and phosphatase inhibitor (Sigma Chemical Co, St. Louis, MO, USA).

2.2. Melatonin evaluation

Melatonin concentrations in ram seminal plasma were quantified using a commercial competitive immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Switzerland, Sensitivity: 0.5 pg/mL, Intra-assay variability: 5.2%, Inter-assay variability: 11.2%), following the manufacturer's instructions. Briefly, 100 µL of each sample, control, and calibrator were loaded in duplicate in a microtiter plate coated with an anti-melatonin antibody and incubated for 16–20 h at 2–8 °C. After incubation, 50 µL of biotinylated melatonin were added to each well and incubated for 3 h at 2–8 °C. After three washes, 100 µL of streptavidin conjugated to horseradish peroxidase (HRP) were loaded into the wells and incubated for a further 60 min in a plate rotator set at 600 rpm at 18–28 °C. The wells were rewashed three times, and 100 µL of tetramethylbenzidine substrate (TMB) were added to each well, and incubated for 30 min in a plate rotator at 600 rpm and 18–28 °C, protected from direct light. After incubation, 100 µL of 0.25 M SO_4H_2 solution were added, and absorbance was measured on a microtiter plate reader (TECAN Spectrafluor plus, Switzerland) at 450 nm.

2.3. Testosterone assays

Testosterone concentrations in ram seminal plasma were quantified by use of a total testosterone commercial ELISA kit assay (Testo-Easia, BioSource Europe, S.A., Belgium; Sensitivity: 0.05 ng/mL; Intra-assay variability: 4.8%, Inter-assay variability: 7.1%), following the manufacturer's instructions. Briefly, 50 µL of each sample, control, and calibrator, along with 100 µL of testosterone labeled with horseradish peroxidase (HRP) were loaded in duplicate in a microtiter plate coated with an anti-testosterone specific antibody and incubated for 1 h at room temperature. After incubation, the wells were washed three times, and 100 µL of chromogenic substrate (TMB) were added to each well and incubated for 30 min at room temperature, protected from direct light. After incubation, 100 µL of 0.2 M HCl solution were added, and absorbance was measured on a microtiter plate reader (TECAN Spectrafluor plus, Switzerland) at 450 nm.

2.4. Antioxidant enzyme activity assays

The seminal plasma antioxidant defense system was assessed by determining the activity of the following enzymes: Glutathione reductase (GRD), glutathione peroxidase (GPx) and catalase (CAT). Measurements were performed as previously described (Casao et al., 2013) with adaption occurring for the microtiter plate using a spectrophotometric method with a microtiter plate reader (TECAN Spectrafluor plus, Tecan Grup Ltd., Männedorf, Switzerland). All samples were loaded in duplicate and analyzed in the same assay.

2.4.1. Glutathione reductase (GRD. EC.1.6.4.2)

The GRD activity was measured by following the decrease in absorbance due to NADPH oxidation as a consequence of the GSSG reduction. The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; 0.5 mM EDTA; 85 µM NADPH; 0.8 mM oxidized glutathione (GSSG) and 5 µL of seminal plasma to complete a final volume of 200 µL. The absorbance change at 340 nm was monitored for 3 min with the microtiter plate reader. One unit induces the oxidation of 1.0 µmole/min of NADPH at 25 °C, pH 7.2.

2.4.2. Glutathione peroxidase (GPx. EC.1.11.1.9)

The GPx activity was measured following the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) as a result of catalysis with GPx and using ter-Butylhydroperoxide (t-BuO_2H) as an electron acceptor, coupled to the recycling of GSSG to GSH utilizing GRD and NADPH. The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; EDTA 0.5 mM, 54 mUI of GRD; 85 μM NADPH; 2 mM GSH; 1.2 mM t-BuO_2H and 6 μL of seminal plasma for a final volume of 200 μL. The absorbance change at 340 nm was monitored for 3 min with the microtiter plate reader. One enzyme unit (IU) is defined as the amount of GPx capable of transforming 1 μmole/min of NADPH at 25 °C, pH 7.2.

2.4.3. Catalase (CAT. EC.1.11.1.6)

Catalase activity was measured by determining the decrease in absorbance due to H_2O_2 reduction to H_2O and O_2 in the presence of catalase. The reaction mixture contained 50 mM sodium phosphate buffer at a pH of 7; 11 mM t-BuO_2H and 4 μL of seminal plasma to complete a final volume of 200 μL. The absorbance change at 240 nm was monitored for 120 s with the microtiter plate reader. One enzyme unit (IU) is defined as the amount of catalase capable of transforming 1.0 μmol/min of H_2O_2.

2.5. Statistical analyses

Monthly data (mean ± S.E.M. of the weekly seminal plasma samples) from each breed were pooled together for illustrative purposes. Also, data from each breed were grouped for the four climatic seasons (Dry season 1: From December to February; Rainy season 1: March to May; Dry season 2: June to August and Rainy season 2: September to November) for statistical analyses. First, normality was evaluated by the Kolmogorov-Smirnov test, and homogeneity of variance by the Levene's test. The effect of the breed and the season on hormonal concentration and antioxidant enzyme activity was subsequently analyzed using a two-way ANOVA followed by use of a Bonferroni or a Games-Howell *post-hoc* test, as appropriate. The possible correlation between the melatonin concentration in seminal plasma and testosterone concentration or antioxidant enzyme activity was evaluated using a Pearson correlation test. GraphPad Software (La Jolla, CA, USA) and SPSS Statistics (IBM Analytic, Armonk, NY, USA) were used, and $P < 0.05$ was considered statistically significant.

3. Results

There were monthly variations in melatonin concentrations in seminal plasma in all three breeds (Fig. 2a), with the least values during the two rainy seasons between March and May and again between October and December. There were no differences among breeds during the yearly evaluations. Thus, when the data were grouped into the four climatological seasons, the use of the two-way ANOVA indicated there was an effect of the season but not of the breed on the melatonin concentration in seminal plasma. During the first rainy season (from March to May), the melatonin concentration was less in the Creole ($P < 0.05$ when compared with the second dry season) and the Hampshire rams ($P < 0.01$ when compared with both dry seasons). In the Romney Marsh rams, the melatonin concentration also was less during the first rainy season and there were no differences during the other seasons of the year (Table 1).

Surprisingly, there was a different pattern in testosterone concentration in ram seminal plasma as compared with that for melatonin with two distinct peaks occurring. The first increase in testosterone concentration was in May–June, and the second in November (Fig. 2b). There was a distinct and sustained increase in testosterone concentration; however, this was less in the Creole rams. There was a difference in testosterone concentration in ram seminal plasma among seasons and breeds. In particular, the testosterone concentration was greater in the second dry season when compared with the first in the Romney Marsh and Hampshire ($P < 0.01$), but not in the Creole (Table 2) rams. Furthermore, the testosterone concentration in Creole rams during the second dry season was less than the values in the rams of the other two breeds ($P < 0.01$).

For the antioxidant enzyme activity, there was only a monthly variation in GPx activity during the year (Fig. 3a), with lesser activity from January to April which increased from May to December. Consequently, GPx activity was less during the first dry and rainy seasons when compared with the second seasons ($P < 0.05$) in all the breeds (Table 3). The results from the statistical analysis indicated there were differences between breeds for GPx activity, or between breed or season for GRD or CAT activity (Tables 4 and 5). There were no differences in the yearly pattern of GRD and CAT activity (Fig. 3b and c).

Correlation analyses indicated that there was no relationship between melatonin concentrations in ram seminal plasma and antioxidant enzyme activity in any of the breeds. There was only a negative correlation ($P < 0.05$) between melatonin and testosterone concentrations in seminal plasma of Hampshire rams (Table 6).

4. Discussion

Even though there is considerable knowledge of the role of melatonin in sheep reproduction in temperate climates, little is known about the involvement of this hormone in tropical sheep breeds. To begin to clarify this subject, the melatonin concentration was quantified, and its relationship with testosterone concentration and antioxidant enzyme activity was determined, in the seminal plasma of Creole, Romney Marsh, and Hampshire rams reared in Colombia when there was an equatorial light regimen (12L:12D) during two dry and rainy seasons.

In the present study, there were no differences in the melatonin concentrations in the seminal plasma of these breeds. These results are consistent with previous reports where there was a lack of differences between sheep breeds in terms of pituitary response

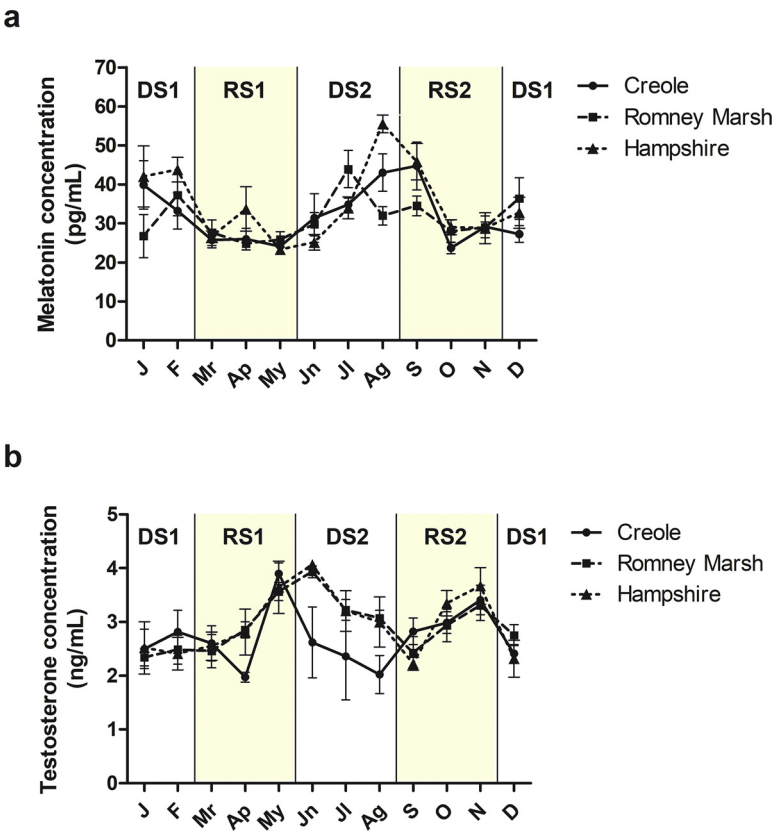

Fig. 2. Monthly values of melatonin (a) and testosterone (b) concentrations in seminal plasma of Creole, Romney Marsh, and Hampshire rams (four rams of each breed) when there was an equatorial photoperiod (12L:12D); Values are depicted as mean ± S.E.M. of four seminal plasma samples/ram and month; Dry (DS) and rainy (RS) seasons are also indicated.

Table 1
Melatonin concentration (pg/mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season; Different letters in the same row indicate differences between seasons.

	Dry season 1 (*n* = 52)	Rainy season 1 (*n* = 52)	Dry season 2 (*n* = 52)	Rainy season 2 (*n* = 60)
Creole	32.98 ± 2.73^{a,b}	25.35 ± 1.08^{a}	36.04 ± 2.97^{b}	32.77 ± 3.38^{a,b}
Romney Marsh	33.68 ± 3.15	26.22 ± 1.34	34.81 ± 2.54	30.14 ± 1.5
Hampshire	38.99 ± 3.09^{b}	26.37 ± 1.14^{a}	38.23 ± 4.04^{b}	34.50 ± 2.80^{a,b}

Table 2
Testosterone concentration (ng/mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are depicted as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season; Different lowercase letters in the same row indicate differences between seasons, whereas different capital letters in the same column indicate differences among breeds.

	Dry season 1 (*n* = 52)	Rainy season 1 (*n* = 52)	Dry season 2 (*n* = 52)	Rainy season 2 (*n* = 60)
Creole	2.56 ± 0.16	2.80 ± 0.25	2.35 ± 0.34^{A}	3.06 ± 0.14
Romney Marsh	2.54 ± 0.16^{a}	2.91 ± 0.18^{a,b}	3.45 ± 0.13^{Bb}	2.89 ± 0.18^{a,b}
Hampshire	2.40 ± 0.12^{a}	2.96 ± 0.24^{a,b}	3.47 ± 0.19^{Bb}	3.10 ± 0.21^{a,b}

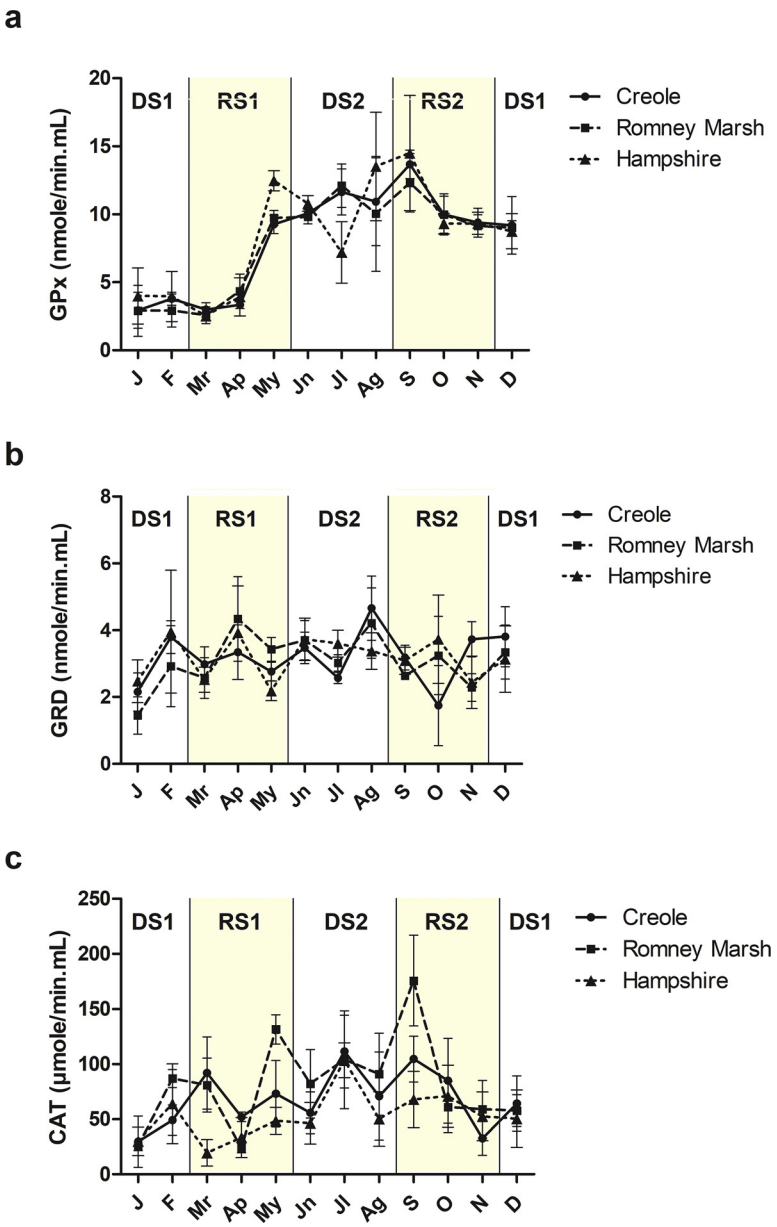

Fig. 3. Monthly values of glutathione peroxidase (GPx, panel a) glutathione reductase (GRD, panel b), and catalase (CAT, panel c) activities in seminal plasma of Creole, Romney Marsh, and Hampshire rams (four rams of each breed) when there was an equatorial photoperiod (12L:12D); Values are depicted as mean ± S.E.M. of four seminal plasma samples/ram and month; Dry (DS) and rainy (RS) seasons are also indicated.

Table 3
Glutathione peroxidase (GPx) activity (nmole/min mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season; Different letters in the same row indicate differences between seasons.

	Dry season 1 (n = 52)	Rainy season 1 (n = 52)	Dry season 2 (n = 52)	Rainy season 2 (n = 60)
Creole	4.66 ± 0.79[a]	5.02 ± 0.89[a]	10.82 ± 1.03[b]	10.61 ± 0.91[b]
Romney Marsh	5.26 ± 1.07[a]	5.32 ± 0.98[a]	10.59 ± 1.3[b]	10.15 ± 0.79[b]
Hampshire	5.81 ± 1.12[a]	6.01 ± 1.33[a]	10.51 ± 1.96[b]	10.56 ± 1.24[b]

Table 4
Glutathione reductase (GRD) activity (nmole/min mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M/ *n* indicates the number of seminal plasma samples per breed and season.

	Dry season 1 (*n* = 52)	Rainy season 1 (*n* = 52)	Dry season 2 (*n* = 52)	Rainy season 2 (*n* = 60)
Creole	3.29 ± 0.44	3.03 ± 0.31	3.56 ± 0.40	2.91 ± 0.47
Romney Marsh	2.63 ± 0.52	3.64 ± 0.54	3.35 ± 0.27	2.73 ± 0.39
Hampshire	3.17 ± 0.67	2.41 ± 0.18	3.58 ± 0.30	3.05 ± 0.51

Table 5
Catalase (CAT) activity (μmole/min mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season.

	Dry season 1 (*n* = 52)	Rainy season 1 (*n* = 52)	Dry season 2 (*n* = 52)	Rainy season 2 (*n* = 60)
Creole	49.41 ± 11.68	50.38 ± 10.62	50.38 ± 10.67	79.41 ± 18.09
Romney Marsh	59.07 ± 15.36	65.70 ± 15.36	82.41 ± 18.94	91.21 ± 19.50
Hampshire	37.15 ± 10.62	29.66 ± 12.80	59.82 ± 12.80	61.86 ± 12.46

Table 6
Pearson's correlation (*r*) between melatonin and testosterone concentrations and antioxidant enzyme activity in seminal plasma of Creole, Romney Marsh, and Hampshire rams; *$P < 0.05$.

Melatonin	Testosterone	GPX	GRD	Catalase
Creole	−0.403	0.309	0.328	0.502
Romney Marsh	0.089	0.470	−0.319	0.415
Hampshire	−0.606*	0.443	−0.077	0.398

to the photoperiod (Poulton and Robinson, 1987). Seasonal sheep breeds subjected to an equatorial light regimen revert to a non-seasonal breeding pattern or a breeding pattern where there is a minimal effect of seasonality similar to what occurs in the local breeds (Wodzicka-Tomaszewska et al., 1967; Jackson et al., 1990).

Furthermore, the melatonin concentration in the seminal plasma of these Colombian rams is less than when these rams are located in temperate climates, even during the non-reproductive season (Casao et al., 2010). Even with the relatively small concentration of melatonin, the results of the present study indicate it fluctuates throughout the year, with lesser values during rainy seasons and increases during the dry seasons. There was the least melatonin concentration during the first rainy season, from March to May, which is coincident with the minimum concentrations observed in rams located in temperate climates (Casao et al., 2010). This initial decrease in the seminal plasma melatonin concentration also occurs at a time coincident with the anovulatory season in Black-Belly sheep (Chemineau et al., 2004) when there are tropical photoperiodic conditions. The melatonin concentration in seminal plasma decreases again a second time in October and November, the months during whichthe concentrations of this hormone are maximum in rams located in temperate climates.

Several factors can affect the melatonin concentration in ram seminal plasma. The concentration of this hormone in this fluid reflects the seasonal variations of blood melatonin (Casao et al., 2010) and there are also increases after exogenous melatonin treatment (Casao et al., 2013). Thus, the changes in seminal plasma concentrations of melatonin between dry and rainy seasons could be due to differences in nocturnal melatonin secretion. The rams used in the present experiment were located at 4°40′N and were subjected to the natural light regimen, with no photoperiodic changes that could modify nocturnal melatonin secretion from the pineal gland. The fluctuation in these factors, therefore, do not explain these seasonal differences. Although in some species, such as the European Hamster, nocturnal melatonin secretion, when there is a long photoperiod, can be affected by temperature (Vivien-Roels et al., 1997) however, there have been no reports that ambient temperatures modulate melotonin secretion in sheep (Wodzicka-Tomaszewska et al., 1967). Furthermore, in the mountain region where the rams used in the present study were located, mean temperatures do not oscillate throughout the year. As previously suggested, this variation in seminal plasma melatonin could also be of testicular origin (Gonzalez-Arto et al., 2016). Previous results with European rams indicated that there was not variation in melatonin synthesis by the testis that was related to season of the year (Cebrian-Perez et al., 2017) which is similar to what occurs in other organs with extra-pineal melatonin (Acuña-Castroviejo et al., 2014).

The most likely source of variation in seminal plasma melatonin in rams located in areas with an equatorial photoperiod could be the variation in content of this hormone in the feed. Vegetal melatonin, known as phytomelatonin, has been detected in a wide variety of plant families (Koca Caliskan et al., 2017). The endogenous phytomelatonin increases when there are stressful conditions,

such as high salinity, cold temperatures or drought (Arnao and Hernandez-Ruiz, 2013a,b). Thus, during the dry seasons, the melatonin concentration in pasture could be greater than during the rainy seasons, therefore, a greater amount of melatonin could be ingested with the feed of the rams and could contribute to increases in the concentrations of circulating melatonin (Hattori et al., 1995) and consequently its concentration in seminal plasma. The presence of phytomelatonin in the feed of the rams would also explain the lack of differences among the breeds in the present study.

Regardless of its origin, the melatonin concentration in the seminal plasma of rams when they were located in an area with an equatorial photoperiod does not correlate positively with the testosterone concentration or antioxidant enzyme activity, unlike what occurs in rams located in temperate climates (Casao et al., 2010). The testosterone concentration in the rams of the present study was affected by breed and season. Only the rams of the British breeds had distinct differences in profiles of testosterone concentrations in both dry seasons with greater values during the second dry season. In temperate regions, the increase in nocturnal melatonin secretion at the beginning of the reproductive season stimulates the hypothalamus-pituitary-testicular axis (D'Occhio et al., 1984; Lincoln et al., 1981), and blood testosterone concentrations increase 2–4 weeks later (Rosa and Bryant, 2003). This hormonal change is reflected in the pattern of testosterone in ram seminal plasma throughout the year (Casao et al., 2010) or after melatonin treatment (Casao et al., 2013). In the Romney Marsh or Hampshire rams in the present study that were located in an area where there was an equatorial photoperiod, the testosterone increase in seminal plasma occurred before the melatonin increase, so a melatonin stimulatory effect is unlikely. It is possible that in these rams there was stimulation by non-photoperiodic cues to synchronize the reproductive functions. Rams subjected to constant short or long photoperiods for several years had an endogenous reproductive rhythm in terms of testicular volume and prolactin secretion (Howles et al., 1982), although the pattern did not occur at the time of year when the natural breeding cycle prevailed. Social factors may also have affected the outcomes in the present study where the rams with British breeding also had greater concentrations of testosterone than the Creole rams. Creole rams are smaller and less aggressive than Hampshire and Romney Marsh rams, and thus probably have a lesser rank in their social group, as indicated by their behavior during semen collection. Results of previous studies indicate that subordinate males have lesser testosterone concentrations than dominant animals (Ungerfeld and Lacuesta, 2015) and fewer increases in testosterone during the breeding season (Aguirre et al., 2007).

For antioxidant enzyme activity, there was only variation in GPx activity during the year, although this variation was not correlated with that of melatonin. This result is consistent with previous findings with Simmental bulls raised in tropical climates, in which there was only a seasonal profile in GPx (Nichi et al., 2006). In the present study, GPx activity increased in May, at the end of the first rainy season, and remained greater during the following dry and rainy seasons. Under tropical conditions, where there is a relatively greater temperature or humidity, antioxidant enzyme activity increases and this minimizes oxidative damage to the spermatozoa (Soren et al., 2016). Although the temperature remains constant throughout the year in the equatorial region where the rams of the present study were located, this increase in GPx activity could be due to the greater ambient humidity during the rainy season. In a previous study with Duroc boars in a tropical region results for sperm morphology patterns indicated that with relatively lesser temperatures and greater humidity there was the same effect that occurred with relatively greater temperatures and lesser humidity (Suriyasomboon et al., 2005).

5. Conclusions

In conclusion, melatonin is present in the seminal plasma of Creole, Romney Marsh, and Hampshire rams when they are located in an area where there is an equatorial photoperiod (12L:12D), with there being differences in concentration between rainy and dry seasons. The melatonin concentration in this fluid, however, does not correlate with the testosterone concentration or antioxidant enzyme activity, unlike what occurs in rams located in areas where that are temperate climates. These findings provide new perspectives on the use of melatonin in ram reproduction when rams are located in areas where there are tropical or equatorial climates.

Conflicts of interest

None.

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