Exogenous melatonin does not improve the freezability of Blanca Andaluza goat semen over exposure to two months of short days

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Abstract

This paper compares the effects of exposure to exogenous melatonin (MEL), short days (SD, 8 h of light) and long days (LD, 16 h of light), on reproductive activity, sperm motility and other reproductive variables, in Blanca Andaluza bucks. Fourteen males were spilt into two groups of seven animals (G1 and G2). They were subjected to five alternations of 2 months of LD followed by 2 months of SD or MEL before the experimental period of three consecutive intervals of: 1) 2 months of SD (G1, N=7) or MEL (G2, N=7); 2) 2 months of LD (G1+G2, N=14); and 3) 2 months of SD (G2, N=7) or MEL (G1, N=7). Plasma testosterone concentration, live weight, testicular weight and fresh semen quality were determined weekly. Semen was also cooled and frozen-thawed every fortnight, and the same quality variables measured as for fresh sperm. When the bucks were under LD treatment, the testosterone concentration was higher than when under MEL or SD treatment ($P<0.01$); values for the semen concentration and total number of sperm per ejaculate were also higher ($P<0.001$). No differences were observed between the MEL and SD treatments in terms of fresh, cooled or frozen-thawed sperm quality. Only some quality variables on fresh semen were improved by MEL and SD treatment (at least $P<0.05$). In conclusion the results of the present experiment showed that MEL improved the fresh semen motility variables, but this did not improve the motility of frozen-thawed sperm over that recorded for either SD or LD treatment.

Keywords: melatonin, photoperiod, semen cryopreservation, bucks

1. Introduction

The photoperiod (or season of the year) has been suggested the main factor influencing the seasonality of reproduction in male goats (Delgadillo et al. 1993). Short or decreasing days (SD) stimulate the secretion of luteinizing hormone (LH), which in turn induces testicular growth and the release of testosterone, and thus a quantitative and qualitative increase in semen production. In contrast, long or increasing days (LD) depress LH secretion, testicular growth and the release of testosterone, causing semen quality and quantity to fall (Muduuli et al. 1979; Rouger, 1974; Pelletier et al. 1988; Delgadillo and Chemineau, 1992; Zarazaga et al. 2009). Artificial photoperiod (2 months of SD:2 months of LD; or 4 months of SD:4 months of LD) has been used to control even to abolish (1 month of SD:1 month of LD) the oscillation in testosterone concentration (Delgadillo et al., 1993, 1995; Delgadillo and Chemineau, 1992) and semen quality in male goats (Delgadillo et al., 1991, 1992, 1993). Indeed, light treatments have been used to ensure the production of more spermatozoa per ejaculate, and thus obtain a larger number of doses for artificial insemination per male and year (Delgadillo et al. 1993).

The freezing of goat semen is a basic requirement of assisted reproduction programmes, and allows samples of high genetic value to be maintained over long periods (Cabrera et al. 2005; Mara et al. 2007; Dorado et al. 2009). However, the processes of freezing and thawing can harm the ultrastructure, biochemistry and functional integrity of sperm cells (Watson, 2000), resulting in their reduced motility, membrane integrity and fertilizing capacity (Purdy, 2006).

Melatonin administration is reported to improve sperm quality in rams during the non-breeding season (Kokolis et al. 2000; Kaya et al. 2001; Casao et al. 2010a). This hormone has a regulatory effect on the hypothalamus-pituitary-testicular axis

(D'Occhio et al. 1984), modulating GnRH pulsatility (Langford et al. 1987) and gonadotrophin and testosterone production (Lincoln et al. 1990). A direct effect of melatonin on semen quality cannot, however, be ruled out since melatonin receptors are present in the epididymal tissue of rats (Shiu et al. 2000) and humans (Van Vuuren et al. 1992), and melatonin is present in human (Luboshitzky et al. 2002) and ram (Casao et al. 2010b) seminal plasma. In a number of species, melatonin may also help protect spermatozoa from different kinds of injury during semen manipulation (Sarabia et al. 2009; Rao and Gangadharan, 2008; Sönmez et al. 2007; Du Plessis et al. 2010; Casao et al. 2010c). The literature, however, contains no information on the potential beneficial effects of exogenous melatonin with regard to the freezing of buck semen.

It was hypothesized that treatment with exogenous melatonin might obtain fresh and frozen-thawed semen of quality similar to that afforded by SD treatment, and that both these treatments would provide better quality semen than LD treatment. The present study therefore examines whether two months of treatment with exogenous melatonin has any beneficial effect on the cryopreservation of buck semen compared to two months of SD or LD treatment.

2. Material and methods

General

All procedures were performed by trained personnel in strict accordance with Spanish guidelines for the protection of experimental animals (RD 53/2013), and in agreement with European Union Directive 86/609. The study was conducted at the University of Huelva experimental farm (latitude 37º 15'N), which meets the requirements of the European Community Commission for Scientific Procedure Establishments (2010/63).

The experimental animals were 14 Blanca Andaluza bucks (1.5 years old at the start of the experiment), previously trained to mount a teaser doe and to ejaculate into an artificial vagina. Bucks were fed daily with barley straw *ad libitum*, and lucerne hay and a commercial concentrate, following INRA standards (Morand-Fehr and Sauvant, 1988) according to the animals' live weights (LW). All animals had free access to water and mineral blocks containing trace elements and vitamins. At the onset of the experiment, the animals were allocated to relatively uniform groups, according to their LW and testicular weight (TW).

Experimental Design

To adapt the bucks to the rapid photoperiodic alternations to which they would be submitted, before the experimental period, all the males were exposed to five alternations of 2 months of LD (16 h of light/dark; lights on 07:00 h, lights off 23:00 h) followed by 2 months of SD (8 h of light/dark; lights on 07:00 h, lights off 15:00 h) or melatonin (MEL) treatment. Thus, by the end of this period, all animals had been subjected to the same pre-treatments: three periods of LD plus one of SD plus one of MEL).

The experimental period proper started on the 8th July, when half of the bucks received three subcutaneous melatonin implants (Group 1, N=7), each containing 18 mg of the hormone (Melovine®, CEVA Salud Animal, Barcelona, Spain), at the base of the left ear. The other seven males (Group 2, N=7) were exposed to artificial SD for a period of 2 months. On the 9th September, all animals were exposed to LD for a period of 2 months. To ensure that the melatonin-implanted animals properly experienced the photoperiod, any remains of the implant were removed. On the 8th November, Group 1

was exposed to SD and Group 2 was implanted with three subcutaneous melatonin implants as described above (Fig. 1).

Testicular weight and plasma testosterone concentration

Testicular weight and the plasma testosterone concentration were recorded weekly throughout the study. The former was assessed by comparative palpation using an orchidometer; all evaluations were made by the same operator (Oldham et al. 1978). Blood samples for determining the plasma testosterone concentration were obtained by jugular venipuncture and using vacuum tubes containing heparin; samples were taken once every week at 09:00 h over the entire observation period. Plasma was obtained by centrifugation at 3000 x *g* for 30 min, and the plasma stored at -20ºC until use. Testosterone concentrations were determined using a commercial enzyme-linked immunoassay (ELISA) kit (Demeditec Diagnostics, Kiel-Wellsee, Germany). The intra- and interassay coefficients of variation were 6.4% and 10.8% respectively.

Semen collection and evaluation of sexual behaviour

Semen collection was performed weekly. On each occasion, the sexual behaviour of each buck was assessed by presenting it with an intact, oestrus-induced doe, allowing 5 min for the male to ejaculate. The induction of oestrus in the teaser doe was achieved via a subcutaneous injection of 2 mg of oestradiol cypionate suspended in oil (Sigma-Aldrich Química, S.A., Spain) (Delgadillo et al. 1999; Zarazaga et al. 2009). The ejaculation latency, the percentage of bucks that ejaculated, and the percentage of active males (bucks that attempted to mount the doe at least twice, but which did not achieve ejaculation within 5 min), were recorded. Animals were always tested in the same order and by the same handlers.

Fresh semen quality

The volume of the ejaculated semen was recorded immediately after collection in a graduated collection vial. Global motility was assessed by transferring a drop of undiluted semen to a warm slide (35ºC), placing a cover slip on it, and immediately observing it under a microscope at 40x. The assessment of overall motility was made using a 0 to 5 scale (0 = no motility, 5 = 100% motility) (Baril et al. 1993). Forward progressive motility (FPM) was assessed by transferring a drop of semen diluted 1:400 with PBS onto a warm slide and estimating the progressive sperm motility on a scale of 0–5, as observed at 400x (Baril et al. 1993). Using this same sample, sperm concentration and motility variables were measured using a computer-assisted sperm analysis (CASA) system (ISAS, Proiser SL, Valencia, Spain). Sperm concentration was measured in a Bürker chamber, again using the CASA system, after diluting an aliquot with a 0.05% formaldehyde saline solution (1:400) and observing at 400x. The total number of spermatozoa per ejaculate was calculated from the volume and sperm concentration values.

The following motility variables were then analysed: percentage of static, motile and progressive motile spermatozoa, curvilinear velocity (VCL, µm/s, or the velocity of the actual trajectory of the sperm), straight-line velocity (VSL, µm/s, i.e., the velocity calculated across the straight-line distance between the beginning and end of the sperm track), average velocity (VAP, µm/s, or the velocity over the average calculated path), linearity coefficient (LIN), the straightness coefficient (STR) and the wobble coefficient (WOB). Sperm were classified as medium-speed spermatozoa when their velocity was between 45 and 75 µm/s, and as rapid spermatozoa when their velocity was >75 µm/s.

Spermatozoa were considered progressively motile when they travelled straight over at least 80% of their trajectory.

Semen cooling and freezing

Semen was chilled and frozen every fortnight. After examination of the semen quality, ejaculates were diluted in washing solution at 1:10 (v:v) (250 mM Tris, 28 mM glucose, 104 mM citric acid, 0.05% streptomycin, 500 UI penicillin/mL) at 37ºC and centrifuged once (700 × *g* for 15 min) to eliminate the seminal plasma. The supernatant was then removed at room temperature (23ºC), and the sperm pellet diluted in a Tris-yolk extender (250 mM Tris, 28 mM glucose, 104 mM citric acid, 6% egg yolk, 0.05% streptomycin, 500 UI penicillin/mL and distilled water to 100 ml). After 5 min, a new dilution was performed by adding a second diluent (250 mM Tris, 28 mM glucose, 104 mM citric acid, 6% egg yolk, 8% glycerol, 0.05% streptomycin, 500 UI penicillin/mL, and distilled water to 100 mL) at room temperature (20ºC) at a volume similar to that used in the first dilution, resulting in a final concentration of $800x10^6$ spermatozoa/mL, 6% egg yolk and 4% glycerol. Egg yolk lecithin was inactivated by subjecting the egg yolk to 56ºC for 30 min.

All diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA).

The semen thus prepared was chilled from room temperature to 5ºC over at least 3 h (in a cooler), and then packed into 0.25 or 0.50 mL plastic straws. These straws were placed horizontally on a rack situated 4 cm above the surface of a liquid nitrogen pool for 15 min, and then plunged into and stored in the liquid nitrogen.

Post-cooling and post-thawing assessment of semen quality

Semen quality was evaluated after the 3 h cooling period and after thawing (thawing was performed no later than one day after freezing). Frozen straws were thawed in a water bath at 37ºC for 30 s. In both cases, a sample of semen was transferred to a warm slide and the motility variables measured as described above using a CASA system.

2.5. Statistical analysis

The effects of the treatments on TW and plasma testosterone concentrations were analysed by repeated measures ANOVA. Treatment effects with respect to time were analysed using the Tukey t-test. ANOVA was used to analyse the effect of the treatments on the quality of fresh, cooled and frozen-thawed semen. Variables expressed as percentages (i.e., LIN, STR, WOB, sperm motility, percentage of bucks that ejaculated, and the percentage of active males) were arcsine-transformed before analysis. When differences among treatments were detected by ANOVA, the Tukey t-test was used to determine their significance. Overall motility and FPM were compared using the Mann-Whitney U test.

Significance was set at $P<0.05$. All analyses were performed using the SPSS package (Statistical Package for the Social Sciences, 2008).

3. Results

3.1. Testicular weight and testosterone concentration

Repeated measures ANOVA showed a definite effect of time (week) on testosterone concentration ($P<0.01$) but not on TW (Fig. 2). However, these changes

occurred in a similar manner in all treatments; no interaction *treatment x week* was observed.

No differences were observed among the treatments in terms of TW (308.58±1.7 g). No differences in testosterone concentration were seen between the MEL and SD treatments, but the LD treatment showed lower testosterone concentrations (14.65±0.86, 14.03±1.04 and 4.21±0.52 ng/mL, for MEL, SD and LD, respectively; $P<0.01$).

3.2. Sexual behaviour and fresh semen characteristics

No differences were seen among treatments in terms of ejaculation latency (57.8±3.0 s for all three treatments as a whole), the percentage of bucks deemed active (89.2%), or the percentage of bucks that ejaculated (83.1%) (Table 1). Neither were any differences seen in terms of overall motility (4.72±0.04) or individual motility (4.54±0.05). However, the ejaculate volume was higher when animals underwent LD compared to MEL treatment (Table 1). In addition, the semen concentration ($P<0.001$) and the total number of sperm per ejaculate ($P<0.001$) were higher during the LD treatment than during the MEL or SD treatments.

3.3. Fresh semen motility variables

No differences were seen between the MEL and SD treatments in terms of fresh sperm motility variables. The VSL, LIN, STR and progressive spermatozoa were, however, higher during the MEL treatment than the LD treatment (at least $P<0.05$). The percentage of medium-speed spermatozoa was higher during the SD treatment than during the LD treatment ($P<0.001$) (Table 2).

No differences were seen between the MEL and SD treatments with respect to the motility variables of the rapid (Table 3) and medium-speed spermatozoa (Table 4).

In these treatments, the STR values recorded for the rapid spermatozoa were higher than those recorded in the LD treatment ($P<0.01$). The VCL, VSL, VAP and LIN values for these rapid sperms were also higher during the MEL than during the LD treatment (at least $P<0.05$) (Table 3). For the medium-speed spermatozoa, the VSL, LIN, STR and WOB values were higher during the MEL and SD treatments than during the LD treatment ($P<0.001$). Moreover, the VAP values recorded for these sperms during the SD treatment were higher than during the LD treatment ($P<0.001$) (Table 4).

3.4. Cooled semen quality variables

No differences were seen between the MEL and SD treatments in terms of cooled semen quality (Table 5).

The VCL was, however, lower during the MEL and SD treatments compared to the LD treatment ($P<0.001$), and the VAP cooled higher during the LD than the SD treatment ($P<0.01$). Finally, the percentage of medium-speed motile sperms was lower during the LD treatment ($P<0.001$) (Table 5).

No difference was seen between the MEL and SD treatments with respect to sperm motility variables. However, the VCL and VAP of the rapid sperm were lower in the SD than in the LD treatment (at least $P<0.01$) (Table 3). For the medium-speed spermatozoa, the VSL, VAP, LIN and WOB values were higher in the MEL treatment than in the LD treatment (at least $P<0.05$) (Table 4).

3.5. Frozen-thawed sperm quality

No differences were seen between the MEL and SD treatments in terms of frozen-thawed sperm quality. The WOB value was, however, higher ($P<0.05$) during the MEL than during the LD treatment (Table 6). No differences were seen among any

of the treatments in terms of motility variables for the rapid or medium-speed spermatozoa (Tables 3 and 4).

4. Discussion

This research supports the hypothesis that two months of SD or MEL treatment provide similar fresh sperm quality results. However, they also reveal that MEL treatment does not protect buck sperm from the harm caused by cryopreservation.

The plasma testosterone concentration was clearly associated with the treatment to which the bucks were subjected. High testosterone concentrations were recorded during the SD and MEL treatments, and low concentrations during the LD treatment. This helps confirm that the photoperiod is the main environmental factor controlling reproductive activity in Blanca Andaluza bucks. However, only small variations in TW were recorded following changes from the LD to melatonin/SD treatment. This finding is similar to that described by Delgadillo et al. (1991), who subjected bucks to two months of SD and then two months of LD. As proposed by Pelletier et al. (1985) in rams, the photoperiod treatments used in the present study probably caused the stimulation (SD/MEL) and relaxation (LD) of the hypothalamo-pituitary axis. However, the inhibitory effect of LD may not have had sufficient time to build up with the rapid photoperiodic schedule changes used. Consequently, a greater TW was maintained during the non-stimulatory photoperiod than might be seen with natural photoperiod changes. The absence of variation in TW indicates high spermatogenic activity in all treatments.

The percentage of bucks that ejaculated, or that were considered sexually active, was very high even during the LD treatment; indeed, no differences were seen among treatments. This suggest that the rapid photoperiod schedule change might have masked

the expected negative effect of LD. This result indicates that the bucks were sensitive to the LD photoperiod, as their testosterone concentrations show, but the reduction in their libido was very low since more than the 80% of the bucks ejaculated or were sexually active during the LD treatment. The values obtained for these variables are similar to those reported for Payoya bucks by our group at the same latitude (Zarazaga et al. 2009). In the latter experiment, differences during the breeding season and seasonal anoestrous of the second year of the study were also scant.

No differences were observed between the MEL and SD treatments with respect to sexual behaviour or semen quality, clearly indicating that both treatments stimulate breeding activity in these males. A succession of LD and SD might therefore be considered an effective way of stimulating breeding activity in males on organic farms, where hormonal treatments are not allowed. Nevertheless, the values for sperm concentration and total sperm number per ejaculate were higher during the LD treatment. Annual variation in these variables has been described in the literature for different goat breeds (Karagiannidis et al. 2000; Pérez and Mateos, 1996; Roca et al. 1992; Zarazaga et al. 2009), with higher sperm concentrations during spring-summer (=LD). However, the latter authors observed a smaller ejaculate volume and a higher spermatozoa count with respect to that volume, during the non-breeding season than during the breeding season. However, in the present experiment, no differences in ejaculate volume were seen among any of the treatments, probably due to the rapid alternations of photoperiod.

The fresh semen motility results are in line with those for sexual behaviour; no differences between the MEL and SD treatments were observed. However, VSL, LIN and STR, and perhaps more importantly the percentage of progressive motile spermatozoa, increased during the MEL treatment compared to the LD treatment. These

results contrast with those of Casao et al. (2010a) in rams. The latter authors observed an increase in progressive motility and VCL, but only in the rapid spermatozoa of semen obtained during seasonal anoestrous in MEL-treated animals (compared to controls). However, they observed no other effect of MEL treatment on any other motility variable, either in rapid or medium-speed spermatozoa. In the present experiment, all motility variables except for WOB in the rapid spermatozoa, and all motility variables except for VCL and VAP in the medium-speed spermatozoa, were higher in the MEL-treatment period. The explanation for this might lie in the species used or the experimental design; Casao et al. (2010a) used different males for each group, but in the present work the same males were moved from one treatment to another. Tuli and Holtz (1995) also observed a higher percentage of progressive motile spermatozoa in Boer bucks during the breeding season than during seasonal anoestrous. Abdelwahab et al. (2006), reported differences in VAP and LIN associated with the season, their values being higher in spring (partially similar to the present LD treatment) than in autumn (partially similar to the present MEL or SD treatment). However, in the present work, no differences in VAP were seen, and the effect on LIN was the opposite to that described by Abdelwahab et al. (2006) (although they used a smaller number of males and recorded a lower number of ejaculates in each season). The increase in progressive motility and most of the other motility variable values induced by MEL or SD might (at least partly) explain the improvement in reproductive variables reported in previous studies on goats by our group (Celi et al. 2013; Zarazaga et al. 2012a, 2012b, 2013) (in these studies the females were mated around 45 days after melatonin implantation in the males).

Despite the above results, neither the MEL nor the SD treatment improved the quality of cooled or frozen-thawed sperm over the values recorded for the LD treatment.

This result differs from that reported in the literature for rams (Succu et al. 2011). Other reports also exist claiming a beneficial effect of melatonin in terms of protecting spermatozoa from different kind of manipulation injury in different species, including humans (Sarabia et al. 2009; Rao and Gangadharan, 2008; Sönmez et al. 2007; Du Plessis et al. 2010; Casao et al. 2010). The reasons for these controversial results may be several and not mutually exclusive. In ram seminal plasma, the presence of melatonin improves sperm quality (Casao et al. 2010) and might do so in bucks too were it not for the fact that buck sperm needs to be washed, which removes most of any melatonin present. Further, Succu et al. (2011) used only three males and their ejaculates, and it is well known that the freezability of semen varies widely depending on the donor male.

Concerning the Blanca Andaluza breed. This breed is not as important economically or in census terms as the Malagueña or the Murciano-Granadina, but it is the one that best represents meat goat production linked to grazing. The majority of goat farms raising this breed are located in Andalusia's mountain zones. Interest in the preservation of autochthonous breeds, raised using extensive or semi-extensive grazing, has also recently increased among Spanish farmers and many of these breeds, such as the Blanca Andaluza goat, are considered as special protection breeds (BOE, 2006). For that reason steps, as the present experiment, should therefore be taken to ensure its preservation.

5. Conclusions

The present study shows that, in Blanca Andaluza bucks, two months of SD treatment provide semen of a quality equal to that achieved with two months of exogenous MEL treatment. Reproductive activity (testosterone concentrations) was

associated with the treatment to which the animals were subjected, with high testosterone concentrations recorded during the MEL and SD treatments, and low concentrations during the LD treatment. The MEL treatment improved the quality of fresh semen, but neither this treatment nor the SD treatment improved the quality of cooled or frozen-thawed sperm over that achieved with LD treatment.

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Figure 1. Experimental procedures.

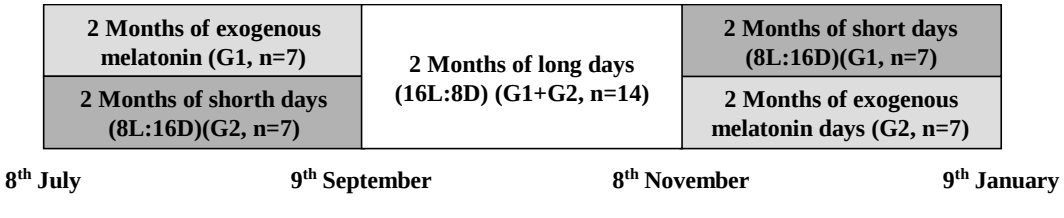

Figure 2. Weekly means (±SEM) for testosterone concentration (ng/mL) (top), and testicular weight (g) (bottom) for Blanca Andaluza bucks submitted to the different treatments over time (shaded area = long day treatment). Different letters indicates differences between weeks.

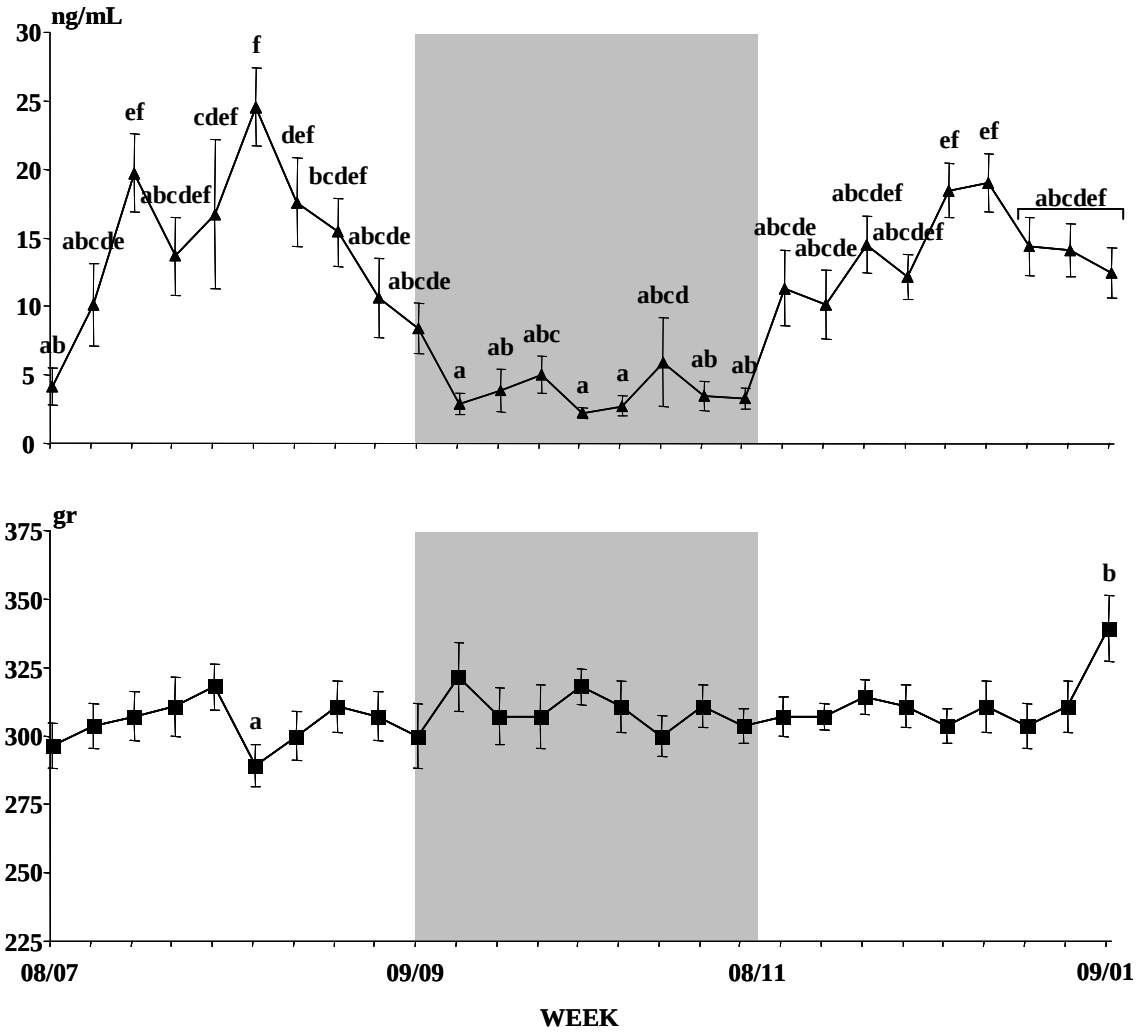

Table 1. Sexual behaviour and fresh semen quality under the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

	MEL	SD	LD
Bucks that ejaculate (%)	85.7	82.7	80.8
Active males (%)	92.1	90.6	84.8
Ejaculation latency (s)	57.9±4.9	64.2±5.9	51.0±4.6
Ejaculate volume (mL)	0.99±0.04a	1.12±0.05ab	1.16±0.04b
Semen concentration (10^6 sperm/mL)	5613.2±196.5a	5845.6±213.99a	6861.4±284.1b
Total sperm per ejaculate (10^6 sperm/ejaculate)	5516.4±292.9a	6231.1±319.2a	7609.4±356.7b
Global motility	4.73±0.06	4.71±0.05	4.72±0.07
Individual motility (FPM)	4.62±0.08	4.54±0.08	4.46±0.09

Different letters in the same line indicate significant differences between groups ($P<0.05$).

Table 2. Sperm motility variables and percentage of different types of spermatozoa in fresh semen during the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

	MEL	SD	LD
VCL (μm/s)	99.4±1.1	96.0±1.2	97.2±1.1
VSL (μm/s)	75.1±1.4a	72.3±1.4ab	69.8±1.4b
VAP (μm/s)	89.6±1.4	86.1±1.4	85.8±1.4
LIN (%)	75.2±0.9a	74.9±0.9a	71.4±1.1b
STR (%)	83.6±0.6a	83.7±0.5a	80.8±0.7b
WOB (%)	89.7±0.6	89.2±0.6	87.9±0.7
Motile spermatozoa (%)	86.7±1.3	85.9±1.1	84.7±1.7
Rapid spermatozoa (%)	70.5±1.6	67.4±1.5	70.2±1.9
Medium-speed spermatozoa (%)	12.4±0.8ab	14.4±0.8b	10.6±0.7a
Progressive spermatozoa (%)	59.3±1.4a	58.9±1.1a	53.2±1.7b

VCL: Curvilinear velocity, μm/s. VSL: Straight-line velocity, μm/s. VAP: Average path velocity, μm/s. LIN: Linearity coefficient, %. STR: Straightness coefficient, %. WOB: Wobble coefficient, %. Different letters in the same line indicate significant differences between groups ($P<0.05$).

Table 3. Motility variables for rapid spermatozoa in fresh, cooled and frozen-thawed semen from bucks undergoing the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

			MEL	SD	LD
Rapid spermatozoa	Fresh semen	VCL	109.3± 1.0a	106.3±1.0ab	105.8±0.9b
		VSL	82.7±1.3a	80.3±1.3ab	76.7±1.4b
		VAP	98.6±1.2a	95.5±1.3ab	93.9±1.3b
		LIN	75.5±0.9a	75.3±0.9ab	72.3±1.1b
		STR	83.6±0.6a	83.8±0.5a	81.0±0.8b
		WOB	90.1±0.6	89.6±0.7	88.6±0.7
	Cooled semen	VCL	100.1±0.9b	99.2±1.1b	105.3±0.7a
		VSL	54.2±1.2	52.1±1.3	55.1±1.2
		VAP	78.6±1.0ab	76.5±1.3b	81.2±1.0a
		LIN	54.2±1.2	52.6±1.5	52.3±1.1
		STR	68.6±0.8	67.5±1.2	67.5±0.9
		WOB	78.6±0.8	77.2±1.1	77.2±0.8
	Frozen-thawed semen	VCL	89.7±0.7	90.4±0.6	90.3±0.4
		VSL	58.9±1.6	57.9±1.4	56.5±1.0
		VAP	74.1±1.4	74.1±1.0	72.9±0.7
		LIN	65.5±1.7	64.0±1.5	62.6± 1.0
		STR	78.9±1.1	77.7±1.1	77.3±0.8
		WOB	82.3±1.3	81.9±0.9	80.7±0.7

VCL: Curvilinear velocity, μm/s. VSL: Straight-line velocity, μm/s. VAP: Average path velocity, μm/s. LIN: Linearity coefficient, %. STR: Straightness coefficient, %. WOB: Wobble coefficient, %. Different letters in the same line indicate significant differences between groups ($P<0.05$).

Table 4. Motility variables for medium-speed spermatozoa in fresh, cooled and frozen-thawed semen in bucks undergoing the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

			MEL	SD	LD
Medium-speed spermatozoa	Fresh semen	VCL	63.6±0.6	64.9±0.2	63.4±0.8
		VSL	46.9±1.0a	48.2±0.7a	41.8±1.0b
		VAP	55.7±0.8ab	57.1±0.5a	53.3±0.9b
		LIN	73.5±1.2a	74.1±1.0a	65.2±1.4b
		STR	83.4±0.9a	84.1±0.7a	77.1±1.3b
		WOB	87.5±0.8a	87.9±0.7a	83.3±1.1b
	Cooled semen	VCL	62.9±0.3	62.7±0.2	62.1±0.2
		VSL	34.6±1.0a	33.0±1.1ab	30.4±0.8b
		VAP	48.5±0.7a	47.4±0.7ab	45.3±0.6b
		LIN	54.8±1.5a	52.5±1.6ab	48.9±1.2b
		STR	70.7±1.2	68.8±1.5	66.8±1.2
		WOB	76.9±0.9a	75.6±0.9ab	72.9±0.8b
	Frozen-thawed semen	VCL	60.7±0.3	60.2±0.3	60.2±0.3
		VSL	41.3±0.8	40.1±0.8	39.1±0.7
		VAP	50.8±0.6	49.9±0.6	49.0±0.5
		LIN	67.9±1.2	66.6±1.4	64.9±1.0
		STR	81.0±0.8	80.0±1.0	79.6±0.7
		WOB	83.6±0.8	82.9±0.8	81.3±0.7

VCL: Curvilinear velocity, μm/s. VSL: Straight-line velocity, μm/s. VAP: Average path velocity, μm/s. LIN: Linearity coefficient, %. STR: Straightness coefficient, %. WOB: Wobble coefficient, %. Different letters in the same line indicate significant differences between groups ($P<0.05$).

Table 5. Sperm motility variables and percentage of different types of spermatozoa in cooled semen during the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

	MEL	SD	LD
VCL (μm/s)	86.7±1.4b	85.5±1.5b	93.5±1.4a
VSL (μm/s)	47.0±1.3	44.8±1.6	48.6±1.3
VAP (μm/s)	67.8±1.3ab	65.5±1.5b	71.6±1.4a
LIN (%)	54.2±1.2	52.3±1.5	51.8±1.1
STR (%)	69.0±0.8	67.7±1.2	67.4±0.9
WOB (%)	78.1±0.8	76.6±1.0	76.5±0.8
Motile spermatozoa (%)	86.1±1.1	83.8±1.2	83.0±2.0
Rapid spermatozoa (%)	60.2±2.2	57.4±2.2	65.1±2.4
Medium-speed spermatozoa (%)	18.0±1.5b	18.3±1.5b	11.9±1.0a
Progressive spermatozoa (%)	31.9±1.5	29.5±1.7	29.6±1.5

VCL: Curvilinear velocity, μm/s. VSL: Straight-line velocity, μm/s. VAP: Average path velocity, μm/s. LIN: Linearity coefficient, %. STR: Straightness coefficient, %. WOB: Wobble coefficient, %. Different letters in the same line indicate significant differences between groups ($P<0.05$).

Table 6. Sperm motility variables and percentage of different types of spermatozoa in frozen-thawed semen during the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

	MEL	SD	LD
VCL (μm/s)	63.3±1.5	61.6±1.4	62.0±1.1
VSL (μm/s)	41.1±1.3	38.8±1.1	38.2±1.0
VAP (μm/s)	52.2±1.5	50.0±1.3	49.4±1.0
LIN (%)	64.8±1.3	63.2±1.3	61.6±1.0
STR (%)	78.6±0.9	77.6±1.0	77.1±0.7
WOB (%)	82.0±0.9	81.1±0.7	79.6±0.6
Motile spermatozoa (%)	65.6±3.1	62.4±3.2	67.8±2.2
Rapid spermatozoa (%)	23.9±2.3	20.2±2.0	20.4±1.6
Medium-speed spermatozoa (%)	27.8±1.7	28.1±1.7	32.1±1.4
Progressive spermatozoa (%)	31.1±1.8	26.5±1.6	30.5±1.4

VCL: Curvilinear velocity, μm/s. VSL: Straight-line velocity, μm/s. VAP: Average path velocity, μm/s. LIN: Linearity coefficient, %. STR: Straightness coefficient, %. WOB: Wobble coefficient, %.