

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

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1 **Abstract**

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

21

22 **Keywords:** melatonin, photoperiod, semen cryopreservation, bucks

1 **1. Introduction**

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

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

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

1 (D'Occhio et al. 1984), modulating GnRH pulsatility (Langford et al. 1987) and
2 gonadotrophin and testosterone production (Lincoln et al. 1990). A direct effect of
3 melatonin on semen quality cannot, however, be ruled out since melatonin receptors are
4 present in the epididymal tissue of rats (Shiu et al. 2000) and humans (Van Vuuren et al.
5 1992), and melatonin is present in human (Luboshitzky et al. 2002) and ram (Casao et
6 al. 2010b) seminal plasma. In a number of species, melatonin may also help protect
7 spermatozoa from different kinds of injury during semen manipulation (Sarabia et al.
8 2009; Rao and Gangadharan, 2008; Sönmez et al. 2007; Du Plessis et al. 2010; Casao et
9 al. 2010c). The literature, however, contains no information on the potential beneficial
10 effects of exogenous melatonin with regard to the freezing of buck semen.

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

17

18 **2. Material and methods**

19 *General*

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

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

9

10 *Experimental Design*

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

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

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

3

4 *Testicular weight and plasma testosterone concentration*

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

15

16 *Semen collection and evaluation of sexual behaviour*

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

1

2 *Fresh semen quality*

3 The volume of the ejaculated semen was recorded immediately after collection
4 in a graduated collection vial. Global motility was assessed by transferring a drop of
5 undiluted semen to a warm slide (35°C), placing a cover slip on it, and immediately
6 observing it under a microscope at 40x. The assessment of overall motility was made
7 using a 0 to 5 scale (0 = no motility, 5 = 100% motility) (Baril et al. 1993). Forward
8 progressive motility (FPM) was assessed by transferring a drop of semen diluted 1:400
9 with PBS onto a warm slide and estimating the progressive sperm motility on a scale of
10 0–5, as observed at 400x (Baril et al. 1993). Using this same sample, sperm
11 concentration and motility variables were measured using a computer-assisted sperm
12 analysis (CASA) system (ISAS, Proiser SL, Valencia, Spain). Sperm concentration was
13 measured in a Bürker chamber, again using the CASA system, after diluting an aliquot
14 with a 0.05% formaldehyde saline solution (1:400) and observing at 400x. The total
15 number of spermatozoa per ejaculate was calculated from the volume and sperm
16 concentration values.

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

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

3

4 *Semen cooling and freezing*

5 Semen was chilled and frozen every fortnight. After examination of the semen
6 quality, ejaculates were diluted in washing solution at 1:10 (v:v) (250 mM Tris, 28 mM
7 glucose, 104 mM citric acid, 0.05% streptomycin, 500 UI penicillin/mL) at 37°C and
8 centrifuged once ($700 \times g$ for 15 min) to eliminate the seminal plasma. The supernatant
9 was then removed at room temperature (23°C), and the sperm pellet diluted in a Tris-
10 yolk extender (250 mM Tris, 28 mM glucose, 104 mM citric acid, 6% egg yolk, 0.05%
11 streptomycin, 500 UI penicillin/mL and distilled water to 100 ml). After 5 min, a new
12 dilution was performed by adding a second diluent (250 mM Tris, 28 mM glucose, 104
13 mM citric acid, 6% egg yolk, 8% glycerol, 0.05% streptomycin, 500 UI penicillin/mL,
14 and distilled water to 100 mL) at room temperature (20°C) at a volume similar to that
15 used in the first dilution, resulting in a final concentration of 800×10^6 spermatozoa/mL,
16 6% egg yolk and 4% glycerol. Egg yolk lecithin was inactivated by subjecting the egg
17 yolk to 56°C for 30 min.

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

21 The semen thus prepared was chilled from room temperature to 5°C over at least
22 3 h (in a cooler), and then packed into 0.25 or 0.50 mL plastic straws. These straws
23 were placed horizontally on a rack situated 4 cm above the surface of a liquid nitrogen
24 pool for 15 min, and then plunged into and stored in the liquid nitrogen.

25

1 *Post-cooling and post-thawing assessment of semen quality*

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

7

8 *2.5. Statistical analysis*

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

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

20

21 **3. Results**

22

23 *3.1. Testicular weight and testosterone concentration*

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

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

3 No differences were observed among the treatments in terms of TW (308.58 ± 1.7
4 g). No differences in testosterone concentration were seen between the MEL and SD
5 treatments, but the LD treatment showed lower testosterone concentrations (14.65 ± 0.86 ,
6 14.03 ± 1.04 and 4.21 ± 0.52 ng/mL, for MEL, SD and LD, respectively; $P < 0.01$).

7

8 *3.2. Sexual behaviour and fresh semen characteristics*

9 No differences were seen among treatments in terms of ejaculation latency
10 (57.8 ± 3.0 s for all three treatments as a whole), the percentage of bucks deemed active
11 (89.2%), or the percentage of bucks that ejaculated (83.1%) (Table 1). Neither were any
12 differences seen in terms of overall motility (4.72 ± 0.04) or individual motility
13 (4.54 ± 0.05). However, the ejaculate volume was higher when animals underwent LD
14 compared to MEL treatment (Table 1). In addition, the semen concentration ($P < 0.001$)
15 and the total number of sperm per ejaculate ($P < 0.001$) were higher during the LD
16 treatment than during the MEL or SD treatments.

17

18 *3.3. Fresh semen motility variables*

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

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

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

8

9 *3.4. Cooled semen quality variables*

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

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

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

21

22 *3.5. Frozen-thawed sperm quality*

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

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

3

4 **4. Discussion**

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

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

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

1 the expected negative effect of LD. This result indicates that the bucks were sensitive to
2 the LD photoperiod, as their testosterone concentrations show, but the reduction in their
3 libido was very low since more than the 80% of the bucks ejaculated or were sexually
4 active during the LD treatment. The values obtained for these variables are similar to
5 those reported for Payoya bucks by our group at the same latitude (Zarazaga et al.
6 2009). In the latter experiment, differences during the breeding season and seasonal
7 anoestrous of the second year of the study were also scant.

8 No differences were observed between the MEL and SD treatments with respect
9 to sexual behaviour or semen quality, clearly indicating that both treatments stimulate
10 breeding activity in these males. A succession of LD and SD might therefore be
11 considered an effective way of stimulating breeding activity in males on organic farms,
12 where hormonal treatments are not allowed. Nevertheless, the values for sperm
13 concentration and total sperm number per ejaculate were higher during the LD
14 treatment. Annual variation in these variables has been described in the literature for
15 different goat breeds (Karagiannidis et al. 2000; Pérez and Mateos, 1996; Roca et al.
16 1992; Zarazaga et al. 2009), with higher sperm concentrations during spring-summer
17 (=LD). However, the latter authors observed a smaller ejaculate volume and a higher
18 spermatozoa count with respect to that volume, during the non-breeding season than
19 during the breeding season. However, in the present experiment, no differences in
20 ejaculate volume were seen among any of the treatments, probably due to the rapid
21 alternations of photoperiod.

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

1 results contrast with those of Casao et al. (2010a) in rams. The latter authors observed
2 an increase in progressive motility and VCL, but only in the rapid spermatozoa of
3 semen obtained during seasonal anoestrous in MEL-treated animals (compared to
4 controls). However, they observed no other effect of MEL treatment on any other
5 motility variable, either in rapid or medium-speed spermatozoa. In the present
6 experiment, all motility variables except for WOB in the rapid spermatozoa, and all
7 motility variables except for VCL and VAP in the medium-speed spermatozoa, were
8 higher in the MEL-treatment period. The explanation for this might lie in the species
9 used or the experimental design; Casao et al. (2010a) used different males for each
10 group, but in the present work the same males were moved from one treatment to
11 another. Tuli and Holtz (1995) also observed a higher percentage of progressive motile
12 spermatozoa in Boer bucks during the breeding season than during seasonal anoestrous.
13 Abdelwahab et al. (2006), reported differences in VAP and LIN associated with the
14 season, their values being higher in spring (partially similar to the present LD treatment)
15 than in autumn (partially similar to the present MEL or SD treatment). However, in the
16 present work, no differences in VAP were seen, and the effect on LIN was the opposite
17 to that described by Abdelwahab et al. (2006) (although they used a smaller number of
18 males and recorded a lower number of ejaculates in each season). The increase in
19 progressive motility and most of the other motility variable values induced by MEL or
20 SD might (at least partly) explain the improvement in reproductive variables reported in
21 previous studies on goats by our group (Celi et al. 2013; Zarazaga et al. 2012a, 2012b,
22 2013) (in these studies the females were mated around 45 days after melatonin
23 implantation in the males).

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

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

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

21

22 **5. Conclusions**

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

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

6

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12 implants.

13 **References**

- 14 Abdelwahab, D., Rivera, M.M., Rodríguez-Gil, J.E., Rigau, T., 2006. Seasonality
15 Affects on Sperm Motility Kinematic Parameters of Murciano-Granadina Bucks.
16 *Reprod. Domest. Anim.*, 41 (Suppl. 2), 103-125.
- 17 Baril, G., Chemineau, P., Cognie, Y., Guerin, Y., Leboeuf, B., Orgeur, P., Vallet, J.C.,
18 1993. *Manuel de formation pour l'insémination artificielle chez les ovins et les*
19 *caprins*. Rome: Ed. F.A.O; 1993. p. 193.
- 20 BOE, 2006. APA/661/2006, Order of 3 March, by replacing the annex to Royal Decree
21 1682/1997 of 7 November, which updates the Catalog Livestock Breeds of
22 Spain. *Boletín Oficial del Estado* 59, 10 March 2006. pp: 9656-9657. [In
23 Spanish].
- 24 Cabrera, F., González, F., Batista, M., Calero, P., Medrano, A., Gracia, A., 2005. The
25 Effect of Removal of Seminal Plasma, Egg Yolk Level and Season on Sperm

1 Freezability of Canary Buck (*Capra hircus*) *Reprod. Domest. Anim.*, 40, 191-
2 195.

3 Casao, A., Vega, S., Palacín, I., Pérez-Pe, R., Laviña, A., Quintín, F.J., Sevilla, E.,
4 Abecia JA, Cebrián-Pérez, J.A., Forcada, F., Muiño-Blanco, T., 2010a. Effects
5 of melatonin implants during non-breeding season on sperm motility and
6 reproductive parameters in rasa aragonesa rams. *Reprod Domest Anim* 45, 425-
7 432.

8 Casao, A., Mendoza, N., Pérez-Pé, R., Grasa, P., Abecia, J.A., Forcada, F., Cebrián-
9 Pérez, J.A., Muino-Blanco, T., 2010b. Melatonin prevents capacitation and
10 apoptotic-like changes of ram spermatozoa and increases fertility rate. *J. Pineal*
11 *Res.*, 48, 39-46.

12 Casao, A., Cebrián, I., Asumpção, M.E., Pérez-Pé, R., Abecia, J.A., Forcada, F.,
13 Cebrián-Pérez, J.A., Muiño-Blanco, T., 2010c. Seasonal variations of melatonin
14 in ram seminal plasma are correlated to those of testosterone and antioxidant
15 enzymes. *Reprod. Biol. Endocrinol.*, 8, 59. doi: 10.1186/1477-7827-8-59.

16 Celi, I., Gatica, M.C., Guzmán, J.L., Gallego-Calvo, L., Zarazaga, L.A., 2013. Influence
17 of the male on the reproductive performance of female Payoya goats implanted
18 with melatonin at the winter solstice. *Anim. Reprod. Sci.*, 137, 183-188.

19 D'Occhio, M.J., Schanbacher, B.D., Kinder, J.E., 1984. Profiles of luteinizing hormone,
20 follicle-stimulating hormone, testosterone and prolactin in rams of diverse
21 breeds: effects of contrasting short (8L:16D) and long (16L:8D) photoperiods.
22 *Biol. Reprod.*, 30, 1039-1054.

23 Delgadillo, J.A., Canedo, G.A., Chemineau, P., Guillaume, D., Malpoux, B., 1999.
24 Evidence for an annual reproductive rhythm independent of food availability in
25 male Creole goats in subtropical northern Mexico. *Theriogenology*, 52, 727-737.

- 1 Delgadillo, J.A., Chemineau, P., 1992. Abolition of the seasonal release of luteinizing
2 hormone and testosterone in Alpine male goats (*Capra hircus*) by short
3 photoperiodic cycles. *J. Reprod. Fertil.*, 94, 45-55.
- 4 Delgadillo, J.A., Hochereau-De Reviere, M.T., Daveau, A., Chemineau, P., 1995. Effect
5 of short photoperiodic cycles on male genital tract and testicular parameters in
6 male goats (*Capra hircus*). *Reprod. Nutr. Dev.*, 35, 549-558.
- 7 Delgadillo, J.A., Leboeuf, B., Chemineau, P., 1991. Decrease in the seasonality of
8 sexual behavior and sperm production in bucks by exposure to short
9 photoperiodic cycles. *Theriogenology*, 36, 755-770.
- 10 Delgadillo, J.A., Leboeuf, B., Chemineau, P., 1992. Abolition of seasonal variations in
11 semen quality and maintenance of sperm fertilizing ability by photoperiodic
12 cycles in goat bucks. *Small Ruminant Res.*, 9, 47-59.
- 13 Delgadillo, J.A., Leboeuf, B., Chemineau, P., 1993. Maintenance of sperm production
14 in bucks during a third year of short photoperiodic cycles. *Reprod. Nutr. Dev.*,
15 33, 609-617
- 16 Dorado, J., Hidalgo, M., Muñoz, A., Rodríguez, I., 2009. Assessment of goat semen
17 freezability according to the spermatozoa characteristics from fresh and frozen
18 samples. *Anim. Reprod. Sci.*, 112, 150-157.
- 19 Du Plessis, S.S., Hagenaar, K., Lampiao, F., 2010. The in vitro effects of melatonin on
20 human sperm function and its scavenging activities on NO and ROS.
21 *Andrologia*, 42, 112-116.
- 22 Karagiannidis, A., Varsakeli, S., Karatzas, G., 2000. Characteristics and seasonal
23 variations in the semen of Alpine, Saanen and Damascus goat bucks born and
24 raised in Greece. *Theriogenology*, 53, 1285-1293.

- 1 Kaya, A., Baspinar, N., Yildiz, C., Kurtoglu, F., Ataman, M.B., Haliloglu, S., 2000.
2 Influence of melatonin implantation on sperm quality, biochemical composition
3 of the seminal plasma and plasma testosterone levels in rams. *Rev. Med. Vet.*,
4 151, 1143-1146.
- 5 Kokolis, N., Theodosiadou, E., Tsantarliotou, M., Rekkas, C., Goulas, P., Smokovitis,
6 A., 2000. The effect of melatonin implants on blood testosterone and acrosin
7 activity in spermatozoa of the ram. *Andrologia*, 32, 107-114.
- 8 Langford, G.A., Ainsworth, L., Marcus, G.J., Shrestha, J.N., 1987. Photoperiod
9 entrainment of testosterone, luteinizing hormone, follicle-stimulating hormone,
10 and prolactin cycles in rams in relation to testis size and semen quality. *Biol.*
11 *Reprod.*, 37, 489-499.
- 12 Lincoln, G.A., Lincoln, C.E., McNeilly, A.S., 1990. Seasonal cycles in the blood
13 plasma concentration of FSH, inhibin and testosterone, and testicular size in
14 rams of wild, feral and domesticated breeds of sheep. *J. Reprod. Fertil.*, 88, 623-
15 633.
- 16 Luboshitzky, R., Shen-Orr, Z., Herer, P., 2002. Seminal plasma melatonin and gonadal
17 steroids concentrations in normal men. *Arch. Androl.*, 48, 225-232.
- 18 Mara, L., Dattena, M., Pilichi, S., Sanna, D., Branca, A., Cappai, P., 2007. Effect of
19 different diluents on goat semen fertility. *Anim. Reprod. Sci.*, 102, 152-157.
- 20 Morand-Fehr, P., Sauvant, D., 1988. Goat nutrition, in: Jarrige, R. (Ed.), *Alimentation*
21 *des Bovins, Ovins et Caprins*. Institut National de la Recherche Agronomique
22 (Paris, France), pp. 281-304.
- 23 Muduuli, S., Sanford, L.M., Palmer, W.M., Howland, B.E., 1979. Secretory patterns
24 and circadian and seasonal changes in luteinizing hormone, follicle stimulating

1 hormone, prolactin and testosterone in the male Pygmy goat. *J. Anim. Sci.*, 49,
2 543-553.

3 Oldham, C.M., Adams, N.R., Gherardi, P.B., Lindsay, D.R., McKintosh, J.B., 1978.
4 The influence of level of food intake on sperm-producing capacity of testicular
5 tissue in the ram. *Aust. J. Agric. Res.*, 29, 173-179.

6 Pelletier, J., Brieu, V., Chesneau, D., Pisselet, C., De Reviers, M., 1985. Abolition
7 partielle des variations saisonnières du poids testiculaire chez les béliers par
8 diminution de la période du cycle lumineux. *CR. Acad. SC. Paris*, 665-668.

9 Pelletier, J., Chemineau, P., Delgadillo, J.A., 1988. Seasonality of sexual activity and its
10 photoperiodic control in the adult ram and he-goat. In: *Proc. 11th Int. Congr.*
11 *Anim. Reprod. & A. I.*, Dublin 5, 211-219.

12 Pérez, B., Mateos, E., 1996. Effect of photoperiod on semen production and quality in
13 bucks of Verata and Malagueña breeds. *Small Ruminant Res.*, 22, 163-168.

14 Purdy, P.H., 2006. A review on goat sperm cryopreservation. *Small Ruminant Res.*, 63,
15 215-225.

16 Rao, M.V., Gangadharan, B., 2008. Antioxidative potential of melatonin against
17 mercury induced intoxication in spermatozoa in vitro. *Toxicol. In Vitro*, 22, 935-
18 942.

19 Roca, J., Martínez, E., Vázquez, J.M., Coy, P., 1992. Characteristics and seasonal
20 variations in the semen of Murciano-Granadina goats in the Mediterranean area.
21 *Anim. Reprod. Sci.*, 29, 255-262.

22 Rouger. Y., 1974. Etude des interactions de l'environnement et des hormones sexuelles
23 dans la régulation du comportement sexuel des Bovidae. Thèse Doctorat es
24 Sciences Naturelles, Université de Rennes, 197 pp.

- 1 Sarabia, L., Maurer, I., Bustos-Obregón, E., 2009. Melatonin prevents damage elicited
2 by the organophosphorous pesticide diazinon on mouse sperm DNA. *Ecotoxicol.*
3 *Environ. Saf.*, 72, 663-668.
- 4 Shiu, S.Y., Li, L., Siu, S.W., Xi, S.C., Fong, S.W., Pang, S.F., 2000. Biological basis
5 and possible physiological implications of melatonin receptor-mediated
6 signaling in the rat epididymis. *Biol. Signals Recept.*, 9, 172-187.
- 7 Sönmez, M., Yüce, A., Türk, G., 2007. The protective effects of melatonin and Vitamin
8 E on antioxidant enzyme activities and epididymal sperm characteristics of
9 homocysteine treated male rats. *Reprod. Toxicol.*, 23, 226-231.
- 10 SPSS 2008: SPSS statistics base user's guide 17.0. SPSS Inc. (Chicago) p. 640.
- 11 Succu, S., Berlinguer, F., Pasciu, V., Satta, V., Leoni, G.G., Naitana, S., 2011.
12 Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-
13 dependent manner. *J. Pineal. Res.*, 50, 310-318.
- 14 Tuli, R.K., Holtz, W., 1995. Effect of season on the freezability of Boer goat semen in
15 the northern temperate zone. *Theriogenology*, 43, 1359-1363.
- 16 Van Vuuren, R.J., Pitout, M.J., Van Aswegen, C.H., Theron, J.J., 1992. Putative
17 melatonin receptor in human spermatozoa. *Clin. Biochem.*, 25, 125-127.
- 18 Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. *Anim.*
19 *Reprod. Sci.*, 60-61, 481-492.
- 20 Zarazaga, L.A., Celi, I., Guzmán, J.L., Malpaux, B., 2012a. Enhancement of the male
21 effect on reproductive performance in female Mediterranean goats with long day
22 and/or melatonin treatment. *Vet. J.*, 192, 441-444.
- 23 Zarazaga, L.A., Gatica, M.C., Celi, I., Guzmán, J.L., 2012b. Reproductive performance
24 is improved during seasonal anoestrus when female and male Murciano-

1 Granadina goats receive melatonin implants and in Payoya goats when females
2 are thus treated. *Reprod. Dom. Anim.*, 47, 436-442.

3 Zarazaga, L.A., Gatica, M.C., Gallego-Calvo, L., Celi, I., Guzmán, J.L., 2013. The
4 influence of the buck effect on the reproductive performance of early postpartum
5 Payoya goat females in seasonal anoestrous could be enhanced by melatonin
6 implants. *Span. J. Agric. Res.*, 11, 997-1003.

7 Zarazaga, L.A., Guzmán, J.L., Domínguez, C., Pérez, M.C., Prieto, R., 2009. Effects of
8 season and feeding level on reproductive activity and semen quality in Payoya
9 buck goats. *Theriogenology*, 71, 1316-1325.

1 **Figure 1.** Experimental procedures.

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2 Months of exogenous melatonin (G1, n=7)	2 Months of long days (16L:8D) (G1+G2, n=14)	2 Months of short days (8L:16D)(G1, n=7)	
2 Months of shorth days (8L:16D)(G2, n=7)		2 Months of exogenous melatonin days (G2, n=7)	
8th July	9th September	8th November	9th January

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

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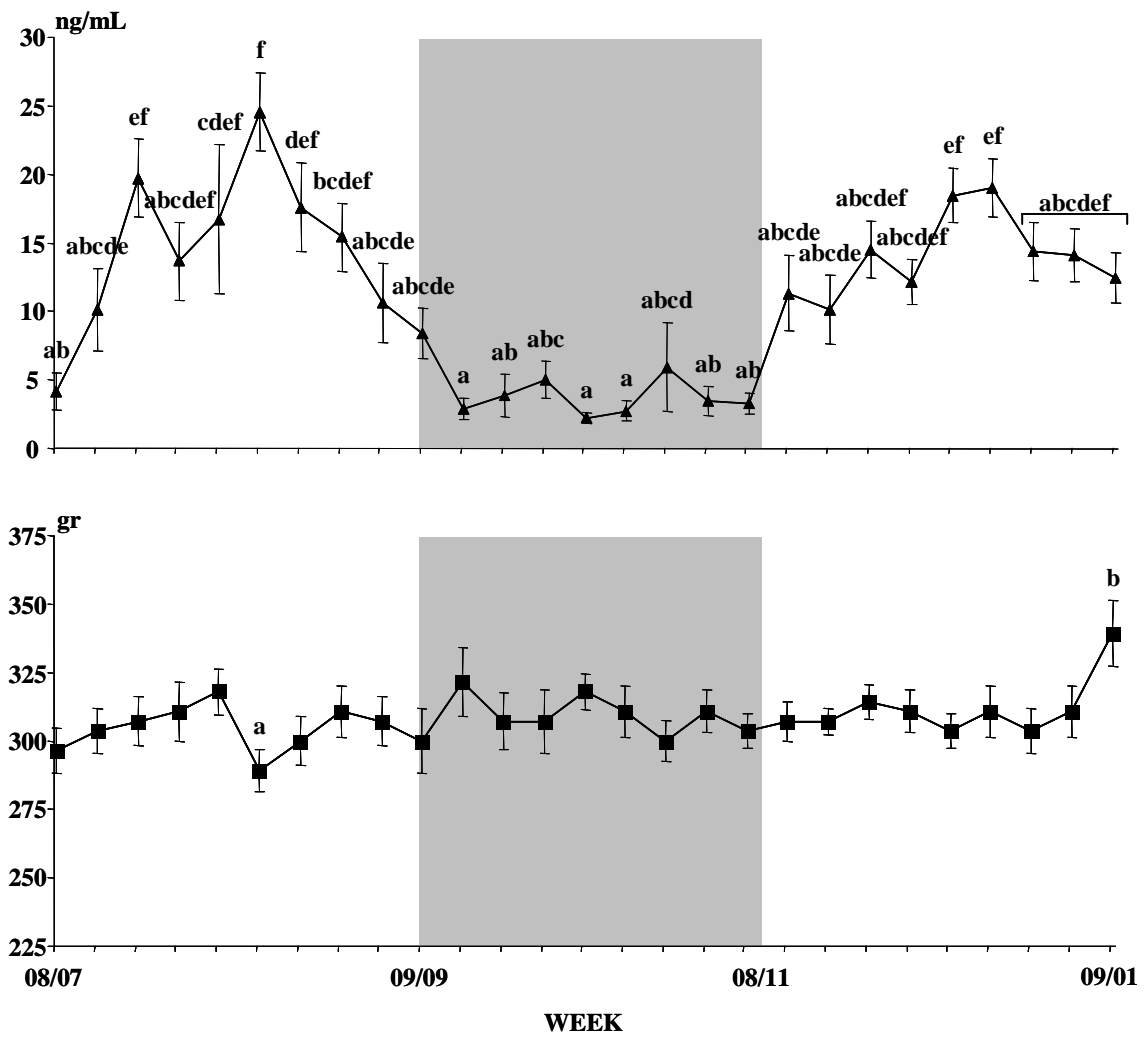


Table 1. Sexual behaviour and fresh semen quality under the melatonin (MEL), short day (SD) and long day (LD) treatments (means±SEM).

	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, $\mu\text{m/s}$. VSL: Straight-line velocity, $\mu\text{m/s}$. VAP: Average path velocity, $\mu\text{m/s}$. 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).

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

3

	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

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

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

3

	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

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

6