Seasonal variations of sperm freezability associated with testicular germinal epithelium changes in domestic (*Ovis aries*) and wild (*Ovis musimon*) rams

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Abstract. The aim of this study was to examine ovine sperm cryoresistance during the rutting season (RS) and its association with sperm head area and seminiferous epithelium proliferation. Small ruminants show fluctuating testosterone levels throughout the year which could interfere with spermatogenesis and sperm cryopreservation. Ejaculates, testicular biopsies and blood were collected during the middle and the end of the RS (Middle-RS *vs* End-RS) during periods of high and low testosterone levels in Merino and Mouflon rams. Fresh and frozen-thawed sperm quality, sperm morphometry, seminiferous tubule morphometry and testicular proliferation markers (proliferating-cell nuclear antigen, Ki67 and GATA-4) were evaluated. Post-thaw sperm viability was higher in the End-RS in Merino (69.9±8.2 *vs* 41.6±7.3%; *P*=0.020) and Mouflon (40.9±3.3 *vs* 24.2±5.0%; *P*=0.008). Mouflons had larger sperm head area at the End-RS (38.3±0.2 *vs* 34.3±0.1 μm^2; *P*=0.029), whereas there was no difference between Merino groups (35.7±0.5 *vs* 34.8±1.0 μm^2). Seminiferous tubule morphometry and proliferation markers showed higher levels of germinal epithelium proliferation in the Middle-RS of both species. In conclusion, sperm freezability is affected during the RS in domestic and wild rams which could be correlated with changes that occur during spermatogenesis since there is an effect of season on cell proliferation in the testis.

Additional keywords: cryopreservation, testis, testosterone, Sertoli cell, spermatogenesis, spermatozoa.

Introduction

Sperm cryopreservation techniques allow the establishment of genetic resource banks, which can be used for commercial purposes in domestic species and to support conservation of wild species (Holt and Pickard 1999). Small ruminants are seasonal breeding mammals that

reproduce during a short period of time in the wild to give birth when offspring survival is most likely. Domestic small ruminant species are considered to have a less marked seasonality pattern while wild species have a shorter and more restrictive period of sexual activity. Nevertheless, males of both domestic and wild species have a seasonal pattern of testosterone secretion (Santiago-Moreno *et al.* 2005). It has long been assumed that the most favorable period to collect semen for its cryopreservation is during the rutting season, coinciding with the increase or peak of testosterone secretion (Holt 2001). However, high testosterone levels have been associated with decreased freezability in domestic (*Capra hircus*) and wild (*Capra pyrenaica*) caprines (Tuli and Holtz 1995; Coloma *et al.* 2011), and thus sperm collection at the end of the rutting season, when sperm quality remains high while testosterone secretion is decreasing, has been recommended (Coloma *et al.* 2011). Therefore, many questions arise regarding the underlying causes of sperm cryotolerance variations throughout the year. Additionally, a seasonal variation of sperm head dimensions has been reported in rams (Bravo *et al.* 2014), which could be correlated with sperm cryoresistance since water and electrolyte membrane transport during the cooling and freezing process are affected by cell size (Thurston *et al.* 2001; Esteso *et al.* 2006).

Testicular function of seasonal breeders is regulated by environmental factors through the hypothalamic-pituitary-testicular axis that controls the secretion of gonadotrophins and consequently the testosterone secretion by Leydig cells in the testis (Young and Nelson 2001). Testosterone plays a crucial role in spermatogenesis that takes place inside the seminiferous tubules of the testis (Ramaswamy and Weinbauer 2014). Due to animal domestication, the seasonal endocrine pattern and sexual activity differ between wild and domestic sheep breeds (Lincoln *et al.* 1990; Santiago-Moreno *et al.* 2005). Testosterone levels in domestic rams

(*Ovis aries*) increase slowly to reach their maximum in July and then decrease gradually until December, whereas wild rams, such as Mouflon *(Ovis musimon),* show a marked peak of testosterone at the beginning of the breeding season before rapidly decreasing and reaching basal levels in January (Santiago-Moreno *et al.* 2005). Seasonal changes of plasma testosterone concentration in rams are accompanied with parallel changes of LH and FSH plasma levels (Pelletier *et al.* 1982; Lincoln *et al.* 1990; Lincoln 1998; Sanford *et al.* 2002) and also with an increase in LH and FSH-receptor concentration within the testis before the onset of the breeding season (Barenton and Pelletier 1983). Testicular regression during the non-breeding season is controlled by a combination of apoptosis and desquamation of germ cells (Young and Nelson 2001; Jimenez *et al.* 2015). Moreover, fluctuations of serum testosterone levels have been correlated with changes of the seminiferous tubules morphometry (Kus *et al.* 2003) and cell proliferation (Bansode *et al.* 2003). The quantification of proliferation markers can be used to evaluate circannual changes of germ cell or somatic cell activity in the testicular parenchyma. The proliferative activity of germ cells has been evaluated by quantifying the proliferating-cell nuclear antigen (PCNA) and Ki67 distribution pattern in pathological and normal human and canine testis (Sarli *et al.* 1994; Steger *et al.* 1998). Moreover, PCNA has been used as a marker of cell proliferation in cultured granulosa cells (McClusky 2005) and Ki67 has been widely studied as a target in cancer therapy since it is overexpressed in cancer cells (Yang *et al.* 2018). In addition, GATA-4 is a transcription factor expressed in Sertoli cells and Leydig cells but not in germ cells (Ramos-Vara and Miller 2009) that has an important role in cardiac development (Molkentin *et al.* 1997), gonadal development and sex differentiation (Hales 2001). The combination of these proliferation

markers provides information about the different cell types activity in the testicular seminiferous epithelium during spermatogenesis.

The study of the seasonality in relation to post-thaw sperm quality provides valuable information to better understand the causes of fluctuations of cryotolerance and allows the identification of the optimal time of the year to collect and freeze sperm for genetic resource banks. We hypothesize that the resistance of ovine sperm cells to freeze-thawing could be affected by season, as reported in caprine (Coloma *et al.* 2011), through testosterone-related changes in the testicular germinal epithelium that could be affecting sperm head size.

The aim of the present work was to examine the association between high and low testosterone levels (middle and end of the rutting season) with post-thaw sperm quality, sperm cell morphometry and changes of the testicular germinal epithelium during the rutting season. A moderate seasonal breeder, the Spanish Merino sheep, and its wild ancestor the Mouflon with a marked rutting season, were used in this study.

Materials and methods

Experimental design

Ejaculates, testicular biopsies and blood samples were collected during the middle of the rutting season (Middle-RS group) coinciding with high plasma testosterone concentration and at the end of the rutting season (End-RS group) coinciding with low plasma testosterone concentration defined by each species (Santiago-Moreno *et al.* 2005). Due to the different endocrine pattern that domestic and wild species show, samples were collected in July and December in Merino rams and November and January in Mouflon rams for the Middle-RS and End-RS groups respectively. Testosterone plasma levels were measured in Merino ram

samples (n = 16 in July and n = 20 in December) and Mouflon ram samples (n = 10 in November and n = 23 in January). Sample collection and analysis were structured in two experiments:

Experiment 1. Effect of rutting season on sperm freezability and sperm head area: A total of 36 ejaculates were collected from 24 Mouflon rams (11 ejaculates collected from 10 Mouflon rams in the Middle-RS and 25 ejaculates collected from 16 Mouflon rams in the End-RS). A total of 17 ejaculates were collected from 6 Merino rams (9 ejaculates collected from 4 Merino rams in the Middle-RS and 8 ejaculates collected from 4 Merino rams in the End-RS). Sperm head area was also assessed in fresh samples (n = 4 for each species and group).

Experiment 2. Effect of rutting season on cell proliferation in the testis: Testicular biopsies were collected in the Middle-RS and End-RS groups from 8 Mouflon rams (n = 4 for each group) and 8 Merino rams (n = 4 for each group) to quantify spermatogenic activity by morphometry of the seminiferous tubules and by proliferation markers.

Animals

Animals were housed at the Animal Reproduction Department of the Spanish National Institute for Agricultural and Food Research and Technology (INIA, Madrid, Spain, 40ºN 25'N latitude). Rams were maintained under natural day length conditions. Animal handling procedures were approved by the INIA Ethics Committee following the European Union Directive 2010/63/UE.

Hormone analysis

Testosterone plasma concentration was measured by radioimmunoassay (RIA; TRI-CARB 2100TR, Liquid Scintillation Analyzer, PerkinElmer Inc., Waltham, USA) based on the competitive binding method between labelled and non-labelled testosterone. Testosterone was extracted from 250 µL plasma with cyclohexane and ethyl acetate. Aliquots of samples and testosterone standards were mixed with a mouse monoclonal anti-testosterone antibody (BM724, OriGene Technologies GmbH, Rockville, USA), [1,2,6,7-^{3}H(N)]-testosterone (PerkinElmer Inc., Waltham, USA) and testosterone (46923, VETRANAL^{TM}, Sigma-Aldrich, Seelze, Germany). Free and bound fractions were subsequently separated with a solution of Norit A (Serva Co., Heidelberg, Germany) and Dextrane (Sigma Chemical Co., St. Louis, USA). A calibration curve was done with dilutions from 0.125 to 8.0 ng/mL testosterone. Samples with high testosterone concentration were included at frequent intervals to determine the coefficient of variation (CV).

Semen collection

Merino semen samples were collected by artificial vagina using a teaser ewe and Mouflon semen samples were collected by the transrectal ultrasound-guided massage of the accessory sex glands (TUMASG) technique previously described (Santiago-Moreno *et al.* 2013). Briefly, the penis was manually protruded, and the accessory sex glands were examined by real-time transrectal ultrasonography (Prosound 2, Aloka CO, Tokyo, Japan). A back-and-forth motion massage was applied on the ampulla of the vas deferens using the ultrasound probe. The penile, perineal and pelvic areas were manually stimulated to facilitate the expulsion and collection of sperm in a 3 mL glass collection tube. When necessary, electrical stimuli (0.2 mA, 6-8 s) were applied using the rectal probe of an electroejaculator (Lane Manufacturing Inc., Denver, USA).

Sperm quality analysis

Semen volume was measured in 10 mL glass collection tubes and sperm concentration was assessed by a photometer for semen concentration analysis (SDM 1 Ovine/Caprine, Minitube, Tiefenbach, Germany) in Merino samples or a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany) in Mouflon samples. Sperm motility was assessed by a computer-assisted sperm analysis system (CASA-mot) using Sperm Class Analyzer® v.4.0. software (Microptic S.L., Barcelona, Spain) coupled to a Nikon microscope (Eclipse 50i, Nikon Corporation, Tokyo, Japan) equipped with a camera (A312fc, Basler AG, Ahrensburg, Germany). Samples were diluted in a TEST based extender (TES 210.6 mM, Tris 95.8 mM, glucose 10.1 mM) and 3 µL drops were placed in a Leja eight-chamber slide (Leja Products B.V., Nieuw Vennep, The Netherlands). A minimum of three fields and 500 sperm tracks were evaluated at 100x for each sample chamber (image acquisition rate 25 frames/s). Total sperm motility (%), progressive motility (PM, %) and the following kinetic parameters were evaluated by CASA: curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm/s) and beat-cross frequency (BCF, Hz). Total motility included all sperm cells in motion regardless of the type of movement, whereas progressive motility was considered when STR > 80%.

Sperm viability and acrosomal status were evaluated by a fluorescence microscope using the fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC) (counting 200 cells). Four classes of spermatozoa can be identified with PI/PNA-FITC: 1) live spermatozoa with intact acrosome (PI-/PNA-FITC-), 2) live spermatozoa with damaged acrosome (PI-/PNA-FITC+), 3) dead

spermatozoa with intact acrosome (PI+/PNA-FITC-), 4) dead spermatozoa with damaged acrosome (PI+/PNA-FITC+). The sum of all the PI-negative sperm was the total viability and the sum of all the PNA-negative-sperm was considered to be the acrosome integrity.

The percentage of spermatozoa with morpho-abnormalities was assessed in 2% glutaraldehyde-fixed samples using a phase-contrast microscope at 400x. The following categories of morpho-abnormalities were evaluated counting 200 cells per sample: abnormal head, decapitated sperm, mid-piece abnormalities, broken neck, coiled tails, broken tails and cytoplasmic droplets.

Sperm cryopreservation and freezability assessment

Fresh sperm samples were diluted to a final concentration of $100x10^6$ sperm/mL in a TEST based extender containing TES 210.6 mM, Tris 95.8 mM, glucose 10.1 mM, 6% v/v clarified egg yolk and 5% v/v glycerol. Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Merck KGaA (Darmstadt, Germany) and Panreac Química S.A. (Barcelona, Spain). Semen samples were cryopreserved in 0.25 mL French straws (L'Aigle Cedex, France) by a conventional freezing technique previously described (Pradiee *et al.* 2016). Briefly, diluted samples were cooled for a total of 3 h at 5°C and finally straws were exposed to liquid nitrogen vapors for another 10 min before being immerse in liquid nitrogen.

Straws were stored between 2 and 12 months before being thawed in a water bath at 37°C for 30 s to assess the post-thaw quality parameters described in the sperm quality analysis section. Post-thaw sperm quality variables were compared between Middle-RS and End-RS groups. Sperm freezability was assessed by calculation of Cryoresistance Ratio (CR) being CR = Post-thaw value/ Fresh value x 100 (Supplementary Table 1).

Sperm head area assessment

Semen smears were prepared with 5 µL of fresh sample dragged across the slide before being air-dried and stained by Hemacolor® (Merck) as previously described (Sancho *et al.* 1998). Slides were sealed with Eukitt® mounting medium (Panreack Quimica S.L.U., Barcelona, Spain) and a coverslip. Sperm cell morphometric parameters were assessed by the morphometry module of CASA (Sperm Class Analyzer® v.4.0. software, Microptic S.L., Barcelona, Spain) in 100 sperm cells per animal using the 60x objective of a Nikon microscope (Eclipse 50i, Nikon Corporation, Tokyo, Japan). For both species, sperm head area values were subjected to *k*-means cluster analysis to identify four subpopulations. Technical details were described by Esteso *et al*. (2015). Features for the cluster analysis are briefly described in the statistical analysis section.

Testicular biopsies collection

Animals were anesthetized with a combination of intravenous detomidine (50 µg/kg; Domosedan®, Pfizer Inc., Amboise Cedex, France), ketamine hydrochloride (0.5 mg/kg; Imalgene-1000, Rhône Mérieux, Lyon, France) and tiletamine-zolazepan (0.5 mg/kg; Zoletil-100, Virbac España S.A., Barcelona, Spain). Animals were maintained with isoflurane (Isobavet, Intervet Schering-Plough Animal Health, Madrid, Spain) during the procedure and anesthesia was reversed with yohimbine hydrochloride (0.7 mg/kg: half intravenous and half intramuscular; Sigma, Zwijndrecht, the Netherlands).

The scrotal circumference was measured prior to the biopsy collection by punch (BP80, HealthLink, Florida, USA). Biopsies were immediately fixed in 4% buffered formalin

(Panreac Química, Barcelona, Spain) for 24 h before being processed and embedded in paraffin wax.

Morphometry assessment of the seminiferous tubules

Five micrometer sections were stained with a standard hematoxylin and eosin stain procedure (Thermo Scientific, Madrid, Spain) for morphometric analysis. Morphometric parameters of 15 seminiferous tubules per animal and condition of study were measured using the Leica QWinPro software (Leica Mycrosystems, Wetzlar, Germany) at 200x magnification (Fig. 1*a*, *b*, *c*, *d*). The following parameters were measured at high and low testosterone levels in Merino (Fig. 1*e*, *f*) and Mouflon rams (Fig. 2*a*, *b*): tubular diameter (µm), tubular area (µm²), seminiferous epithelium height (µm), number of cell nuclei inside the tubule (including germ cells and Sertoli cells) and tubular area occupied by cell nuclei (µm²). The ratio "tubular area occupied by cell nuclei/ tubular area" and the ratio "seminiferous epithelium height/ tubular diameter" were calculated.

Quantification of proliferation markers in the seminiferous tubules

The expression of tubular PCNA, Ki67 and GATA-4 proteins was detected by an indirect ABC immunohistochemical procedure using a commercial kit (Dako EnVision Flex, Dako, Agilent Technologies, Madrid, Spain). Briefly, after deparaffinization and rehydration of 5 µm-sections, an antigen retrieval procedure was performed (Target Retrieval Solution High pH, Dako, USA) at 98ºC for 30 min, following by endogenous peroxidase blocking using a commercial solution (Dako, USA). Sections were then incubated overnight at 4°C with primary monoclonal mouse anti-PCNA (1:5000; SC-56; Santa Cruz), rabbit anti-Ki67 (1:100; ab16667; Abcam) and mouse anti-GATA-4 (1:2000; SC-25310; Santa Cruz). Control slides

were incubated without the primary antibody to confirm the immunolabeling specificity. Antibodies were diluted in Antibody Diluent (Dako, USA). To amplify the GATA-4 immunostaining, sections were incubated with Mouse Linker Solution (Dako, USA) for 15 min at 37ºC. Slides were incubated with the anti-mouse or anti-rabbit secondary labelled polymer (Dako, USA) for 30 min at 37ºC. Sections were finally incubated with 3-3´Diaminobencidine DAB (Dako, USA) for 5 min at room temperature, contrasted with hematoxylin, dehydrated, cleared and mounted (Neo-Mount, Merck, Germany). The immunolabeling quantification was performed by counting positive cells in 10 fields per animal at 400x magnification. For the PCNA and GATA-4 reactivity quantification, only positive cells located at the intratubular basal level (spermatogonia and Sertoli cells) were quantified while all the intratubular Ki67 positive cells (germ cells and Sertoli cells) were quantified (Fig. 3*a*, *b*, *c*, *d*).

Statistical analysis

Statistical analysis was assessed by the STATISTICA software for Windows version 12.0 (StatSoft, Inc., Tulsa, OK, USA). Data distribution was determined by the Shapiro-Wilk test and homogeneity of variance was assessed by Levene test. Testosterone levels, sperm quality parameters, mean sperm head area (Experiment 1), scrotal circumference, seminiferous tubules morphometric parameters and immunolabeling quantification (Experiment 2) were analyzed by the t-test or the Mann-Whitney-U-test when data did not follow a normal distribution. For the *k*-means cluster analysis to identify four subpopulations of sperm head area, STATISTICA specifically uses Lloyd's method to implement the k-means algorithm. The right number of clusters was determined by a v-fold cross-validation algorithm included in the STATISTICA package. The morphometric descriptors for the subpopulations and

subpopulation proportions were compared between groups by the t-test (Experiment 1). *P* values lower than 0.05 was considered statistically significant. Data are expressed as mean ± standard error of the mean (s.e.m.).

Results

Plasma testosterone concentration of Merino rams was higher in July than in December (6.7±1.2 *vs* 2.0±0.4 ng/mL; $P=0.001$) and plasma testosterone concentration of Mouflon rams was higher in November than in January (4.6±1.4 *vs* 0.5±0.1 ng/mL; $P<0.001$). The RIA analysis had a CV inter-assay of 11% and CV intra-assay of 7%.

Experiment 1: Effect of rutting season on sperm freezability and sperm head area

Fresh sperm quality parameters such as semen volume, sperm concentration, acrosome integrity, total motility, most of the kinetic CASA parameters and morpho-abnormalities did not differ between Middle-RS and End-RS groups in both species (Tables 1 and 2). Only sperm viability ($P=0.025$) and the ALH ($P=0.001$) of Merino ram fresh ejaculates differed between groups (Table 1).

Post-thaw sperm parameters were affected by season in Merino and Mouflon rams and the viability of frozen-thawed spermatozoa was lower in the Middle-RS groups in both species ($P=0.020$ and $P=0.008$; Tables 1 and 2). Frozen-thawed sperm of the Middle-RS Merino group had lower acrosome integrity ($P=0.021$) and higher LIN ($P=0.049$), WOB ($P=0.038$) and BCF ($P=0.004$) than the End-RS group (Table 1). Frozen-thawed sperm of the Middle-RS Mouflon group had lower total motility ($P=0.041$), VCL ($P=0.005$), VAP ($P=0.047$), ALH ($P<0.001$) and BCF ($P=0.011$) than the End-RS group (Table 2). There were no differences between groups in other kinematic parameters nor in the percentage of morpho-abnormalities

in both species. Cryoresistance Ratios showed higher sperm freezability in the End-RS groups of both species (Supplementary Table 1), supporting results of Tables 1 and 2.

Regarding sperm head area, mean values did not differ between Merino groups (35.7±0.5 *vs* 34.8±1.0 μm^2) while it was found to be larger in the End-RS than in the Middle-RS Mouflon group (38.3±0.2 *vs* 34.3±0.1 μm^2; P=0.029; Table 3). Sperm subpopulations of Merino rams did not differ between groups (Table 3) whereas Mouflon sperm subpopulations of the End-RS group had larger mean sperm head area (P<0.0001; Table 3). No differences were found between groups regarding the percentages of subpopulations in both species (Table 3).

Experiment 2: Effect of rutting season on cell proliferation in the testis

Scrotal circumference was higher in the Middle-RS than in the End-RS in Merino rams (32.6±5.0 *vs* 24.8±1.4 cm; P=0.001) and Mouflon rams (24.5±2.7 *vs* 20.6±0.6 cm; P=0.001).

Morphometric parameters of the seminiferous tubules were affected during the RS in Merino rams (Fig. 1) and the seminiferous epithelium height (P=0.001), the tubular area occupied by cell nuclei (P=0.011) and the number of cell nuclei (P=0.023) were higher in the Middle-RS group than in the End-RS group (Fig. 1*g*, *h*, *i*). The ratios “tubular area occupied by nuclei/ tubular area” and “epithelium height/ tubular diameter” were also higher in the Middle-RS group of Merino rams (P<0.001; Fig. 1*j*). Regarding the morphometric parameters of Mouflon rams (Fig. 2), the tubular area occupied by cell nuclei was higher in the Middle-RS than in the End-RS (P=0.033; Fig. 2*d*) whereas the rest of the parameters did not differ between groups (Fig. 2*c*, *e*, *f*). The tubular diameter and tubular area did not differ between groups of both species.

Regarding immunohistochemistry results (Fig. 3), a seasonal pattern of cell-cycle activity inside the seminiferous tubules was identified in both Merino (Fig. 3*a*, *b*, *e*) and Mouflon rams (Fig. 3*c*, *d*, *f*). The PCNA protein was detected in Sertoli cells, Leydig cells and germ cells except elongated spermatids. The PCNA expression of Sertoli cells and spermatogonia was higher in the Middle-RS than in the End-RS in Merino rams (P=0.004) whereas there was no significant difference between Mouflon groups. The quantitative distribution pattern of Ki67-positive-germ cells showed higher expression in the Middle-RS than in the End-RS in Merino rams (P<0.0001) and Mouflon rams (P<0.001). GATA-4 reactivity was identified in Sertoli and Leydig cells nuclei while early and late spermatids had paranuclear labelling. The GATA-4 quantification in Sertoli cells showed higher expression levels in the End-RS than in the Middle-RS in Mouflon rams (P<0.001) whereas there was no difference between Merino groups. Control slides were immunonegative (Supplementary Figure 1).

Discussion

Sperm freezability was lower in the Middle-RS than in the End-RS in both domestic and wild rams, thus the initial hypothesis is accepted. Our findings revealed that testosterone-related changes in testicular germinal epithelium, and in subsequent sperm morphometric characteristics, happened in a relative short time, at the end of the rutting season, coinciding with decreasing testosterone concentration. Although many sperm characteristics, such as membrane structure, are modified considerably during epididymal maturation (Jones 1998) and after sperm contact with secretions of the accessory sex glands (Manjunath *et al.* 2007), our data support the fact that sperm changes affecting cryoresistance also occur during spermatogenesis. The changes in the proliferative activity of germ cells and Sertoli cells that

were seen throughout the rutting season may explain the consequent changes of sperm freezability.

Season of ejaculate collection influences sperm cryoresistance of other mammals such as buck (Coloma *et al.* 2011), bull (Koivisto *et al.* 2009), boar (Barranco *et al.* 2013) or stallion (Janett *et al.* 2003). Variation in sperm cryosurvival may be explained by chemical and physical differences in unsaturated fatty acids and phospholipids which affect sperm membrane fluidity (Jones 1997; Ladha 1998). Sperm susceptibility to cold-shock and the freezing process has been associated with the cholesterol and protein sperm content (Darin-Bennett and White 1977; Moce *et al.* 2010; He *et al.* 2016; Salmon *et al.* 2016). Since there are seasonal variations of sperm cholesterol (Argov-Argaman *et al.* 2013) and sperm protein composition (van Tilburg *et al.* 2015), further investigations are needed to clarify their role on sperm cryosurvival seasonality. Moreover, seasonal variations of the seminal plasma composition have been reported (Smith *et al.* 1999; Dominguez *et al.* 2008) which could be affecting not only the sperm cryoresistance, but also the sperm capacitation status (Ledesma *et al.* 2016). It is known that the freezing-thawing process leads to capacitation-like changes in the sperm plasma membrane (Leahy and Gadella 2011). Sperm hyperactivation is part of the capacitation process and is associated with vigorous (Yanagimachi 1970) non-progressive movement (Mortimer 2000). The flagellum develops high-amplitude waves that lead to changes in the kinematic parameters such as the increase of ALH and a decrease of LIN (Mortimer and Maxwell 1999). Both species showed an increase of ALH accompanied by a decrease of LIN in frozen-thawed sperm at the End-RS, suggesting an effect of season on the hyperactivation status after the freezing-thawing process. Additionally, differences in sperm head size may influence sperm water volume, membrane permeability to water and

cryoprotectant and thus sperm cell freezing capacity (Curry 2000). Variations among sperm head size may be responsible for variations in the velocity of water exchange across the plasma membrane during freezing-thawing (Yaniz *et al.* 2015). Cryopreservation is known to affect the morphometry of the sperm head and reductions in sperm head size after freezing-thawing have been reported in bull (Gravance *et al.* 1998), red deer (Esteso *et al.* 2003), and stallion (Arruda *et al.* 2002). Moreover, there is some evidence that sperm head dimensions may be an indicator of sperm cryosurvival (Gravance *et al.* 1998), 1998). Sperm head area was larger in the End-RS Mouflon group, with a similar tendency observed in Merino rams. Because both species had better sperm freezability at the End-RS, our findings would not support the hypothesis that smaller sperm size is associated with better cryosurvival rates as has been suggested by Esteso *et al.* (2006). Nevertheless, the effect of season was not investigated in their study. In this study we confirm seasonal variations on sperm proliferation in the testis and Sertoli cell activity which could be affecting protein and cholesterol membrane content during spermatogenesis. Thus, the combination of all these factors together plays an important role in sperm cryotolerance.

Reproductive seasonality varies depending on photoperiod and other environmental factors. Domestic species have been selected in order to improve their sexual performance (Bench *et al.* 2001; Ambrosi *et al.* 2018) and to extend their breeding season (Santiago-Moreno *et al.* 2000; Vincent *et al.* 2000). In accordance with this, domestic Merino rams were able to produce high quality ejaculates in July and December. Mouflon fresh semen quality parameters did not differ between testosterone groups probably because both groups of samples were collected during the rutting season (November and January). Nonetheless, both species showed a seasonal pattern of testosterone secretion that affects cell proliferation inside

the seminiferous tubules as shown by the morphometry and immunohistochemistry results. Although previous studies in rodent (*Rattus norvegicus; Rattus fuscipes*) and pika (*Ochotona curzoniae*) testes found an effect of season on the diameter of seminiferous tubules (Hodgson *et al.* 1979; Kus *et al.* 2003; Liu *et al.* 2016), no differences on the tubules diameter or the area were found between experimental groups of wild and domestic rams in the present work. The seminiferous tubules area was not affected by season, but the scrotal circumference was higher in the Middle-RS groups as other authors reported previously (Schanbacher and Ford 1979; Toledano-Diaz *et al.* 2007; Zamiri *et al.* 2010). A positive correlation between testosterone secretion and the volume of interstitium, Leydig cell and lymphatic space in the testis were reported by Mendis-Handagama *et al*. (1988), therefore an increase of the interstitial volume could be the reason for the scrotal circumference fluctuations. The assessment of the morphometric parameters of the seminiferous tubules showed a higher cell density in the Middle-RS group of Merino rams with a similar tendency in Mouflon rams which is in accordance with the proliferation markers quantification obtained in the present work. Immunohistochemistry results of Ki67 showed a higher level of germ cell proliferation in the testis of Merino and Mouflon Middle-RS groups. These findings support the fact that high levels of testosterone stimulate spermatogenesis in the time of the year prior to the natural breeding season of small ruminants. Animal domestication has attenuated the seasonal breeding activity of domestic rams; however, in the present study, a marked testicular regression was quantified in testicular biopsies of low-testosterone groups of both domestic and wild rams. The End-RS Mouflon group showed a decrease of the proliferative marker Ki67 in germ cells accompanied with an increase in Sertoli cells' GATA-4 immunolabeling. Sertoli cells are well known to give support to germ cells during spermatogenesis and are also

in charge of the phagocytic removal of residual bodies detached from spermatids and apoptotic spermatogenic cells (Blanco-Rodriguez and Martinez-Garcia 1999; Nakanishi and Shiratsuchi 2004). The phagocytosis of apoptotic spermatogenic cells induce lipid droplet formation in Sertoli cells (Wang *et al.* 2006), and accumulation of lipid droplets in the cytoplasm of Sertoli cells during testicular regression has also been reported (Hodgson *et al.* 1979). Additionally, GATA-4 plays an essential role in the lipid and cholesterol uptake, transport and metabolism in the intestine (Battle *et al.* 2008). All together suggest that the increase of GATA-4 levels in Sertoli cells in seasonal breeders could be related to the higher phagocytic activity that Sertoli cells must undergo during testicular regression when the apoptosis of spermatogenic cells is increased. Although Sertoli cell population was considered to be fixed and stable after puberty (Hochereau-de Reviers *et al.* 1987), some studies showed that Sertoli cells are not terminally differentiated and can be altered in the adult male (Hotzel *et al.* 1998; Tarulli *et al.* 2006). Moreover, a cyclic activity of Sertoli cells has been reported in other seasonal breeders such as the red deer (Pintus *et al.* 2015) and the pikas (Liu *et al.* 2016). Our findings suggest a seasonal pattern of Sertoli cell activity and, since Sertoli cells are essential for spermatogonial differentiation (Zhang *et al.* 2007), its action could be affecting sperm membrane composition and thus fluctuations of sperm cryotolerance.

In conclusion, seasonal variations of sperm freezability in domestic and wild rams within the rutting season were confirmed. Sperm cryopreserved in December for Merino rams and January for Mouflon rams (End-RS groups) have better quality after freeze-thawing. Hence, the optimal time for sperm collection and cryopreservation in domestic and wild rams is at the end of the rutting season, when testosterone secretion is decreasing but sperm have better quality after freeze-thawing. Also, the fall of testosterone levels at the end of the natural

breeding season in both species entails testicular regression, despite semen production being maintained. The germinal epithelium of the seminiferous tubules shows a seasonal pattern of proliferation in both species, which could affect sperm membrane properties and the subsequent cryotolerance in small ruminants.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure legends

Fig. 1. Seminiferous tubules morphometric parameters assessment in hematoxylin-eosin stained testicular sections at 200x magnification (*a*, *b*, *c*, *d*): the number of cell nuclei inside the tubule (*b*), tubular area occupied by cell nuclei (*b*), tubular area (*c*), tubular diameter (*d*) and seminiferous epithelium height (*d*) were assessed in Merino testis in the middle (Middle-RS; *e*) and in the end (End-RS; *f*) of the rutting season. Results are shown in graphs (*g*) to (*j*) (mean ± s.e.m.). Asterisks indicate statistically significant differences between groups (*P<0.05; **P<0.001). Scale bar = 50 µm.

Fig. 2. Seminiferous tubules morphometric parameters were assessed in hematoxylin-eosin stained sections of Mouflon testis in the middle (Middle-RS; *a*) and in the end (End-RS; *b*) of the rutting season (200x magnification). Results are shown in graphs (*c*) to (*f*) (mean ± s.e.m). Asterisks indicate statistically significant differences between groups (*P=0.033). Scale bar = 50 µm.

Fig. 3. Comparative immunostaining pattern of PCNA (first column), Ki67 (second column) and GATA-4 (third column) at 400x magnification in seminiferous tubules of Merino (*a*, *b* rows) and Mouflon (*c*, *d* rows) ram testis parenchyma in the middle (Middle-RS) and in the end (End-RS) of the rutting season. PCNA immunolabeling was quantified in Sertoli cells (thick arrows) and spermatogonias (thin arrows), Ki67 in spermatocytes (arrowheads) and round spermatids (asterisks) and GATA-4 in Sertoli cells (thick arrows). Results of positive cells quantification are shown in graphs (*e*) and (*f*) (mean ± s.e.m.). Asterisks indicate statistically significant differences between groups (*P=0.004; **P<0.001; ***P<0.0001). Scale bar = 20 µm.

Supplementary Figure 1. Negative controls of PCNA (*a*), Ki67 (*b*) and GATA-4 (*c*) in seminiferous tubules of testicular biopsies at 400x magnification. Scale bar = 20 µm.

Table 1. Merino rams' sperm quality parameters (mean ± s.e.m.) of fresh and frozen-thawed samples collected in the middle (Middle-RS; n = 9) and in the end (End-RS; n = 8) of the rutting season. Asterisks indicate statistically significant differences between groups of the same species (*P<0.05).

	Merino ram fresh sperm		**Merino ram frozen-thawed sperm**	
Group	**Middle-RS**	**End-RS**	**Middle-RS**	**End-RS**
Month of collection	**July**	**December**	**July**	**December**
Levels of testosterone	**High**	**Low**	**High**	**Low**
Ejaculate volume (mL)	1.0±0.1	0.9±0.1		
Concentration (mill/mL)	4568.7±83.4	3950.8±307.7		
Sperm viability and acrosomal status:				
PI-/PNA-FITC- (%)	85.9±3.1*	94.4±1.2*	40.6±7.2*	68.6±8.2*
PI+/PNA-FITC- (%)	9.8±2.0*	3.9±1.0*	24.0±3.3	22.1±5.1
PI-/PNA-FITC+ (%)	0.2±0.2	0.4±0.2	1.0±0.4	1.3±0.7
PI+/PNA-FITC+ (%)	4.1±1.2	1.4±0.4	34.4±9.1*	8.0±3.9*
Total viability (%)	86.1±3.1*	94.8±1.3*	41.6±7.3*	69.9±8.2*
Acrosome integrity (%)	95.7±1.2	98.3±0.3	64.6±9.9*	90.8±3.9*
Motility variables:				
Total motility (%)	86.8±1.5	88.3±2.4	39.3±4.2	55.3±9.8
PM (%)	32.2±4.3	37.1±4.9	19.9±3.4	20.6±4.8
VCL (μm/s)	157.2±5.8	155.5±6.5	106.7±8.1	89.5±8.4
VSL (μm/s)	76.5±8.0	84.8±7.9	69.1±9.2	48.2±5.8
VAP (μm/s)	112.8±8.8	124.9±9.5	90.2±9.7	68.0±7.7
LIN (%)	48.0±4.0	54.8±5.3	63.1±3.6*	53.3±2.3*
STR (%)	66.8±2.9	68.1±4.2	75.5±2.3	70.6±1.7
WOB (%)	71.0±3.4	80.0±4.4	83.2±2.7*	75.4±1.8*
ALH (μm)	5.5±0.2*	3.9±0.3*	2.6±0.2	3.0±0.1
BCF (Hz)	7.8±0.3	8.0±0.3	8.5±0.3*	6.4±0.5*
Morpho-abnormalities (%)	3.7±1.4	4.0±1.2	8.8±1.9	6.0±2.3

PI: propidium iodide; PNA: peanut (*Arachis hypogaea*) agglutinin. PI-/PNA-FITC-: live spermatozoa with intact acrosome; PI-/PNA-FITC+: live spermatozoa with damaged acrosome; PI+/PNA-FITC-: dead spermatozoa with intact acrosome; PI+/PNA-FITC+: dead spermatozoa with damaged acrosome. CASA: computer-aided sperm analysis system; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

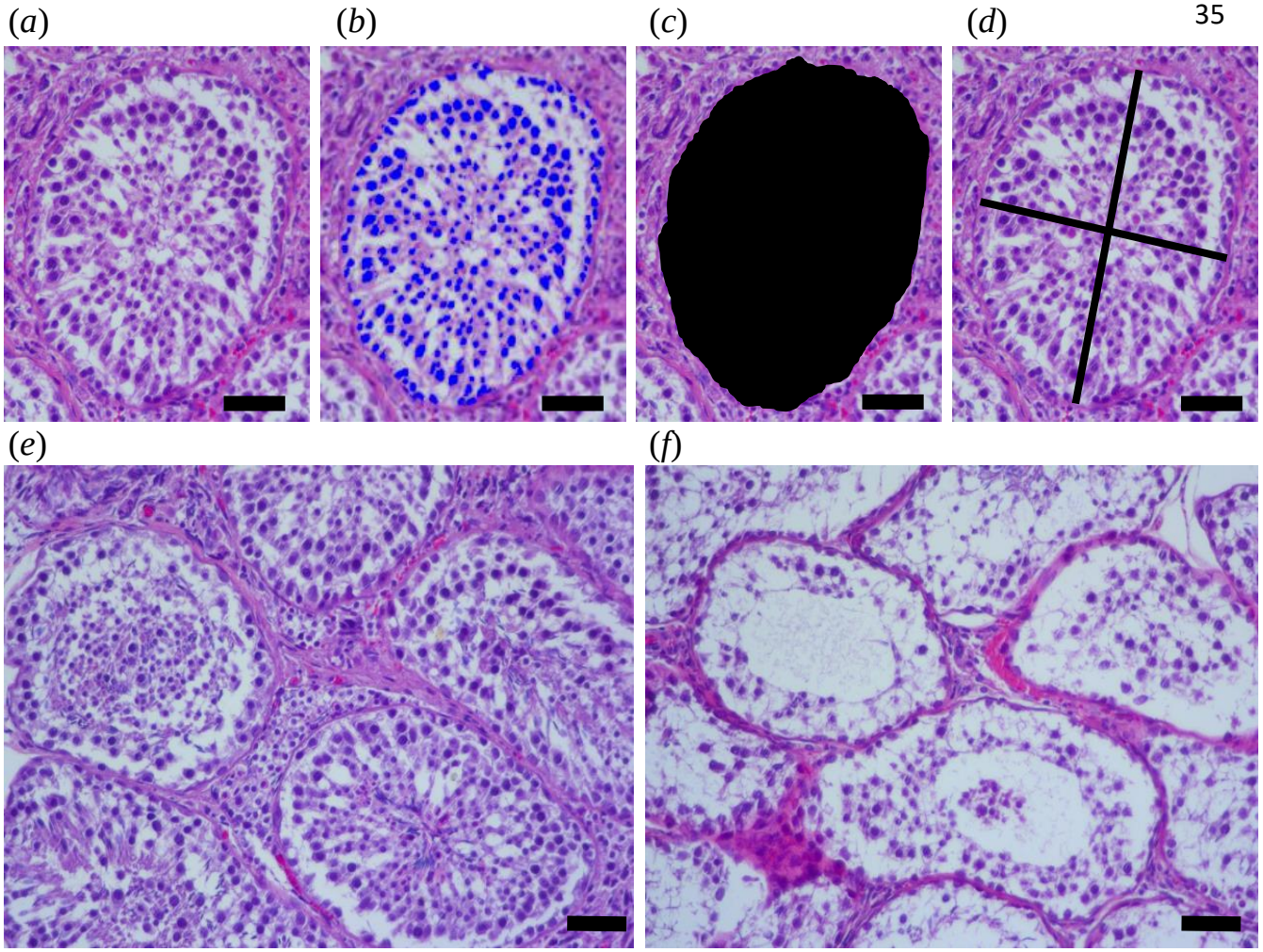
Table 2. Mouflon rams' sperm quality parameters (mean ± s.e.m.) of fresh and frozen-thawed samples collected in the middle (Middle-RS; n = 11) and in the end (End-RS; n = 25) of the rutting season. Asterisks indicate statistically significant differences between groups of the same species (**P*<0.05; ***P*<0.001).

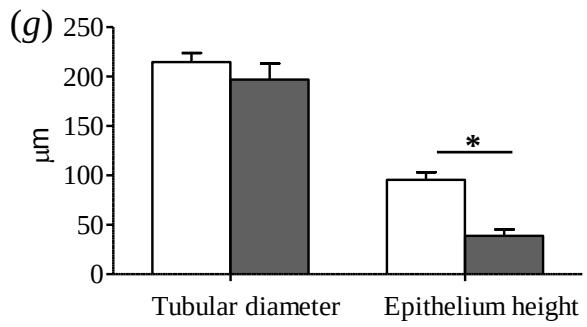
	Mouflon ram fresh sperm		**Mouflon ram frozen-thawed sperm**	
Group	**Middle-RS**	**End-RS**	**Middle-RS**	**End-RS**
Month of collection	**November**	**January**	**November**	**January**
Levels of testosterone	**High**	**Low**	**High**	**Low**
Ejaculate volume (mL)	1.2±0.3	0.8±0.1		
Concentration (mill/mL)	565.6±254.1	1398.8±246.9		
Sperm viability and acrosomal status:				
PI-/PNA-FITC- (%)	69.0±3.5	78.1±3.9	23.7±5.0*	40.0±3.2*
PI+/PNA-FITC- (%)	25.8±3.2	15.3±2.3	53.1±4.5*	42.0±2.7*
PI-/PNA-FITC+ (%)	0.0±0.0	0.4±0.3	0.5±0.3	1.0±0.3
PI+/PNA-FITC+ (%)	5.3±2.3	6.2±2.1	22.7±5.1	17.0±2.2
Total viability (%)	69.0±3.5	78.4±3.8	24.2±5.0*	40.9±3.3*
Acrosome integrity (%)	94.8±2.3	93.4±2.2	76.8±5.1	82.0±2.2
Motility variables:				
Total motility (%)	62.1±9.0	62.2±5.0	22.0±5.0*	34.8±3.3*
PM (%)	26.2±10.3	20.5±3.7	8.5±2.9	13.5±1.6
VCL (μm/s)	75.7±20.1	72.9±5.9	58.8±7.0*	83.3±4.5*
VSL (μm/s)	45.5±17.0	36.4±4.4	38.8±6.3	46.3±2.5
VAP (μm/s)	57.6±19.8	50.9±5.5	48.6±7.4*	63.8±3.7*
LIN (%)	55.2±6.5	48.1±3.3	63.3±4.9	56.4±2.0
STR (%)	76.6±2.4	70.1±2.0	78.1±3.5	73.69±2.0
WOB (%)	71.6±6.3	67.6±3.1	79.7±3.9	76.5±1.4
ALH (μm)	2.8±0.2	2.6±0.2	1.8±0.2**	2.8±0.1**
BCF (Hz)	9.2±0.3	8.2±0.4	6.9±0.6*	8.4±0.3*
Morpho-abnormalities (%)	58.6±9.3	38.1±5.7	49.4±7.4	42.0±5.2

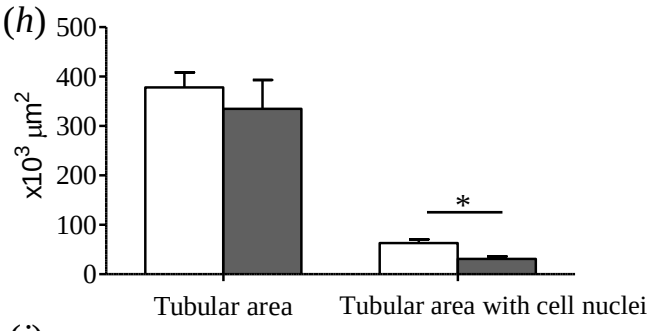
PI: propidium iodide; PNA: peanut (*Arachis hypogaea*) agglutinin; PI-/PNA-FITC-: live spermatozoa with intact acrosome; PI-/PNA-FITC+: live spermatozoa with damaged acrosome; PI+/PNA-FITC-: dead spermatozoa with intact acrosome; PI+/PNA-FITC+: dead spermatozoa with damaged acrosome; CASA: computer-aided sperm analysis system; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

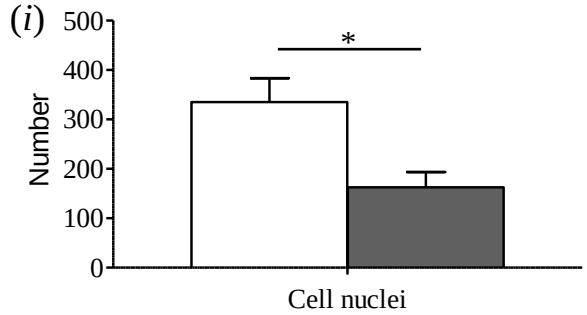
Table 3. Sperm head area (mean ± s.e.m) in Merino and Mouflon fresh sperm samples in the middle (Middle-RS; n = 4) and in the end (End-RS; n = 4) of the rutting season. Asterisks indicate statistically significant differences between groups of the same species (*P<0.05; **P<0.001; ***P<0.0001).

	Sperm head area			
	Merino ram		**Mouflon ram**	
Group	**Middle-RS**	**End-RS**	**Middle-RS**	**End-RS**
Mean (μm^2)	34.8±1.0	35.7±0.5	34.3±0.1*	38.3±0.2*
Subpopulation 1 (μm^2)	37.7±0.8	38.5±0.6	37.4±0.2***	40.7±0.2***
(%)	12.5±1.2	16.5±3.9	13.8±3.8	19.3±2.1
Subpopulation 2 (μm^2)	35.7±0.9	36.5±0.6	35.2±0.2***	39.0±0.2***
(%)	38.0±3.3	33.5±2.8	28.0±3.0	33.3±2.3
Subpopulation 3 (μm^2)	34.2±1.0	34.9±0.6	33.7±0.3***	37.6±0.2***
(%)	33.3±2.3	36.0±4.2	36.8±1.9	28.3±3.0
Subpopulation 4 (μm^2)	32.0±1.4	32.6±0.5	32.1±0.4**	35.7±0.2**
(%)	16.3±1.1	14.0±3.0	21.5±5.9	19.3±3.7









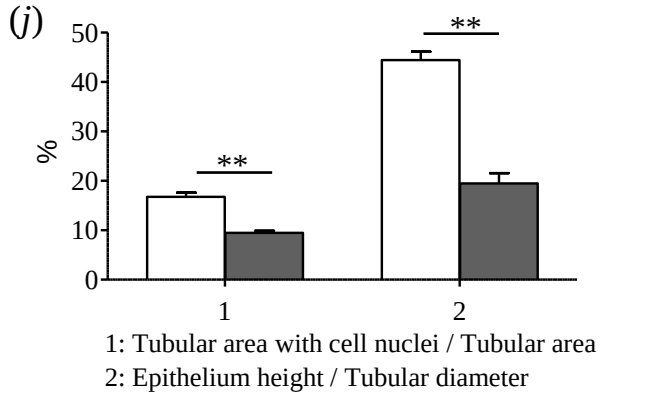


Fig. 1. Merino Middle-RS Merino End-RS

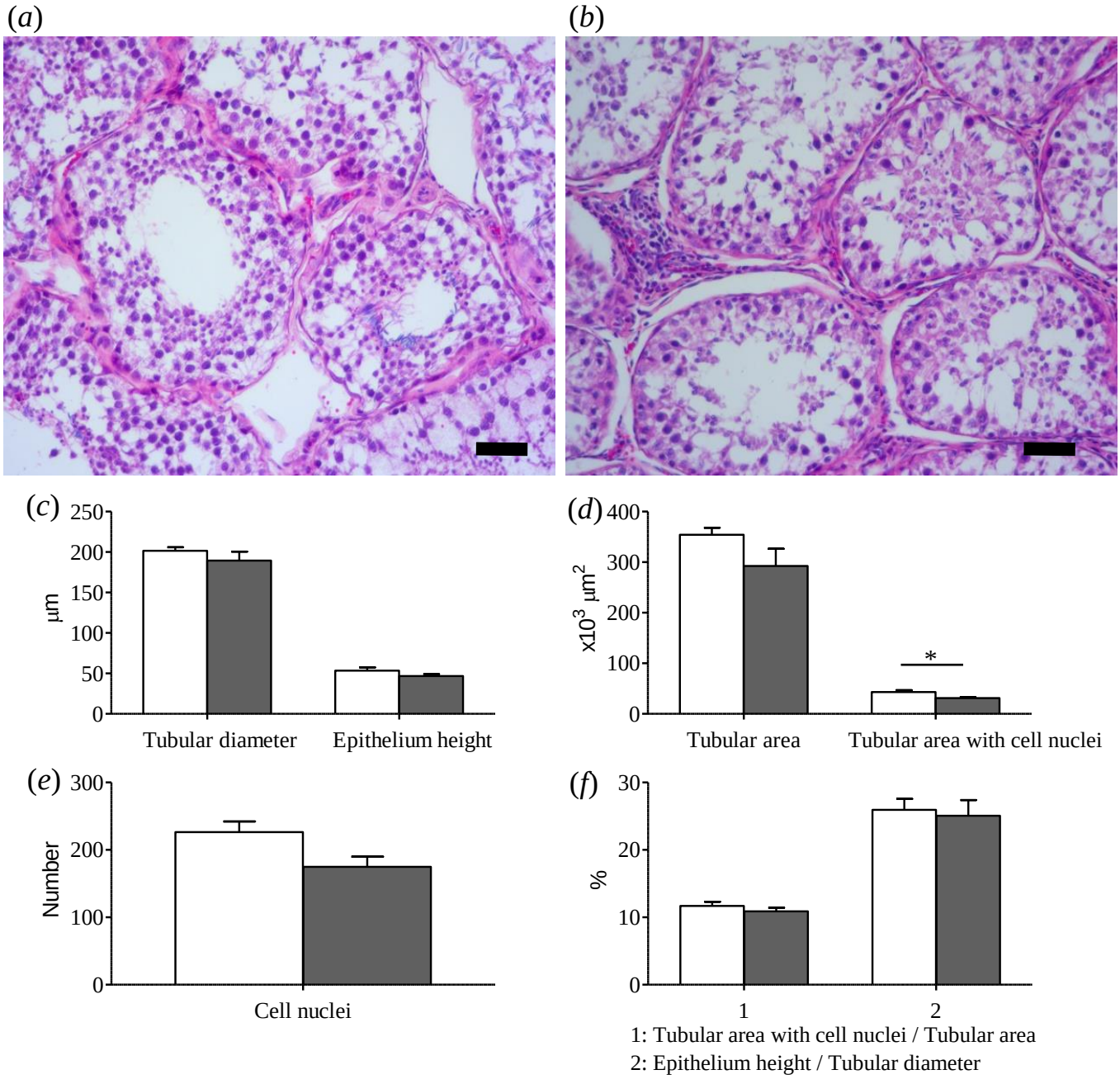
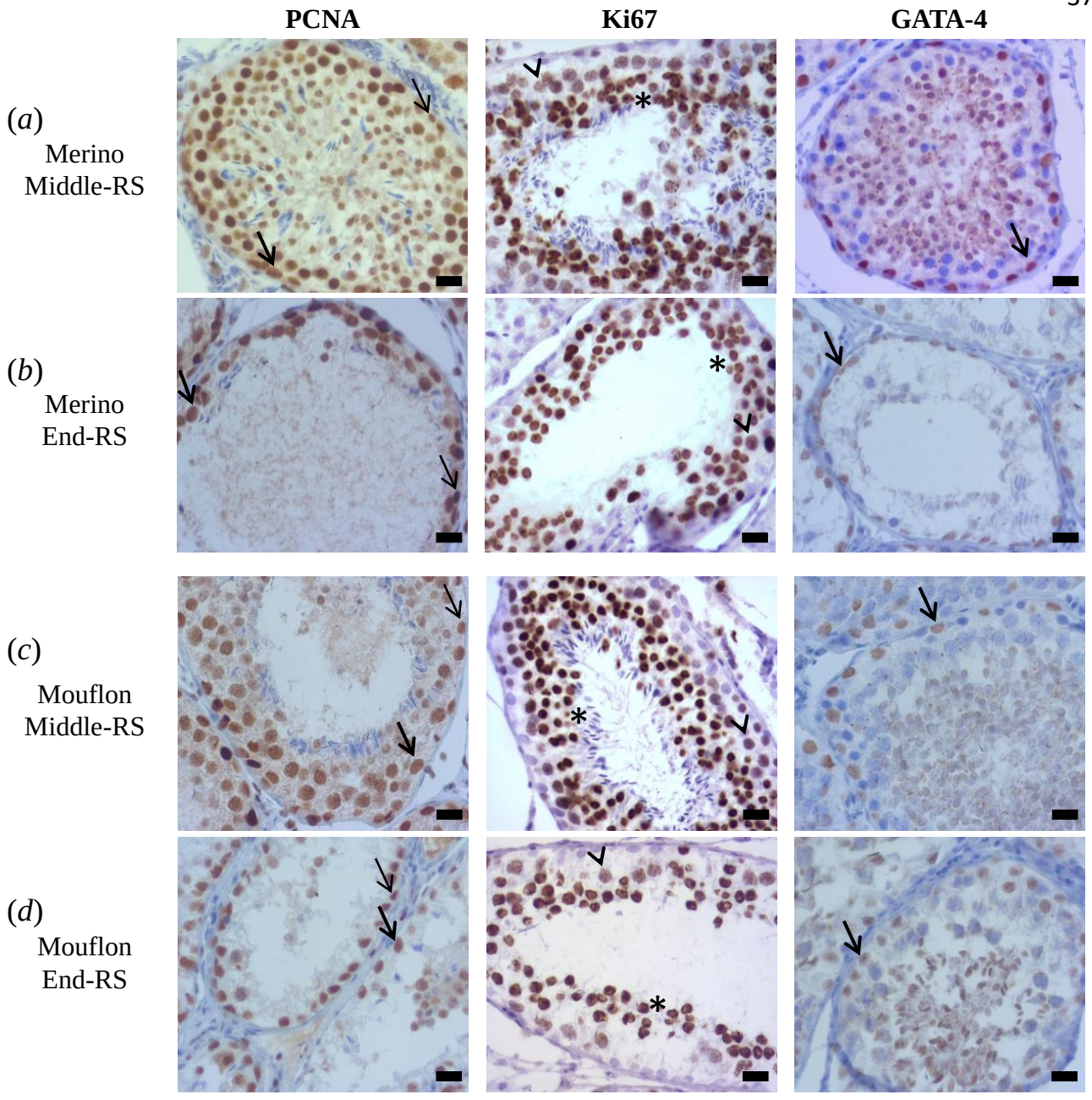

(a)
(b)
(c)
250
200
150
100
50
0
µm
Tubular diameter
Epithelium height
(d)
400
300
200
100
0
x10³ µm²
*
Tubular area
Tubular area with cell nuclei
(e)
300
200
100
0
Number
Cell nuclei
(f)
30
20
10
0
%
1
2
1: Tubular area with cell nuclei / Tubular area
2: Epithelium height / Tubular diameter
Mouflon Middle-RS
Mouflon End-RS

Fig. 2.


PCNA
Ki67
GATA-4
(a)
Merino
Middle-RS
(b)
Merino
End-RS
(c)
Mouflon
Middle-RS
(d)
Mouflon
End-RS

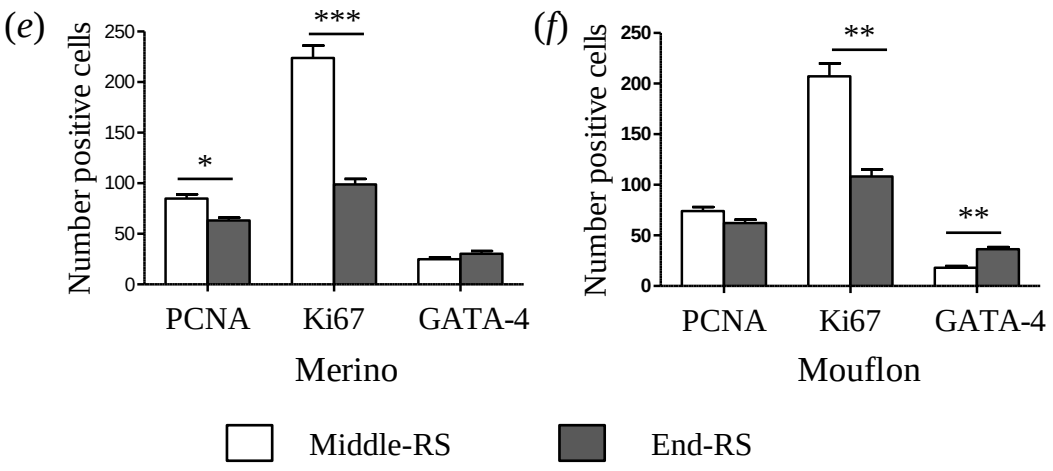
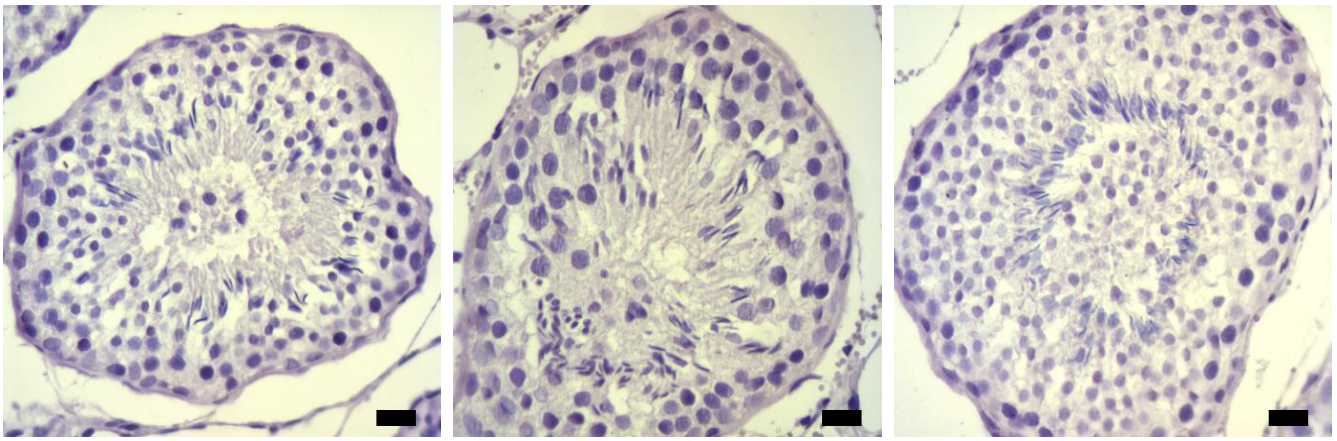

(e)
Number positive cells
250
200
150
100
50
0
*
***
PCNA
Ki67
GATA-4
Merino
(f)
Number positive cells
250
200
150
100
50
0
**
**
PCNA
Ki67
GATA-4
Mouflon
Middle-RS
End-RS

Fig. 3.

(*a*) (*b*) (*c*)



Supplementary Figure 1