Spanish Journal of Agricultural Research
16 (2), e0403, 13 pages (2018)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2018162-12882
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, O.A, M.P. (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Sperm quality and seminal plasma proteins in three sheep breeds under high altitude and tropical conditions

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Abstract

We tested the hypothesis that sheep breed can influence the sperm quality and seminal plasma (SP) composition and investigated any potential relationship between SP proteins and antioxidant enzyme activities (AO) with sperm quality. Ejaculates from twelve rams of three breeds were obtained during the rainy season at high altitudes, and sperm quality was automatically evaluated (CASA-Hamilton Thorne). The AO of superoxide dismutase, glutathione peroxide and glutathione reductase (GR) in SP was evaluated and total proteins were separated by SDS-PAGE. Comparative analyses of semen quality parameters between breeds revealed that Creole and Hampshire breeds had a higher sperm quality compared with Romney Marsh ($p<0.05$), although no difference in AO was found. GR activity was negatively correlated ($p<0.05$) with several kinematic variables and positively ($p<0.05$) with morphological abnormalities. The highest SP protein concentration was found in semen collected from Hampshire males compared with that from Creole and Romney Marsh ($p<0.05$). SDS-PAGE analysis showed the presence of 32 protein bands in SP with molecular weights between 334 and 10 kDa. Differences ($p<0.05$) between breeds in bands of 43, 25, 22 and 20 kDa were observed. These results evidence a relationship between the protective effect of the antioxidant enzyme system in SP of three ram types under high altitude and tropical conditions and semen quality. Our findings also suggest that the identified proteins might play an important role in sperm physiology and quality.

Additional keywords: antioxidant defense enzymes; high-tropic; rainy season.

Abbreviations used: ALH (head lateral amplitude); AO (antioxidant); AV (Artificial Vagina); BCF (beat frequency); BT (bent tail); CASA (computer-assisted sperm analysis); CT (coiled tail); DCD (distal cytoplasmic droplet); GPx (glutathione peroxidase); GR (glutathione reductase); GSH (Glutathione); GSSG (Glutathione disulphide); LIN (linearity); PCD (proximal cytoplasmic droplet); ROS (Reactive Oxidative Species); SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis); SOD (superoxide dismutase); SP (seminal plasma); STR (straightness); VAP (path velocity); VCL (track speed); VSL (progressive velocity); WOB (wobble); XTT (tetrazolium salt (3-{1-[(phenylamino)- (1, 2)).

Authors' contributions: Conceived, designed and performed the experiments: JAC, MCS, and HGL. Analyzed the data: JAC, MCS and TMB. Contributed reagents, materials and facilities: HGL and JACP. Wrote the paper: MCS, TMB and JACP. All authors revised and approved the final manuscript.

Citation: Carvajal-Serna, M.; Cardozo, J. A.; Grajales-Lombana, H.; Cebrián-Pérez, J. A.; Muiño-Blanco, T. (2018). Sperm quality and seminal plasma proteins in three sheep breeds under high altitude and tropical conditions. Spanish Journal of Agricultural Research, Volume 16, Issue 2, e0403. https://doi.org/10.5424/sjar/2018162-12882

Received: 18 Jan 2018. **Accepted:** 21 Jun 2018.

Funding: National University of Colombia, under the "Strategic Program for genetic and reproductive improvement and determining the characteristics and quality of the carcass and meat in sheep production systems in five regions of Colombia" (Ref: 110 157 635 854 Project, funded by Colciencias in the call Col576 2012); MINECO-FEDER (AGL 2013-43328-P).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Located in the equatorial zone of South America, Colombia has a long-time tradition of sheep production with strong links with its culture. In the last decade, the number of herds as well as animals increased with a significant impact in local economy. An economic advantage of sheep production in the equatorial countries and other tropical and sub-tropical environments is that reproduction is unseasonal and meat production is stable throughout the year (Rosa & Bryant, 2003). However, ambient temperature, humidity, solar radiation, and rainfall can affect livestock production systems by environmental stressors on sheep physiology (Marai *et al.*, 2007; Abecia *et al.*, 2016) and the annual rain-drought cycle can modify the food availability (Rosa

& Bryant, 2003). The rainfall effect on fertility rate has been studied in ewes on temperate zones (Galina *et al.*, 1996; Arrebola *et al.*, 2009, 2016). Low rainfall has a detrimental effect on the offspring after artificial insemination in spring (Abecia *et al.*, 2016) summer and autumn (Palacios & Abecia, 2014), and a possible explanation for the effect of annual rainfall was associated with the nutritional status of animals (Arrebola *et al.*, 2009). Therefore, in this study we have investigated the possible sperm quality differences among breeds in the raining season. Creole is a local sheep breed adapted to environmental conditions that constitutes an important genetic resource for the small-scale agriculture in certain ecoregions (Soma *et al.*, 2012; Bravo *et al.*, 2015). Hampshire and Romney Marsh are genetic-selected breeds introduced in Colombia by direct importation from European countries since 1960 to improve the local sheep breed. All these breeds display an unseasonal reproduction under equatorial environment. Research related to the diversity and adaptation of local and foreign breeds may help to identify the most appropriate animal genetic resources for each region (Bravo *et al.*, 2015).

Sperm quality is the main factor that limit the male reproductive efficiency. In addition, the influence of breed, geographical location and differences among individual rams have been reported (Karagiannidis *et al.*, 2000; Kasimanickam *et al.*, 2007). Likewise, differences in sperm functionality, progressive motility and volume of the ejaculate have also been observed between genetically selected breeds in temperate zones (Kasimanickam *et al.*, 2007) and between local breed in arid zones (Ibrahim, 1997). Conversely, no significant differences have been found in sperm quality parameters between local breeds in the Mediterranean area (Karagiannidis *et al.*, 2000).

Mammalian sperm cell physiology is supported by seminal plasma (SP) (Desnoyers & Manjunath, 1992; Manjunath & Thérien, 2002; Muiño-Blanco *et al.*, 2008), a complex biological fluid composed of multiple secretions from the testis, epididymis, prostate, seminal vesicles and bulbourethral glands (Domínguez *et al.*, 2008). SP provides metabolic maintenance as an energy source for the sperm cell, and modulates sperm functionality in terms of motility, viability (Maxwell *et al.*, 1998) and resistance to damage by keeping spermatozoa in a decapacitated-state both in fresh (Luna *et al.*, 2015) and frozen-thawed (Domínguez *et al.*, 2008; Bernardini *et al.*, 2011) semen. The beneficial components of SP are difficult to define because of its complexity and the presence of both stimulating and inhibitory factors (Muiño-Blanco *et al.*, 2008; Leahy, *et al.*, 2010a). Certain proteins isolated from SP stabilise the sperm membrane and reduce stress damage, increasing the membrane resistance to cold-shock (Barrios *et al.*, 2000; Perez-Pe *et al.*, 2001; Colás *et al.*, 2009) and cryopreservation (Leahy *et al.*, 2009; Bernardini *et al.*, 2011). Other SP proteins are enzymes, which are very important for the maintenance of the sperm fertilizing ability after ejaculation. Among these enzymes, the antioxidant defence system plays a critical role given that, under physiological conditions, spermatozoa generate small amounts of reactive oxygen species (ROS). Increased ROS and decreased antioxidant activity in SP have been related with male infertility (de Lamirande *et al.*, 1997; Aitken & Baker 2004). The SP antioxidant enzyme defence system includes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT), and plays an essential role in the sperm protection from the oxidative stress (Aitken & Roman, 2008; Agarwal *et al.*, 2014), working synergistically to remove the oxidizing components (Marti *et al.*, 2007; Shiva *et al.*, 2011).

Taking into account the wide range of roles of SP protein components in sperm functions, it might be assumed that the absence, presence or differences in content of specific SP proteins and enzymes might be reflected in altered sperm functionality and fertilizing ability (Agarwal *et al.*, 2014; Sharma *et al.*, 2015). Techniques such as enzyme kinetics and proteomics allow the characterization or identification of these SP proteins at molecular level and are useful to reveal differences between breeds. It has been shown that SP composition is related to sperm membrane-binding proteins and is highly conserved between seasonal breeds in the southern hemisphere (Bernardini *et al.*, 2011). Conversely, differences in the protein composition of SP have been found in crossbred rams, and it has been suggested that they could be influenced by season, breeds, age and SP management (Asadpour, 2012). These analyses have provided knowledge of the biological pathways involved in fertility (du Plessis *et al.*, 2011) and are the basis for the identification of fertility biomarkers (Agarwal *et al.*, 2014; Muhammad Aslam *et al.*, 2014; Soleilhavoup *et al.*, 2014).

Information on characteristics and differences of sperm functionality (Ibrahim, 1997; Karagiannidis *et al.*, 2000; Kasimanickam *et al.*, 2007) and SP composition (Bernardini *et al.*, 2011; Asadpour, 2012) of several breeds of ram has been reported. Nevertheless, little is known about sperm quality, SP composition and antioxidant defense of the Creole (a local breed) and other introduced sheep breeds under high altitude and tropical conditions in Colombia. In this study, we tested the hypothesis that sheep breed can influence SP composition. Thus, the objectives of this study were 1) to evaluate differences in sperm quality; 2) to evidence

changes in the SP protein content and antioxidant enzyme activity; and 3) to determine whether there is any relationship between the antioxidant enzyme activity and SP proteins with sperm quality. The study was carried out on three sheep breeds under the high altitude and tropical conditions in Colombia.

Material and methods

Animals and locations

All animals used in this study were handled in strict accordance with the Colombian Animal Protection legislation (Law 84/1989, modified by Law 1774/2016). Males were housed under uniform nutritional conditions at the Center for ovine research, technological development and extension of the National University of Colombia, located in Mosquera (4°40'57'' N. 74°12'50'' W) at 2510 m above sea level. Evaluation of the antioxidant enzyme, protein concentration and protein profile of SP was carried out in the Laboratory of Molecular Microbiology (LMM) at the Center of Biotechnology and Bioindustry (CBB - Tibaitatá) of the Colombian corporation for agricultural research (CORPOPICA) in the Department of Cundinamarca.

Semen collection and evaluation

Semen was obtained with an artificial vagina (AV) according to the protocol approved by the Bioethics committee of the National University of Colombia (CB-074-2014). All experiments were carried out with fresh semen obtained from twelve mature rams (2-5 years old) of three breeds (4 Creole; 4 Romney Marsh; 4 Hampshire). We have already shown no significant difference in sperm quality between the first and second ejaculates in these specific breeds, under the same experimental conditions of the present study (Carvajal-serna *et al.*, 2018). Therefore, to ensure enough volume for all intended evaluations, two successive ejaculates were collected and pooled once a week from each ram individually, during 6 weeks between September and November (rainfall season).

After collection, each pooled semen sample was diluted at a ratio of 1/75 with a holding medium [250 mM sucrose, 0.1 mM EGTA, 4 mM sodium phosphate, 5 mM glucose, 10 mM HEPES and 2 mM KOH; final pH 7.5 (Mendoza *et al.*, 2012), at 37 ºC]. After the first dilution, aliquots were diluted at a ratio (1:1) with a vital stain Viadent (10 µg/mL freshly prepared before the experiment; supplied by Hamilton Thorne, Beverly, MA, USA) containing the fluorescent dye Hoechst 33258, and then incubated for 2 min at 37 ºC in the dark. These successive dilutions (1:75) and (1:1) were carried out to guarantee a final concentration of $40 \cdot 10^6$ spz/mL, that fits into the operational range recommended by the IVOS II CASA system (Hamilton Thorne, Beverly, MA 01915, USA).

Immediately after incubation, samples (3 µL) were placed onto pre-warmed 20-mm Leja slides (Leja Luzernestraat 10, 2153 GN Nieuw-Vennep, Netherlands). Sperm concentration, motility and viability were sequentially analysed using the IVOS II CASA system, which consisted of an integrated optical system that provides automatic field selection and precise control of temperature (37 ºC) during analysis. Standard motility analysis is performed on five fields under typical phase contrast illumination, and viability analysis is then performed on the same fields under fluorescent illumination. For the assessment of sperm velocities and motion parameters [path velocity (VAP), progressive velocity (VSL), track speed (VCL), head lateral amplitude (ALH), beat frequency (BCF), straightness (STR), linearity (LIN) and wobble (WOB)] an image capture rate of 31 frames/sec and visible light (blue light-emitting diode) was used. Sperm viability assessment was performed using the VIADENT™ stain biz-benzimide trihydrochloride (Hoeschst 33259, 5 µg/mL) that was implemented to determine the vitality of the sample, since it penetrates only cells with damaged membranes and adheres to the DNA emitting fluorescence (detected using the Viadent filter).

Furthermore, the computerized system (IVOS II) can objectively identify morphological defects only related to the sperm tail. The morphological parameters are classified into coiled tail (CT), bent tail (BT), proximal cytoplasmic droplet (PCD) and distal cytoplasmic droplet (DCD). The total sum of the sperm percentages with these morphological defects is considered as abnormal spermatozoa by the IVOS II system. Therefore, based on this criterion the system estimates the percentage of cells with normal morphology (NM) in the sample.

Collection of seminal plasma (SP)

SP was obtained by centrifugation of each pooled sample separately at 12000 X g for 5 min in a microfuge (HERMLE Labortechnik GmbH, Siemensstr 25, D-78564 Wehingen, Germany) at 4 ºC. The supernatant was centrifuged again and the SP recovered and filtered through a 0.22 mm Millipore membrane (© Merck KGaA, Darmstadt, Germany). After adding 10% protease and phosphatase inhibitor (Phenylmethanesulfonyl fluoride, Sigma Chemical Co, St. Louis, MO, USA) it was kept at -20 ºC. The protein concentration was determined following the Bradford´s (1976) method, using a

Coomassie dye which upon binding to proteins undergoes a change in absorbance (Bio-Rad, Hercules, CA, USA).

Antioxidant enzyme assays

The antioxidant defence system of SP was assessed by determining the activity of the following enzymes: Superoxide dismutase (SOD), GPx and GR. Measurements were performed as previously described (Casao *et al.*, 2013), adapted for the microtiter plate with a spectrophotometric method using an ELISA plate Reader (Elx BioTek Instruments, Inc., Winooski, VT, USA). All samples were loaded in duplicate and analysed in the same assay.

• *Superoxide dismutase (Mn SOD and Cu/Zn SOD. EC 1.15.1.1)*

The activity was measured as a decrease in the XTT (3'-(1-[(phenylamino)-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzenesul-phonic acid hydrate (Sigma Chemical Co, St. Louis, MO, USA) reduction by the superoxide anion generated by xanthine oxidase. The reaction mixture contained 40.5 mM sodium phosphate buffer at pH 7.8; 0.15 mM xanthine; 0.15 mUI xanthine oxidase; 30 mM XTT and 10 µL of SP to complete a final volume of 200 µL. The reaction was initiated by the addition of xantine oxidase, and the absorbance change at 470 nm was monitored for 3 min with a microtiter plate reader. One enzyme unit (IU) was defined as the amount of SOD capable of transforming 1.0 µmol/min of $O_2^{\bullet-}$.

• *Glutathione reductase (GR. EC.1.6.4.2)*

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

• *Glutathione peroxidase (GPx. EC.1.11.1.9)*

The activity was measured following the oxidation of GSH to GSSG catalysed by GPx and using ter-butylhydroperoxide (t-BuO2H) as an electron acceptor, coupled to the recycling of GSSG back to GSH utilizing GR and NADPH. The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; EDTA 0.5 mM, 54 mUI of GR; 85 µM NADPH; 2 mM GSH; 1.2 mM t-BuO2H and 6 µL of SP to complete a final volume of 200 µl. The absorbance change at 340 nm was monitored for 3 min with the microtiter plate reader. One IU is defined as the amount of GPx capable of transforming 1 µmol/min of NADPH at 25 ºC, pH 7.2.

Protein separation by polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the molecular weight and relative content of the main SP proteins as previously described (Laemmli, 1970) using a 8-16% polyacrylamide gel. A concentration of 35 µg SP protein was mixed with 0.1% bromophenol blue containing 5% SDS, 20% glycerol and 10% β-mercaptoethanol. The molecular weight was estimated using low-molecular weight protein standards. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (0.15%) including 50% methanol and 10% acetic acid, and de-stained in a mixture of 25% methanol, 10% acetic acid and distilled water until no background was detectable.

Capture and image analysis

Gels were digitized with an image documentation system (Molecular Imaging Gel Doc, Bio-Rad, Hercules, CA, USA). The gel images were analysed to determine the molecular weight and the relative protein content using the Quantity One computer program (version 4.6.5, Bio-Rad, Hercules, CA, USA).

Statistical analysis

The results are expressed as means ± SEM of the number of samples indicated in each case (Creole n=24; Romney Marsh n=24; Hampshire n=24). The normal distribution of sperm quality parameters, activity of SP antioxidant enzymes and concentration of total SP proteins were tested using the Kolmogorov-Smirnov test. Differences between data were analysed by a GLM procedure test (PROC GLM) with a Nested model taking into account the rams within breed [Breed (ID)]. To test the hypothesis, we used the Pr > F of the Type I error of the sum of squares, followed by a Tukey-Kramer comparison. Additionally, a Pearson correlation test between semen quality, enzymatic activity and concentration of proteins was carried out using the statistical package SAS 9.0 (SAS®; SAS Inst. Inc., Cary, NC, USA, 2006).

Results

Differences in sperm quality

Sperm quality and kinematic parameters of fresh ejaculates from Creole, Romney Marsh and Hampshire breeds were measured. The results obtained from four

mature rams of each of the three breeds (24 ejaculates from each breed) are shown in Table 1. Significant differences ($p<0.05$) in volume, sperm concentration, total motility, progressive motility, ALH and viability were found between breeds. Differences ($p<0.05$) between rams within breeds were also found in sperm quality and kinematic parameters. Romney Marsh and Hampshire breeds presented significant differences ($p<0.01$) among rams within each breed in volume, total and progressive motility and vitality (Table 1). Additionally, the motion parameters ALH, STR, VAP and VSL presented differences ($p<0.05$) among rams within each breed in at least one of the breeds evaluated.

The Hampshire breed produced significantly ($p<0.05$) higher volume than the Romney Marsh and Creole breeds (Table 1). All other parameters were also significantly higher in the Hampshire than in the Romney Marsh, while no significant differences ($p>0.05$) were found in sperm quality between the Hampshire and Creole breeds (Table 1).

The sperm percentage with normal morphology and with both BT and DCD was significantly different ($p<0.05$) between breeds during the rainy season (Table 2). Spermatozoa from the Romney Marsh breed had lower values of normal morphology than the other two breeds, a higher content of distal cytoplasmic droplet (4.79%) and the highest proportion of BT (3.35 %).

Antioxidant enzyme activity in semen of different breeds

Antioxidant enzyme activities were simultaneously determined in the SP of the three breeds during the rainfall season. Medium values (± S.E.M) for Creole, Romney Marsh, Hampshire of SOD (21.79±3.83, 15.84±4.67, 18.92±5.38 mmol/min·mL, respectively), GR (1.80±0.162, 2.15±0.307, 1.88±0.143 nmol/min·mL, respectively) and GPx (173.03±39.18, 168.90±37.11, 117.07±39.72 nmol/min·mL, respectively) activities of each breed. Significant differences were neither found between breeds nor between rams of each breed ($p>0.05$).

We then tested whether there was any correlation between the antioxidant enzyme activities in SP and either sperm quality or semen kinematic parameters. No correlation was observed between the sperm quality variables and the activity of the antioxidant enzymes. However, significant correlations ($p<0.05$) were found between GR activity and several kinematic variables in Creole and Romney Marsh breeds, while none was found in the Hampshire breed (Table 3).

Protein concentration and proteomic analysis of seminal plasma (SP) proteins

The highest SP protein concentration was found in semen collected from Hampshire males (60.66±2.63

Table 1. Sperm quality and kinematic parameters of Creole, Romney Marsh and Hampshire males during the rainfall season. All values are mean ± SEM of 24 assays.

Parameter[1]	Creole (n=24)	Romney Marsh (n=24)	Hampshire (n=24)
Volume (mL)	1.85±0.09^{c}	2.29±0.19^{b}*	2.81±0.17^{a}*
Total motility (%)	84.56±1.37^{a}	75.49±2.75^{b}*	85.45±1.55^{a}*
Prog. motility (%)	66.96±1.88^{a}	53.50±3.14^{b}*	62.69±2.51^{a}*
Conc. (spz · 10^{6}/mL)	2.793.99±280.07^{a}*	2.164.21±168.01^{b}*	2.389.06±163.22^{ab}
Viability (%)	87.64±1.45^{a}	79.91±2.75^{b}*	87.88±1.47^{a}*
ALH µm	7.92±0.21^{a}	7.37±0.20^{b}*	7.42±0.18^{ab}
BCF Hz	44.35±0.84	45.85±1.03	44.99±0.56
LIN %	53.50±2.43	54.90±1.20	53.93±1.06
STR %	87.28±0.67*	85.94±0.65	85.90±0.63*
VAP µm/s	158.87±4.20	146.80±4.21	149.53±4.54*
VCL µm/s	248.82±6.42	237.58±7.26	240.08±4.90
VSL µm/s	141.40±4.19	130.53±4.94	129.45±4.76*
WOB %	62.98±0.67	62.59±0.95	62.21±1.01

[1]ALH (head lateral amplitude); BCF (beat frequency); LIN (linearity); STR (straightness); VAP (path velocity); VCL (track speed); VSL (progressive velocity); WOB (wobble). Different letters within rows indicate significant differences ($p<0.05$) between breeds. *:significant difference ($p<0.01$) within breed

Table 2. Differences between breeds in the sperm proportion (mean ± SEM of 24 assays) with normal morphology and with the following alterations: bent tail (BT), coiled tail (CT), proximal cytoplasmic droplet (PCD) and distal cytoplasmic droplet (DCD) during the rainfall season. All values are mean ± SEM of 24 assays. Different letters within rows indicate significant differences ($p<0.05$).

Breed	Normal morphology (%)	BT (%)	CT (%)	PCD (%)	DCD (%)
Creole (n=24)	93.40±0.71[a]	2.20±0.31[a]	0.34±0.10	3.71±0.47	1.31±0.17[a]
Romney Marsh (n=24)	88.34±1.85[b]	3.35±0.44[b]	0.53±0.11	4.42±0.71	4.79±1.09[b]
Hampshire (n=24)	94.07±0.85[a]	1.57±0.17[a]	0.19±0.04	2.06±0.30	1.17±0.19[a]

Table 3. Pearson's correlation between sperm kinematics parameter and glutathione reductase (GR) activities in fresh ejaculates of Creole and Romney Marsh males during the rainy season.

Kinematic parameter[1]	Romney Marsh (n=24)	Hampshire (n=24)	Creole (n=24)
ALH	-0.176	-0.310	-0.449*
VAP	-0.511*	-0.162	-0.430*
LIN	-0.4192*	-0.184	-0.155
STR	-0.4223*	-0.052	0.050
BT	0.5387*	-0.142	0.117
CT	0.4453*	-0.028	0.282

[1]ALH (head lateral amplitude); BCF (beat frequency); BT (bent tail); CT (coiled tail); DCD (distal cytoplasmic droplet); LIN (linearity); STR (straightness); VAP (path velocity). Significant correlation (*) was considered with a $p<0.05$

mg/mL) compared with that from Creole and Romney Marsh (52.43±3.02 and 54.97±2.19 mg/mL, respectively; ($p<0.05$)).

Comparative SDS-PAGE analysis of SP of the three breeds was carried out. The profile revealed the presence of 32 protein bands with molecular weights ranging from 10 up to 334 kDa. The most intense bands, according to the highest densitometric intensities, were those of 25, 24, 22, 20, 14 and 13 kDa bands, which represent 39.71% of the total SP proteins. Significant differences ($p<0.05$) between breeds were found in several bands of approximate molecular weight 43 kDa (P43), 25 kDa (P25), 22 kDa (P22) and 20 kDa (P20) (Fig. 1). No significant difference ($p>0.05$) was observed between rams within each breed for any protein band.

In addition, some SP protein bands were significantly correlated with sperm quality parameters. We found a positive correlation between the 13 kDa band and both BT ($r=0.60164$; $p=0.0031$) and CT ($r=0.65083$; $p=0.0010$) in Romney Marsh.

Furthermore, the possible correlation between the antioxidant enzyme activities and the concentrations of the protein bands identified in each breed was evaluated. GR correlated inversely with at least one protein band in each race: Creole (P20, $p=0.011$; $r=-0.504$), Romney Marsh (P20, $p=0.013$; $r=-0.643$) and Hampshire (P13, $p=.038$; $r=-0.393$). Likewise, GPx correlated with the P13 band (13 kDa, $p=0.030$; $r=-0.411$) in the Hampshire breed.

Discussion

Although information on the semen characteristics of rams is widely available, little is known about the semen of ovine breeds adapted to tropical conditions. This study is focused on three sheep breeds during the rainy season in high altitude tropical conditions in Colombia.

The reproductive capacity and seminal quality of rams may be affected by several factors such as breed (Kasimanickam *et al.*, 2007), seasonality (Ibrahim, 1997; Avdi *et al.*, 2004), photoperiod (Pérez & Mateos, 1996), nutrition and environmental variables (Galina *et al.*, 1996). Our results showed that, although the semen characteristics of the three breeds were considered satisfactory, Creole and Hampshire breeds had a higher sperm quality compared with Romney Marsh. The ejaculate volume of Hampshire was higher than that of Creole, which had the highest sperm concentration. A possible physiological reason for the higher ejaculate volume rather than concentration in Hampshire is due to the volume of SP in the ejaculate (Ibrahim, 1997). It has been reported that the amount of SP surrounding each spermatozoon in an ejaculate varies depending on the sperm concentration in ram (Gundogan *et al.*, 2010) and boar (Kommisrud *et al.*, 2002). Furthermore, it is well known that, in mammalian species, SP and its components play essential roles in the regulation of sperm functionality (Rodríguez-Martínez *et al.*, 2011).

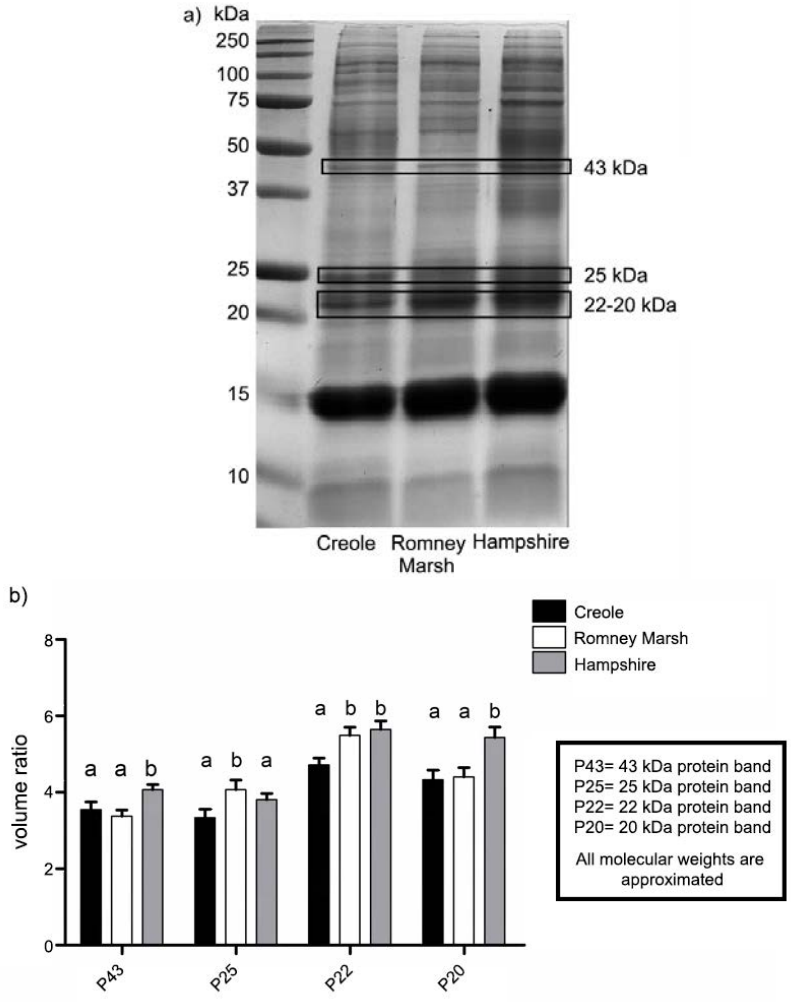

Figure 1. SDS-PAGE analysis of seminal plasma (SP) obtained from ram semen samples of Creole, Romney Marsh and Hampshire males during the rainfall season. (a) SP proteins separated by SDS-PAGE. Image analysis by Quantity One revealed significant differences between breeds in four protein bands (identified by a box and the respective approx. molecular weight). (b) Difference in the concentration (peak intensity) of the four protein bands (P43, P25, P22 and P20). a,b: significant difference (p<0.05) between breeds. Each experiment was performed 12 times, and presented data are means ± SEM of relative intensity units.

Improve motility characteristics (El-Hajj Ghaoui *et al.*, 2007), increase the sperm resistance to stress (Barrios *et al.*, 2000, 2005; Muiño-Blanco *et al.*, 2008; Mendoza *et al.*, 2013) and to aging during storage (Gundogan *et al.*, 2010), and reduce the deleterious effects of cryopreservation (El-Hajj Ghaoui *et al.*, 2007; Leahy *et al.*, 2011). Therefore, we could suggest that a higher volume of SP in the ejaculate might be one of the reasons for the better sperm quality of the Hampshire breed.

Regarding the kinematic parameters, our data showed that the differences between breeds were generally not significant, similarly to what has been reported for the kinematic values of Suffolk and Katahdin rams (Kasimanickam *et al.*, 2007). Although the ALH (amplitude of lateral head displacement) was significantly higher in the Creole breed than in Romney Marsh and Hampshire, no difference in LIN was found. It is worth noting that Hamilton Thorne instruments use ALH_{max} for kinematic analysis (Mortimer, 2000). Therefore, based on the criteria defined by Mortimer & Maxwell (1999) for hypermotility of ram spermatozoa ($ALH_{max} \geq 9$ μm, LIN 30 %), the ALH values were lower and the LIN values were higher in the three breeds than the established threshold values, which indicates that these spermatozoa do not have a hyperactivated movement.

Comparison among rams within breeds revealed no significant differences in sperm quality in Creole, while Romney Marsh and Hampshire breeds presented significant differences among males. These results would indicate that the introduced breeds would be less adapted to the environmental conditions.

Concerning morphology, the Romney Marsh semen had the lowest sperm rate with normal characteristics; additionally, a significantly higher value of bent tail and distal cytoplasmic droplet was found. This might be related to the presence of immature cells in the ejaculate (Belleannée *et al.*, 2011), which is indicative of oxidative stress (Marti *et al.*, 2007) and the generation of free radicals, as already reported in human semen (Aitken *et al.*, 1998). Specifically, a positive correlation between GR activity and the sperm percentages with bent and coiled tail was observed in Romney Marsh breed. Likewise, a negative correlation between GR and ALH (head lateral amplitude) and VAP (path velocity) was observed in the Creole breed. The fact that excessive antioxidant activity may be detrimental to sperm functions (Maxwell & Stojanov, 1996; Calamera *et al.*, 2003) must be taken into account. The GR enzyme is part of the GR/GPx system and its substrate (GSH), which functions as a defence system against the production of hydrogen peroxide (Alvarez & Storey, 1989; Storey *et al.*, 1998). The action of this system implies a balance between the production of ROS and scavengers (Câmara *et al.*, 2011), which involves preserving the sperm metabolic activity (De Lamirande & Gagnon, 1995). This toxicity would be related to the action of the antioxidant enzymes, given that high levels of GSH cause an early decrease in motility and kinetics parameters in ovine sperm cooled at 5 °C (Câmara *et al.*, 2011) and can affect cell homeostasis (Sikka, 1996). This would be the explanation for the negative correlation between GR activity and the VAP and ALH variables in the Creole breed. Our findings would indicate that GR may be utilized to counter the possible oxidative damage due to spermatozoa with cytoplasmic droplet retention (Kasimanickam *et al.*, 2006).

Several studies have shown a positive relationship between sperm quality parameters and ram seminal plasma protein levels (Taha *et al.*, 2000; Almadaly *et al.*, 2016), and that the semen collection procedure has no significant effect on protein concentration (Marco-Jiménez *et al.*, 2008; Ledesma *et al.*, 2014) although differential protein profile was observed between artificial vagina and electroejaculation (Ledesma *et al.*, 2014). A correlation between seminal plasma proteins and male fertility has been widely shown (Almadaly *et al.*, 2016), and although information regarding breed comparisons is scarce, differences between hair and wool breeds have not been associated with fertility (Taha *et al.*, 2000; Marco-Jiménez *et al.*, 2008). The SP total protein concentration found in the three breeds analysed here was higher than that described for Rasa Aragonesa rams in breeding season (Marti *et al.*, 2007), and lower than that of Corriedale in the southern hemisphere (synthetic breed created in New Zealand) (Ledesma *et al.*, 2014). These differences would suggest that the SP total protein levels of local breeds are not usually very high, as already described for Awassi and Barki Egyptian breeds (Taha *et al.*, 2000). Environmental and seasonal factors can affect both ram physiology (Marai *et al.*, 2007) and SP total protein concentration (Smith *et al.*, 1999) and profile (Cardozo *et al.*, 2006).

It has been shown that SP proteins are adsorbed onto the mammalian sperm surface and modify its properties (Desnoyers & Manjunath, 1992; Manjunath & Thérien, 2002), stabilize the plasma membrane and increase the sperm fertilizing potential (Mendoza *et al.*, 2013; Luna *et al.*, 2015). Furthermore, it has been suggested that some of the coating proteins are essential for maintaining the stability of the membrane up to the process of capacitation (decapacitating factors) (Desnoyers & Manjunath, 1992; Manjunath *et al.*, 2002). However, the role of SP in ram sperm function still remains controversial (Leahy & de Graaf, 2012) as both inhibitory (de Graaf *et al.*, 2007; Leahy, *et al.*, 2010b) and stimulating (Barrios *et al.*, 2000; Pérez-Pé *et al.*, 2002) effects have been found. Additionally, specific protein fractions have been isolated from ram SP and identified as responsible for preventing (Barrios *et al.*, 2005; Cardozo *et al.*, 2008; Rickard *et al.*, 2015) and repairing (Barrios *et al.*, 2000; Bernardini *et al.*, 2011) cold-shock damage. These specific fractions (Barrios *et al.*, 2005) have a similar protein profile to those identified by other authors in ram SP of different breeds (Bergeron *et al.*, 2005; Jobim *et al.*, 2005; Bernardini *et al.*, 2011; Soleilhavoup *et al.*, 2014), and are named RSVP-14, RSVP-20 and RSVP-22 (Barrios *et al.*, 2005; Fernandez-Juan, 2006; Cardozo *et al.*, 2008) given that they are exclusively synthesized by the seminal vesicles. These proteins belong to the binding of sperm protein (BSP's) family (Manjunath *et al.*, 2008; Serrano *et al.*, 2015) due to the presence of two fibronectin type II domains (FN2 domain) which confer many binding properties on them (Desnoyers & Manjunath, 1992). BSP's play an important function in the stabilization of the sperm membrane as they bind to the sperm surface during ejaculation (Manjunath & Thérien, 2002). The gene codification for these BSP proteins is BSP5a (25 kDa band protein), BSP5c (RSVP-22), BSP5b (RSVP-20) and BSP1 (RSVP-14) (Serrano *et al.*, 2015). RSVP-20 and -22 function as decapacitating factors binding to the membrane domains in a first step (Barrios *et al.*,

2005; Cardozo *et al.*, 2008). Both proteins are released from the membrane once capacitation is initiated, contributing to the process in a similar way to RSVP-14 (Barrios *et al.*, 2005). In this study, we found significant differences between breeds in the content of several SP proteins. Three protein bands of approximately 25, 22 and 20 kDa that we named P25, P22 and P20, respectively presented the lowest levels in Creole breed. It is likely that P22 and P20 could match with the RSVP's described in ovine, and thus they may play a role in membrane stabilization. Likewise, the 43 kDa protein band (P43) showed the lowest concentration in Romney Marsh, while Hampshire showed the highest value. One might speculate that P43 match with clusterin, which has been described as a glycoprotein in mammalian SP (Blaschuk *et al.*, 1983). This is the most plentiful glycoprotein found in the testis network and has the characteristics of an acidic protein (Jobim *et al.*, 2005). In denaturing conditions, clusterin has an approximate molecular weight of 40-43 kDa. Its function in the male reproductive tract varies depending on its tertiary structure and posttranslational modifications (glycosilations/sulphatations-state) (Ibrahim *et al.*, 1999), origin (testicular or epididymis) (Tung & Fritz, 1985; O'Bryan *et al.*, 1990), and location (fluid or cell) (Sensibar *et al.*, 1993; Ibrahim *et al.*, 1999). Clusterin has been reported to be involved in spermatogenesis (Fritz *et al.*, 1983; Sylvester *et al.*, 1991), protection of spermatozoa against the complement attack from epididymal and uterine secretions (O'Bryan *et al.*, 1990; Ibrahim *et al.*, 1999), or agglutination of abnormal spermatozoa (O'Bryan *et al.*, 1994), and acting like an extracellular chaperone against heat shock on membrane molecules (Humphreys *et al.*, 1999) and against apoptosis (Bailey *et al.*, 2002). However, clusterin has been identified in morphologically abnormal human (Muciaccia *et al.*, 2012) and bull (Ibrahim *et al.*, 2000; Shojaei Saadi *et al.*, 2013) spermatozoa, while it has been associated with all ram spermatozoa in fresh ejaculates, showing different staining patterns (Ibrahim *et al.*, 2001). SP clusterin (sCLU) negatively correlates with human sperm abnormal morphology, protamine deficiency, and DNA fragmentation as lower sCLU concentrations have been detected in the SP of infertile (Salehi *et al.*, 2013) and vasectomized (O'Bryan *et al.*, 1990) men. Therefore, the low relative presence of this potential clusterin in the SP of Romney Marsh is consistent with a lower sperm quality and a high number of spermatozoa with membrane alterations. Therefore, differences in the presence of these proteins in the SP would be related to differences in sperm quality in the three studied breeds and, possibly, to other molecular events related to fertilization in which these proteins are involved.

In conclusion, our findings show significant differences in the sperm quality of fresh ejaculates from Creole, Romney Marsh and Hampshire breeds. The antioxidant enzymatic defence system in SP is related with semen quality and can affect kinematic sperm parameters in the Creole and Romney Marsh breeds. Certain proteins that might play an important role in sperm physiology and quality have also been identified. These results can be useful for improving the identification of SP proteins able to act as biomarkers of fertilizing potential and the semen-based reproductive technologies in mammalian species.

Acknowledgements

Special thanks to Dr. Cesar Terán for the provision of the environmental information for the development of the study.

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