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SEMEN EVALUATION OF TWO SELECTED LINES OF RABBIT BUCKS

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Abstract: Twenty rabbit bucks of 9 months of age were used to evaluate semen quality of two lines of New Zealand rabbit bucks selected for litter size at weaning (A line) and growth rate from weaning to slaughter (R line). The morphological semen characteristics indicated that the A line spermatozoa had greater acrosome integrity (+3.6 percentage units; $P<0.01$) and smaller sperm head size (for example, −1.46 µm2 for sperm head area) than in the R line. Seminal functional traits were also significantly higher for the A line (+13.4 percentage units for viability, +10.6 percentage units for hypo-osmotic swelling test (HOST) and +3.3 g/L for seminal plasma protein. However, no differences were detected between lines for motility parameters and seminal plasma protein electrophoretic profiles. Both lines had the same twelve bands with the following molecular weights to the nearest 1 kD: 124, 117, 99, 86, 75, 62, 40, 32, 21, 19, 10 and 6 kD. A relationship (r=0.308 for A line and 0.359 for R line; $P<0.01$) was found between the integrity of the plasmatic membrane (viability rate) and tail membrane (HOST) of the spermatozoa in the A line, but not in the R line, which had greater sperm head size. There was also a significant positive correlation coefficient between sperm concentration and either viability or some kinetic traits (r=0.567 and 0.575 for VCL, r=0.584 and 0.561 for VSL and r=0.588 and 0.588 for VAP, for A and R lines, respectively; $P<0.001$). We concluded that the A line seems to have better semen characteristics than the R line. We also found an interesting correlation among the seminal morphological, functional and kinetic traits, which could possibly be used to facilitate semen evaluation.

Key words: rabbit, semen profile, selection criteria, hypo-osmotic swelling test, seminal plasma protein.

INTRODUCTION

The control of rabbit reproduction has experienced great changes in the last decade, mainly as a consequence of the development of new techniques such as commercially applicable artificial insemination (AI). AI is widely used as a routine procedure in European rabbit farms (Alabiso *et al.*, 1996; Alvariño, 2000; Lavara *et al.*, 2005). This technique makes possible new production methods such as "cycled production", in which all the does of a batch are inseminated on the same day (Theau-Clément, 2000). Male rabbits are undoubtedly the basic element in reproductive and productive success, but have not received the attention they deserve. This is particularly true if we consider that one single male can affect the fertility and prolificacy of about one hundred females when AI is performed routinely in rabbit farms (Alvariño, 2000). In addition, the male genetic value should be considered because the maternal or growth traits have a direct effect on the productive traits of the offspring.

The true fertilizing potential of an ejaculate can only be determined after insemination, but this practice is time-consuming and expensive. Laboratory assays have therefore been developed to estimate seminal quality *in vitro* and correlate seminal quality parameters with fertility *in vivo*. In regard to this, finding

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a laboratory test that could reliably predict the fertility of a semen sample would be highly desirable (Rodríguez-Martínez, 2003; Carluccio *et al.*, 2004). Seminal morphological traits, such as the percentage of abnormal sperm or acrosome damage, have been correlated with fertility in rabbits (Courtens *et al.*, 1994; Lavara *et al.*, 2005). Recently, morphometric parameters (length, width, area and perimeter) of sperm heads using ad hoc software (assisted sperm morphometry analysis, ASMA) have offered new opportunities to identify fertility predictors of rabbit sperm (Marco-Jiménez *et al.*, 2005). Mocé *et al.* (2004), Daader and Seleem (2005) and Safaa *et al.* (2008) have indicated that the hypo-osmotic swelling test (HOST) is more reliable in assessing the outcome of *in vitro* fertilization in rabbits than other semen parameters. Viudes de Castro *et al.* (2004) have suggested that the seminal plasma protein profile could be used to differentiate between rabbit breeds. Estimated motility, or kinetics parameters measured by using Computer-Assisted Sperm Analysis (CASA), has also been correlated with fertility in rabbits (Farrell *et al.*, 1993; Brun *et al.*, 2002).

The aim of this study is to characterize the semen parameters of two rabbit lines selected by two different criteria (litter size at weaning and growth rate from weaning to slaughter) by determining morphological, functional, and kinetic sperm parameters, as well as seminal plasma protein composition and electrophoretic profiles in order to further verify the effect of these lines on cryopreservation and fertility rate.

MATERIALS AND METHODS

Animals

The experiment was performed between April and June 2004. Ten males from two different rabbit lines were selected randomly at 9 months of age from those raised at the Animal Breeding farm of the Department of Animal Science of the Polytechnic University of Valencia. The first line has been selected during 33 generations for litter size at weaning (Maternal line, A line) and the second has been selected during 24 generations for growth rate from weaning to slaughter (Growth line, R line) in accordance with the selection methodologies described by Estany *et al.* (1989 and 1992, respectively). Animals were housed under a photoperiod of 16L:8D, in individual cages, fed a commercial diet *ad libitum* (17.5% of crude protein, 3.5% of ether extract, 16.7% of crude fiber on DM basis) which covered the nutritional requirements of rabbits' mature phase in accordance with De Blas and Mateos (1998) recommendations. All animals were kept under the same managerial and hygienic conditions. Fresh tap water was freely available at all times from stainless steel nipples in each cage.

Semen collection and evaluation

Two ejaculates per male were collected weekly by artificial vagina, with an interval of 15-30 min between successive ejaculates, as described by Mocé *et al.* (2000). The following measurements were taken from fresh spermatozoa:

Morphological examination: The volume (mL) of each ejaculate was measured in a graduated conical tube. Both ejaculates were then pooled and diluted (1:25) in a fixation solution of Phosphate Buffer Saline (PBS) glutaraldehyde (2%) to estimate the concentration (spermatozoa million/mL) by Thoma–Zeiss cell counting chamber. The percentage of abnormal spermatozoa or spermatozoa with normal apical ridge (NAR, percentage of acrosome integrity) were measured in the pooled spermatozoa sample fixed in a PBS glutaraldehyde solution, at a magnification of 500× by using a microscope with differential interference contrast (Nomarski contrast) and phase contrast. Sperm morphometry was determined from the fixed pooled sample in a phase microscope at 400× using the Sperm-Class Analyzer software (Microptic, Barcelona, Spain) to detect the head of the spermatozoa and to measure automatically the morphometric parameters (length (μm), width (μm), area (μm^2), perimeter (μm) and Fun1 (length/width)) of 100 spermatozoa from each sample.

Viability and HOST percentages were measured in a 1:25 dilution in a HOST solution (75 mOsm) according to Mocé *et al.* (2004) with Hoechst stain (1 µg/100 mL; H33258, Sigma Aldrich, Madrid, Spain) at 25-30 ºC for 15 min. Viability was represented by the percentage of non-stained head sperm/total sperms and the HOST percentage was calculated as the percentage of non-stained spermatozoa with swollen coiled tails/total spermatozoa.

Seminal plasma protein profile: The rest of the ejaculates were centrifuged at 4,500 *g* for 20 min at 5 ºC to obtain the seminal plasma. The supernatant was then re-centrifuged in a micro-centrifuge at 10,000 g for 5 min and thereafter stored at −20 ºC until the seminal plasma protein and protein electrophoretic profile analyses. Total seminal plasma proteins (g/L) were measured by the Biuret method and separated in a poliacrilamide gradient electrophoresis (4-15%), stained with a solution 0.1% of Comassic R-250 in methanol, acetic acid and distilled water (5:1:5). The protein profile was analyzed by Genetools software (Syngene, IZASA, Spain).

Motility percentage and kinetic traits were assessed using the CASA system, which has a specific set-up for rabbit sperm evaluation (Viudes de Castro *et al.*, 1999 and Castellini *et al.*, 2002). The percentages of total motile sperm cells, curvilinear velocity (VCL, µm/s; the average velocity measured over the actual point to point track followed by the cell), straight-line velocity (VSL, µm/s; the average velocity measured in a straight line from the beginning to the end of the track), average path velocity (VAP, µm/s; the average velocity of the smoothed cell path), linearity index (LIN, %; the average value of the ratio VSL/VCL), straightness (STR, %; the average value of the VSL/VAP ratio), amplitude of lateral head displacement (ALH, µm; the mean width of the head oscillation as the sperm cells swim) and beat cross-frequency (BCF, Hz; the frequency of sperm head crossing the average path in either direction) were evaluated. The software system settings were as follows: frame at frame rate (s), 16–25; minimum contrast, 10; minimum data point, 7; low VAP (mm/s), 15; medium VAP (mm/s), 30; threshold straightness (%), 80. To measure motility rate, a 1:25 dilution was made with a tris–citric acid–glucose extender (pH: 6.8, 300 mOsm). Two drops of 10 µl were placed on a slide and covered with a cover slip (20×20mm). Motility from at least four fields was examined at 37ºC under a microscope with phase contrast optics, at 100× and connected to a monitor through a camera.

Statistical analysis

The effect of the two selection lines on morphological, functional, kinetic sperm traits and seminal protein concentration was analyzed by using a General Linear Model (GLM) procedure, while Pearson's correlation coefficients among viability, HOST, sperm concentration, sperm head perimeter, VCL, VSL, VAP, LIN and ALH were calculated by (CORR) procedure of the SAS (1997). Percentage values were transformed to arcsine before being statistically analyzed. The results are presented in tables as least square means (LSM)±(standard error of means). All differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Morphological traits

No differences were detected between the A and R lines in any morphological traits except for acrosome integrity and sperm morphometry (Table 1). In fact, no differences were observed for total ejaculate volume, sperm concentration and percentage of sperm abnormality between the lines. The overall means of these parameters were similar to the previous studies for ejaculate volume (Vicente *et al.*, 2000) in the R line, for spermatozoa concentration (Pascual *et al.*, 2004) in the R line during the spring season and for sperm abnormalities (García-Tomás *et al.*, 2006) in two rabbit sire lines and their reciprocal crosses. However, Theau-Clément *et al.* (2007) reported that the selection for high 63-d body weight of rabbit

Table 1: LSM±SEM of seminal morphological traits for two different rabbit lines (number of observations between brackets).

Trait	A line	R line	*P*-value[1]
1st ejaculate volume, mL	0.51±0.031 (85)	0.64±0.041 (55)	*
2nd ejaculate volume, mL	0.53±0.031 (85)	0.39±0.034 (65)	***
Total ejaculates volume, mL	1.04±0.052 (80)	1.02±0.064 (52)	NS
Concentration, N×10^6 sperm/mL	232±15.3 (80)	220±17.4 (59)	NS
Sperm abnormality, %	20.7±1.54 (84)	18.4±1.73 (60)	NS
Acrosome integrity, %	93.6±0.94 (84)	90.0±1.01 (60)	**
Morphometry:			
Length, µm	8.12±0.007 (7662)	8.39±0.008 (5323)	***
Width, µm	3.97±0.004 (7662)	4.13±0.005 (5323)	***
Area, µm^2	26.75±0.033 (7662)	28.21±0.040 (5323)	***
Perimeter, µm	21.56±0.019 (7662)	22.23±0.023 (5323)	***
Fun1[2]	2.063±0.0027 (7662)	2.051±0.0033 (5323)	**

[1] NS = Not significant (P>0.05); * (P<0.05); ** (P<0.01); *** (P<0.001).
[2] Fun1 = Length/width.

bucks reduced semen volume and increased sperm concentration without affecting the fertilizing capacity of the rabbit semen.

In the current research, a significant increase (P<0.01) was detected in acrosome integrity in the A *vs*. R line (93.6% and 90.0%, respectively). Roca *et al.* (2005) registered similar values for acrosome integrity (90.0%) in hybrid male rabbits. However, Lavara *et al.* (2005) observed lower acrosome integrity (88.2%) and sperm abnormality (10.1%) in R line bucks than in the present study. Similar results for ejaculate volume (0.63 mL), sperm concentration (171 million sperms/mL) and percentage of both abnormal sperms and NAR (19.9 and 84.6%, respectively) were reported by Lavara *et al.* (2007a) who examined a total of 1022 ejaculates from 85 R line rabbit bucks. In contrast, values for sperm-cell concentration were lower than those reported previously by the same authors. These differences could be explained by genetic, age and environmental factors and also by the different evaluation criteria, sample size, and semen processing methodologies applied.

In genral sperm morphometry parameters were significantly higher (P<0.001) for the R line (8.39, 4.13, 28.21 and 22.23) than the A line (8.12, 3.97, 26.75 and 21.56 for length (µm), width (µm), area (µm^2) and perimeter (µm), respectively). Marco-Jiménez *et al.* (2005), applying a multivariate method of ASMA morphometric parameters, classified the bucks into two groups; bucks with sperms having small head size showing lower fertility (45.0%) than bucks with sperms having large head size (77.9%). These findings indicate that the morphometric parameters of rabbit sperm could be important in predicting bucks' fertility

since they remain fairly constant with time. Each buck has individual characteristics, which means that the pathological and/or environmental factors that affect sperm size can be studied.

Functional measurements

In general, seminal functional traits were significantly higher in the A than in R line males, except for the sperm motility percentage which was not affected (Table 2). Similar data for viability were recorded previously by Nagy *et al.* (2002), whereas García-Tomás *et al.* (2006) found a higher value (81.1%) than that observed in the current research (56.1%) in two sire lines and their reciprocal crosses. These differences may be due to the bucks' age and/or the different methodologies used. In fact, in this study, the sperm plasmatic membrane was evaluated immediately after ejaculation using a fluorescent stain, whereas García-Tomás *et al.* (2006) studied the stored ejaculates using a vital nigrosin-eosin stain.

HOST is a simple and accessible method that provides useful information on the functionality of plasma membranes and can be used as an additional test for routine semen analysis (Neild *et al.*, 1999; 2000). The percentages of sperms with swollen head and coiled trails in response to HOST are in good agreement with those observed in white New Zealand rabbit bucks during the winter season (Daader and Seleem, 2005; Safaa *et al.*, 2008).

In the current research, total seminal plasma protein was higher in the A line than in the R line (22.1 *vs*. 18.8 g/L, respectively, Table 2). These findings were lower than those reported by Viudes de Castro *et al.* (2004) for these lines in the winter season (30.5 *vs*. 36.6 g/L, respectively). These differences might be explained by the different environmental conditions (spring in the current experiment *vs*. winter in the previous ones), which could affect the plasma protein composition.

No differences in total sperm motility were observed between male lines, reaching an overall mean of 66.4% (Table 2). This value is consistent with those found by Roca *et al.* (2000 and 2005) in hybrid rabbits, Brun *et al.* (2002) in purebred and crossbred rabbit bucks selected for litter size, and Lavara *et al.* (2007a and b) in R line selected bucks. On the other hand, Pascual *et al.* (2004) reported lower values in young (6-9 months) R line bucks during the spring season for the total sperm motility percentage (ranging from 30.6 to 47.0%) than those observed in the current research. This discrepancy may be ascribed to the different ages of the bucks. In a previous study (Lavara *et al.* 2005), sperm motility of R line bucks was 80.5%, much higher than that found in the present research. These contrasting data could have arisen from a bias in choosing the bucks for insemination since, to avoid fertility problems, only the best ejaculate samples were examined. Also, Theau-Clément *et al.* (2007) reported that the selection of rabbit bucks for high 63-d body weight impaired sperm motility.

Table 2: LSM±SEM of seminal functional traits for two different rabbit lines (number of observations between brackets).

Trait	A line	R line	*P*-value[1]
Viability, %	65.5±2.6 (87)	42.1±3.0 (58)	***
HOST[2], %	77.9±2.3 (87)	67.3±2.9 (55)	**
Seminal plasma total protein, g/L	22.1±0.6 (67)	18.8±0.8 (43)	**
Total sperm motility, %	65.4±3.5 (41)	67.5±3.6 (39)	NS

[1] NS = Not significant ($P>0.10$); ** ($P<0.01$); *** ($P<0.01$).
[2] HOST = Hypo osmotic swelling test using 75 mOsm solution.

Table 3: LSM±SEM of seminal kinetic traits[1] for two different rabbit lines.

Trait	A line	R line	*P*-value[2]
No. of observations	41	39	
VCL, µm/sec	71.4±4.7	76.9±4.8	NS
VSL, µm/sec	29.8±2.9	33.3±2.4	NS
VAP, µm/sec	40.7±2.9	44.3±3.0	NS
LIN, %	49.2±1.4	51.7±1.4	NS
STR, %	68.5±1.2	71.3±1.2	†
ALH, µm	2.2±0.1	2.5±0.1	NS
BCF, Hz	6.9±0.3	7.7±0.3	*

[1] VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity index, STR: straightness, ALH: amplitude of lateral head displacement, BCF: beat cross frequency.
[2] NS = Not significant ($P>0.10$); † ($P<0.10$); * ($P<0.05$).

Kinetic parameters

Sperm kinetic traits for R line were greater than those for the A line, but none of the differences were significant except for BCF (Table 3). Viudes de Castro *et al.* (2004) achieved similar results and detected no differences between A and R lines in VCL, LIN and ALH. The values of the kinetic parameters obtained in the current study are similar to those obtained by Lavara *et al.* (2007a and b) for R line rabbit bucks. Moreover, in the current study the overall LIN mean was 50.4%, a value between those reported by Farrell *et al.* (1993) and Theau-Clément *et al.* (1996). However, according to Castellini and Lattaioli (1999), VAP was 59.8-73.2 µm/sec and LIN 56.7-60.6% in rabbit semen at different sperm concentrations with 60.0 to 82.5% motility. Pascual *et al.* (2004) also reported lower values for kinetic traits in the R line during the spring season, except for VSL and BCF, which were higher than those recorded in the current study. Theau-Clément *et al.* (2007) reported that bucks selected for high body weight at 63 days of age produced semen with lower VAP and ALH than non selected bucks whereas, both lines produced semen with similar VSL and LIN. These differences could be explained by individual variations in the bucks used, the different CASA system (Jasko *et al.*, 1990) and analysis conditions, such as medium, times, etc.

Seminal plasma protein profile

Gels for seminal plasma protein electrophoretic profile show that there are twelve clear bands for both selection lines, having the following molecular weights to the nearest 1 kD: 124, 117, 99, 86, 75, 62, 40, 32, 21, 19, 10, and 6 kD. No differences in the protein bands from individual ejaculates were observed in the current study, whereas Viudes de Castro *et al.* (2004) detected two different bands in the protein profile between A and R lines. These bands of seminal plasma protein were around 41 kD for the A line, and around 13 kD for the R line. These difference could be explained by the different environmental conditions (the current trial was carried out in spring while, Viudes de Castro *et al.* (2004) made their study in winter), which could have affected plasma protein composition and profiles. Unpublished data on individual ejaculates from these lines indicated that there was a variable seasonal effect on the relative volume of seminal protein bands, which confirms our hypothesis. Further studies will be needed to focus on the correlation between the seminal plasma protein electrophoretic profile and seminal morphological, functional and kinetic traits.

Correlation coefficient

There is a positive correlation coefficient (r=0.388; $P<0.001$) between sperm viability and HOST in the A line, but not in the R line (Table 4). Moreover, a positive correlation coefficient was detected between

Table 4: Correlation coefficients between some of sperm parameters for A line (up diagonal) and R line (down diagonal) rabbit bucks (number of observations between brackets).

	Viability	HOST	CONC	PERI	VCL	VSL	VAP	LIN	ALH
		***	**	NS	NS	NS	NS	NS	NS
Viability		0.388 (87)	0.308 (80)	0.065 (82)	0.206 (41)	0.089 (41)	0.140 (41)	-0.101 (41)	0.040 (41)
	NS		NS	NS	NS	NS	NS	†	NS
HOST	0.079 (55)		0.108 (80)	-0.103 (82)	0.127 (41)	0.182 (41)	0.170 (41)	0.306 (41)	-0.013 (41)
	**	NS		NS	***	***	***	NS	***
CONC	0.359 (56)	-0.161 (53)		-0.026 (79)	0.567 (39)	0.584 (39)	0.588 (39)	-0.204 (39)	0.534 (39)
	NS	NS	NS		*	*	*	†	NS
PERI	0.017 (56)	0.080 (53)	0.020 (59)		-0.386 (38)	-0.355 (38)	-0.378 (38)	-0.283 (38)	-0.264 (38)
	NS	NS	***	†		***	***	NS	***
VCL	0.219 (36)	-0.261 (33)	0.575 (37)	-0.284 (37)		0.885 (41)	0.959 (41)	-0.023 (41)	0.892 (41)
	NS	NS	***	NS	***		***	†	***
VSL	0.229 (36)	-0.112 (33)	0.561 (37)	-0.224 (37)	0.892 (39)		0.978 (41)	0.270 (41)	0.782 (41)
	NS	NS	***	NS	***	***		NS	***
VAP	0.220 (36)	-0.172 (33)	0.588 (37)	-0.263 (37)	0.953 (39)	0.985 (39)		0.155 (41)	0.844 (41)
	NS	*	NS	NS	NS	NS	NS		NS
LIN	-0.002 (36)	0.401 (33)	0.105 (37)	-0.010 (37)	-0.098 (39)	0.191 (39)	0.091 (39)		-0.216 (41)
	NS	†	***	NS	***	***	***	NS	
ALH	0.180 (36)	-0.337 (33)	0.622 (37)	-0.231 (37)	0.903 (39)	0.762 (39)	0.835 (39)	-0.231 (39)	

Viability: sperm viability (%), HOST: hypo osmotic swelling test using 75 $mOsmKg^{-1}$ osmotic solution, CONC: concentration ($N\times10^6$ sperm/mL), PERI: sperm head perimeter (µm), VCL: curvilinear velocity (µm/sec), VSL: straight line velocity (µm/sec), VAP: average path velocity (µm/sec), LIN: linearity index (%), ALH: amplitude of lateral head displacement (µm).
NS: Not significant ($P>0.10$); † ($P>0.05$); * ($P<0.05$); ** ($P<0.01$); *** ($P<0.001$).

HOST and LIN (r=0.306 in A line; $P>0.05$ and r=0.401 in R line; $P<0.05$). In both lines, a significant positive correlation is evident between sperm concentration and either viability or kinetic traits (VCL, VSL, VAP and ALH), suggesting a relationships among seminal morphological, functional and kinetic traits. These findings are in agreement with those of Brun *et al.* (2002), who observed a positive correlation coefficient (r=0.42; $P<0.05$) between sperm concentration and motility of purebred and crossbred rabbit bucks selected for litter size.

In conclusion, A line bucks have better semen characteristics than those of the R line. Also, ASMA morphometric parameters prove useful in characterizing both sperm sub-population and individual bucks, whereas HOST provides a good indication of rabbit buck reproductive ability. There was also an interesting correlation among seminal morphological, functional and CASA kinetic traits, which might prove to be useful in facilitating semen evaluation. However, more studies are needed to focus on the relationships between semen characteristics and different nutritional factors, seasons and sperm conservation techniques.

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