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Male Fertility in Natural Populations of Red Deer Is Determined by Sperm Velocity and the Proportion of Normal Spermatozoa[1]

Aurelio F. Malo,[3] J. Julián Garde,[4] Ana J. Soler,[4] Andrés J. García,[4] Montserrat Gomendio,[3] and Eduardo R.S. Roldan[2,3]

Reproductive Ecology and Biology Group,[3] Museo Nacional de Ciencias Naturales (CSIC), 28006-Madrid, Spain
Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCLM),[4] 02071-Albacete, Spain

ABSTRACT

Male reproductive success is determined by the ability of males to gain sexual access to females and by their ability to fertilize ova. Among polygynous mammals, males differ markedly in their reproductive success, and a great deal of effort has been made to understand how selective forces have shaped traits that enhance male competitiveness both before and after copulation (i.e., sperm competition). However, the possibility that males also may differ in their fertility has been ignored under the assumption that male infertility is rare in natural populations because selection against it is likely to be strong. In the present study, we examined which semen traits correlate with male fertility in natural populations of Iberian red deer (*Cervus elaphus hispanicus*). We found no trade-offs between semen traits. Our analyses revealed strong associations between sperm production and sperm swimming velocity, sperm motility and proportion of morphologically normal spermatozoa, and sperm viability and acrosome integrity. These last two variables had the lowest coefficients of variation, suggesting that these traits have stabilized at high values and are unlikely to be related to fitness. In a fertility trial, our results show a large degree of variation in male fertility, and differences in fertility were determined mainly by sperm swimming velocity and by the proportion of morphologically normal sperm. We conclude that male fertility varies substantially in natural populations of Iberian red deer and that, when sperm numbers are equal, it is determined mainly by sperm swimming velocity and sperm morphology.

acrosome reaction, gamete biology, male reproductive tract, sperm, sperm motility and transport

INTRODUCTION

In most species, male reproductive success shows a high degree of variation within populations, because males differ to a great extent in the number of ova that they fertilize during their lifetime [1]. Such variation between males reaches extreme values in polygynous mammals, where a few males in the population father the majority of offspring during each breeding season (for red deer, see [2]; for fur seals, see [3]). Male reproductive success is determined in part by the number of females to which they gain sexual access and in part by their ability to fertilize the available ova. Among mammals, competition between males to copulate with females is intense, leading to the evolution of traits that improve the chances to win agonistic encounters with other males, such as large body size or weapons [4]. In those species where sperm competition is prevalent, traits that enhance the ejaculate's competitiveness after copulation also have evolved, such as increased sperm numbers [5, 6].

A great deal of effort has been made to understand how selective forces have shaped behavioral, morphological, and physiological traits that enhance male competitiveness both before and after copulation [4–7]. Most of these studies, however, have ignored the possibility that males in natural populations also may differ in their fertility and that these differences may have a considerable influence on male reproductive success. In fact, it generally is assumed that male infertility is uncommon in natural populations, because it would be strongly selected against (for review, see [7, 8]). This assumption may apply to sterile males, because they would leave no descendants and, thus, would be at an evolutionary dead end. However, little attention has been paid to the fact that males may show varying degrees of fertility as a result of their genetic makeup (e.g., inbreeding [9–11]) or may be temporarily infertile because of environmental causes, such as food scarcity, stress, and pathogens (for review, see [12, 13]). The few field studies that have addressed this issue have shown that reduced male fertility or temporary male infertility may be more common among natural populations than previously thought [14–17]. However, the nature of the data obtained from natural populations often makes it difficult to disentangle the role played by male and female factors or an interaction between both, as in the case of genetic incompatibility [18].

Because male fertility has been dismissed as a significant component of male reproductive success, no efforts have been made to understand which semen traits determine male fertility in natural populations. In contrast, this issue has received much attention in two other contexts: livestock breeding, and treatment of human infertility. The economic benefits derived from maximizing the efficiency of livestock breeding have led to major efforts to identify which semen traits determine male fertility. Most of these studies have failed to link specific sperm traits to fertility, however, and the results have been rather contradictory or inconclusive [19–21]. On the other hand, treatment of human male infertility has led to the search for ejaculate traits that can explain reproductive failures and predict success in assisted conception. These studies have shown that in the subpop-

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[2]Correspondence: E.R.S. Roldan, Museo Nacional de Ciencias Naturales (CSIC), c/Jose Gutierrez Abascal 2, 28006-Madrid, Spain.
FAX: 34 91 564 5078; e-mail: roldane@mncn.csic.es

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TABLE 1. Reproductive parameters of Iberian red deer from natural populations.

	Mean ± SEM	Range	n
Total testes volume (ml)	65.78 ± 2.087	(18.9–191.4)	182
Sperm suspension volume (ml)	1.34 ± 0.023	(0.8–2.4)	188
Sperm concentration ($\times 10^6$ per ml)	1319.20 ± 67.110	(20–4840)	187
Total sperm number ($\times 10^6$)	1937.85 ± 128.124	(24–10 648)	187
% Motile sperm	62.11 ± 1.347	(24–10 648)	187
Quality of motility	2.77 ± 0.051	(0–4)	187
% Viable spermatozoa	90.10 ± 0.446	(62–100)	188
% Normal spermatozoa	77.02 ± 1.137	(12–97)	188
% Acrosome integrity	86.51 ± 0.605	(41–100)	186
% ARIC30	85.79 ± 0.793	(44–100)	188
VCL (μm/sec)	112.96 ± 1.494	(80–161)	133
VSL (μm/sec)	68.95 ± 1.467	(25.7–108.8)	133
VAP (μm/sec)	82.58 ± 1.532	(40.1–124.8)	133

ulation of infertile men who seek medical assistance, sperm concentration in the ejaculate, sperm motility, sperm morphology, and acrosomal status are some of the semen traits known to influence male fertility among patients [22, 23]. It is unknown, however, if these same traits would account for fertility differences among males in healthy populations.

The difficulty in identifying which semen traits determine male fertility may lie in the fact that efforts have concentrated in two areas that represent opposite extremes. Livestock breeders have been artificially selecting fertile males for many generations, which may have resulted in little variability between males, particularly regarding those traits linked more closely to fertility. On the other hand, clinical studies have been concerned mainly with the study of a subpopulation of males whose fertility is compromised. Semen traits responsible for such infertility may not be representative of fertility determinants in less-biased populations. Thus, the results may have little applicability to natural populations of mammals, which likely lie somewhere in between these two extremes.

In the present study, we examined which semen traits correlate with male fertility in natural populations of Iberian red deer. We evaluated several semen traits in a sample of 188 males, and we analyzed the relationships between these traits to test if trade-offs exist between different variables, the nature of the associations between different semen traits, and the coefficient of variation (CV) for each of them. We also carried out a fertility trial, inseminating sets of females with the semen of individual males to evaluate their fertility, together with an investigation of which semen traits are related more strongly to male fertility. Because the number of spermatozoa is an important determinant of fertility [24–27], all females were inseminated with the same number of spermatozoa to assess the importance of other semen variables. The results of these studies are presented here.

MATERIALS AND METHODS

Animals

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD223/1988, which conforms to European Union Regulation 86/609 and adheres to guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

The study sample included 188 Iberian red deer (*Cervus elaphus hispanicus*) stags culled during the mating season (October–December) in different wild populations from the south of Spain during 1999–2001. In this region, the reproductive season begins at the end of September and lasts for 3 mo [28–30]. Culls were undertaken following Spanish laws that, in turn, conform to European Union regulations. Measurements of body size were taken in the field. Both testes were removed (in the scrotum) and transported at 20–21°C to the laboratory. Time elapsed between animal death and sperm analyses ranged from 3 to 6 h, an adequate and reliable time interval for evaluating sperm parameters, because a decrease in the quality of sperm traits begins to take place 12 h after the death of a male [31].

Evaluation of Male Reproductive Parameters

A total of 13 reproductive variables were quantified. Testes and epididymides were removed from the scrotum, and diameters of left and right testes were measured with a caliper. Relative testes size was calculated by

TABLE 2. Results of the PCA analysis performed on 13 reproductive traits of Iberian red deer stags.[a]

	Factor score 1		Factor scores 2	
Reproductive variables	Factor loadings	*P*	Factor loadings	*P*
Relative testes size	0.476	<0.0001	−0.295	<0.001
Relative sperm suspension volume	0.659	<0.0001	−0.373	<0.0001
Sperm concentration	0.863	<0.0001	−0.229	<0.05
Relative sperm number	0.884	<0.0001	−0.245	<0.01
% Motile sperm	0.118	NS	0.753	<0.0001
Quality of motility	0.281	<0.0001	0.655	<0.0001
% Viable sperm	0.397	<0.0001	0.155	NS
% Normal sperm	0.017	NS	0.659	<0.0001
% Acrosome integrity	0.334	<0.001	0.250	<0.01
% ARIC30	0.414	<0.0001	−0.290	<0.001
VCL	0.392	<0.0001	0.230	<0.01
VSL	0.578	<0.0001	0.368	<0.0001
VAP	0.576	<0.0001	0.324	<0.001
Eigenvalue	3.544		2.236	
Variance	0.272		0.172	

[a] Factors were not rotated. NS, Not significant.

TABLE 3. Correlations of the 13 male reproductive traits analyzed.[a]

	Relative testes size	Relative sperm suspension volume	Sperm concentration	Relative sperm number	% Motile sperm	Quality of motility	% Viable sperm	% Normal sperm	% NAR	% ARIC30	VCL	VSL	VAP
Relative testis size		**0.43**	**0.34**	**0.42**	0.08	0.13	**0.26**	−0.10	**0.30**	**0.22**	0.05	0.10	0.10
Relative sperm suspension volume	***		**0.47**	**0.63**	−0.07	0.01	**0.23**	−0.09	**0.19**	**0.21**	−0.01	0.16	0.08
Sperm concentration	***	***		**0.95**	0.00	0.09	**0.22**	−0.17	0.12	**0.34**	0.16	**0.28**	**0.27**
Relative sperm number	***	***	***		0.02	0.11	**0.23**	−0.15	0.16	**0.31**	0.14	**0.29**	**0.27**
% Motile sperm	—	—	—	—		**0.82**	**0.29**	**0.27**	**0.25**	−0.10	−0.01	0.11	0.03
Quality of motility	—	—	—	—	***		**0.25**	**0.28**	**0.28**	−0.06	−0.10	0.18	0.04
% Viable sperm	**	*	*	*	**	**		0.02	**0.49**	0.14	0.16	0.07	0.12
% Normal sperm	—	—	—	—	***	***	—		**0.25**	−0.07	0.02	0.13	0.05
% NAR	***	*	—	—	**	**	***	**		**0.21**	−0.04	0.05	0.00
% ARIC30	*	*	***	***	—	—	—	—	*		−0.01	0.08	0.05
VCL	—	—	—	—	—	—	—	—	—	—		**0.04**	0.78
VSL	—	—	*	**	—	—	—	—	—	—	***		
VAP	—	—	*	*	—	—	—	—	—	—	***	***	0.89

[a] Pearson correlation coefficients for sperm velocities were calculated with a smaller sample size (n = 121 stags) than for the rest of sperm traits (n = 173 stags). *P = 0.01; **P = 0.001; ***P = 0.0001. Correlations with P = 0.05 are not shown in the table, as the significance level was adjusted to P = 0.01 to avoid type I error. Bold numbers indicate significant results.

FIG. 1. Cluster diagram showing how closely different reproductive traits are associated with each other. This cluster diagram is based on the raw correlation matrix (Table 3), with complete linkage joining rule (distance metric = 1 − Pearson r).

adding up major diameters of both testes and dividing by body length. Spermatozoa were recovered by cutting the caudae epididymides with a surgical blade, collecting the oozing sperm mass, and placing it in 1 ml of Dulbecco PBS containing 0.5% BSA. Spermatozoa recovered from caudae epididymides are functionally mature and have a fertilizing potential equivalent to that of ejaculated sperm [32].

Because the sperm masses collected from epididymides varied between males, the sperm suspension volume recovered was measured in graduated test tubes. Subsamples were taken for the assessment of various sperm parameters. Sperm concentration was estimated using a hemocytometer. Total sperm number was calculated taking into account the actual volume of sperm recovered. Because this variable exhibited an allometric relation, it was corrected for body length (distance in centimeters from the tip of the nose to the sacrococcygeal articulation). Proportion of motile spermatozoa was assessed subjectively at 37°C between a slide and a coverslip and using phase-contrast optics. The quality of motility was assessed using a scale from zero (lowest) to five (highest). Sperm viability, morphology, and acrosome integrity were assessed in smears stained with eosin-nigrosin and, subsequently, with Giemsa and counting 200 spermatozoa under a bright field as described previously [33]. Proportion of viable spermatozoa reflects live spermatozoa (i.e., those excluding eosin) over the total number of spermatozoa. Proportion of normal spermatozoa reflects spermatozoa not showing abnormal morphology of the sperm head, midpiece, or principal plus terminal pieces. Finally, proportion of spermatozoa with intact acrosomes (acrosome integrity or percentage of normal apical ridge) accounts for intact spermatozoa not showing damaged or lost apical ridges.

To examine the functional capacity of deer spermatozoa, an acrosome reaction assay was carried out. The acrosome reaction is an absolute requirement for fertilization, and induction with natural agonists (progesterone or zona pellucida) or molecular probes (the Ca^{2+} ionophore 23187) can be used to assess this sperm function [34, 35]. The responsiveness to inducers of the acrosome reaction is related to reproductive quality [36–38], although the acrosome reaction triggered by natural agonists or molecular probes may not necessarily assess identical aspects of acrosomal exocytosis and its underlying pathways. For induction of the acrosome reaction, spermatozoa were suspended in a saline solution with the following composition: 142 mM NaCl, 2.5 mM KOH, 10 mM glucose, 2 mM $CaCl_2$, 20 mM Hepes, 1 mg/ml of polyvinyl alcohol, and 1 mg/ml of polyvinyl pyrrolidone (pH adjusted to 7.55 at 20°C with NaOH and osmolality of 305 mOsmol/kg) [39]. Spermatozoa were stimulated to undergo the acrosome reaction by treating them with 1 μM of the Ca^{2+} ionophore A23187. The occurrence of the reaction was monitored by phase-contrast microscopy of glutaraldehyde-fixed samples taken before treatment (time 0) and after 30 min of incubation at 37°C under air. For each sperm sample, the increase in acrosome-reacted spermatozoa from 0 to 30 min was calculated (%ARIC30) [38].

Objective measures of sperm velocity were recorded in spermatozoa suspended in Dulbecco PBS with 0.5% BSA and using a computer-aided sperm analyzer (Sperm Class Analyzer; Microptic, Barcelona, Spain). The three descriptors of sperm motility employed, scored by analyzing a minimum of 100 tracks per sample, were curvilinear velocity (VCL; velocity of the actual track of the sperm), average path velocity (VAP; velocity over a calculated, smoothed path), and straight-line velocity (VSL; veloc-

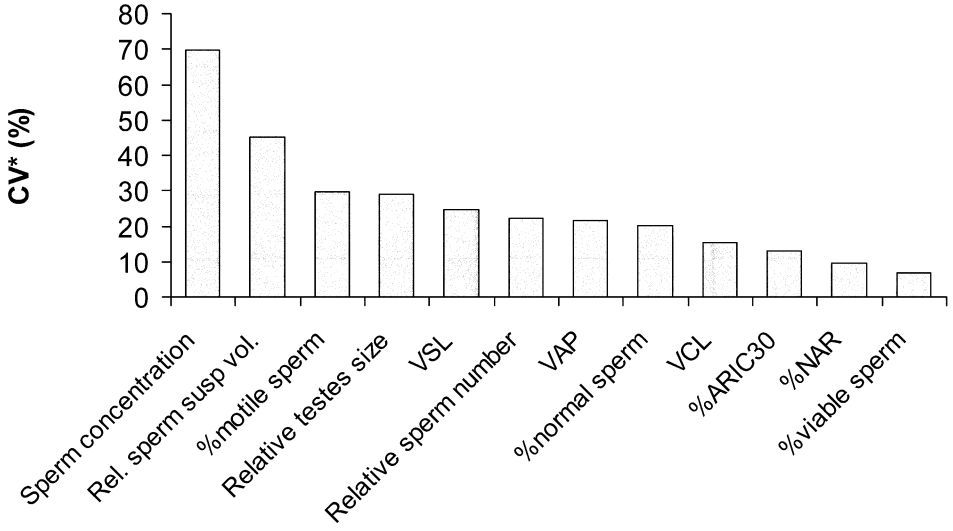

FIG. 2. Coefficients of variation corrected for sample bias (CV*) for several reproductive traits.

ity calculated using the straight-line distance between the beginning and end of the sperm track).

Artificial Insemination Trials

Sperm samples from 11 red deer males from natural populations were used to inseminate a total of 247 hinds. Each female was inseminated once with spermatozoa from one male. The red deer population of hinds has been kept for five generations in captivity. Sperm samples from each male were used to inseminate between 3 and 69 females. Spermatozoa were obtained from males culled in natural populations as described above. To allow the insemination of a large number of females with spermatozoa from the same male, sperm samples were cryopreserved in liquid nitrogen after collection. A detailed description of methods used for sperm freezing and artificial insemination can be found elsewhere [40]. After sample thawing and before insemination, subsamples were taken for evaluation of several sperm parameters. The following sperm parameters were assessed: proportion of motile spermatozoa, quality of motility, proportion of viable spermatozoa, proportion of normal spermatozoa, proportion of spermatozoa with intact acrosome, and three sperm velocity descriptors (VCL, VAP, and VSL). To control for the effects of sperm number on fertility, a relation that has been known for a long time [24–27], the total number of spermatozoa inseminated (100×10^6 spermatozoa) was kept constant for all the artificial inseminations performed. No effect of number of females inseminated by each male was found on the individual fertility rate (linear regression, $F_{1,9} = 0.15$, $P = 0.70$).

To minimize the potential influence of female fertility on the results obtained, all hinds used for artificial inseminations had to meet two general conditions: to be in good physical condition, as assessed by subjective evaluation of fat deposits; and to have given birth the previous year. In addition, all hinds had their estrous cycles synchronized to avoid the confounding effects of hinds being at different stages of the reproductive cycle (for details, see [40]).

We considered that a male scored a successful fertilization when the female became pregnant. Fertilization success for every male was calculated as follows: number of hinds pregnant/number of hinds inseminated $\times$ 100.

TABLE 4. General linear model for male fertility.[a]

Source	*df*	Mean square	*F*	*P*
Multiple regression model for male fertility ($F_{3,7} = 5.027$, $P = 0.036$; $R^2 = 0.683$)				
Intercept	1	50.049	0.481	0.510
% Normal sperm	1	17.044	0.164	0.698
VCL	1	694.412	6.680	0.036
No. of females inseminated/male	1	19.336	0.186	0.679
Error	7	103.948		
Linear regression model for male fertility ($F_{1,9} = 18.019$, $P = 0.002$, $R^2 = 0.667$)				
Intercept	1	54.996	0.647	0.442
VCL	1	1530.755	18.019	0.002
Error	9	764.579		

[a] Data were obtained from the insemination of 247 hinds by sperm from 11 Iberian red deer free-ranging stags. Full model and reduced model are shown after deleting nonsignificant terms.

Statistical Analyses

Given that testes size, sperm suspension volume, and total sperm number were significantly correlated with body size ($F_{1,175} = 79.09$, $P < 0.00001$; $F_{1,181} = 15.62$, $P < 0.001$; and $F_{1,180} = 8.97$, $P = 0.003$, respectively), residuals were employed for multivariate and correlation analyses. The three variables showed normal distributions (Kolmogorov-Smirnov normality test; relative testes size, $d = 0.041$, $P > 0.2$; relative sperm suspension volume, $d = 0.054$, $P > 0.2$; relative sperm number, $d = 0.076$, $P > 0.2$).

All variables were transformed to enhance or attain a normal distribution. Proportions were arcsine-squareroot transformed, and the rest were log transformed.

Principal Component Analysis

We performed a principal component analysis (PCA) to explore relationships between variables and to assess how much of the overall variance is shared by the different sperm traits [41]. All 13 reproductive variables were included in the analysis. Kolmogorov-Smirnov test for goodness of fit [42] was used to test for variable normal distribution. All the variables introduced in the multivariate analyses, except for quality of motility, satisfied this test or had positive kurtosis. Kolmogorov-Smirnov values for all variables (and for kurtosis, if normality was absent) were as follows: relative testes size, $d = 0.041$, $P > 0.2$; relative sperm suspension volume, $d = 0.054$, $P > 0.2$; relative sperm number, $d = 0.076$, $P > 0.2$; percentage of motile sperm, $d = 0.195$, $P < 0.01$, positive kurtosis; quality of motility, $d = 0.236$, $P < 0.01$; percentage of viable sperm, $d = 0.079$, $P < 0.2$; percentage of normal sperm, $d = 0.134$, $P < 0.01$, positive kurtosis; percentage of acrosome integrity, $d = 0.057$, $P > 0.2$; %ARIC30, $d = 0.065$, $P > 0.2$; VCL, $d = 0.764$, $P > 0.2$; VSL, $d = 0.056$, $P > 0.2$; and VAP, $d = 0.040$, $P > 0.2$.

Correlation Analysis

We performed two-way correlations of all variables to explore further the bivariate relationships between reproductive variables. Given that sperm velocity variables had a smaller sample size than the rest (n = 121 vs. 173), two sets of comparisons were performed to allow maximum sample size. For simplicity, all bivariate correlations are shown together in Table 3. To avoid a type I error, the level of test significance was adjusted to $P < 0.01$. In this way, only 1 of 100 correlations would be expected to be significant by chance.

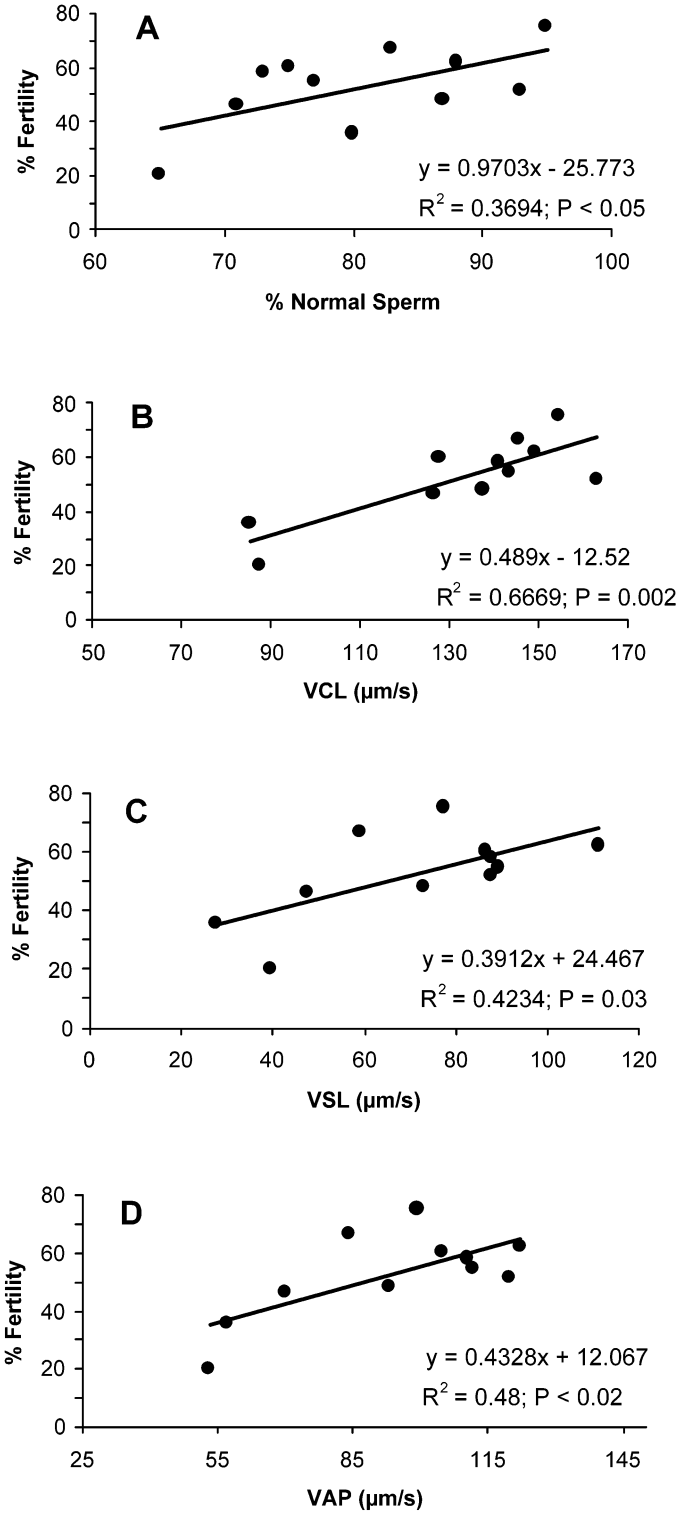

FIG. 3. Relationships of in vivo fertility with sperm morphology (**A**) and with three sperm velocity parameters (**B**, VCL; **C**, VSL; and **D** VAP) of 11 male red deer obtained after artificial insemination of 247 hinds.

Cluster Analysis

A cluster diagram was constructed with the sperm-traits raw correlation matrix with complete linkage-joining rule (distance metric = 1 − Pearson *r*). We aimed to identify visually the different groups of sperm traits and the hierarchical relationships between them.

Coefficients of Variation

The use of CVs allows a comparison of the variability of certain traits adjusting for differences in magnitude of trait means [43]. The CV for a particular trait in a population thus is the SD divided by the mean. Here, we calculated CVs following the Haldane correction for sample bias [44]:

$$CV^* = (1 + 1/4n)s/\bar{Y}$$

where n = sample size, s = SD, and $\bar{Y}$ = mean.

The CVs were not calculated for quality of motility because of its categorical nature [43]. For the calculation of CVs, variables must not have negative values. Given that testes size and total sperm number were corrected for body size, residuals had to be rescaled to eliminate negative values before CV calculation.

RESULTS

Reproductive Traits in Iberian Red Deer Males

Iberian red deer males had testes with a mean volume of 65.7 ml. Analyses of samples recovered from caudae epididymides revealed a mean sperm suspension volume of 1.34 ml, a sperm concentration of 1319×10^6 spermatozoa/ml, and a total sperm number of 1937×10^6 spermatozoa, with the latter two variables showing marked differences between minimum and maximum values, which fluctuated between two and three orders of magnitude, respectively (Table 1).

The percentage of motile sperm showed a great deal of variation, with an average value of 62% (range, 0–90%). Quality of motility was intermediate, with an average of 2.77 (range, 0–4). The percentages of viable sperm, acrosome integrity, and morphologically normal sperm showed high average values (90%, 86%, and 77%, respectively). The percentage of viable sperm was consistently high between individuals (range, 62–100%), whereas the percentage of acrosome integrity showed a somewhat larger range of values (41–100%). However, the percentage of morphologically normal sperm showed a large degree of variation between individuals (range, 12–97%).

The occurrence of sperm acrosome reactions in response to a 30-min ionophore stimulation (%ARIC30) showed a high mean value of 85% and a wide range of individual responses (44–100%). Finally, assessments of sperm swimming velocities by means of a computerized system revealed values of VCL, VAP, and VSL of 113, 83, and 69 μm/sec, respectively, with a wide range of values among males (Table 1).

Multivariate Analysis of Semen Quality

The PCA performed with the 13 variables rendered two significant factor scores that explained 44% of the variance (Table 2). Factor score 1 explained 26% of the variance, and 11 of 13 variables contributed significantly to it: relative testes size, relative sperm suspension volume, sperm concentration, relative sperm numbers, quality of motility, percentage of viable sperm, percentage of acrosome integrity, %ARIC30, and the three sperm velocity variables (VCL, VSL, and VAP). The percentage of morphologically normal sperm and the percentage of motile sperm did not correlate significantly with factor score 1.

The fact that the PCA accounted for 44% of the total variance suggests that a considerable amount of information remains unexplained and that more detailed analyses are needed to gain a deeper understanding of the relationships between different sperm traits.

Correlations

We used correlations to detect associations between variables and to infer the existence of groups of closely related variables. Given the nature of some variables, three groupings were expected a priori and were, indeed, identified in the present: First, a strong relation was found between sperm production variables. Testes size showed significant correlations with sperm suspension volume, sperm concentration, and relative sperm number. Sperm suspension volume correlated significantly with sperm concentration and relative sperm number, and the correlation between sperm concentration and relative sperm number was very high. Second, the proportion of motile sperm and the quality of motility showed a high correlation. Third, sperm velocity variables also presented high correlations between them (Table 3).

New associations between sets of variables were revealed that provide further information concerning the relationships between semen traits. First, sperm production traits showed significant correlations with %ARIC30, with the associations with sperm concentration and relative sperm number being particularly strong. Second, these two sperm production variables also were significantly correlated with two sperm swimming velocities, VSL and VAP, and were not far from significance with VCL. Third, both the proportion of motile sperm and quality of motility correlated significantly with the proportion of normal sperm, revealing an association between sperm morphology and motility. Fourth, proportion of acrosome integrity and %ARIC30 also were correlated. Fifth, two variables, percentage of viable sperm and proportion of acrosome integrity, showed positive correlations with a large number of semen variables. Viability correlated with all the sperm production variables, with the proportion of motile sperm and quality of motility, and particularly strongly with acrosome integrity. On the other hand, proportion of acrosome integrity correlated with testes size, proportion of motile sperm, quality of motility, and proportion of normal sperm.

The three sperm swimming velocities did not correlate with any other variable besides the association with sperm production variables mentioned above.

No differences were found in the level of significance of the correlation coefficients when the three sperm production variables, which were corrected for body size, were analyzed using the absolute values.

Cluster Analysis

The construction of a cluster diagram, based on Pearson *r* values obtained from the correlation matrix, allows a visual representation of each set of variables and how closely they are associated to each other (Fig. 1). This cluster diagram shows that all the sperm production variables are strongly associated. In turn, this cluster is associated with the proportion of acrosome-reacted sperm after a 30-min exposure to ionophore (%ARIC30).

Similarly, the three sperm swimming velocities are strongly associated. This cluster shows a clear relationship with the cluster formed by the sperm production variables and %ARIC30.

The proportion of motile sperm and the quality of motility are strongly associated, and both are clearly associated with the proportion of normal sperm. In turn, this cluster is associated with the group conformed by the percentage of viable sperm and the percentage of acrosome integrity.

Analysis of CVs

Figure 2 shows the CVs for 12 of 13 variables included in the present study. The only variable excluded from this analysis was quality of motility, because it was given a subjective score of between zero and five and, thus, was not appropriate for this kind of analysis. Sperm concentration and sperm suspension volume showed the highest CVs (70% and 45%, respectively). Next in the ranking are a group of variables that showed intermediate CVs, ranging from 30% to 15%. In decreasing order of CV, these are proportion of motile sperm, relative testes size, VSL, relative sperm number, VAP, proportion of normal sperm, and VCL. The lowest CVs corresponded to %ARIC30 (13%), percentage of acrosome integrity (10%), and percentage of viable sperm (7%).

Sperm Quality and Fertility

Spermatozoa of 11 males from natural populations were cryopreserved to perform a fertility trial. Freezing several sperm samples from each male was necessary to allow the insemination of a large number of females by each male. The proportion of cases in which females inseminated with spermatozoa from the same male became pregnant was used as the male's fertility rate. On the whole, 247 inseminations were carried out, and in each, the same number of spermatozoa were inseminated. After thawing and before insemination, several sperm traits were evaluated to examine the associations between each sperm trait and male fertility.

Male fertility rates varied from 20% to 75%. The three sperm velocity parameters (VCL, VSL, and VAP) and, to a lesser extent, the proportion of normal sperm showed significant associations with fertility (Table 4 and Fig. 3). However, the proportion of motile spermatozoa and the quality of motility did not show a significant association with fertility, and neither did the proportion of acrosome integrity nor the proportion of viable sperm.

DISCUSSION

Our results show that males from natural populations of Iberian red deer vary markedly in their fertility even when sperm numbers are kept constant. Differences in fertility rates between males related strongly to three sperm swimming velocity parameters (VCL, VSL, and VAP) as well as to the percentage of morphologically normal spermatozoa.

Given the difficulties found in previous studies, it is surprising that such clear associations between specific semen traits and male fertility have emerged. This likely results from males in natural populations showing a wide degree of variation in semen traits, a situation unlikely to be found in domestic species that have been subjected to artificial selection for improved fertility for many generations and in animals that are kept under uniform conditions. Such variation also may be absent from clinical studies that focus exclusively on patients with fertility problems.

From an evolutionary perspective, these findings imply that differences between males in fertility should be taken into account when considering which factors influence male reproductive success in natural populations. The four variables found to determine male fertility had intermediate CVs. This raises the question as to why males differ in these crucial semen traits, namely the percentage of morphologically normal spermatozoa and three sperm swim-

ming velocity parameters (VCL, VSL, and VAP). The percentage of morphologically normal spermatozoa has been shown to decrease with inbreeding [10, 11], which therefore could be a primary determinant of male fertility in natural populations. It has been suggested that sperm swimming velocity is determined mainly by the mitochondria inherited from the mother and that, for this reason, it should show no associations with other semen traits [45]. However, our finding that sperm swimming velocity and sperm production parameters are clearly associated does not support this hypothesis.

Theory predicts that there should be trade-offs between different sperm traits, given that sperm is costly to produce [46–48]. The main trade-offs suggested include sperm numbers versus sperm size [49, 50] and sperm size and velocity versus sperm longevity [51–53]. Our results show no trade-offs between the semen parameters included in the present study, suggesting that such traits coevolve to maximize fertilizing efficiency. However, we did not include sperm size in the present study, so that question remains open.

Our analyses revealed, to our knowledge for the first time, the strength of the associations between different male reproductive traits and the hierarchy of the links between them. First, the close association between sperm production and sperm swimming velocity revealed a strong link between the two variables, which seem to have a greater impact on male fertility. The number of spermatozoa transferred to the female has an important effect on fertility [5, 25], and our fertility trials show that this also is the case with sperm velocity. This finding suggests that males with high fertility in red deer populations have both high sperm numbers and spermatozoa that swim at high speed. Second, the proportion of motile spermatozoa and quality of motility were associated closely with the proportion of normal spermatozoa, an association that has been postulated frequently [54] but for which no strong data were available. Third, sperm viability and acrosome integrity were strongly associated. Both parameters show a low CV, all males showed high values, and they were not associated with male fertility. These results suggest that such variables have stabilized at high values because they are so essential for fertilization and that males with lower values have been intensively selected against in natural populations.

We conclude that males from natural populations differ in their fertility and that, when sperm numbers remain constant, sperm swimming velocity and sperm morphology are the main determinants of fertility. On the other hand, sperm viability and acrosome integrity have no effect on fertility, probably because strong selection in natural populations has stabilized these parameters at high values. These results demonstrate that it is possible to identify specific semen traits that determine fertility in natural populations, despite the lack of success when working with domestic species. They also show that some of the traits known to determine fertility among infertile human patients, such as acrosome integrity [35], are not associated with fertility in natural populations, because selection has favored uniformly high values. Third, sperm traits believed to play an important role exclusively in the context of sperm competition, such as sperm velocity [55, 56], are primary determinants of fertility in males from natural populations in the absence of sperm competition. This implies that among mammals, there may be no specific sperm traits favored by sperm competition but, rather, that the same traits that are important for fertilization experience an even greater selection under sperm competition.

Our findings suggest that differences in fertility between males may contribute significantly to generating differences in reproductive success. It is widely assumed that the large differences in male lifetime reproductive success observed among polygynous mammals, such as red deer, mainly result from differences in their ability to win agonistic encounters with other males and in their ability to defend females from other males. Our results suggest that such a scenario is incomplete, because once a male defends a harem or a territory, his ability to fertilize plays an important role in determining his reproductive success.

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