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Sperm Population Structure and Male Fertility: An Intraspecific Study of Sperm Design and Velocity in Red Deer[1]

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ABSTRACT

Sperm design and velocity play key roles in influencing sperm performance and, therefore, can determine fertilization success. Several interspecific studies have demonstrated how these features correlate, and it has been hypothesized that selection may drive changes in these sperm traits. Here, we examine the association between sperm design and swimming velocity in a study conducted at an intraspecific level in Iberian red deer (*Cervus elaphus hispanicus*). We addressed how the structure of different sperm subpopulations, based on sperm morphometry and velocity, are interrelated and, in turn, how they associate with fertility. Our results show that males with high fertility rates have ejaculates with high percentages of spermatozoa exhibiting fast and linear movements and that these are highly correlated with a large proportion of spermatozoa having small and elongated heads. On the other hand, males with low fertility are characterized by a subpopulation structure in which slow and nonlinear as well as small and wide spermatozoa are predominant. These findings provide insight regarding how sperm size and velocity are interrelated and how they both are associated with fertility.

ejaculate heterogeneity, fertility, sperm, sperm length, sperm size, sperm subpopulations, sperm velocity

INTRODUCTION

Numerous studies have attempted to explain how sperm parameters determine male fertility, mostly focusing on sperm velocity as a trait that may influence a male's fertilizing capacity. Sperm velocity has been related to fertility in a wide range of species, including mammals [1–3], fish [4], birds [5], or insects [6]. The diversity in sperm size and shape across species is also thought to influence sperm velocity and, therefore, possibly determine fertility. A number of studies have addressed how sperm design and sperm velocity could be interrelated [7–10] and how both could affect the reproductive success of males [3, 4, 11–12]. Controversy exists, however, about the way diversity in sperm design translates into variation in sperm velocity. Thus, whereas the results of several studies have supported associations between sperm design and sperm velocity [7, 10, 13, 14], others have not detected such associations [8].

Most studies have been conducted at the interspecific level, and the large degree of variation between species has allowed identification of an association between sperm morphometrics and velocity [10]. However, this relationship has been more difficult to appreciate at the intraspecific level (i.e., within species) given that differences between males are usually smaller. A recent intraspecific analysis showed that when data on sperm size and swimming velocity are matched for the same sperm cells, the relationship is strong and clear [15]. However, when data on size and velocity are collected from different sperm subsamples (as in most studies), such relationships might not arise.

One key characteristic of mammalian ejaculates is heterogeneity [16–18]. This heterogeneity has been related to different aspects of male reproductive performance, with the realization that fertilization ability may vary depending on the characteristics that spermatozoa show at the time of fertilization [17, 19]. The majority of studies have addressed the relations between sperm traits and male fertility based on average values of sperm parameters, but to our knowledge, no attention has been paid to sperm heterogeneity and how it could affect the fertilization ability of a male. The characterization of such ejaculate heterogeneity would allow a more detailed analysis of the relationship among sperm features and their role in determining male fertility.

To characterize sperm heterogeneity of an ejaculate, clustering statistical methods have been used to identify groups or subpopulations of spermatozoa sharing common characteristics [20]. The sperm traits used to characterize this heterogeneity should be those with a role in determining the fertility of a male. The identification of sperm parameters of interest for fertility requires not only a sperm-quality assessment but also a definition of the best conditions for fertility evaluation [21]. It is now clear that fertilization success does not simply depend on the absolute number of vital, motile, morphologically normal spermatozoa inseminated in the female [19]. Moreover, measures of individual sperm traits or the results of single functional tests are poorly correlated with fertility [22]. As a result, the emphasis is now focused on analyses that incorporate multiple variables to examine how different sperm parameters interact to determine fertility. To achieve these goals, a supervised learning statistical methodology [23–25] has recently been incorporated in these analyses. The advantage of this methodology lies in the use of prior

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sources of information when characterizing spermatozoa, which ultimately will allow a more comprehensive interpretation of functional relationships. Although this methodology has been used in other biological systems [24, 26, 27], its use in the field of spermatology is novel [28, 29].

In the present study, we examined, at the intraspecific level in red deer, the relationship between sperm design and velocity in males with different fertility. To account for sperm heterogeneity of an ejaculate, we identified and characterized different sperm subpopulations based on sperm design and velocity features. For the characterization of sperm subpopulations, we took advantage of supervised learning methods as a way to consider multiple sources of information jointly in a single analysis. The sperm subpopulation distribution defined in this way was then used to assess how changes in sperm population structure reflect on male fertility.

MATERIALS AND METHODS

Ethics

The present study was approved by the ''Comité de Ética en Investigación de la Universidad de Castilla-La Mancha.'' All animal handling was done following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

Animals

The study sample included 12 Iberian red deer (*Cervus elaphus hispanicus*) stags culled during the mating season (October–December) in three different wild populations from the south of Spain [30]. Culls were performed following Spanish laws that in turn conform to European Union regulations. Medianilla SL allowed the insemination of hinds at Finca Las Lomas (Vejer de la Frontera, Cádiz, Spain).

Sperm Collection and Evaluation

Both testes in the scrotum were removed and transported at 20–21°C to the laboratory. Time elapsed between the animal's death and sperm analysis and processing ranged from 3 to 6 h, which is an adequate and reliable interval for evaluating sperm parameters in this species as the decrease in quality of sperm traits only begins 12 h after the death of the male [31]. Sperm samples were cryopreserved as described by Soler et al. [32] and then used to inseminate hinds. Only samples with a minimum of 80% motile sperm and a wave motion of 4 (on a linear scale from 0 [no movement] to 5 [strong wave motion]), assessed subjectively, were used. Just after sample thawing and before insemination, subsamples were taken to assess sperm morphometric and velocity parameters. Methods for analyses of sperm morphometry and velocity have been described previously [7, 33]. Briefly, spermatozoa were diluted in Dulbecco PBS with 0.5% bovine serum albumin. Objective measures of sperm velocity were recorded using a computer-aided sperm analyzer (Sperm Class Analyzer [SCA]; Microptic). For the assessment of sperm morphometry, microscope slides were prepared by placing 5 μl of sperm diluted in PBS on the clear end of a frosted slide and then dragging the drop across the slide. Semen smears were air-dried and stained using the commercial kit Hemacolor (catalog no. 11661; Merck). Next, stained samples were permanently mounted to the slide with a coverslip and DPX. The morphometric module of the SCA was used to capture sperm head dimensions. A total of five sperm head dimension parameters were assessed: head length (HL), head width (HW), head area (HA), head perimeter (HP), and the shape factor known as perimeter to area (p2a; calculated as $HP^2/4 \times \pi \times HA$) (Fig. 1) [34]. In addition, six motility descriptors were quantified: curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH), and beat cross frequency. Data for sperm morphometry were collected from eight stags, whereas velocity descriptors were obtained from 12 stags.

Fertility Data

Data on male reproductive performance were also gathered. Sperm samples from the 12 red deer males were used to inseminate a total of 257 hinds. To allow insemination of a large number of females with spermatozoa from the same male, sperm samples were cryopreserved and stored in liquid nitrogen after collection. Each female was inseminated once with spermatozoa from one male, and samples from each male were used to inseminate between 4 and 69 females. No effect of the number of females inseminated by each stag was found on individual male fertility rate. To eliminate female influence on fertility success and retain the intermale variation on fertility rates, the experiment was designed so that 1) no preferential matings were conducted, 2) all females were in good physical conditions and had given birth the previous year, 3) the estrous cycles of all females were synchronized, and 4) the total number of spermatozoa inseminated (100×10^6) was kept constant for all the artificial inseminations performed [11]. We considered that a male had scored a successful fertilization when the female became pregnant. Fertilization success for every male was calculated as the number of hinds made pregnant divided by the number of hinds inseminated and expressed as percentage. In the present study, male fertility was of 55% ± 4.9% (mean ± SEM).

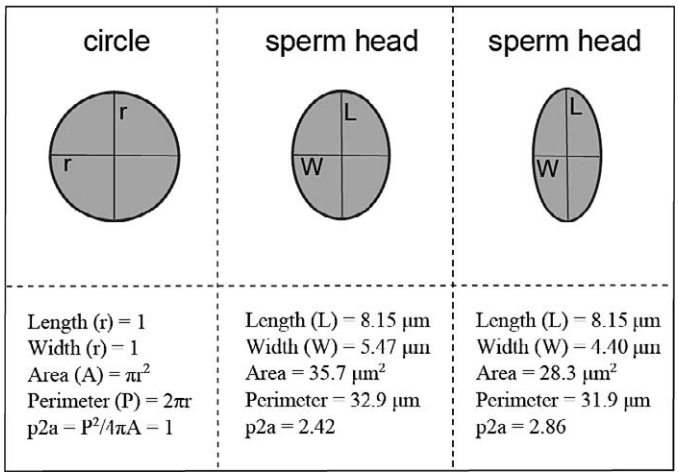

FIG. 1. Schematic drawings of the perimeter to area (p2a) shape factor. For a circle, p2a equals 1 (**left**). As the value of p2a increases, the shape becomes more elongated (**middle** and **right**).

Statistical Analysis

A supervised learning method was used to characterize sperm subpopulation structures based on motility and morphological information. The statistical procedure used to characterize sperm subpopulations was the support vector machine (SVM) methodology [23–25]. The basis of supervised learning methodology is as follows: The goal is to predict the value of an outcome measure based on a number of input measures, so the presence of the outcome variable guides the learning process. A training set of data is used to observe the outcome and feature measurements for a set of objects. Using these data, we build a prediction model, or learner, that will enable us to predict the outcome for new, unseen objects. A good learner is one that accurately predicts such an outcome. When translating this idea to the field of spermatology and, more specifically, the aim of the present study, the outcome measure was the subpopulation to which spermatozoa belong, and the input measures were all the characteristics of sperm design and velocity assessed. As a training data set, we used two subset of male deer, one with low fertility rates and one with high fertility rates. Clear differences were observed in the sperm subpopulation distribution among the groups of high and low fertility (Fig. 2). For each of these males in the training data set, individual sperm tracks and morphometry were assessed visually and assigned to one of the different subpopulations as described by Goodson et al. [28] and Ramón et al. [29]. Then, the SVM methodology was used to generate a model that, using the information on sperm shape and velocity, allowed an accurate classification of sperm from a male ejaculate into the subpopulation to which they belonged. This model was then used to characterize the sperm subpopulations in the ejaculates of the other males not included in the training set.

In the present study, information on sperm design and velocity from four males, two with low fertility and two with high fertility, was used as source of prior information in a training data set (Fig. 2). Individual sperm from these four males were assigned to different subpopulations based on their head morphometry and velocity parameters and then used by the supervised learning procedure to generate the SVM model for the automated characterization of sperm subpopulations. The sperm characteristics used as initial classificatory variables were HL and p2a for head morphometry [18] and VAP and LIN for velocity [17]. Once SVM equations were generated, we performed the

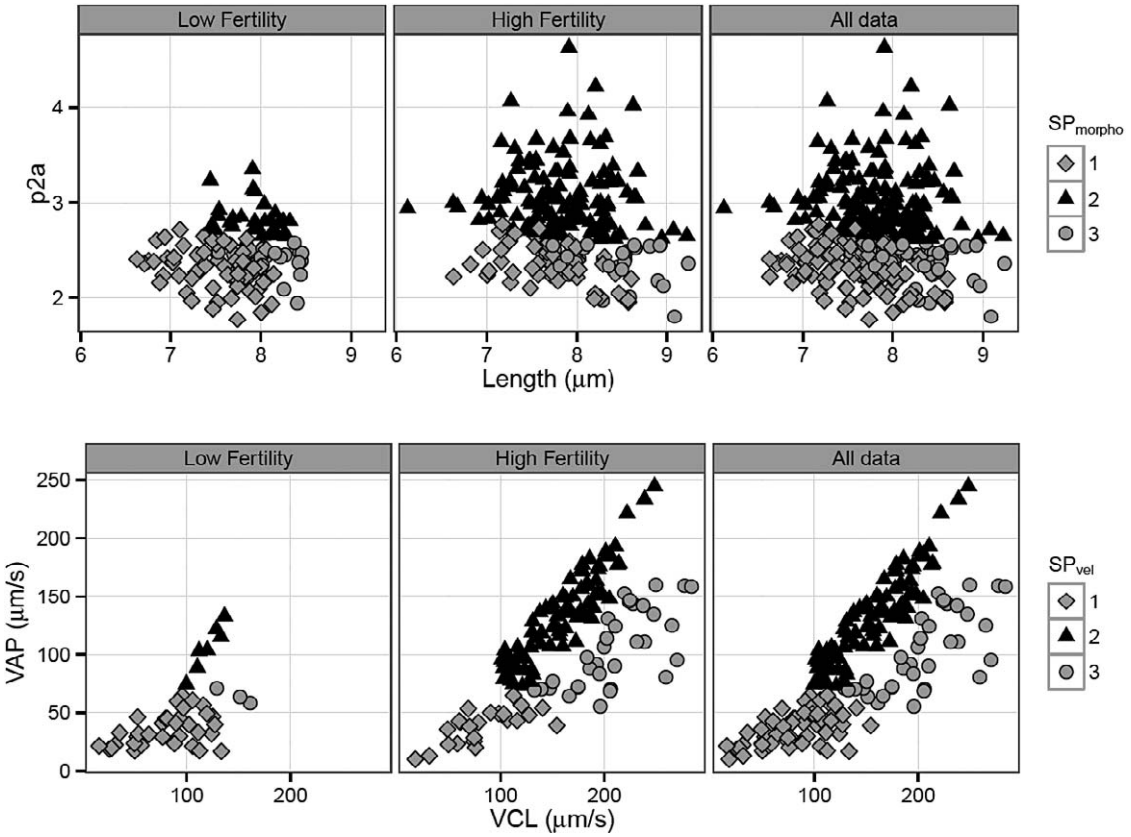

FIG. 2. Distribution of sperm subpopulations in Iberian red deer ejaculates based on head morphometry and velocity parameters. Sperm distribution for males with low fertility, males with high fertility, and all males included in the present study are shown. Three head morphometry sperm subpopulations were identified: small and wide (SP1$_{morpho}$), small and elongated (SP2$_{morpho}$), and long and wide (SP3$_{morpho}$). Three motile sperm subpopulations were identified: slow and nonlinear (SP1$_{vel}$), fast and linear (SP2$_{vel}$), and fast and nonlinear (SP3$_{vel}$).

characterization of the sperm subpopulations for the sperm samples of the other eight males.

After characterizing sperm subpopulations, we performed a two-way correlation analysis including all sperm subpopulations to explore possible associations between both sperm head morphometry and sperm velocity. Finally, we performed regression analyses to explore the relationships between the proportion of each sperm subpopulation in the sperm sample and fertility.

All statistical analyses were performed using the R statistical environment [35]. Package e1071 [36] were used to perform SVM analysis. *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Graphical display of sperm head morphometry and velocity characteristics for males with large differences in their fertility rates (Fig. 2) showed that, for males with low fertility, sperm heads were mostly small and wide, with predominantly slow movement, whereas for males with high fertility, sperm heads were mostly small and long, with fast movement. According to differences observed in the p2a parameter (values of p2a close to 1 indicate a shape similar to a circle, and as this value increases, a more elongated shape is observed; see Fig. 1), males with low fertility were characterized by spermatozoa with a round head, whereas males with high fertility rates were characterized by spermatozoa with an elongated head (Fig. 2). When all males were considered, three sperm morphometric subpopulations could be defined. This classification is in agreement with results reported earlier on sperm head morphometry in red deer [18].

Figure 2 also presents the distribution of subpopulations based on sperm velocity parameters. Males with low fertility were characterized by spermatozoa with a slow movement (lower VCL and VAP). Conversely, males with high fertility rates exhibited a decrease in the percentage of spermatozoa with slow movement, with an increase in VCL. Within spermatozoa with high VCL, two groups could be distinguished based on their VAP. Overall, three velocity subpopulations were obtained, which matched subpopulations previously identified for red deer [17].

TABLE 1. Summary of sperm subpopulation characteristics based on spermatozoa head dimensions.[a]

	Fertility (%)			Sperm characteristics			
Subpopulation	Low	High	All males (range)	Length (μm)	Width (μm)	Area (μm^2)	p2a
SP1$_{morpho}$	67.75 ± 1.89	29.86 ± 5.10	37.96 ± 7.20 (16–70)	7.82 ± 0.02	4.62 ± 0.02	30.25 ± 0.17	2.26 ± 0.01
SP2$_{morpho}$	24.94 ± 3.51	65.00 ± 7.27	52.32 ± 7.22 (21–80)	8.14 ± 0.03	4.46 ± 0.02	29.37 ± 0.14	2.98 ± 0.02
SP3$_{morpho}$	7.31 ± 1.62	5.14 ± 2.17	9.72 ± 2.33 (3–22)	8.70 ± 0.03	4.78 ± 0.04	33.81 ± 0.39	2.24 ± 0.02

[a] Data are presented as the mean ± SEM.

TABLE 2. Summary of sperm subpopulation characteristics based on spermatozoa velocity.[a]

	Fertility (%)			Sperm characteristics				
Subpopulation	Low	High	All males (range)	VCL (μm/sec)	VSL (μm/sec)	VAP (μm/sec)	LIN (%)	ALH (μm)
$SP1_{vel}$	80.45 ± 9.02	17.48 ± 3.20	34.74 ± 11.29 (5–95)	83.77 ± 2.99	29.37 ± 1.57	40.39 ± 1.29	37.91 ± 1.66	4.60 ± 0.22
$SP2_{vel}$	13.34 ± 8.08	56.39 ± 9.84	53.35 ± 10.07 (0–77)	153.86 ± 2.69	112.10 ± 2.59	128.09 ± 2.46	73.20 ± 1.09	5.21 ± 0.16
$SP3_{vel}$	6.20 ± 0.94	26.12 ± 6.63	11.90 ± 2.87 (3–26)	179.55 ± 6.33	40.50 ± 2.14	95.61 ± 3.07	23.18 ± 1.30	8.71 ± 0.44

[a] Data are presented as the mean ± SEM.

The use of information on sperm morphometry and velocity from males with considerable differences in their fertility rates in an SVM procedure resulted in the classification of spermatozoa in three morphometric sperm subpopulations (Table 1) and three velocity sperm subpopulations (Table 2). The three sperm subpopulations obtained based on sperm head morphometry were as follows: small and wide ($SP1_{morpho}$), small and elongated ($SP2_{morpho}$), and long and wide ($SP2_{morpho}$). Morphometric subpopulations were classified as small or long based on whether their HL and HA were below or above average (average HL, 8.15 μm; average HA, 30.3 μm^2); in addition, morphometric subpopulations were classified as wide or elongated based on whether their p2a was below or above average (average p2a, 2.63). Regarding sperm velocity, the characteristics of the three sperm subpopulations were as follows: slow and nonlinear ($SP1_{vel}$), fast and linear ($SP2_{vel}$), and fast and nonlinear ($SP3_{vel}$). Subpopulations based on sperm velocity were classified as slow or fast based on whether their VCL was below or above average (average VCL, 136.7 μm/sec); moreover, velocity subpopulations were classified as nonlinear or linear based on whether their LIN was below or above average (average LIN, 54.07).

We searched for possible correlations between morphometry and velocity subpopulations (percentages of each) to assess if spermatozoa in a particular morphometric sperm subpopulation had a characteristic movement (Table 3). We found that the proportion of spermatozoa with small and wide heads ($SP1_{morpho}$) showed a strong positive correlation ($r = 0.83$, $P < 0.01$) with the proportion of spermatozoa having a slow and nonlinear movement ($SP1_{vel}$) and a strong negative correlation ($r = -0.76$, $P < 0.05$) with the proportion of spermatozoa having a rapid and linear movement ($SP2_{vel}$). On the other hand, the proportion of spermatozoa with an elongated head ($SP2_{morpho}$) showed a strong positive correlation ($r = 0.69$, $P < 0.05$) with the rapid and linear subpopulation ($SP2_{vel}$) and a strong negative correlation of ($r = -0.76$, $P < 0.05$) with the slow and nonlinear subpopulation ($SP1_{vel}$).

Finally, we examined how the males' sperm subpopulation distribution related to their fertility. Four regression analyses including the small and wide ($SP1_{morpho}$), small and elongated ($SP2_{morpho}$), slow and nonlinear movement ($SP1_{vel}$), and rapid and linear ($SP2_{vel}$) variables were carried out. The percentages of spermatozoa with a small and wide head ($SP1_{morpho}$) and a slow and nonlinear movement ($SP1_{vel}$) showed a negative relation with the fertility rates of males, explaining 70% and 66%, respectively, of the variation in fertility rates (Fig. 3, a and c). On the other hand, the percentages of spermatozoa with a small and elongated head ($SP2_{morpho}$) and a rapid and linear ($SP2_{vel}$) movement were positively correlated with fertility and explained 69% and 48%, respectively, of the variation of fertility (Fig. 3, b and d).

DISCUSSION

The present study examined, at the intraspecific level, relations between ejaculate subpopulations defined on the basis of sperm design and velocity and revealed how changes in sperm population structure are associated with differences in male fertility. Our findings show that an increase in the percentage of spermatozoa with a rapid and linear movement is associated with an increase in male fertility. This motion pattern is strongly correlated with spermatozoa having a small and elongated head. On the other hand, high percentages of spermatozoa with a slow and nonlinear movement strongly correlates with an increase in the percentage of spermatozoa with small and wide heads and an associated reduced fertility of males. Overall, we found that sperm head form is the most important feature in determining the fertility potential. Thus, males presenting high fertility rates are characterized by a sperm subpopulation with elongated heads. Interestingly, these results agree with the observation that sperm competition selects for spermatozoa with more elongated sperm heads, which are thought to result in more hydrodynamically efficient cells [10]. Therefore, small changes in sperm head shape could have a major impact on sperm swimming velocity and, therefore, on sperm fertilization ability [7, 37].

Previous studies have examined correlations between sperm design and sperm function [7, 38, 39] and their role as determinants of fertilization success [3, 4, 12]. However, although some studies showed significant correlations between these traits, others found no evidence for these associations [8]. All these studies concentrated on analyses of sperm linear dimensions and, more importantly, used average values for the different traits examined, and this may have hindered the possibility of uncovering the relations between traits because average values do not capture the variation existing among spermatozoa in an ejaculate. A large degree of variation exists among species, and such variation may be required for the identification of associations between traits, as seen in interspecific comparisons. Such associations may be more difficult to appreciate at the intraspecific level given the small magnitude of differences between males [8]. Interestingly, when data on dimensions and velocity were collected from the same sperm cells, the relationship between these traits was clear [15]. However, although this approach is potentially very useful to accurately assess relationships between sperm design and function, it may not be applied to all species. Thus, whereas Fitzpatrick et al. [15] obtained morphometry and motility data for the same sperm cells in the sea urchin

TABLE 3. Correlations among percentages of sperm subpopulations.[a]

Subpopulation	$SP1_{morpho}$	$SP2_{morpho}$	$SP3_{morpho}$
$SP1_{vel}$	0.83**	−0.76*	-0.21^{NS}
$SP2_{vel}$	−0.76*	0.69*	0.21^{NS}
$SP3_{vel}$	-0.61^{NS}	0.58^{NS}	0.07^{NS}

[a] NS, nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

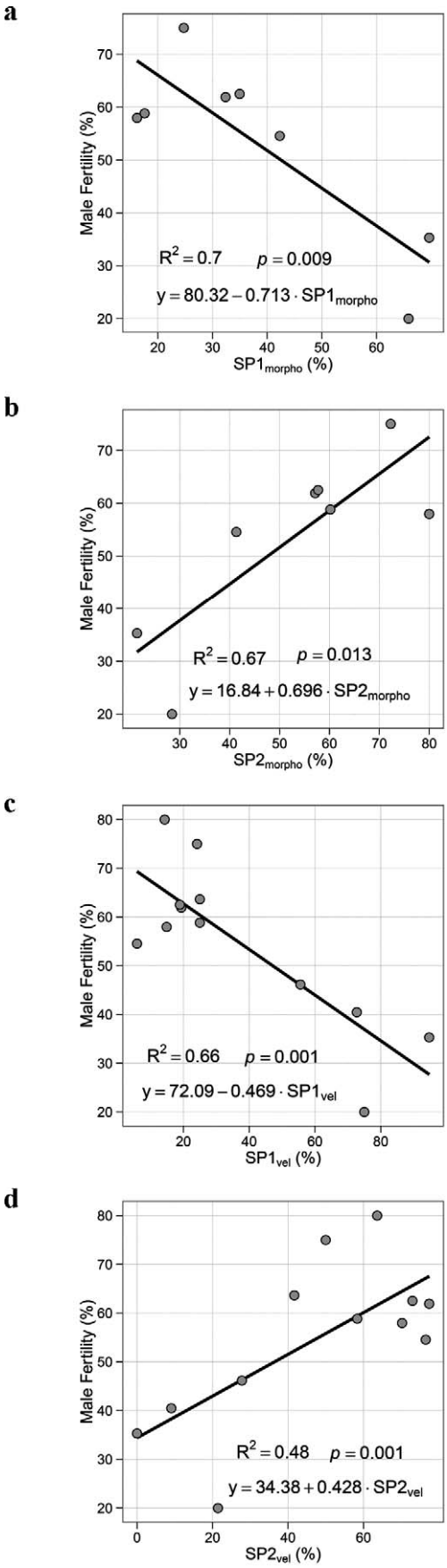

FIG. 3. Relationships between male fertility (number of hinds pregnant/number of hinds inseminated × 100) and head morphometry and velocity of red deer spermatozoa. **a**) Relation between male fertility and the proportion of small and wide ($SP1_{morpho}$) sperm. **b**) Relation between male fertility and the proportion of small and elongated ($SP2_{morpho}$) sperm. **c**) Relation between male fertility and the proportion of slow and nonlinear ($SP1_{vel}$) sperm. **d**) Relation between male fertility and the proportion of fast and linear ($SP2_{vel}$) sperm.

(*Heliocidaris erythrogramma*), a species with large and slow-moving spermatozoa, in others species (i.e., ungulates) spermatozoa are smaller in size and have a very fast movement, which prevents detailed examination of sperm morphometry and velocity in single sperm cells.

The present study examined the relationship between sperm design and velocity by making use of sperm population structure as an indicator of the heterogeneity of semen. Sperm heterogeneity is a widely recognized feature with a key role in the reproductive performance of males [17–19]. Spermatozoa in a single ejaculate have different structural and functional characteristics that may be associated to differences in fertility potential. Here, we characterized sperm subpopulation structure of ejaculates based on sperm head morphometry and sperm velocity, features that were both assessed objectively by means of computer-assisted sperm analyses. Earlier studies that assessed this possible relationship only considered sperm dimensions (length and width) for sperm morphometry [12, 40] and proportions between sperm components (e.g., ratio of HL to HW or of sperm HL to flagellum length) [7]. In our analyses, we included several morphometric parameters not only to quantify sperm head dimensions but also to approximate measures of sperm head shape. Thus, we sought to obtain as much information as possible on sperm morphometry to allow a better characterization of sperm form. Given that relatively subtle differences in sperm design seem to have a great impact upon sperm performance [10], our approach to collect information on sperm size and an estimation of form allowed us to identify relationships between sperm traits and fertility that may not have been detected with the analysis of basic sperm dimensions.

For the characterization of sperm subpopulations, we have taken advantage of a joint analysis of different sperm parameters together with fertility in a supervised learning procedure [23–25]. This methodology has been used previously to understand changes in mouse sperm motility patterns taking place during the transition from progressive to hyperactivated motility during capacitation [28] and to examine differences in red deer motile sperm subpopulation structure occurring as a result of cryopreservation [29]. In the present study, we used this methodology to identify subpopulation structures of both sperm form and velocity that best distinguishes males with low and high fertility.

Earlier attempts to establish relations between sperm design, sperm velocity, and fertility have been limited by the use of average values for these sperm traits. Methods to analyze and quantify ejaculate heterogeneity (i.e., within-male variability) were not available for those studies, and complex relations between sperm form, sperm velocity, and fertility could not be explored. The characterization of sperm subpopulations, as developed in the present study, has proven useful to capture sperm heterogeneity in an objective and accurate analysis that can reveal, if present, relationships between sperm form and velocity. In this way, it is possible to capture the covariance between sperm design and velocity within males, with the opportunity to analyze these traits in the same sperm sample, which thus allowed us to assess the relationships between sperm design and velocity more accurately at the intraspecific

level. This highlights the importance of accounting for all sources of variation in sperm traits given the substantial within- and between-male variance in traits, and it supports the idea that sperm characteristics should be assessed in the same sperm samples when examining sperm design-velocity correlations [15]. In the present study, using information on sperm subpopulation structure, we have shown how sperm design and sperm velocity interrelate and how this subpopulation structure differs between males with different fertility.

Finally, it has been hypothesized that sperm design and velocity are likely to evolve jointly [41], and support for this idea has been presented [9, 10, 13, 14, 42]. The present study, which found a strong association between subpopulation structures defined by sperm morphometry and sperm velocity, adds support to the hypothesis that these traits evolve together. If joint evolution of sperm morphometry and sperm velocity had occurred, we would expect males with high fertility to show higher percentages of spermatozoa that have more elongated heads and that swim faster. Our results revealed that this was, indeed, the case. Previous studies have already found a strong positive correlation between all sperm components and how they evolve in an integrated manner into more elongated spermatozoa [10, 43]. The increase in sperm length has been found to be associated with enhanced swimming velocity and, therefore, higher fertility. The present study also represents a first attempt to uncover the relationships between sperm design and velocity in a species with internal fertilization, thus addressing the question raised by previous work focused on an externally fertilizing species [15]. These two studies have found a strong correlation between sperm characteristics using, in both cases, an approach that allows the evaluation of sperm design and sperm velocity in the same sample.

In conclusion, our approach for capturing within-male sperm heterogeneity through the characterization of sperm subpopulation structure revealed a strong association between sperm head design and velocity and the role that both may play in male fertility. Males with high fertility showed high percentages of spermatozoa with rapid and linear movement that, in turn, were strongly correlated to high percentages of spermatozoa with elongated heads. Analyses of sperm subpopulation structure thus represent an important tool that will contribute to further characterization of ejaculate complexity.

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