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High Levels of DNA Fragmentation in Spermatozoa Are Associated with Inbreeding and Poor Sperm Quality in Endangered Ungulates[1]

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ABSTRACT

Inbreeding is known to cause deleterious effects upon reproduction and survival, but its effects upon sperm DNA integrity have not been examined. In the present study, we analyzed this relationship among three endangered ungulates: *Gazella cuvieri*, *Gazella dama mhorr,* and *Gazella dorcas neglecta*. In addition, we examined whether levels of sperm DNA fragmentation are associated with semen quality. The magnitude of sperm DNA damage in the two species with high levels of inbreeding (*G. cuvieri* and *G. dama mhorr*) was extremely high when compared to the species with low levels of inbreeding (*G. dorcas neglecta*) and to values previously reported for outbred populations. Levels of sperm DNA fragmentation significantly increased with inbreeding and age. Increased DNA damage in sperm was associated with increased sperm head abnormalities, lower percentage of sperm with an intact acrosome, and poor motility. Our findings suggest that the link between inbreeding and semen quality is mediated by the effects of inbreeding upon sperm DNA damage. The deleterious effects of inbreeding upon the paternal genome likely decrease male fertility and may cause genetic damage to future generations. Because inbreeding is common among endangered species, high levels of sperm DNA damage may have considerable impact upon the viability of their populations.

conservation, endangered species, gamete biology, inbreeding, SCSA, semen quality, sperm, sperm DNA fragmentation

INTRODUCTION

Among endangered species, small population size and lack of gene flow lead to increased levels of inbreeding, which have deleterious effects upon the reproduction and survival of individuals. Understanding the full impact of these genetic factors as well as the underlying mechanisms is crucial to minimize their deleterious consequences among endangered species, in which these factors can rarely be avoided. Inbreeding increases genome-wide homozygosity, leading to the expression of deleterious recessive alleles [1], which in turn results in high juvenile mortality [2], low female reproductive success [3], and increased vulnerability to parasites [4, 5].

A number of studies have also revealed links between high levels of inbreeding and poor semen quality, either using the pedigree coefficient of inbreeding [6–8] or by estimating levels of genetic variability [9–12] (for review, see [13]). Inbreeding has also been associated with an increased incidence of cryptorchidism [10] and a reduction in testes size and sperm production [11, 14]. In competitive contexts, inbred males have lower fertilization success than outbred males [15–17]. These studies show that inbreeding influences an array of sperm traits, such as morphology, motility, and acrosome integrity, and that its effects are widespread across taxa. One possible explanation is that the increases in the expression of deleterious recessive alleles associated with inbreeding commonly influence genes regulating sperm formation, energy production, and sperm function. This would imply than despite differences between taxa in reproductive physiology, genetic architecture, and environmental conditions, inbreeding always influences the same reproductive genes, resulting in similar effects upon semen quality. Perhaps a more parsimonious alternative is that inbreeding disrupts some basic cellular mechanism that in turn influences many aspects of sperm performance. The most likely underlying mechanism that could mediate the relationship between high levels of inbreeding and decreased sperm quality is increased levels of sperm DNA fragmentation, which influences many aspects of sperm quality (including morphology, motility, and fertilizing ability) and results in decreased male fertility [18–23].

To our knowledge, no study has examined whether inbreeding influences the integrity of sperm DNA. However, the implications of a potential link between inbreeding and sperm DNA damage are huge, because the integrity of the male genome has a major impact upon the reproductive success of individuals. DNA fragmentation in spermatozoa is associated with poor semen quality, lowered fertilization rates, impaired preimplantation, and poor pregnancy outcomes [18–23]. In addition, it may have further consequences for the offspring when the pregnancy is successful. When spermatozoa carrying damaged DNA do fertilize, such damaged DNA may be repaired by the oocyte, but if this process fails, all cells in the body of the offspring, including the germ line, may be affected, leading to the transmission of mutations to future generations [20, 21, 24].

Male germ cells are particularly susceptible to DNA damage because of the down-regulation of DNA-repair systems during late spermatogenesis and because of progressive loss of the ability to undergo apoptosis [21]. In addition, once released by the germinal epithelium, spermatozoa may spend weeks in the epididymis, where they experience a maturational process and, once ejaculated, have to swim along the female tract and overcome several barriers before reaching the ovum. During

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these processes, sperm lack some of the protective mechanisms that somatic cells have and are vulnerable to DNA damage by a number of environmental factors [20]. It has been proposed that the high degree of DNA compaction within the sperm head has evolved to protect sperm DNA from exogenous assault, but despite these defensive measures, DNA damage seems to be more frequent among spermatozoa than in somatic cells [21].

Several causes for DNA fragmentation are possible, including oxidative stress, abortive apoptosis, incorrect recombination, and defective chromatin packaging [21, 25, 26]. The available evidence suggests that oxidative stress is the most likely cellular pathway generating DNA damage in spermatozoa [20, 25]. However, it is also possible that different causal agents may act at different stages, such as sperm formation in the testes, sperm maturation in the epididymis, or transit along the female tract.

Factors influencing levels of DNA fragmentation are poorly known but include paternal age, exposure to xenobiotics, and male genital tract infection [27, 28]. To our knowledge, the possibility that levels of inbreeding may influence levels of DNA damage during spermatogenesis (affecting sperm formation) has not been explored. Homozygosity in defective alleles of DNA-repair genes may have consequences in terms of the level of DNA strand breaks found in the cells, as evidenced by studies of knockout mice carrying targeted mutations of DNA-repair genes [29]. Thus, the genetic background of the individuals may play an important role in sperm DNA fragmentation, either increasing their susceptibility to suffer damage or diminishing their ability to repair damaged DNA.

The available evidence suggests that increased levels of DNA damage in sperm would lead to decreased male fertility and offspring survival and, thus, to further declines in population size, which could threaten the viability of endangered species. To our knowledge, however, no studies have investigated whether high levels of inbreeding among endangered species are associated with increased DNA damage in sperm. In the present study, we examined the relationships between inbreeding and the degree of sperm DNA fragmentation of three endangered gazelle species for which captive breeding programs have been established. These three species are under different levels of threat: *Gazella dama mhorr* is extinct in the wild (and *Gazella dama* is critically endangered), *Gazella cuvieri* is endangered, and *Gazella dorcas neglecta* is vulnerable [30]. In addition, the size of the founding populations was different for the three species, leading to high levels of inbreeding among *G. cuvieri* and *G. dama mhorr*, whereas levels of inbreeding are low for *G. dorcas neglecta* [31]. By studying three closely related species under the same environmental conditions, we are able to compare populations that are severely endangered and suffering from high levels of inbreeding with others that are under a smaller degree of threat and do not experience high levels of inbreeding.

MATERIALS AND METHODS

Study Populations

The present study was carried out in three species of endangered gazelles—*G. cuvieri* Ogilby, 1841; *G. dorcas neglecta* Lavauden, 1926; and *G. dama mhorr* Bennett, 1833—for which captive breeding programs have been established at the Parque de Rescate de Fauna Sahariana (CSIC), Almeria, Spain. A total of 49 males were included in the present study (14 *G. cuvieri*, 24 *G. dama mhorr,* and 11 *G. dorcas neglecta*).

Pedigree Coefficient of Inbreeding

Pedigree information obtained from the international studbooks of the three species [32–34] was analyzed following the Stevens-Boyce algorithm [35] implemented in PEDSYS software (Southwest Foundation for Biomedical Research). Previous work has shown that the inbreeding coefficients for *G. cuvieri* and *G. dama mhorr* were underestimated during earlier studies in which coefficient of inbreeding was calculated in the traditional way, because their founding populations do not conform to conventional assumptions [31]. In the present study, we use the ''realistic'' coefficient of inbreeding for *G. cuvieri* and *G. dama mhorr*, which was calculated considering that founders were related and had a moderate inbreeding coefficient ($f = 0.125$) (for further details, see [31]).

Males analyzed in the present study are a subsample of individuals included in a previous study that examined the relationships between inbreeding coefficient and individual genetic variability [31]. Levels of inbreeding and genetic variability in this subsample are no different from those in the larger sample, so we are confident that this subsample is representative of the study populations.

Semen Collection and Evaluation

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65, and following guidelines established in the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction. Semen was collected by electroejaculation under surgical anesthesia, and sperm traits were evaluated and quantified as described previously [36]. Briefly, semen volume and wave motion were assessed shortly after collection (within 40–60 min). Sperm concentration was estimated using a hemocytometer. Semen aliquots were diluted in PBS with 5 mg/ml of bovine serum albumin and used to assess individual sperm motility, which was assessed by placing 10 μl of sperm suspension between a glass slide and a coverslip (22 × 22 mm), both prewarmed to 37°C. Percentages of individual and progressively motile sperm were estimated, and quality of motility was assessed using a scale of 0 (lowest) to 5 (highest). This sperm suspension was also used to assess sperm morphology and acrosome integrity, which were determined in smears stained with eosin-nigrosin and Giemsa and examined using bright field. The following semen parameters were thus assessed: percentages of head, midpiece, and principal piece plus terminal piece abnormalities; percentage of spermatozoa with intact acrosomes; and percentages of individual and progressive motility. Because analyses of DNA fragmentation in sperm had to be carried out at a later stage, spermatozoa were stored by cryopreservation using a diluent based on Tes and Tris buffers (TEST) and containing 0.4% glucose, 5% egg yolk, and 6% glycerol as described previously [36].

Analyses of Sperm DNA Fragmentation

Several methods have been developed to assess DNA damage and chromatin integrity [37, 38]. We used the sperm chromatin structure assay (SCSA), a flow cytometry-based method, because it is highly correlated to male fertility and sperm competitive ability [39], is highly repeatable between different measures of the same individual and between fresh and cryopreserved semen, and has been successfully used in a wide range of species [37, 38, 40]. The SCSA method involves denaturing sperm chromatin by exposure to low pH and then staining the treated cells with acridine orange. After the treatment, sperm with double-stranded DNA shows green fluorescence, whereas sperm with single-stranded DNA (i.e., fragmented DNA) shows red fluorescence. The DNA fragmentation index (DFI) is calculated as the ratio of red fluorescence to total fluorescence.

For SCSA analyses, cryopreserved spermatozoa were thawed and diluted in cold (4°C) TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, and 1 mM disodium ethylenediaminetetra-acetic acid; overall, pH 7.4) to a concentration of 1–2 × 10^6 sperm/ml. Analyses were carried out as described previously [38, 39]. The raw data were processed using the SCSAsoft program (SCSA Diagnostics, Inc., Brookings, SD) for statistical analyses. The output was a cytogram of the native DNA versus the fragmented DNA, the total DNA versus the DFI, and a histogram of the DFI frequencies. These measures allowed us to quantify DNA integrity of sperm by calculating the percentage of sperm with a high level of DNA fragmentation in each male's semen sample (%DFI; hereafter referred to as DFI) (Supplemental Fig. S1, available online at www.biolreprod.org). Two samples per individual were analyzed.

Statistical Analyses

To compare levels of DNA fragmentation in the three species under study, we calculated mean DFI as well as range and standard deviation for each species, and we used a one-way ANOVA to test for significant differences between species. DFI was arcsine-transformed to attain a normal distribution

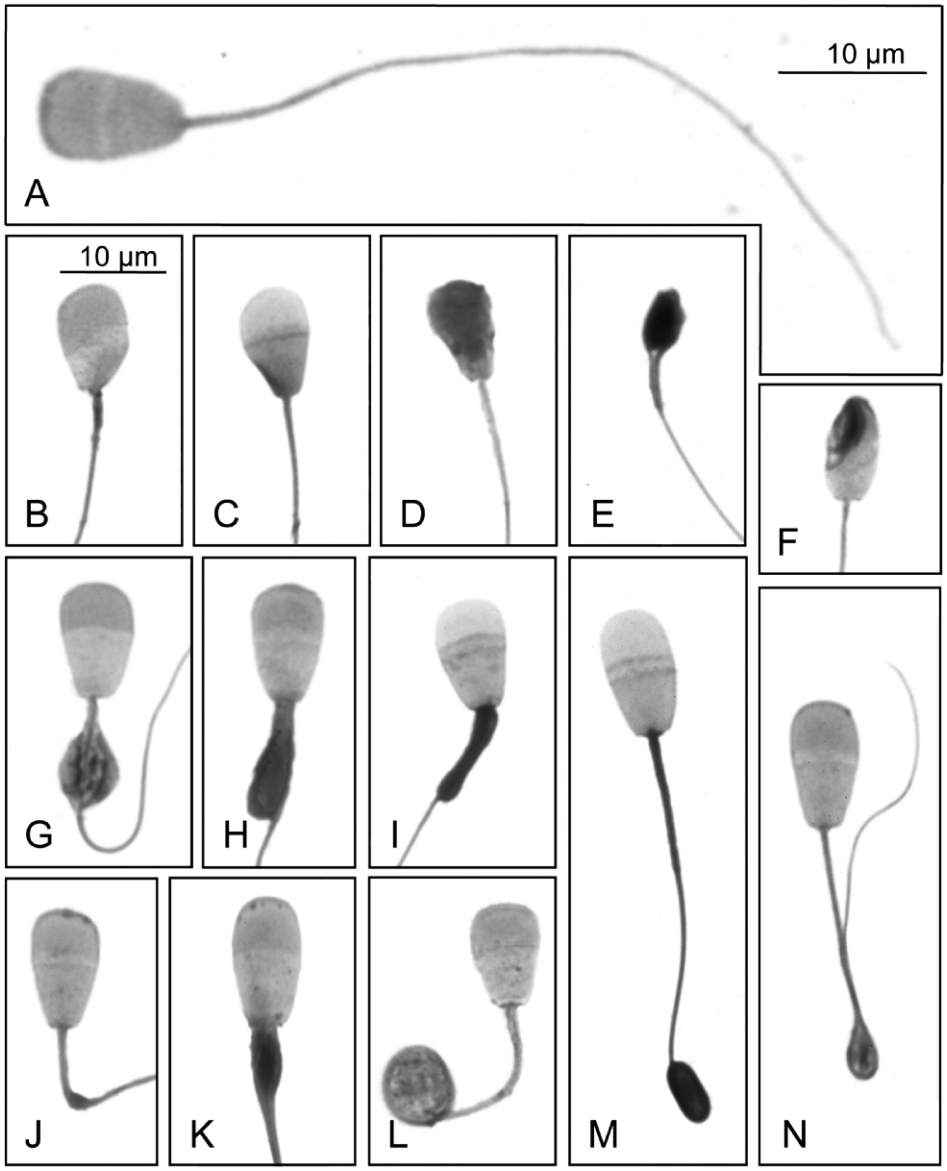

FIG. 1. Sperm abnormalities in gazelles. A normal spermatozoon and abnormalities in the head, midpiece, and principal piece of *Gazella dama mhorr* spermatozoa are shown; abnormalities in the other two species (*Gazella cuvieri* and *Gazella dorcas neglecta*) follow similar patterns. **A**) Normal spermatozoon. **B**–**F**) Sperm head abnormalities. **G**–**K**) Midpiece abnormalities. **L**–**N**) Abnormalities in the sperm principal piece. Bar = 10 μm; spermatozoa in panels **B**–**N** have the same magnification.

(Shapiro-Wilks > 0.05). If a significant difference was found, a Tukey test was used to determine which species had significantly different levels of DNA fragmentation.

We used General Linear Models to analyze factors affecting DNA fragmentation levels in the spermatozoa and the relationship between DNA fragmentation in the spermatozoa and several semen parameters. In both cases, analyses were first carried out for the three species together, so species was included in the model as a fixed factor with three levels. To control for possible differences among species, we included the interaction between species and the main terms as detailed below for each analysis. When the interaction was not significant, we used the simplest additive model, including only the main terms. Otherwise, we repeated the analyses at the intraspecific level. In all the models, we analyzed whether the residuals fitted a normal distribution. If they did not, Monte-Carlo simulations were employed to generate a new distribution to test the significance [41]. The program Matlab 7.0 (The MathWorks, Natick, MA) was used, and the simulations were repeated 1000 times.

Factors affecting DNA fragmentation levels in spermatozoa. The models included DFI as a dependent variable. The independent variables were species,

TABLE 1. Age, inbreeding, and sperm parameters of males of *Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta* used in this study.[a]

	Gazella cuvieri (N = 14)			*Gazella dama* (N = 24)			*Gazella dorcas* (N = 11)		
Parameters	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Age (days)	2229.00	1235.95	879–5033	2484.83	771.78	823–3575	2188.64	1227.81	667–5042
Coefficient of inbreeding	0.31	0.05	0.19–0.39	0.30	0.03	0.27–0.40	0.04	0.04	0.00–0.14
Head abnormalities (%)	8.14	13.44	0–53	7.23	4.39	2–17	3.82	1.54	1–6
Midpiece abnormalities (%)	5.43	5.06	1–21	9.58	8.10	2–28	2.36	1.63	1–6
Principal piece abnormalities (%)	1.57	1.09	0–4	2.38	1.54	1–8	1.18	1.08	0–3
Intact acrosomes (%)	84.69	14.89	39–95	87.26	9.88	63–98	93.43	8.26	76–99
Individual motility (%)	65.36	26.05	5–95	76.46	22.86	25–95	80.00	12.45	60–95
Progressive motility (%)	32.50	28.13	0–80	67.08	23.68	20–90	76.36	14.51	50–95

[a] N = number of males for each species.

TABLE 2. Sperm DFI in endangered gazelles, *Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta*.

Parameter	*G. cuvieri*	*G. dama*	*G. dorcas*
DFI			
Mean ± SEM	22.30 ± 4.49	14.31 ± 3.84	2.57 ± 0.57
SD	16.80	18.83	1.90
Range	7.35–72.22	1.63–64.41	0.58–7.06
DFI interval[a]			
0%–5%	0	29.3	81.8
5%–10%	14.3	37.5	18.2
10%–20%	57.1	16.4	0
20%–30%	7.2	4.3	0
>30%	21.4	12.5	0

[a] Percentage of males in different intervals of DFI values.

age (included because it is known to influence DFI [27]), inbreeding, and the interaction between species and inbreeding.

Relationship between DNA fragmentation in spermatozoa and semen parameters. The models included the seminal parameters as dependent variables and species, DFI, inbreeding, and age as independent variables. The first term interaction between DFI and species was also included. Analyses for each species were carried out, including in the model three independent variables: DFI, inbreeding, and age. For *G. dorcas neglecta*, we carried out simple regressions between each of the seminal parameters and DFI because of the low sample size.

RESULTS

Inbreeding, Age, and Semen Parameters

Both *G. cuvieri* and *G. dama mhorr* are highly inbred compared with *G. dorcas neglecta* (Table 1). In general terms, semen quality was worse in *G. cuvieri* and *G. dama mhorr* and best in *G. dorcas neglecta* (Table 1). In addition, considerable individual variations were found in semen quality. Abnormalities seen in the sperm head, midpiece, and principal piece plus terminal piece are shown in Figure 1. The age of the males sampled did not differ between the three species (Table 1).

Comparison of Levels of DNA Fragmentation

Sperm DFI differed between species ($F_{2,46} = 8.684$, $P < 0.0001$), being higher in *G. cuvieri* and *G. dama mhorr* and lower in *G. dorcas neglecta* (Table 2). The Tukey test revealed that mean DFI was significantly lower for *G. dorcas neglecta* when compared with *G. cuvieri* ($P < 0.001$) and *G. dama mhorr* ($P < 0.05$), but no significant differences were found between *G. cuvieri* and *G. dama mhorr*. These results follow the same pattern as levels of inbreeding.

When individuals were arranged in different categories according to values of sperm DFI, no individuals of the species *G. cuvieri* had DFI levels below 5%, and 21% of the males analyzed had DFI values over 30%. For the species *G. dama mhorr,* more than 30% of males had DFI values above 10%, and 12% of males had DFI values over 30%. In contrast, for the species *G. dorcas neglecta,* no individual had DFI values over 8% (Table 2).

Factors Affecting Levels of DNA Fragmentation

We analyzed the effects of inbreeding upon DFI, taking into account the potential effects of age and species (Table 3). The first term interaction between species and inbreeding was not significant and, therefore, was taken out of the model (species × inbreeding: $F_{2,42} = 0.105$, $P > 0.1$). The results showed that both inbreeding and age had a significant effect upon DFI but that species did not (Table 3). Although the factor species was not significant, it was kept in the model to control for its effect.

TABLE 3. GLMs showing the relationship between sperm DFI and inbreeding, and age (independent variables) for *Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta*.

Parameter	*df*	*F*	R^{2a}	P^b
Inbreeding	1,44	4.20	0.05	<0.05
Age	1,44	4.32	0.05	<0.05
Species	2,44	2.73	0.07	ns

[a] The proportion of the total variance explained by each variable included in the model.
[b] Values are shown as nonsignificant (ns), $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Relationship Between DNA Fragmentation Levels in Spermatozoa and Semen Parameters

The relationship between sperm DNA fragmentation and semen traits was first analyzed considering the three species together. A significant relationship was found between DFI and percentage of sperm with head abnormalities, percentage of spermatozoa with intact acrosome, and percentage of spermatozoa with individual and progressive motility (Table 4). In all cases, only DFI was significant, and no effect of inbreeding or age was detected. No significant relationships were found between DFI and abnormalities in midpiece and in principal piece plus terminal piece. The interaction between species and DFI was significant for percentage of sperm head abnormalities, suggesting that the relationship between this trait and DFI varied between species (Table 4). In contrast, the interaction between DFI and species was not significant for percentage of spermatozoa with intact acrosome and percentage of spermatozoa with individual and progressive motility ($P > 0.05$), so the simplest additive model was chosen (Table 4). The factor species had a significant effect upon percentage of sperm head abnormalities and percentage of progressive motility.

TABLE 4. Relationship between sperm DFI and different semen traits for *Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta*.

Parameters	*df*	*F*	R^{2a}	P^b
Sperm head abnormalities (%)				
DFI	1,41	7.809	0.057	<0.01
Inbreeding	1,41	0.076	0.000	ns
Age	1,41	0.818	0.006	ns
Species	2,41	27.148	0.396	<0.001
Species × DFI	2,41	28.489	0.415	<0.001
Intact acrosome (%)[c]				
DFI	1,35	36.116	0.447	<0.001
Inbreeding	1,35	0.115	0.001	ns
Age	1,35	1.879	0.023	ns
Species	2,35	1.035	0.025	ns
Individual motility (%)[c]				
DFI	1,43	47.482	0.434	<0.001
Inbreeding	1,43	1.423	0.000	ns
Age	1,43	0.037	0.000	ns
Species	2,43	1.750	0.032	ns
Progressive motility (%)[c]				
DFI	1,43	17.875	0.182	<0.001
Inbreeding	1,43	0.036	0.000	ns
Age	1,43	1.043	0.010	ns
Species	2,43	6.027	0.123	<0.01

[a] The proportion of the total variance explained by each variable included in the model.
[b] Values are shown as nonsignificant (ns), $P < 0.05$, $P < 0.01$, or $P < 0.001$.
[c] Results of the simplest additive model, after removing the non-significant interaction between DFI and species, are shown.

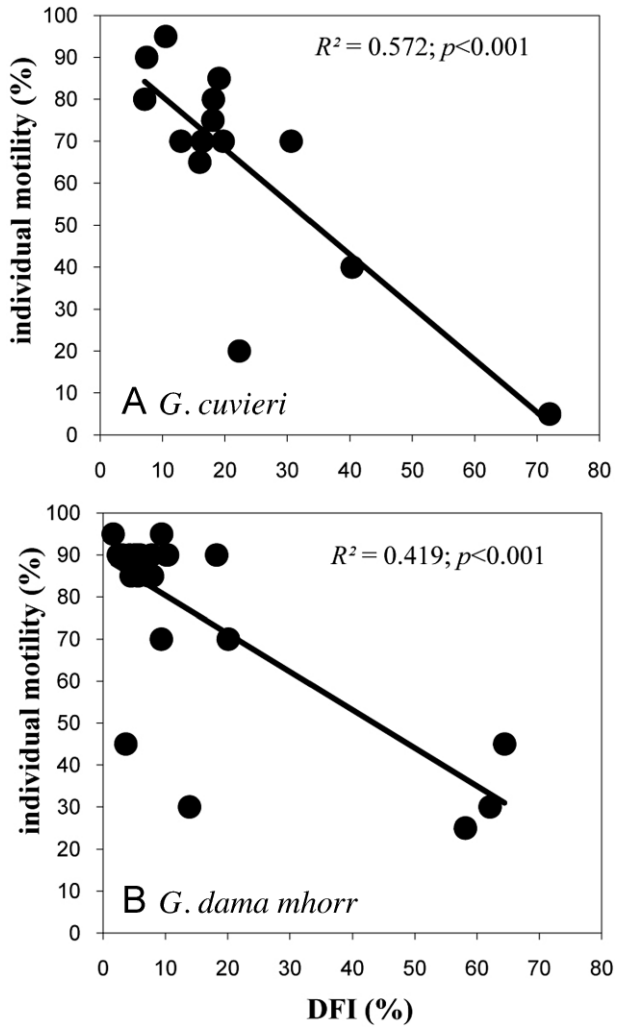

FIG. 2. Relationship between sperm DFI and percentage of sperm with individual motility for *Gazella cuvieri* (**A**) and *Gazella dama mhorr* (**B**).

Because a significant interaction was found between species and DFI for percentage of sperm head abnormalities, we carried out the analyses for each species separately. In *G. cuvieri,* high levels of DFI were associated with a reduction in the percentage of spermatozoa with intact acrosome and sperm motility (Fig. 2A) and with an increase in percentage of sperm head abnormalities (Table 5). No effect of inbreeding or age was found on these sperm traits when they were in the same model as DFI.

For *G. dama mhorr,* an increase in DFI was associated with a reduction in the percentage of spermatozoa with intact acrosomes and a reduction in motility (both progressive and individual) (Table 5 and Fig. 2B). No effect of inbreeding or age was found for *G. dama mhorr* in the semen traits that were associated to DFI (Table 5).

In *G. dorcas neglecta,* the model with three variables did not show any significant effect (probably because of the low sample size). However, when the model was fitted including DFI only, it was negatively related to individual motility ($F_{1,9} = 5.71$, $P < 0.05$). No other variable showed any significant relationship with DFI.

TABLE 5. Relationship between sperm DFI and different semen traits in *Gazella cuvieri* and *G. dama mhorr.*

Parameters	*df*	*F*	R^{2a}	P^b
G. cuvieri				
Sperm head abnormalities (%)				
DFI	1,10	63.591	0.695	<0.001
Inbreeding	1,10	0.212	0.002	ns
Age	1,10	0.458	0.005	ns
Intact acrosome (%)				
DFI	1,9	14.974	0.455	<0.01
Inbreeding	1,9	0.667	0.020	ns
Age	1,9	0.246	0.007	ns
Individual motility (%)				
DFI	1,10	19.794	0.540	<0.01
Inbreeding	1,10	0.050	0.001	ns
Age	1,10	0.859	0.023	ns
G. dama mhorr				
Intact acrosome (%)				
DFI	1,17	28.516	0.612	<0.001
Inbreeding	1,17	0.131	0.002	ns
Age	1,17	0.318	0.007	ns
Individual motility (%)				
DFI	1,20	20.026	0.399	<0.001
Inbreeding	1,20	0.005	0.000	ns
Age	1,20	2.097	0.041	ns
Progressive motility (%)				
DFI	1,20	15.977	0.379	<0.001
Inbreeding	1,20	0.123	0.003	ns
Age	1,20	1.103	0.026	ns

[a] The proportion of the total variance explained by each variable included in the model.
[b] Values are shown as nonsignificant (ns), $P < 0.05$, $P < 0.01$, or $P < 0.001$.

DISCUSSION

The present study has revealed a link between levels of inbreeding and levels of sperm DNA fragmentation among endangered ungulates. We have studied three species of gazelles from North Africa (*G. cuvieri*, *G. dama mhorr,* and *G. dorcas neglecta*) for which captive breeding programs were established more than 30 yr ago. Current populations of these three species differ in their levels of inbreeding because of differences in the size of the founding populations, in the degree of threat suffered by natural populations at the time the captive breeding programs started, and in the degree of admixture throughout the captive breeding program [31]. In these three species, levels of inbreeding and levels of sperm DNA fragmentation follow the same pattern: high in *G. cuvieri* and *G. dama mhorr*, and low in *G. dorcas neglecta*. When the three species are analyzed in the same model, age and inbreeding have a significant effect upon levels of sperm DNA fragmentation, but the factor species does not. Furthermore, because the interaction between inbreeding and species was not significant, we conclude that the relationship between inbreeding and DFI is similar for the three species.

We also analyzed the relationships between levels of sperm DNA fragmentation and semen traits. Models in which the three species were included showed significant relationships between DFI and sperm morphological abnormalities and motility. Our findings agree with those of previous studies showing a link between sperm DNA fragmentation and these sperm traits [19, 27, 42, 43] but only weak relationships with other semen traits (for review, see [21]). The significant interaction between species and DFI for one sperm trait implied that the relationship between sperm DFI and semen parameters differed between species. Intraspecific analyses revealed that in species with high inbreeding and high levels of DNA damage (*G. cuvieri* and *G. dama mhorr*), the latter is strongly associated with a decrease in several sperm traits, whereas in the species with low levels of inbreeding and low levels of DNA damage in sperm (*G. dorcas neglecta*), only a weak

association between sperm DNA fragmentation and sperm motility was found.

One possible explanation is that inbreeding may influence levels of sperm DNA damage and other sperm traits simultaneously, as suggested by evidence showing that factors which damage sperm DNA, such as chemical genotoxic agents and heat stress, also cause changes in sperm head morphology [44–46]. Our findings, however, suggest an alternative pathway. When inbreeding and age were considered jointly with levels of sperm DNA fragmentation, significant relationships were only found between sperm DNA damage and sperm morphology and motility. This suggests that the link between inbreeding and poor semen quality may be mediated by sperm DNA fragmentation. In other words, inbreeding may affect sperm DNA integrity, giving rise to changes in sperm head morphology and sperm motility [47]. This would explain why inbreeding is so often found to be associated with a decrease in the proportion of normal sperm and motile sperm but less so with other sperm traits [13].

At this stage, we can only speculate about the mechanisms through which inbreeding may lead to increased sperm DNA damage. The most important factor protecting sperm DNA from damage is the high degree of condensation within the sperm head. This is achieved by a complex process that occurs during the final postmeiotic phases of spermatogenesis, when histones are replaced first by transition proteins and then by protamines [48]. The end result is that DNA within the sperm head achieves a much higher degree of compaction than it does in somatic cells. The increased expression of deleterious recessive alleles could lead to errors in this process, leading to inefficient DNA packaging within the sperm head that in turn would increase levels of sperm DNA damage and affect sperm performance. In particular, problems associated with DNA compaction are known to influence the sperm head, leading to abnormal morphologies that influence acrosome integrity and decrease sperm swimming performance along the female tract [48].

The potential consequences of the effects of inbreeding upon sperm DNA damage and semen quality are twofold. On the one hand, increases in sperm DNA fragmentation result in decreased male fertility [37, 21, 39, 49]. In humans, males who exceed 30% DFI suffer a considerable reduction in fertility [37, 39, 49]; however, these studies were carried out on patients attending fertility clinics and, therefore, the samples may be biased. In other species, the subfertility threshold seems to be lower. Male fertility declines when DFI values are more than 10–20% in bulls and more than 8% in boars (for review, see [39]). The two species of endangered ungulates with high levels of inbreeding included in the present study showed much higher levels of sperm DNA damage than those reported for domestic ungulates, with male *G. cuvieri* having an average DFI of 22% and *G. dama mhorr* an average DFI of 14%. In contrast, *G. dorcas,* which has low levels of inbreeding, had an average DFI of 3%. Furthermore, in *G. cuvieri*, 85% of the individuals in the present study had a DFI higher than 10%, whereas in *G. dama mhorr,* 33% of the individuals had values above this threshold. In contrast, in *G. dorcas neglecta,* no individual had levels of DNA fragmentation over 8%. Extreme values reached 72% DFI among *G. cuvieri* males and 64% DFI among *G. dama mhorr* males. The magnitude of the level of sperm DNA damage found in the species with high levels of inbreeding is thus enormous when compared to the levels in outbred populations. Such high levels of sperm DNA fragmentation thus are likely to have a considerable impact upon male fertility.

On the other hand, males with high levels of sperm DNA damage may fertilize under optimal conditions (e.g., given enough time and repeated sexual access to females) and in the absence of competition from other males [50, 51], as is the case in captive breeding programs. In these cases, the damage in sperm DNA may result in deleterious effects upon offspring [20, 21]. Previous studies of inbreeding have rarely considered the possibility that inbreeding in males may affect offspring viability, because most studies have focused on the effects of maternal inbreeding [2, 52, 53].

In conclusion, the present study highlights the important role that the integrity of the paternal genome may play when levels of inbreeding are high, as is commonly the case among endangered species. Increased levels of sperm DNA damage may both reduce male fertility and result in the transmission of genetic disease to offspring. These effects may be exacerbated among polygynous species, because each reproductive male will have a considerable impact upon the reproduction of several females. As male fertility decreases and offspring mortality rates increase, the decline in population size will accelerate, potentially leading such populations to an extinction vortex.

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