Paternal levels of DNA damage in spermatozoa and maternal parity influence offspring mortality in an endangered ungulate

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Understanding which factors influence offspring mortality rates is a major challenge since it influences population dynamics and may constrain the chances of recovery among endangered species. Most studies have focused on the effects of maternal and environmental factors, but little is known about paternal factors. Among most polygynous mammals, males only contribute the haploid genome to their offspring, but the possibility that sperm DNA integrity may influence offspring survival has not been explored. We examined several maternal, paternal and individual factors that may influence offspring survival in an endangered species (*Gazella cuvieri*). Levels of sperm DNA damage had the largest impact upon offspring mortality rates, followed by maternal parity. In addition, there was a significant interaction between these two variables, so that offspring born to primiparous mothers were more likely to die if their father had high levels of sperm DNA damage, but this was not the case among multiparous mothers. Thus, multiparous mothers seem to protect their offspring from the deleterious effects of sperm DNA damage. Since levels of sperm DNA damage seem to be higher among endangered species, more attention should be paid to the impact of this largely ignored factor among the viability of endangered species.

Keywords: sperm DNA damage; offspring mortality; parity; endangered species

1. INTRODUCTION

A major issue in evolutionary and conservation biology is to understand which factors influence offspring mortality rates because these have a major impact upon male and female lifetime reproductive success, population dynamics and may hinder the chances of recovery among declining populations (Clutton-Brock *et al.* 1988; Caughley & Gunn 1996). Among polygynous mammals, such as most ungulates, long-term studies have identified environmental and maternal factors that influence offspring survival rates through their effects on levels of investment during pregnancy and lactation (Clutton-Brock *et al.* 1982, 1988; Festa-Bianchet *et al.* 1995; Clutton-Brock & Pemberton 2004). However, although males differ to a large extent in the proportion of their offspring that survive (Clutton-Brock *et al.* 1988), the paternal factors that may play a role are poorly understood since in most mammalian species, males do not invest in offspring beyond fertilization.

The contribution of the paternal genome to offspring viability may depend on levels of sperm DNA integrity. However, despite growing concerns about potential deleterious effects of sperm DNA damage in offspring, the

evidence remains weak and is mostly indirect. Studies in humans have shown that factors that affect sperm DNA integrity (such as exposure to xenobiotics, smoking and old age) also increase the incidence of certain childhood diseases (Fraga *et al.* 1996; Ji *et al.* 1997; Wyrobeck *et al.* 2006; reviewed in Aitken *et al.* 2004, 2009). However, a direct link between differences in levels of DNA damage between males and the viability of their offspring has not been established.

Spermatozoa are highly susceptible to DNA damage despite the evolution of protective mechanisms, such as the high degree of condensation of the DNA within the sperm head (Lewis & Aitken 2005). Sperm seems particularly vulnerable to DNA damage compared with other types of cell owing to the progressive loss of the ability to undergo apoptosis and the decrease in the regulation of DNA-repair systems that occurs during spermatogenesis, as well as the challenging environments faced by sperm in the male and female reproductive tracts (Aitken *et al.* 2004; Lewis & Aitken 2005). Oxidative stress, abortive apoptosis, incorrect recombination and/or defective chromatin packaging have been identified as the main causes of DNA fragmentation (Laberge & Boissonault 2005; Lewis & Aitken 2005).

The effects of sperm DNA fragmentation upon male fertility have been widely studied among humans and domestic species. Previous studies have reported a link

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between high values of sperm DNA fragmentation and poor semen quality, lowered fertilization rates, impaired preimplantation and poor pregnancy outcomes (Aitken & Krausz 2001; Singh *et al.* 2003; Aitken *et al.* 2004; Lewis & Aitken 2005; Fatehi *et al.* 2006; Lin *et al.* 2008; Zini *et al.* 2008). However, males with low fertility may enhance their chances of achieving fertilization under optimal conditions. When spermatozoa carrying damaged DNA do fertilize, and such damaged DNA fails to be correctly repaired by the oocyte, it may result in all cells in the body of offspring being affected, including the germ line, leading to the transmission of mutations to future generations (Shimura *et al.* 2002; Aitken *et al.* 2004; Lewis & Aitken 2005).

Since sperm DNA integrity may influence male fertilization success and offspring survival, its effects upon the viability of endangered populations could be far-reaching. For endangered species, captive breeding programmes generally provide males with ideal conditions for reproduction, such as exclusive sexual access to several females for long periods of time and, thus, males carrying spermatozoa with fragmented DNA may improve their chances of reproducing successfully. These conditions offer a unique opportunity to study the effects of sperm DNA integrity upon offspring survival, since males with a wide range of sperm DNA damage will sire offspring.

In this study, we analyse the factors that influence offspring survival rates in *Gazella cuvieri*, an endangered species for which a captive breeding programme was started over 30 years ago. Detailed records have been kept, which allow the study of paternal and maternal factors that may influence offspring viability. Since these populations are under no food limitation or environmental stress, we can assume that these effects are minimized. The captive population of *G. cuvieri* had a small founding population and suffers from inbreeding depression (Roldan *et al.* 1998; Gomendio *et al.* 2000), so it can be considered representative of other endangered mammals.

2. MATERIAL AND METHODS

(a) Study population

This study was carried out on the endangered gazelle *G. cuvieri* (Ogilby 1841) for which a captive breeding programme has been established at the Parque de Rescate de Fauna Sahariana (CSIC) (Spain). Owing to the small size of the founding population, levels of inbreeding are high and semen quality shows clear signs of inbreeding depression (Roldan *et al.* 1998; Ruiz-López *et al.* 2009). For the present study, the life-history records and reproductive data were obtained from the international studbook (Moreno & Espeso 2008), which contains detailed information of all births, deaths and parentage from 1975 until 2008.

Since *G. cuvieri* is a polygynous species, the individuals are distributed in three different types of herds: (i) reproductive herds, (ii) bachelor groups, and (iii) solitary males. The first type of social group consists of about 10 individuals, one being an adult male and the others adult females with their offspring. The young males are removed from the group where they are born when they are around six months old to be placed in the bachelor groups. Since reproductive herds have only one reproductively mature male with each group of females, paternity assignment is likely to

be accurate. At birth, individuals are marked with ear tags, which make individual recognition possible.

(b) Juvenile survival

Offspring survival was assessed for 106 individuals born between 1992 and 2007. We analysed survival during the first year of life. We divided this period into three time intervals: (i) from birth to one month, (ii) from one month to six months, and (iii) from six months to 1 year, and calculated mortality rates in each period. Since offspring mortality rates were much higher during the first month of life than later on, we compared calves that survived with calves that died during their first month of life.

(c) Sperm DNA fragmentation

Semen samples were obtained during the breeding season from reproductively mature males as described (Garde *et al.* 2003). Semen was collected by electroejaculation under surgical anaesthesia. Sperm DNA damage was evaluated using the sperm chromatin structure assay (SCSA) (Evenson & Wixon 2006; Evenson *et al.* 2007) using cryopreserved spermatozoa. The SCSA is a flow cytometry method that involves denaturing sperm chromatin by exposure to low pH and then staining the treated cells with acridine orange. This assay is based on the differences in the fluorescence of acridine orange after binding to double- and single-stranded DNA. Sperm with double-stranded DNA shows a green fluorescence and sperm with single-stranded DNA a red fluorescence after staining with acridine orange. The percentage of spermatozoa with high levels of red fluorescence (fragmented DNA) and high levels of green fluorescence (immature spermatozoa) were quantified. The DNA fragmentation index (DFI) was calculated as the ratio of red fluorescence to total fluorescence. The raw data were processed using the SCSASOFT program for statistical analyses. The percentage of sperm with a high DFI in each male's semen sample was calculated (%DFI). For each individual, two samples from a single ejaculate were analysed and, since they were highly consistent, the mean was used as the final DFI value.

(d) Non-genetic terms

We included in our analysis variables that have already been shown to influence offspring survival.

- *Parity* (categorical variable, two levels). Primiparous mothers tend to invest less in offspring, which, as a consequence, suffer slower growth rates and higher mortality rates than offspring born to multiparous mothers (red deer, Clutton-Brock *et al.* 1983, 1987; Soay sheep, Clutton-Brock & Pemberton 2004).
- *Calf sex* (categorical variable, two levels). Among sexually dimorphic mammals, males tend to suffer higher mortality rates than females (Clutton-Brock *et al.* 1988; Clutton-Brock & Pemberton 2004). The mortality rate of males in this population is slightly higher than that of females during the first year of life (Moreno & Espeso 2008).
- *Litter size and sex* (categorical variable, five levels). When females produce singletons and twins, offspring mortality rates tend to be lower among singletons and, among twins, they depend on the sex ratio (Cassinello & Gomendio 1996; Clutton-Brock & Pemberton 2004; Korsten *et al.* 2009). In the study population, 39.11 per cent of the births were twins (Moreno & Espeso 2008).

This variable included the following categories: female, male, female–female, male–female and male–male.

- *Inbreeding coefficient of the calf, mother and father.* High levels of inbreeding are associated with an increase in offspring mortality rates (Ralls *et al.* 1979; Coltman *et al.* 1998; Coulson *et al.* 1999; reviewed in Keller & Waller 2002). In the population under study, high levels of inbreeding are associated with a decrease in sperm quality (Roldan *et al.* 1998; Gomendio *et al.* 2000) and a reduction in longevity (Cassinello 2005). Previous work has shown that the inbreeding coefficients for the species *G. cuvieri* were underestimated in previous studies in which coefficient of inbreeding was calculated in the traditional way, because its founding population does not conform to conventional assumptions about founders (Ruiz-López *et al.* 2009). In this study, we used the ‘realistic’ coefficient of inbreeding for *G. cuvieri*, which was calculated considering that founders were related and had a moderate inbreeding coefficient ($f = 0.125$) (for further details, see Ruiz-López *et al.* 2009). Pedigree information was analysed following the Stevens–Boyce algorithm (Boyce 1983) implemented in PEDSYS software (Southwest Foundation for Biomedical Research, San Antonio, TX, USA).
- *Age of the mother and the father.* Offspring born to young and old parents are less likely to survive (Guinness *et al.* 1978; Clutton-Brock 1984; Aitken *et al.* 2004). Parents’ age at the time of conception was calculated by subtracting the duration of gestation (165 days) from the age of parents when the calf was born (Moreno & Espeso 2008).

(e) Statistical modelling of survival

We used generalized linear models with a logit link function and a binomial error to examine the terms that affected juvenile survival. The significance of the DNA fragmentation levels was assessed in a model including the non-genetic terms. This model included also the interaction between maternal parity and sperm DFI, since the transmission of DNA damage to offspring also depends on the ability of the oocyte to repair, which is condition dependent (Agrawal & Wang 2008). The significance of terms was assessed by their Wald statistics, which are distributed as a χ^2 . We used STATISTICA 6.0 (StatSoft 2001) for all analyses.

3. RESULTS

During the first year of life, offspring mortality rates were highest during the first month (23.6%), decreased substantially during the following six months (7.6%) and were even lower during the second half of the first year (1.9%). Thus, we concentrated on examining which factors explain offspring mortality rates during the first month of life.

Primiparous females in the study were significantly younger than multiparous females (ANOVA $F_{1,104} = 59.3904$; $p < 0.001$; mean $\pm$ s.e.m.: primiparous = 836.35 ± 172.19 days; multiparous = 2363.84 ± 98.16 days). Thus, primiparous females were between 2 and 3 years old, and multiparous between 6 and 7 years old.

For females, mean reproductive age was 5 years, but there were females reproducing before they reached their first year of life up to 12 years old (age at conception: mean age $\pm$ s.e.m. = 1989.17 ± 106.38 days,

Table 1. Generalized linear model for offspring mortality ($n = 106$). (d.f. (degrees of freedom), Wald statistics and p -values are shown for each independent variable included in the model. Significant p -values are shown in bold with a superscript indicating: * $p < 0.05$ and ** $p < 0.01$.)

	d.f.	Wald statistic	p -value
sex	1	0.313586	0.575488
parity	1	5.059727	0.024488*
litter size and sex	4	2.787820	0.593937
inbreeding	1	0.006105	0.937723
inbreeding father	1	2.142742	0.143246
inbreeding mother	1	0.237267	0.626187
DFI	1	7.698769	0.005526**
father age	1	1.352828	0.244784
mother age	1	1.182324	0.276883
parity*DFI	1	5.020423	0.025050*

range 208–4530 days). For males, mean reproductive age was also 5 years, and males sired offspring from the age of 2 years up to 14 years (age at conception: mean age $\pm$ s.e.m. = 2120.47 ± 79.47 days, range 735–5430 days).

Gazella cuvieri females may give birth to singletons or twins, and the percentage of each type of litter was: 31.13 per cent single females, 19.81 per cent single males, 11.32 per cent female–female, 15.09 per cent male–male and 22.64 per cent female–male.

Inbreeding coefficient of calves ranged between 0.31 and 0.50 (mean inbreeding $\pm$ s.e.m. = 0.352 ± 0.004). Mean coefficient of inbreeding for fathers was 0.28 (mean inbreeding $\pm$ s.e.m. = 0.279 ± 0.006 , range 0.1875–0.354) and for mothers was 0.36 (mean inbreeding $\pm$ s.e.m. = 0.358 ± 0.005 , range 0.1948–0.445).

In this study sample, *G. cuvieri* males had average values for sperm DNA fragmentation of 19 per cent (mean % DFI $\pm$ s.e.m. = 19.01 ± 0.74), and there was considerable variation between males (range: 7.35–30.83% DFI). There were no males with DFI levels below 5 per cent, most males (77.7%) had DFI values over 10 per cent and a substantial proportion of males (22%) had DFI values over 20 per cent.

In order to understand which factors influence offspring mortality rates in this population, we analysed together the following variables: offspring sex, maternal parity, litter size and sex, inbreeding (mother, father and offspring), age of the mother and the father, and degree of sperm DNA fragmentation. The results of this analysis show that offspring survival was significantly associated with paternal sperm DFI, maternal parity and the interaction between these two variables (table 1). Thus, a higher percentage of calves that died were born to fathers with higher DFI (figure 1). In addition, calves were more likely to die if born to primiparous than to multiparous mothers. A detailed analysis of the interactions showed that calves born to primiparous females were more likely to die if their father had high levels of sperm DNA fragmentation than those born to fathers with low levels of DNA fragmentation. By contrast, calves born to multiparous mothers were more likely to survive, but levels of paternal sperm DNA damage did not seem to have a significant influence. Thus, primiparous mothers suffered a much larger increase in offspring mortality rates when they

Figure 1. Mortality rates for offspring born to fathers with high (greater than 15%) and low (less than 15%) levels of sperm DNA fragmentation. Sample sizes are shown above bars.

reproduced with males with high levels of sperm DNA damage than did multiparous mothers.

4. DISCUSSION

Our findings reveal a major impact of paternal sperm DNA integrity upon offspring survival. In addition, our results show that maternal parity also has a significant effect upon offspring mortality and, more importantly, that it interacts with paternal sperm DNA integrity. This study was carried out on an endangered species that has already been shown to suffer from inbreeding depression (Roldan *et al.* 1998; Gomendio *et al.* 2000) and for which a captive breeding programme has been established where environmental stress is minimal. When all maternal, paternal and individual factors known to influence offspring mortality were taken into account, levels of paternal sperm DNA damage had the greatest effect upon offspring survival, followed by maternal parity and the interaction between both. By contrast, no significant effects upon offspring mortality rates were found for litter size and sex, levels of inbreeding (father, mother and calf) and age of the parents.

Levels of sperm DNA damage in our sample of reproductive *G. cuvieri* males are higher than values reported for non-endangered species. Among domestic ungulates, both bulls and boars have mean levels of sperm DNA fragmentation that are lower than those found among *G. cuvieri* males, and the maximum values reported for these species are similar to the mean value for *G. cuvieri* (Rybar *et al.* 2004). Furthermore, male fertility declines when the proportion of sperm DNA fragmentation (%DFI) is above 8 per cent in boars and 10–20% in bulls (reviewed in Evenson & Wixon 2006). However, in the study population, *G. cuvieri* males with high levels of sperm DNA fragmentation sired offspring, showing that under optimal conditions, such as repeated and exclusive sexual access to females, such males can achieve successful pregnancies.

Given that males with different levels of sperm DNA damage are able to reproduce in this population, it

represents an ideal model to study its effects upon offspring survival. So far, indirect evidence comes from studies on humans where it has been argued that factors which influence sperm DNA integrity also seem to be associated with an increase in certain childhood diseases (reviewed in Aitken *et al.* 2004, 2009). Our results show that offspring born to males with high levels of sperm DNA damage were more likely to die during the first month of life, strongly suggesting that genetic damage was transmitted to offspring, and that these mutations made offspring less likely to cope with the demands associated with early development.

Maternal parity also had a significant effect upon offspring survival, in line with previous work showing that offspring born to primiparous mothers are less likely to survive because their small body size, poor body condition and the requirements of their own growth, result in lower levels of investment in their offspring during pregnancy and lactation (Clutton-Brock *et al.* 1982; Gomendio 1989; Festa-Bianchet *et al.* 1995; Clutton-Brock & Pemberton 2004). Furthermore, levels of paternal sperm DNA damage and maternal parity interacted, so that offspring born to primiparous mothers suffered higher mortality rates if their fathers had high levels of sperm DNA damage than if they had low levels, but offspring born to multiparous mothers did not show differences in survival rates according to their fathers' levels of sperm DNA damage. Recent work has shown that females in better physical condition have oocytes that are better at repairing DNA damage (Agrawal & Wang 2008), and this might be the case among multiparous mothers. An alternative explanation is that young females have oocytes that are not mature enough and lack the appropriate repair mechanisms. Full developmental competence of the oocytes requires a series of nuclear and ooplasmic changes. Different studies in prepubertal oocytes have established that these modifications include organelle redistribution, changes in activity of mitogen-activated protein kinase and maturation-promoting factor, development of the Ca^{2+} release mechanism, capacity to decondense the chromatin of the fertilizing sperm and appropriate levels of genome methylation (O'Brien *et al.* 1996; Salamone *et al.* 2001; Leoni *et al.* 2006; Ptak *et al.* 2006). Thus, our findings strongly suggest that the extent of genetic damage transmitted to offspring is the result of a combination of two factors: the levels of sperm DNA damage and the ability of the female to repair such damage. Multiparous mothers seem able to protect their offspring from paternal sperm DNA damage while primiparous mothers are not.

Our findings have major implications. First, assisted reproductive techniques are being increasingly used in humans (to allow patients of fertility clinics to reproduce), and among endangered species (to facilitate gene flow between isolated populations, see Roldan & Gomendio 2008). Because these techniques bypass processes of selection in the female reproductive tract against low-quality sperm, the use of these techniques may increase the chances that sperm with high levels of DNA damage will fertilize, potentially leading to the transmission of genetic disease to offspring. Among humans, infants born by assisted conception are more likely to suffer health problems (e.g. Hansen *et al.* 2008). More specifically, a recent meta-analysis has found evidence that,

when assisted reproduction techniques are used among infertile patients, DNA damage in spermatozoa is associated with increased rates of pregnancy loss (Zini *et al.* 2008). Thus, levels of DNA damage should be evaluated to prevent these undesirable consequences.

Second, captive breeding programmes should balance the advantages of reproducing as many individuals as possible, to avoid inbreeding and loss of genetic variability, and the avoidance of the spread of genetic damage to future generations. Among endangered species, males should be tested for levels of sperm DNA damage in order to incorporate this information in the decision-making process about which individuals are allowed to reproduce, and to be able to choose the appropriate partners since primiparous females and low-condition females seem more prone to transmit mutations.

Finally, levels of sperm DNA damage seem to be more pronounced among endangered species and are therefore likely to have a major impact on their chances of recovery through its effect on offspring survival. It is likely that the effects of paternal sperm DNA integrity on offspring survival will be even more acute under natural conditions where females are likely to be under worse physical conditions owing to environmental stress and food constraints. More attention should be paid to the impact of paternal sperm DNA integrity upon the viability of wild populations of endangered species.

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