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Inbreeding, fluctuating asymmetry, and ejaculate quality in an endangered ungulate

E. R. S. Roldan[1], J. Cassinello[2], T. Abaigar[3] and M. Gomendio[2*]

[1]*Instituto de Bioquímica (CSIC-UCM), Ciudad Universitaria, 28040-Madrid, Spain*
[2]*Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), c/José Gutierrez Abascal 2, 28006-Madrid, Spain*
[3]*Estación Experimental de Zonas Aridas (CSIC), c/General Segura 1, 04001-Almería, Spain*

An ever-increasing number of species are suffering marked reductions in population size as a consequence of human activities. To understand the impact of these changes it is essential to assess how small population size affects individual fitness and the viability of populations. This issue acquires special relevance among endangered species in which numbers have decreased to such an extent that captive breeding must be established with a few founders. A major risk associated with small population size is inbreeding depression. The effects of inbreeding upon male reproductive traits are the subject of an ongoing controversy, since the evidence linking lack of genetic variability and poor ejaculate quality at the population level has been criticized recently by several authors. We report that among *Gazella cuvieri* males, inbreeding coefficient shows a strong inverse relationship with ejaculate quality. Furthermore, the degree of fluctuating asymmetry is positively related to the coefficient of inbreeding and negatively related to the proportion of normal sperm, suggesting that it is a reliable indicator of genetic stress and of ejaculate quality.

Keywords: ejaculate; spermatozoa; inbreeding; fluctuating asymmetry; ungulates; gazelles

1. INTRODUCTION

The importance of inbreeding depression as a risk to small populations is currently a matter of debate. It is thought that inbreeding may reduce reproductive fitness as a result of the unmasking of deleterious recessive alleles or, alternatively, loss of heterosis (Charlesworth & Charlesworth 1987; Thornhill 1993; Frankham 1995). Most of the evidence relating inbreeding and a decrease in fitness components (mainly juvenile survival) comes from captive populations (Ralls *et al*. 1979, 1988; reviews in Thornhill 1993), generating a bias which has led some authors to question the relevance of such findings for natural populations (e.g. Shields 1993; Caro & Laurenson 1994). It has proven difficult to study the effects of inbreeding in the wild because there are few populations for which there is detailed long-term information on mating patterns and, even when such data exist, mating with multiple males by females makes it impossible to determine paternity on the basis of behavioural information. However, the release of inbred animals into the wild has revealed that the deleterious effects of inbreeding are more pronounced in the wild than in captivity (Jimenez *et al*. 1994).

The recent use of molecular techniques in field studies has allowed the determination of paternity and of genetic similarity between parents. These studies have shown that, although mating between close relatives seems rare, a high degree of genetic similarity between parents results in a higher proportion of unhatched eggs among birds (Bensch *et al*. 1994; Kempenaers *et al*. 1996). Among mammals, matings between genetically similar individuals result in offspring which suffer high mortality rates (Stockley *et al*. 1993). These findings suggest that even in outbred natural populations, mating between genetically related individuals does occur, and has fitness-reducing consequences. When levels of inbreeding are low, the effects of inbreeding may only become apparent under extreme environmental stress (Keller *et al*. 1994).

As an ever-increasing number of species suffer drastic reductions in population size, the threat of inbreeding depression acquires a new and important dimension. The problem is particularly acute among endangered species in which captive breeding is an important component of the strategies developed to avoid extinction. In this context, high levels of inbreeding are often unavoidable and it is important to understand in which way it affects individual fitness. Most studies have looked for fitness-reducing effects among females, which are easier to evaluate since they influence either fecundity or juvenile survival (Ralls *et al*. 1979; Lacy *et al*. 1993). How inbreeding affects male reproductive performance is poorly understood. However, if inbreeding does compromise male reproduction it would have far reaching consequences, particularly among populations with polygynous mating systems, which are prevalent among mammals.

Almost all the evidence linking male reproductive performance and inbreeding in endangered species comes from studies on carnivores. The cheetah has been the subject of a series of influential studies (Wildt *et al*. 1983, 1987*a*; O'Brien *et al*. 1983, 1985, 1987; Menotti-Raymond & O'Brien 1993) which have postulated that loss of

*Author for correspondence (mcnc125@fresno.csic.es).

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genetic variation in this species may be the causal factor underlying the poor semen quality observed among males. It has been suggested that genetic variation in this species has been lost as a consequence of population bottlenecks followed by inbreeding (O'Brien *et al.* 1983, 1985, 1987). These interpretations rest on a series of assumptions that have been questioned on the following grounds (Caro & Laurenson 1994; Caughley 1994; Merola 1994; May 1995): (a) levels of genetic variability were determined by using levels of heterozygosity at a few allozyme loci, which may not be a good indicator of heterozygosity at the genomic level; (b) there is no evidence that there was a high level of genetic diversity prior to the hypothetical bottleneck, and this assumption is unrealistic given that low genetic variability is common among terrestrial carnivores; (c) the lack of a control population with high genetic variability precludes the establishment of a causal link between low genetic variability and poor semen quality; and (d) poor reproductive performance may be limited to captive populations and may be the consequence of husbandry practices rather than lack of genetic diversity. In a comparison of three lion populations with different levels of genetic variability, the proportion of abnormal sperm was lower in an outbred population than in a geographically isolated population, but no clear differences were found between the latter and a zoo population which was monomorphic at all the typed loci (Wildt *et al.* 1987*b*). Thus, it is unclear what degree of differences in genetic variability are needed to influence ejaculate features. All these studies compare levels of genetic variability and semen quality at the population level, and cannot control for differences between populations in factors such as history, environment (e.g. diet, seasonality, wild versus captive populations) and behaviour (e.g. patterns of social organization, mating system, copulatory behaviour), which may well play an important role.

When inbreeding does reduce individual fitness, it would be extremely useful to detect it at the phenotypic level. It has been suggested that fluctuating asymmetry may be a reliable tool in this context, since it may be a sensitive indicator of genetic stress and could be monitored easily (Leary & Allendorf 1989; Parsons 1992; Clarke 1995). The evidence presented so far is controversial (reviewed in Palmer & Strobeck 1986; Parsons 1990; Markow 1995; Møller 1997).

The captive population of *Gazella cuvieri* kept at the Estación Experimental de Zonas Aridas (CSIC) in Almería (in the south of Spain) provides a unique opportunity to study the effects of inbreeding upon reproductive performance at the individual level, thus overcoming the limitations and criticisms of previous studies. Given that all the individuals in this population are exposed to the same environmental factors, differences between individuals are likely to be of genetic origin. Since the original population was first established in 1975, detailed records have been kept. Furthermore, paternity could always be assigned with certainty because only one male was allowed to mate with each group of females. Thus, complete pedigrees have been reconstructed and coefficients of inbreeding determined for all individuals. The extremely low number of founders has led to high levels of inbreeding in the population. *Gazella cuvieri* has been categorized as 'endangered' (IUCN 1996) and wild populations are believed to have been reduced to small and scattered groups. The future of this species therefore relies heavily on the success of the captive breeding programme established at the EEZA and, in this context, understanding the consequences of inbreeding and determining which phenotypic features may be sensitive indicators of genetic stress are clear priorities.

The aim of this study is to determine whether inter-individual differences in levels of inbreeding are related to differences in ejaculate quality. In addition, we examine whether the degree of fluctuating asymmetry can be used as a reliable indicator of levels of inbreeding and of ejaculate quality.

2. MATERIALS AND METHODS

(a) *Animals*

The original population of captive *G. cuvieri* was established in 1975, and the total number of founders consisted of two males and two females. Records, including date of birth and the identity of the mother and the father, have been kept for all individuals, pedigrees have been reconstructed, and coefficients of inbreeding determined for all individuals using the additive relationship method (see Ballou 1983).

(b) *Semen collection and analyses*

Semen was collected from 11 healthy and reproductively mature males between October and December 1996. This time of the year corresponds to the mating season. Semen collection was performed by electroejaculation under surgical anaesthesia (Holt *et al.* 1996). Sperm volume, motility, and concentration, were assessed within 60 min of collection. Aliquants were diluted in a modified Tyrode's medium and used to assess motility and viability, and sub-samples were taken to analyse sperm morphology, and acrosome integrity. Motility (wave motion, individual and progressive motility) was evaluated subjectively. Vigour of sperm movement was scored using a scale from 0–5 (0, non-motile; 5, highly motile). Sperm viability, morphology (structural abnormalities) and acrosome integrity were quantified by staining sperm with eosin–nigrosin and then with Giemsa (Tamuli & Watson 1994). Sperm morphology was categorized either as normal, or with abnormalities in the various sperm components. Coiled tails and presence of cytoplasmic droplets were also noted. For acrosomal status, three categories were used: (a) sperm with a normal apical ridge (NAR); (b) sperm with a damaged or modified apical ridge, or damaged acrosomal cap (DAR); and (c) sperm with a missing apical ridge or lost acrosomal cap (MAR). For each preparation 100–200 spermatozoa were counted. Dimensions of spermatozoa were obtained by image analysis of eosin–nigrosin–Giemsa-stained cells. Images were captured using a Nikon Labophot-2 microscope fitted with a 40 × objective and bright-field illumination, and a CCD monochrome video camera (Sony SSC-M370CE). Images were taped using a videotape recorder (Sony SVO-1500P) and were then digitalized and analysed using an IBM-compatible computer with Visilog software (Visilog v. 4.1.3 Rev 6, Noesis, Vélizy, France). Twenty-five spermatozoa from each individual were measured.

(c) *Phenotypic analyses*

Body condition was scored subjectively using a scale from 0–5. Body weight and testicular dimensions were obtained during

anaesthesia just before electroejaculation. Testes' weights were calculated as described (Harcourt *et al*. 1995) using the maximum length and width of each testis. For the analysis of horn asymmetry, and tests of fluctuating asymmetry, horn measures were always taken by the same operator (J.C.). Three measures were taken on the left and right horns of the 11 males included in the study sample: (a) length (to the nearest millimetre along the horn on the front side using a flexible ruler), (b) greatest latero-medial diameter at base, and (c) greatest oro-aboral diameter at base (the latter two to the nearest 0.1 mm with a caliper). To test for fluctuating asymmetry, measures were also taken from horns of 39 *G. cuvieri* sexually mature males whose skulls belong to the collection of the EEZA. Thus, on the whole, the horns of 50 males were measured. All skulls were measured a second time for assessment of measurement errors. The repeatability of horn length ($r=1.00$, $p<0.0001$), greatest latero-medial diameter at base ($r=0.99$, $p<0.0001$), and greatest oro-aboral diameter at base ($r=0.99$, $p<0.0001$) were very high, as was the repeatability of absolute fluctuating asymmetry in horn length ($r=1.00$, $p<0.0001$), greatest latero-medial diameter at base ($r=0.88$, $p<0.0001$) and greatest oro-aboral diameter at base ($r=0.94$, $p<0.0001$). Hence, measurement errors were insignificant for both size and asymmetry measurements. We tested if horn characters demonstrated directional asymmetry or antisymmetry by determining whether signed right-minus-left character values deviated significantly from normal frequency distributions with a mean of zero (Palmer & Strobeck 1986). None of the mean right-minus-left values differed significantly from zero (one-sample *t*-tests), and none of the skewness or kurtosis values differed significantly from expectations for normal distributions. Thus, we can be confident that the characters used show fluctuating asymmetry. Since absolute character asymmetry was unrelated to character size, we used this value (unsigned right-minus-left character size) rather than relative asymmetry.

(d) *Statistics*

Relationships between coefficient of inbreeding and ejaculate traits were analysed by simple regression analysis on transformed variables, as were relationships between the coefficient of inbreeding and the degree of fluctuating asymmetry. Sperm volume, concentration and sperm numbers were log-transformed; for percentages, arcsin-square root tranformation was used. Spearman rank correlations were used to analyse the relationship between the degree of fluctuating asymmetry and ejaculate quality.

3. RESULTS AND DISCUSSION

Eleven reproductively mature males were selected on the basis of their coefficients of inbreeding, and care was taken to include the whole range of coefficients of inbreeding present in the current population. The high level of inbreeding prevalent in this group (average=0.138; range=0.062–0.226; $n=11$) does not seem to have had a major impact upon average values for ejaculate features at the population level (table 1). Thus, variables such as number of sperm, percentage of progressive motility, percentage of viable sperm, and percentage of normal sperm, show no obvious differences with average values from other less inbred populations of related species of gazelles such as *G. dama* and *G. dorcas* (J. Cassinello, T. Abaigar, M. Gomendio and E. R. S. Roldan, unpublished data).

Table 1. *Semen parameters in* Gazella cuvieri

(Assessments were made on independent ejaculates from 11 individuals. No relationship was found between age of males and ejaculate traits. Abbreviations: NAR, normal apical ridge; DAR, damaged or modified apical ridge; MAR, missing apical ridge or lost acrosomal cap.)

parameter	mean±s.e.m.
volume (μl)	642.8±147.63
concentration ($\times 10^6$ ml^{-1})	419.1±76.65
total sperm count ($\times 10^6$)	351.7±111.21
wave motion (0–5)	2.0±0.54
% motile sperm	52.6±12.67
total number of motile sperm ($\times 10^6$)	285.1±104.05
% sperm with progressive motility	38.1±10.66
total number of progressive sperm ($\times 10^6$)	194.5±85.23
quality of progressive motility (0–5)	2.1±0.54
% viable sperm	65.1±8.32
total number of viable sperm ($\times 10^6$)	279.3±95.10
sperm morphology:	
% normal sperm	77.0±3.67
% with abnormal head	5.1±0.87
% with abnormal midpiece	2.5±1.02
% with abnormal principal/terminal piece	9.4±1.79
% with coiled tail	1.1±0.35
% with cytoplasmic droplet	4.7±1.74
total number of normal sperm ($\times 10^6$)	297.7±95.67
acrosome integrity:	
% NAR	75.3±7.12
% DAR	17.6±4.59
% MAR	7.9±2.81
total number of sperm with normal acrosomes ($\times 10^6$)	310.8±100.79
sperm dimensions (μm) and ratios:	
head length	9.75±0.07
head width	6.34±0.07
midpiece length	11.07±0.14
principal+terminal piece length	47.14±0.63
total length	67.98±0.71
head length/width	1.54±0.02
midpiece/total flagellum	0.19±0.00

However, if we look at differences between individuals, the picture is radically different. Individual coefficient of inbreeding is inversely related to (a) ejaculate volume ($r^2=0.446$, $p=0.02$); (b) the number of motile sperm ($r^2=0.356$, $p=0.05$); (c) both the number of progressive sperm ($r^2=0.398$, $p=0.04$) and the proportion of progressive sperm ($r^2=0.396$, $p=0.04$); (d) both the number of normal sperm ($r^2=0.394$, $p=0.04$) and the proportion of normal sperm ($r^2=0.713$, $p=0.001$) (figure 1*a*); and (e) the number of sperm with normal acrosomes ($r^2=0.388$, $p=0.04$).

The inbreeding coefficient is also inversely related to sperm midpiece length ($r^2=0.447$, $p=0.024$) (figure 1*b*).

Among the different types of abnormalities, the most common are anomalies of the flagellum (45% of all abnormalities), the head (23%), the presence of a cytoplasmic droplet (17%), an abnormal midpiece (10%) and coiled tails (6.1%). However, inbreeding is positively

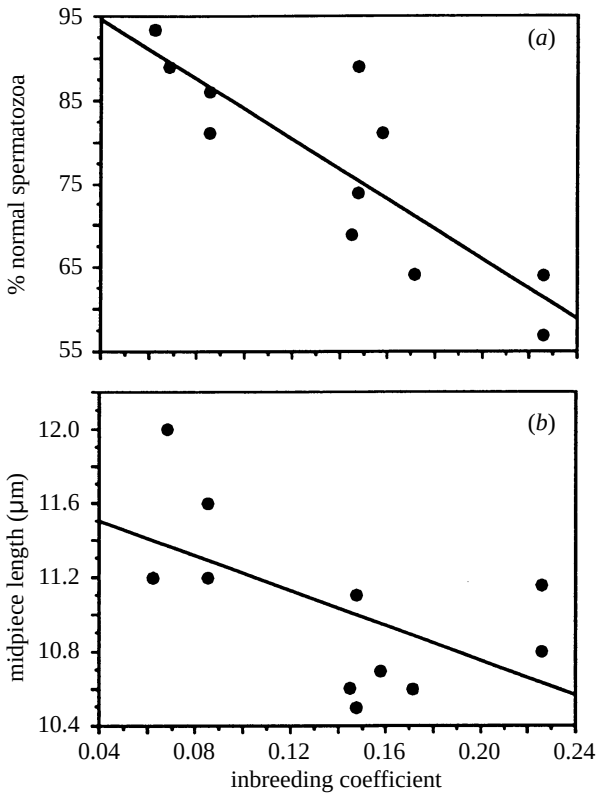

Figure 1. Relationship between inbreeding coefficient and percentage of normal spermatozoa in the ejaculate (*a*) or the length of the sperm midpiece (*b*).

related only to the percentage of sperm with abnormal heads ($r^2=0.369$, $p=0.05$), and the percentage of sperm with a cytoplasmic droplet ($r^2=0.488$, $p=0.02$).

The number of motile and morphologically normal sperm are the two most important determinants of male fertility in mammals. This is because only motile sperm can reach the site of fertilization and penetrate the ova vestments (Drobnis & Overstreet 1992), and morphologically abnormal sperm show defective motility and are strongly selected against by various 'barriers' in the female reproductive tract (Saacke *et al.* 1994; Liu & Baker 1994). The following most important determinant of fertilization success is acrosomal integrity (Drobnis & Overstreet 1992), since the ability to undergo the acrosome reaction at the right time and place is also required in order to penetrate the ova vestments (Roldan *et al.* 1994). There is a strong correlation between sperm motility or percentage of morphologically normal sperm and fertility *in vivo* and *in vitro* in a variety of species including man (Soderquist *et al.* 1991; Sofikitis *et al.* 1994; Eggertkruse *et al.* 1996).

The shortening in midpiece length as a result of inbreeding may also negatively affect fertilization success. In fact, a strong positive correlation between midpiece length and success of fertilization *in vitro* has been found (Sofikitis *et al.* 1994). Midpiece length is under tight genetic control, and shortening of the midpiece results in a decrease in the quantity of mitochondria contained in each spermatozoon (Wooley & Beatty 1967; Wooley 1970). This in turn may diminish energy production and reduce sperm swimming speed or shorten sperm lifespan (Gomendio & Roldan 1993).

Thus, high inbreeding has a negative influence upon those ejaculate features which are most likely to influence fertilization success. To understand the impact of the reductions in sperm quality observed in this study, it is worth pointing out that even in ungulate populations in which none of the sperm abnormalities exceeded 4%, links between male fertility and the proportion of normal sperm have been detected (Soderquist *et al.* 1991). Recent evidence suggests that, when ejaculate quality is poor, even morphologically normal sperm perform badly (Pukhazhenti *et al.* 1996), suggesting that sperm function is depressed in the whole ejaculate. Given that sperm development and morphology are under strict genetic control (Handel 1987; Desjardins & Ewing 1993), it is likely that in this population inbreeding has led to the enhanced expression of deleterious recessive alleles which substantially reduce ejaculate quality, thus hindering male reproduction.

Once we have established that inbreeding depresses ejaculate quality, it is important to determine if inbreeding can be detected at the phenotypic level. Such a link would provide a means by which females could assess male quality, and could also provide a useful tool for the management of populations in which inbreeding coefficients cannot be determined. We found no relationship between inbreeding and physical condition, body weight, body size, body weight/size ratio, testes weight or relative testes weight. Thus, the negative effects of inbreeding upon ejaculate features were unrelated to differences in male body condition due to inbreeding. Inbreeding was also unrelated to horn length and width.

It has been suggested that fluctuating asymmetry is an index of genetic and environmental stress, although this proposal is controversial (Palmer & Strobeck 1986; Parsons 1990; Markow 1995; Møller 1997). Attempts to relate levels of inbreeding and the degree of fluctuating asymmetry have yielded contradictory results. In our study, inbreeding was positively related to the degree of fluctuating asymmetry in horn width (greatest latero-medial diameter at base: $r^2=0.469$, $p=0.03$; greatest oro-aboral diameter at base: $r^2=0.505$, $p=0.02$) (figure 2). Furthermore, the degree of fluctuating asymmetry in horn width (greatest oro-aboral diameter at base) showed a negative correlation with the proportion of normal sperm in the ejaculate ($r_s=-0.85$, $p=0.01$) (figure 3). This appears to be the first time that male ejaculate quality has been related to a phenotypic trait; a link which previous studies have failed to show (e.g. Birkhead & Fletcher 1995).

Taken together our findings have important implications both from fundamental and applied perspectives. The results show that, within a single population, inbreeding has adverse effects upon ejaculate quality influencing those features most likely to affect reproductive performance. The negative relationship between inbreeding and ejaculate quality also suggests a simple mechanism by which females could avoid being fertilized by inbred males: by promoting sperm competition differences in ejaculate quality would ensure the low success of inbred males, without additional mechanisms of female selection. Thus, avoiding being fertilized by inbred males could be an important benefit of promoting sperm competition for females.

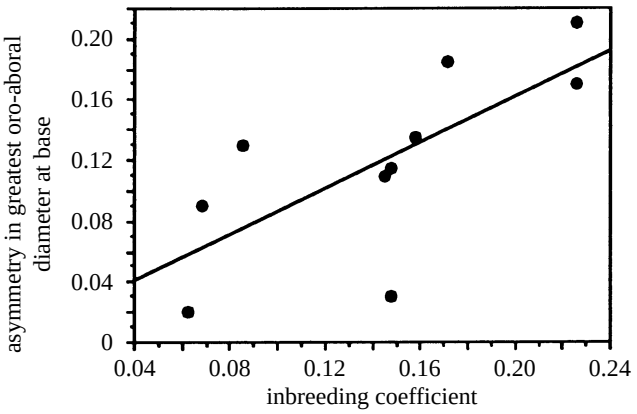

Figure 2. Relationship between inbreeding coefficient and the asymmetry in the greatest oro-aboral diameter at the base of the horn.

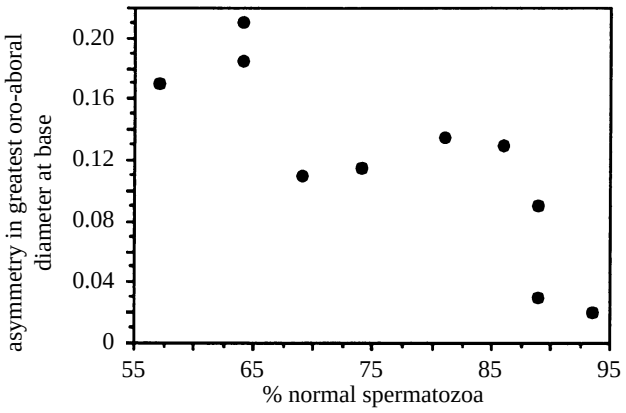

Figure 3. Relationship between asymmetry in the greatest oro-aboral diameter at the base of the horn and percentage of normal spermatozoa in the ejaculate.

We also show that individuals with higher coefficients of inbreeding have greater fluctuating asymmetry in horn width, suggesting that fluctuating asymmetry is a reliable measure of male genetic quality. Furthermore, we found that fluctuating asymmetry seems to be a good indicator of ejaculate quality, hence providing the first evidence in favour of the phenotype-linked fertility insurance hypothesis (Sheldon 1994). Thus, females could use fluctuating asymmetry in males as an index of both male genetic quality and of ejaculate quality. These two factors are likely to be the most important variables influencing mate choice among female mammals. It is possible that bird studies have failed to support the phenotype-linked fertility insurance hypothesis (e.g. Birkhead & Fletcher 1995) because in this group it is also important for females to assess males as care-givers.

Our findings are also relevant for conservation breeding programmes. They show that 22 years after the establishment of this population the effects of inbreeding are still strong, suggesting that the genetic load has not been 'purged'. They also show that, even in populations in which average values do not reveal major effects of inbreeding, individual differences in levels of inbreeding should be taken into account when decisions have to be made as to which males should be chosen to reproduce, which males should participate in genetic resource banks, and which males should be used in reintroduction programmes. Fluctuating asymmetry could be a powerful tool in this context, both in the field and in captive populations in which the use of more invasive means should be minimized. Management decisions of this kind will have far reaching consequences for the future of many species.

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