

Genetic diversity affects testicular morphology in free-ranging lions (*Panthera leo*) of the Serengeti Plains and Ngorongoro Crater

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Reduced genetic variability is known to adversely affect ejaculate quality in inbred lions (*Panthera leo*) physically isolated in the Ngorongoro Crater compared with outbred lions inhabiting the adjacent Serengeti Plains in East Africa. This study compared the histomorphology of testicular biopsies from these two lion populations. Ngorongoro Crater lions had fewer ($P < 0.05$) seminiferous tubules with spermiogenesis and fewer ($P < 0.05$) spermatids per seminiferous tubular cross-section than Serengeti Plains lions, although seminiferous tubular diameter did not differ ($P > 0.05$) between populations. Interstitial areas were greater ($P < 0.05$) in Crater than in Plains lions, but no qualitative differences were evident, suggesting that proportionately less testicular area was occupied by seminiferous tubules in Crater lions. None of the lions in either population had evidence of testicular degeneration. Overall results suggest that inbred Crater lions have reduced spermiogenesis and less total seminiferous tubular area per testis. These data further support the premise that genetic homogeneity compromises reproductive traits in free-living, male African lions.

Introduction

Low genetic variability has been associated with poor seminal quality in lions (*Panthera leo*; Wildt *et al.*, 1987), cheetahs (*Acinonyx jubatus*; Wildt *et al.*, 1983; O'Brien *et al.*, 1985) and Florida panthers (*P. concolor coryi*; Barone, *et al.*, 1994; Roelke *et al.*, 1993). In a study of outbred lions free-ranging in the Serengeti National Park (Tanzania) and an inbred population physically isolated within the Ngorongoro Crater in the same ecosystem, there was a direct relationship between loss of genetic variability and an increasing number of structurally abnormal spermatozoa per electroejaculate (O'Brien *et al.*, 1987; Wildt *et al.*, 1987). Declining reproductive performance with increased inbreeding also has been detected in the Crater lion population (Packer *et al.*, 1991). However, the mechanism by which compromised genetic variability influences these physiological processes has largely gone unstudied in this as well as other species.

In an earlier evaluation, we suspected that impaired spermatogenic function may be related to an endocrine imbalance, so basal and GnRH-stimulated LH, FSH and testosterone secretion were compared between lions living in the two locations (Brown *et al.*, 1991). No significant differences were found in pituitary or testicular hormone production.

The present study was conducted to determine whether the histomorphological characteristics of the testes of lions with high versus low genetic diversity correlated with observed seminal differences. Testis biopsies were evaluated in detail for normal microanatomy and spermatogenesis to determine whether poor semen quality in the less genetically diverse Crater lions was caused by abnormal spermatogenesis.

Materials and Methods

Animals

Male lions were descendants of prides studied consistently since 1966 in the Serengeti Plains ecosystem (Schaller, 1972; Bertram, 1975) and since 1974 in the adjacent Ngorongoro Crater (Packer *et al.*, 1988). The latter is an extinct volcanic caldera that restricts migration and interbreeding with lions of the nearby Serengeti Plains. All study animals were resident males of established prides, and ages of lions were known. Individual lions in both populations were identified by facial and ear scars, whisker patterns and natural markings (Pennycuik and Rudnai, 1970). All lions in this study were considered sexually mature (3.5–9.75 years of age) (Schaller, 1972; Bertram, 1975; Packer *et al.*, 1988). None of the males had been mating on the day they were sampled.

Revised manuscript received 6 June 1996.

Testicular biopsies and histopathological evaluations

All animals were located from a vehicle and anaesthetized with Telazol (tiletamine-HCl and zolazepam-HCl; Warner Lambert, Ann Arbor, MI; 500–600 mg per animal) delivered via a projectile dart (Brown *et al.*, 1991). Testicular biopsies were obtained in October and November immediately after electroejaculation, the seminal data being the primary subject of an earlier report (Brown *et al.*, 1991). Because no seasonal effect on reproduction has been observed in this species (Schaller, 1972; Bertram, 1975), these lions were considered representative of average breeding males.

The scrotum was shaved and prepared for aseptic surgery under field conditions, and the scrotum, tunica vaginalis and tunica albuginea were incised with a sterile scalpel blade. Pressure was applied gently to the sides of the testis, and testicular parenchyma protruding from the surface was excised (approximately 250 mg) and fixed in Bouin's solution.

Testicular samples were dehydrated in graded ethanols, embedded with paraffin wax, sectioned at 7 µm and stained with haematoxylin and eosin or Masson's trichrome. Testicular function was evaluated by examining mean diameter of essentially round seminiferous tubules (Amann, 1986), percentage of seminiferous tubules with spermatids, mean number of spermatids per seminiferous tubule (Amann, 1986), mean number of degenerate cells per seminiferous tubule and histological characteristics of Leydig cells and other interstitial components. Direct assessment of all pre-spermatid stages was not conducted because tissue-handling artifacts distorted distinctive cellular characteristics. Therefore, seminiferous tubule diameter was used as an indirect assessment of spermatogenesis (Amann, 1986). Mean seminiferous tubular diameter was determined in Masson's trichrome-stained sections by measuring 50 round seminiferous tubules per animal on a Micro-Tech 100 Morphometer and Densitometric System (Analytical Imaging Concepts, Irvine, CA). Spermiogenesis was evaluated in Masson's trichrome-stained sections by determining the proportion of 100 seminiferous tubular cross-sections with developing spermatids per animal and the number of spermatids per ten round seminiferous tubular cross-sections (Berndtson, 1989). Degenerate cells were distinguished from residual bodies in haematoxylin- and eosin-stained sections by pyknosis or karyorrhexis of spermatocyte nuclei and hyalinization of cytoplasm. Spermatid counts were performed on all Ngorongoro Crater lions and on an equal number of Serengeti Plains lions matched for ages and basal testosterone concentrations (Brown *et al.*, 1991). Interstitial evaluations of haematoxylin- and eosin-stained sections included the distribution of stroma, pattern of vascularization and Leydig cell distribution and morphology. Interstitial areas were measured in ten adjacent fields at ×100 magnification on the morphometer. Data on spermatozoa concentration, number of spermatozoa per ejaculate, percentage of normal spermatozoa, combined testes volume and circulating testosterone concentrations from our previous report (Brown *et al.*, 1991) were re-calculated for the males that had been biopsied and correlated with histological findings in this study. Calculated values (mean ± SEM) for the ten biopsied Serengeti Plains lions were $62.8 \pm 51.8 \times 10^6$ spermatozoa per ejaculate, $10.9 \pm 8.6 \times 10^6$ spermatozoa ml⁻¹ ejaculate, 59.1 ± 7.5% normal sper-

Table 1. Histomorphometry of testes from two populations of free-ranging lions in the Serengeti ecosystem

	Serengeti Plains lions	Ngorongoro Crater lions
	(n = 10)	(n = 6)
Number of spermatids per seminiferous tubule	134.6 ± 26.6 ^a	88.8 ± 13.8 ^b
Percentage of seminiferous tubules with spermiogenesis	84.2 ± 8.6 ^a	67.8 ± 6.8 ^b
Number of degenerate cells per seminiferous tubule	1.3 ± 0.2	1.1 ± 0.5
Seminiferous tubule diameter (µm)	68.9 ± 5.1	69.4 ± 4.8
Interstitial area (10 ⁴ µm ²)	2.0 ± 0.3 ^a	2.5 ± 0.3 ^b

Values are means ± SEM.

^{a,b}Within rows, values with different superscripts are significantly different ($P < 0.05$).

matozoa, testis volume of 94.6 ± 17.0 cm³ and a basal circulating testosterone concentration of 0.6 ± 0.5 ng ml⁻¹. Calculated values (mean ± SEM) for the six biopsied Ngorongoro Crater lions were $64.6 \pm 52.0 \times 10^6$ spermatozoa per ejaculate, $11.5 \pm 15.5 \times 10^6$ spermatozoa ml⁻¹ ejaculate, 32.8 ± 8.6% normal spermatozoa, testis volume of 62.9 ± 26.8 cm³ and a basal circulating testosterone concentration of 0.74 ± 0.5 ng ml⁻¹. Differences in testes volumes and proportions of normal spermatozoa ($P < 0.05$) between Crater and Plains lions that were previously reported (Brown *et al.*, 1991) were maintained in these subpopulations of biopsied lions.

Statistical analysis

Mann-Whitney two sample tests were used for comparing seminiferous tubule diameter, number of spermatids, numbers of degenerate cells and interstitial areas between locations. Relationships between reproductive variables were determined by least squares linear regression analyses. Data are presented as means ± SEM.

Results

Active spermatogenesis was evident in biopsies from all lions sampled in the Ngorongoro Crater and Serengeti Plains populations, and all lions had mature spermatids. Although seminiferous tubule diameters did not differ ($P > 0.05$) between populations, Crater lions had fewer ($P < 0.05$) seminiferous tubules with spermatids compared with their Plains counterparts (Table 1). Crater lions also had fewer ($P < 0.01$) spermatids per seminiferous tubular cross-section than did Plains lions (Fig. 1). For both populations, the number of spermatids per tubular cross-section did not correlate with the number of degenerate cells per tubule ($r = 0.43$; $P > 0.05$), total sperm numbers per ejaculate ($r = 0.56$; $P > 0.05$), or the mean seminiferous tubular diameter ($r = 0.47$; $P > 0.05$). Furthermore, there was no correlation between combined testis volume and the seminiferous tubular diameter ($r = -0.06$; $P > 0.05$). Although spermatid numbers were lower in Crater lions, the

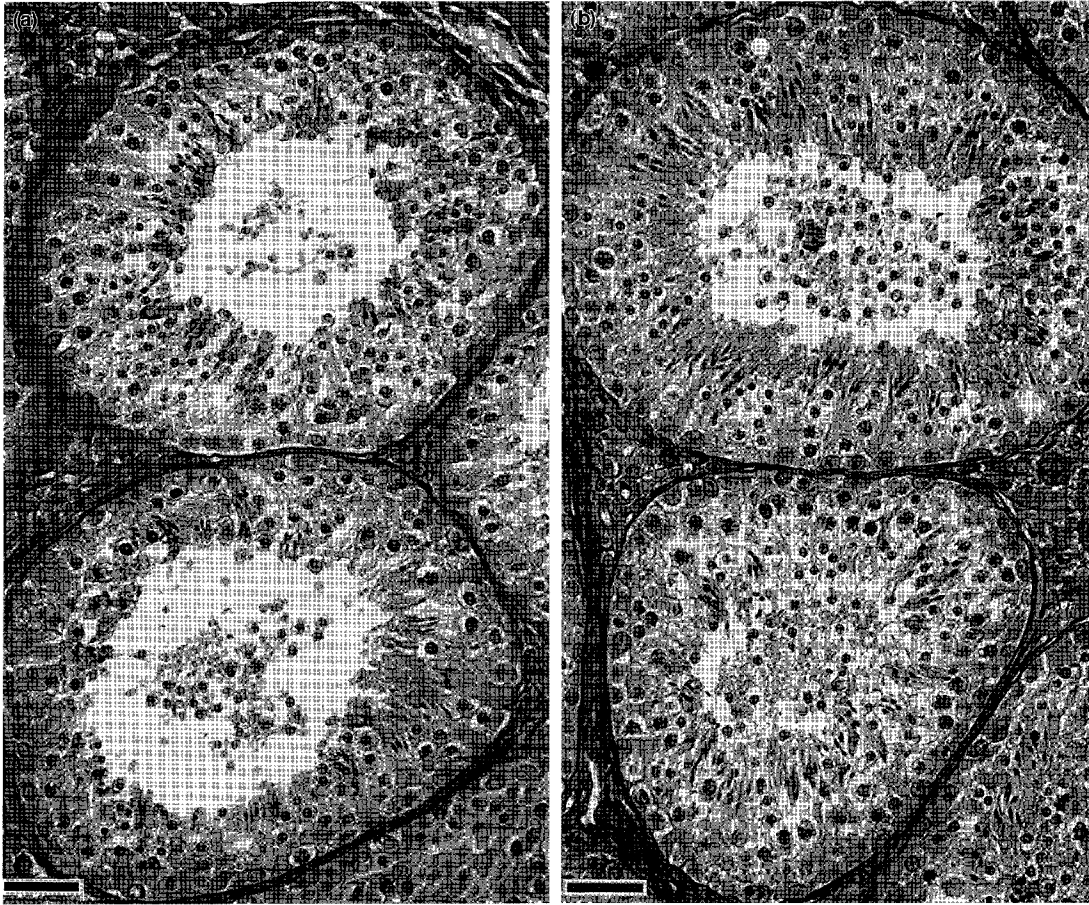


Fig. 1. Photomicrograph of adult lion testes from (a) the Ngorongoro Crater and (b) Serengeti Plains. Crater lions have fewer spermatids per seminiferous tubule than do Serengeti Plains lions, although mean tubular diameters did not differ between populations. Masson's trichrome stain. Scale bars represent 10 μ m.

number of degenerate cells per seminiferous tubule did not differ between populations. Specific morphological defects of ejaculated spermatozoa, such as coiled flagellum or bent midpiece with droplet (Brown *et al.*, 1991), were not identifiable in tissue sections.

Mean interstitial areas including Leydig cells were proportionally greater ($P < 0.05$) in Crater than in Plains lions (Table 1). There was no correlation between the combined testis volume and the interstitial area ($r = -0.32$; $P > 0.05$). Leydig cells (interstitial cells) were arranged in clusters of five to > 50 cells in the intertubular interstitium in both populations. Some, but not all, Leydig cell clusters surrounded blood vessels. No morphological differences were observed between the Leydig cells of Plains and Crater lions or between lions with high and low basal testosterone concentrations. The width of the interlobular septa ranged from 10 μ m to 70 μ m within individual biopsies. Some degree of interstitial variation within and among lions was caused by biopsy-induced oedema

and haemorrhage and regional clustering of Leydig cells. No thickening of peritubular basement membranes or abnormal matrix deposits in the lamina propria, characteristic of testicular degeneration or hypoplasia, were noted.

Discussion

Previous comparisons of these two populations of free-ranging lions in the Serengeti ecosystem measured decreased sperm motility and markedly higher proportions of structurally abnormal spermatozoa in electroejaculates of the more genetically monomorphic Ngorongoro Crater lions (Wildt *et al.*, 1987; Brown *et al.*, 1991). Our previous analyses of circulating gonadotrophin (LH and FSH) and testosterone concentrations revealed that sperm quality differences between locations is unrelated to endocrine dysfunction (Brown *et al.*, 1991). The present study took a more direct approach by assessing

testicular structure and spermatogenesis. Total spermatozoa per tubular cross-sectional area was comparable between the lions living in these two locations, because seminiferous tubular diameters were similar. However, morphometric analyses of testicular biopsies indicated that the Crater lions produced fewer spermatids than did Plains lions. Also, Crater lions had greater interstitial areas and lower testicular volumes than did Plains lions, suggesting that proportionately less testicular parenchyma was occupied by seminiferous tubules in Crater lions. Taken together, these findings could account for the reduced sperm production in the ejaculates of Crater lions (Brown *et al.*, 1991).

In other species, seminiferous tubular diameter is diminished if fewer spermatogonia undergo spermatogenesis or if spermatogenic arrest occurs during early stages (de Kretser and Kerr, 1988; McEntee, 1990; Trainer, 1992). This implies that reductions in spermatid numbers in Crater lions occurred during terminal stages of differentiation (spermiogenesis) and not from spermatogonia or spermatocyte loss. All stages of spermatogenesis were observed in both populations, but direct quantification of the specific stages was not possible for this study because cell morphology was distorted by crush artifacts. In our study, direct quantification of spermatid numbers appeared to provide a more reliable index of testicular function (Berndtson, 1989) than did seminiferous tubular diameter, which is used in other species (Krishnalingham *et al.*, 1982; Amann, 1986). Indeed, seminiferous tubular diameter did not correlate with lower spermatid production in Crater lions, suggesting that it may be an unreliable index of testicular function when spermatogenic rates are normal but terminal differentiation is compromised.

Normal maturation and morphogenesis of spermatozoa are under inherent genetic controls with epigenetic modification by Sertoli cells (Bardin *et al.*, 1988; de Kretser and Kerr, 1988). Sperm cell loss can result from intrinsic lethal genes or a lack of paracrine support during development (Bardin *et al.*, 1988; McEntee, 1990). An inherited developmental block, such as the spermatid-to-spermatozoal arrest reported in hypospermic bulls (McEntee, 1990), is unlikely to be the basis of low spermatid numbers in Crater lions, because all stages of spermatogenesis (including late spermatids and spermatozoa) were found. Crater lion ejaculates also contained high proportions of spermatozoa with defects occurring from abnormal maturation in the seminiferous epithelium, such as coiled flagellum and mid-piece abnormalities (Wildt *et al.*, 1987; de Kretser and Kerr, 1988; Brown *et al.*, 1991). Thus, lower spermatid numbers in seminiferous tubular cross-sections of Crater lion testes may indicate that developmentally defective spermatozoa are released prematurely from Sertoli cells.

Testicular volumes tended to be smaller in Crater than Plains lions, but size disparities and seminal quality (Brown *et al.*, 1991) were not reflected in reduced seminiferous tubular diameter. Smaller testes size in Crater lions may have been caused by fewer or shorter seminiferous tubules because (1) the major determinants of testicular volume are seminiferous tubule diameter, number, and length (Mori and Christensen, 1980; Setchell and Brooks, 1988), (2) Ngorongoro Crater lions had normal seminiferous tubular diameters and (3) Crater lions had relatively more testicular area occupied by interstitium than Plains lions. No Crater lions had lesions typical of testicular

degeneration, such as thickening of the basement membrane or increased interstitial matrix (McEntee, 1990), to account for this smaller testes size. Also, interstitial area did not correlate with testicular volume, further indicating that total seminiferous area must be the main determinant of testis volume. If the assumption that Crater lions had fewer or shorter tubules is correct, then reduced total seminiferous tubular surface area would result in less total sperm production in Crater lions. Because lions normally copulate frequently (Seager and Demorest, 1978; Packer and Pusey, 1983), low sperm reserves may be a contributing factor in the decline of Crater lion numbers (Packer *et al.*, 1991). Although small tissue samples precluded the assessment of seminiferous tubular numbers and lengths in this study, future evaluations could include this parameter.

These morphological findings confirm previous functional data indicating that genetic diversity influences reproductive characteristics of free-ranging lions (Wildt *et al.*, 1987; Brown *et al.*, 1991; Wildt, 1994). Assuming that the data from the genetically diverse lions of the Serengeti Plains represent the norm for this species, then it is apparent that loss of genetic diversity in Crater lions has profound effects on spermatid numbers, testis volume and overall ejaculate quality. Because these are nonlethal traits, they will be perpetuated in the population and more highly expressed as the population diminishes. The reduced testicular function in the homogenetic population of Crater lions may be adequate to maintain the population under normal conditions, but provides limited reserves during periods of environmental stress, disease epidemics or other catastrophic events.

The authors thank A. Pusey, S. O'Brien, D. Gilbert and S. Monfort for assistance with sample collection. They also thank K. N. Hirji, Coordinator of the Serengeti Wildlife Research Institute and the Government of Tanzania for support. This study was supported, in part, by grants from The National Geographic Society, Friends of the National Zoo and the National Science Foundation (No. 8507087).

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