

Reduced heterozygosity impairs sperm quality in endangered mammals

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Inbreeding causes increases in homozygosity and is commonly associated with reductions in fertility and embryogenesis. Although the mechanisms underlying such effects are unknown, recent work has suggested that inbred males may suffer impaired ejaculate quality, thus providing a functional explanation for reductions in reproductive function in inbred populations. However, the relationship between inbreeding and sperm quality remains controversial, particularly in wild populations where the level of inbreeding is typically estimated using neutral molecular markers. Such markers are thought to reflect genome-wide levels of heterozygosity only under restricted conditions, and rarely in outbred populations. Here we employ a comparative approach that takes account of these criticisms and evaluates the evidence linking inbreeding to reductions in sperm quality in 20 mammal species. We focus on sperm abnormalities and sperm motility, which are key determinants of male fertility in many species. We show that species with reduced mean heterozygosity have impaired ejaculated quality, although subsequent analyses revealed that these effects were confined to endangered populations. Our findings therefore support the notion that inbreeding can severely impair sperm quality while concomitantly addressing criticisms surrounding the use of heterozygosity estimates to estimate the level of inbreeding.

Keywords: heterozygosity fitness correlation; abnormal sperm; fertility; ejaculate; sperm competition

1. INTRODUCTION

Inbreeding depression is commonly associated with reductions in fertility and embryogenesis (Keller & Waller 2002). While reduced sperm quality has been implicated as a likely mechanistic explanation underlying these effects (Gage *et al.* 2006), the effect of inbreeding on sperm quality remains controversial. Following initial reports of inbreeding depression for sperm quality in endangered felids (e.g. Wildt *et al.* 1987), subsequent studies of captive and wild species revealed that inbreeding reduces sperm quality (Gomendio *et al.* 2000; Gage *et al.* 2006; van Eldik *et al.* 2006; Asa *et al.* 2007). However, studies examining captive-reared species with known pedigrees

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generally lack an appropriately controlled outbred comparison population (e.g. Caro & Laurenson 1994), and inbreeding depression for sperm quality is not universal (e.g. Gomendio *et al.* 2000). Furthermore, studies of wild populations, which have relied on molecular estimates of inbreeding (multilocus heterozygosity), have come under intense scrutiny (Balloux *et al.* 2004; Slate *et al.* 2004). Although inbred individuals are expected to have more homozygous genomes (Keller & Waller 2002), in generally outbred species there is a tenuous relationship between marker heterozygosity and inbreeding estimated from known pedigrees (Balloux *et al.* 2004; Slate *et al.* 2004). Therefore, researchers interested in understanding how inbreeding influences sperm quality face two key challenges: finding outbred populations for comparisons and accurately estimating the degree of inbreeding present in a species.

Here we employ a comparative approach to examine the association between inbreeding and sperm quality among 10 endangered and 10 non-endangered populations of mammal. We focused on two sperm parameters that are known to reflect male fertility and competitive fertilization performance: sperm abnormalities (e.g. Oettle 1993; Bartoov *et al.* 2002) and the proportion of motile sperm in the ejaculate (e.g. Denk *et al.* 2005). We examined the relationship between these traits and inbreeding using marker heterozygosity to estimate the level of inbreeding. The strength of the correlation between marker heterozygosity and genome-wide heterozygosity is dependent on the individual pedigree in question and the strength of historic inbreeding in the populations (Aparicio *et al.* 2007; Grueber *et al.* 2008). In endangered species, where reduced effective population sizes make inbreeding more common, marker heterozygosity should more accurately reflect genome-wide heterozygosity, and thus act as an appropriate measure of the degree of inbreeding (e.g. Hedrick *et al.* 2001; Aparicio *et al.* 2007; Grueber *et al.* 2008). Therefore, after first assessing the relationship between sperm characteristics and heterozygosity across all species, we performed separate analyses to evaluate the relationship between heterozygosity and sperm quality in endangered and non-endangered species. We predict that any relationship between marker heterozygosity and sperm quality will be confined to endangered species.

2. MATERIAL AND METHODS

(a) Data collection

We obtained data from published sources for sperm characteristics and expected heterozygosity (H_e) for 20 mammalian species (mean number of males per species \pm s.e. 16.3 ± 4.6 , range 4–97; see caption of figure 1 and the electronic supplementary material for species names and further details). Values for H_e were highly correlated with those for observed heterozygosity in the subset of studies ($n=15$) that reported both estimates ($r=0.91$, $p<0.0001$). Since the results were qualitatively similar irrespective of the measure used, we maximized statistical power by focusing on H_e in our subsequent analysis. For 18 species, data on the percentage of motile sperm were also available. We preferentially used sperm characteristics obtained at the peak of the reproductive season. Sperm abnormalities and motility were assessed using species-specific methodologies. However, generally sperm were classified as abnormal based on the structural defects of the acrosome, head, midpiece or flagellum, while sperm motility was estimated as the percentage of motile sperm within each sample (see electronic supplementary material).

For H_e , we used studies where the greatest numbers of micro-satellite loci were examined (mean loci number \pm s.e. 36.7 ± 8.3 , range 4–93; Balloux *et al.* 2004; Slate *et al.* 2004), matching the geographical locations where sperm characteristics and heterozygosity were measured as closely as possible. When data were available from multiple published sources, we used mean values.

Species conservation status was determined based on the International Union for Conservation of Nature's (IUCN) Red List (IUCN 2008). Species classified as 'critically endangered', 'endangered' or 'vulnerable' were considered endangered in our analyses, while those classified as 'near threatened' or 'least concern' were considered non-endangered. The only exception was the American bison (*Bison bison*), which we classified as endangered because this species is undergoing conservation programmes to maintain its near-threatened IUCN status.

Mating system, a proxy measure of sperm competition, did not affect the relationship between sperm parameters and heterozygosity when added as a covariate to the full model. Therefore, mating system was not considered in subsequent analyses.

(b) Phylogenetic analyses

To control for the effects of shared ancestry (Harvey & Pagel 1991), we determined the phylogenetic relationships among species using the phylogeny described in Bininda-Emonds *et al.* (2007). Because this phylogeny did not distinguish between lion subspecies, we placed lions (*Panthera leo*) from the Ngorongoro Crater and the Serengeti as sister subspecies and placed these African lions basal to the Asiatic lion (*Panthera leo persica*) (Yamaguchi *et al.* 2004). All branch lengths were set equal to one. Percentage data (abnormal and motile sperm) were angular transformed. All data were log transformed prior to phylogenetic analyses, which were performed using the R program (v. 2.6.1: R Foundation for Statistical Computing 2007).

Data were analysed using generalized least-squared (GLS) regressions while controlling for phylogenetic effects (Freckleton *et al.* 2002). We used likelihood ratio tests to assess the degree of phylogenetic dependence in the data by calculating the scaling parameter λ (Freckleton *et al.* 2002). To account for the independent examination of heterozygosity estimates on sperm characteristics, we calculated the effect size, r , from each test and used the non-central 95 per cent confidence interval (CI) for r to determine the statistical significance (Nakagawa & Cuthill 2007). Effect sizes were calculated from t values generated from the phylogenetic GLS models (Nakagawa & Cuthill 2007). See ESM for effect sizes, CIs and λ values for regressions.

3. RESULTS

Although there were no significant differences in heterozygosity between endangered and non-endangered species (mean H_e ; endangered: 0.45 ± 0.07 , non-endangered: 0.68 ± 0.06 , $t = -1.36$, $p = 0.19$), endangered species had fewer motile sperm ($t = -2.1$, $p = 0.047$) and more abnormal sperm ($t = 2.49$, $p = 0.02$) than non-endangered species (endangered species: percentage of motile sperm 66.7 ± 5.1 , percentage of abnormal sperm 47.8 ± 8.28 ; non-endangered species: percentage of motile sperm 77.4 ± 1.75 , percentage of abnormal sperm 30.0 ± 5.20).

Across all species, heterozygosity was negatively associated with the percentage of abnormal sperm ($r^2_{\text{adjusted}} = 0.17$, $p = 0.04$; figure 1a) and positively associated with the percentage of motile sperm per ejaculate ($r^2_{\text{adjusted}} = 0.25$, $p = 0.02$; figure 1b). When we considered only endangered species, the relationships between heterozygosity and sperm quality remained significant (sperm abnormalities: $r^2_{\text{adjusted}} = 0.43$, $p = 0.02$; sperm motility: $r^2_{\text{adjusted}} = 0.47$, $p = 0.02$; figure 1). However, for non-endangered species, heterozygosity was not related with either of the two sperm traits (sperm abnormalities: $r^2_{\text{adjusted}} = -0.12$, $p = 1.0$; sperm motility: $r^2_{\text{adjusted}} = -0.13$, $p = 0.68$; figure 1).

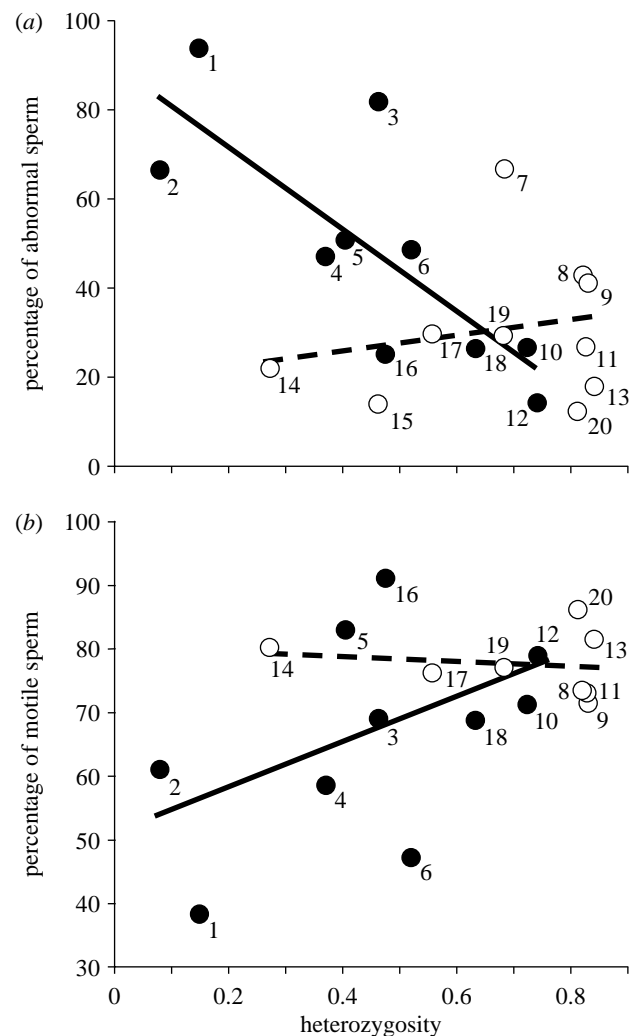


Figure 1. Relationship between the proportion of (a) abnormal and (b) motile sperm in endangered (filled circles, solid line) and non-endangered (open circles, broken line) mammals. Data in the figures are not controlled for phylogeny. Numbers indicate the species: (1) Florida panther, (2) Indian lion, (3) cheetah, (4) black-footed ferret, (5) Ngorongoro Crater lion, (6) black bear, (7) South American panther, (8) margays, (9) tigrinas, (10) red wolf, (11) jaguar, (12) giant panda, (13) ocelot, (14) brown bear, (15) European rabbit, (16) Serengeti lions, (17) howler monkey, (18) bison, (19) domestic cat, (20) coyotes. See electronic supplementary material for additional details.

4. DISCUSSION

Our results demonstrate that extensive inbreeding leads to depressed sperm quality. However, while the negative relationship between species heterozygosity and sperm quality was evident when all species were examined, it is clear that this pattern was generated by the endangered species in the dataset. When we confined our analysis to endangered species, criteria that arguably most closely meet the conditions where heterozygosity reflects inbreeding (e.g. Slate *et al.* 2004; Aparicio *et al.* 2007; Grueber *et al.* 2008), more homozygous species had reduced sperm quality. There was no association between sperm quality and heterozygosity in non-endangered species. Thus, our results support the previously reported link between heterozygosity and sperm quality in European rabbits (*Oryctolagus cuniculus*; Gage *et al.* 2006) while

bolstering the argument that estimates of heterozygosity may not accurately reflect the level of inbreeding unless certain restrictive conditions are met (Balloux *et al.* 2004; Slate *et al.* 2004; Grueber *et al.* 2008).

As argued elsewhere (Gage *et al.* 2006), spermatozoa may be particularly susceptible to inbreeding depression because spermatogenesis relies on highly regulated developmental genes, the disruption of which causes male infertility (e.g. Hargreave 2000). Consequently, exposure of deleterious alleles or losses of heterozygote advantage resulting from inbreeding will directly affect sperm quality. However, given the correlative nature of studies reporting relationships between heterozygosity and fitness, it is not clear whether heterozygosity leads to reduced sperm quality, or alternatively whether species with poor sperm quality suffer from greater rates of infertility, which in turn reduces heterozygosity (Slate & Pemberton 2006). The available evidence suggests, however, that sperm quality is directly influenced by inbreeding rather than *vice versa*. For example, experimentally inbred male domestic cats and dogs produce ejaculates containing fewer (Wildt *et al.* 1982) and more abnormal sperm (Pukazhenthil *et al.* 2006), and recent experimental work on guppies has suggested that highly inbred males produce ejaculates that are competitively inferior than their outbred counterparts (S. R. J. Zajitschek, A. K. Lindholm, J. P. Evans & R. C. Brooks 2008, unpublished data). Impaired sperm quality therefore appears to be a consequence of inbreeding.

Our analyses focused on mammals, as appropriate data were not available from other taxa. Yet inbreeding depression has been demonstrated in many non-mammalian species (Darwin 1876; Keller & Waller 2002) and we anticipate that similar reductions in sperm quality in response to inbreeding will be widespread. Regardless of the focal species, however, our finding that inbreeding is associated with potentially important declines in male reproductive function will have obvious implications for the management of endangered populations, where both genetic viability and reproductive potential are important considerations.

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