Sex-biased inbreeding effects impact upon reproductive success and home range size in the critically endangered black rhinoceros

Supporting Information for Online Publication Only

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Supporting Information provides a detailed description of the study sites, genotyping methods and additional results (Appendix S1) and a reanalysis of the parentage study on a black rhino population in Zimbabwe (data from Garnier et al. 2001) (Appendix S2).

Supporting Information Appendix S1

Additional Methods and Results

Site characteristics

Lewa Wildlife Conservancy (Lewa) is a 267 km^2 wildlife conservancy in Isiolo District. It was formerly a 162 km^2 private ranch, within which a 20 km^2 fenced black rhinoceros sanctuary was established in 1984 with a founding population of 4 females and 3 males from the north of Kenya and Solio Game Reserve. In 1987 another female was moved into the population from Solio Game Reserve. In 1994, the rhino protected area was expanded to include the entire ranch area, and another 4 males were moved into the population from Solio Game Reserve. LWC now includes 37 km^2 of additional land and the 57 km^2 Ngare Ndare Forest which forms a wildlife corridor between the Mount Kenya forest and the Samburu lowlands.

Mugie Rhino Sanctuary (Mugie) is a 93 km^2 part of the privately-owned Mugie Ranch located in northern Laikipia (0°74'N, 36°65'E). The ranch is 200 km^2 and subdivided into a working cattle ranch and a black rhino sanctuary, the two areas are bisected by the unpaved road which runs between Rumeruti and Maralal in Samburu District. In 2004, the sanctuary was founded by 20 black rhinos from Nairobi National Park, Lake Nakuru National Park and Solio Game Reserve.

Ol Pejeta Conservancy (Pejeta) (36°55'E, 00°02'N) is a 365 km^2 wildlife conservancy in the Laikipia District of Kenya. In 1989, 96 km^2 of land was designated as a game reserve predominantly for the conservation of 19 founding black rhinoceros that were received between 1989 and 1993 from Solio Game Reserve or Nairobi National Park, and with one male from Lewa Wildlife Conservancy.

Sample collection

The Kenya Wildlife Service (KWS) maintains a comprehensive database of all black rhinoceros within the sanctuary system with extensive training given to sanctuary personnel on the identification and monitoring of individual animals. One hundred and seven individually-identified black rhinoceros were sampled from the three sanctuaries. Faeces was the predominant source of DNA from Lewa (n=33) and for the new calves born in Mugie (n=7) with OPC sampled through either faecal samples (n=22) or tissue (n=19). Faecal sampling involved locating animals on foot and identifying individuals by either distinctive ear notches or horn shape. Once an animal was located and identified it was followed until the animal either defecated or ran away. For every positively-identified animal, two ~5 g samples of faeces were collected from the outside of the fresh dung pile. Samples were preserved with an approximately 5:1 ratio of desiccating silica:faeces and kept for up to six weeks at room temperature prior to DNA extraction. Several independent faecal sampling events occurred for most animals to ensure accurate identification. For the founder Mugie population KWS made available all of the serum samples (n=20) collected at the time of translocation. We also collected faecal samples from 21 animals (n=15 from Pejeta; n=6 from Lewa) and genotyped all material to assess the reliability of genotypes derived from faecal DNA extractions.

DNA extraction and genotyping

DNA was extracted from faecal samples using a QIAamp® DNA Stool Mini Kit (Qiagen) with minor modifications to the manufacturers' protocol of (1) extending the initial lysis at 55°C to overnight and (2) making two 50 µl elutions in 1xTE buffer after a 15 min incubation; three separate extractions were performed on each faecal sample. DNA extractions from tissue (Qiagen DNeasy Blood & Tissue Kit) and serum (Zymo Research Serum DNA Kit™) were performed according to the manufacturers' instructions.

For faecal DNA, the four replicate extracts with the highest DNA concentration were used to generate genotypes, with the added precaution for low-copy DNA of genotyping every sample six times at all loci. PCRs were performed in 25-µL final reaction volume containing 2 µl of faecal DNA extract (200 ng of tissue/serum DNA), 0.1µg/µl BSA, 200 µM each dNTP, 2.0-2.5 mM $MgCl_2$, 2.5 µl 10X Qiagen® PCR Buffer, 0.625U Qiagen® HotStarTaq™, and 0.5-1.0 µM each primer (forward primers were 5'-labelled with NED, PET, 6-FAM or VIC). Thermal cycling conditions were 96°C for 15 min, followed by 30 (tissue) or 40 cycles (faecal and serum) of (94°C, 1 min; T_a°C, 30 s; 72°C for 1 min), where T_a is the locus-specific annealing temperature (Brown et al. 1999; Cunningham et al. 1999; see Supporting Information Appendix S1 Table 1).

Analyses of genetic diversity

Genotype data were examined for allelic dropout and null alleles using MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004); additional estimates of allelic dropout for faecal DNA samples were derived by comparing genotypes obtained from faecal and tissue samples collected from the same animal (n=21). We used GENEPOP v.4.0 (Raymond & Rousset 1995; Rousset 2008) to calculate exact tests to identify whether there were any significant deviations from expected Hardy-Weinberg equilibrium conditions within each population (Markov chain parameters of 1,000 dememorisations, 100 batches and 1,000 iterations per batch) and to test for linkage disequilibrium between all pairs of microsatellite loci within populations. Because of this multiple testing, sequential Bonferroni corrections were applied to maintain a population-specific error rate of α=0.05 (Rice 1989).

Comparisons between samples that had complementary tissue and faecal samples (n=21) indicated a low genotyping error rate over all microsatellite loci (mean=0.13%; range=0.0-0.24%), with most (>99%) discrepancies due to allelic dropouts in one of the genotyping rounds. The multiple tubes genotyping approach thus allowed any ambiguous genotypes to be identified and resolved by two or more additional rounds of PCR. Independent faecal sampling events and multiple genotyping ensured that complete genotypes were derived for almost all individuals; eight loci were scored for two of the offspring at Lewa, and for 5 animals at Mugie (representing only one mature individual), and all individuals from Pejeta had complete genotypes at 9 microsatellite loci.

Null alleles were detected at one locus (DB44) that was excluded from the analyses. There was no significant deviation from expected Hardy-Weinberg Equilibrium conditions (P>0.05) for the nine microsatellite loci that did not suffer from null alleles (*i.e*. all loci except DB44). There was no evidence of significant (P>0.05) linkage disequilibrium among any pairs of loci, with the one exception of one pair of loci (Br17 & Br4) in one location (Mugie Rhino Sanctuary). Measures of genetic diversity for each reserve are provided in Supporting Information Appendix S1 Table 2.

Parentage analysis

We determined the number of offspring produced by each mature black rhino by parentage analysis. The 62 observations of mother-calf pairings were checked by parentage assignment using CERVUS v.3.0.3 (Marshall et al. 1998). Maternal candidates included all females aged 2 years or more at the time of offspring conception within the same sanctuary (Garnier et al. 2001). Critical LOD scores were determined by simulation for 100,000 offspring and a conservative (see Results) genotyping error rate of 1%. Mother-offspring pairings with >95% certainty were accepted. Paternity assignment was undertaken using the confirmed mother-calf pairings and a simulation of 100,000 offspring to determine critical delta scores, with a genotyping error rate of 1% and a conservative estimate of 80% probability of the true parent being sampled (we sampled ~92-95% of animals based on the Kenyan census). Parentage was accepted for all trio (mother-father-calf) estimates of >95% certainty. The genotype data were checked to ensure that all accepted fathers had compatible genotypes and that we had not overlooked any "next-best" fathers. Given the intensive monitoring of animals in these closed populations, the matches between genetic data and observed maternal-

calf pairings and the high probabilities obtained for parentage assignment it is unlikely our paternity data are biased by levels of genetic diversity (Wang 2010).

Best predictors of the numbers of offspring

To determine that multicollinearity among predictors did not affect our analyses we calculated variance inflation factors (VIFs) using the CORVIF function in the AED package (Zuur *et al*. 2009), where VIF>3 indicates a potential problem with multicollinearity (Zuur *et al*. 2009).

All VIFs for the female predictors were less than 3 therefore were retained for model selection; the correlations between all pairs of predictors were less than +/-0.3, except between home range size (HOM) and the number of females with overlapping home ranges (FOH) (r=0.763, df=25, p=3.77x10^{-6}). VIFs varied between 1.06 and 2.33 for the male predictors, with low correlations (r=+/-0.4 or less) between all pairs of variables except between IR and HOM (R=-0.740, df=15, p=6.86x10^{-4}) and MLH and HOM (R=0.664, df=15, p=0.0036).

Residuals of the final models did not exhibit a significant departure from normality (Shapiro-Wilk test; for female AGE – W=0.978, p=0.807; for male IR - W=0.948, p=0.392; for male MLH - W=0.948, p=0.401) or nor did the show any significant homoscedasticity (Breusch-Pagan test; for male IR - BP=0.007, p=0.932; for male MLH - BP=1.066, p=0.302). A similar outcome is obtained when examining the residuals for the final models that are based on standardised offspring number (Shapiro-Wilk test; for female AGE – W=0.964, p=0.444; for male IR - W=0.946, p=0.365; for male MLH - W=0.933, p=0.223) or homoscedasticity (Breusch-Pagan test; for female AGE – BP=1.533, p=0.216; for male IR - BP=0.0136, p=0.907; for male MLH - BP=0.817, p=0.775), with the exception that the final model for standardised female age depart from heteroscedasticity (Breusch-Pagan test; for female AGE – BP=4.693, p=0.030), although this test would not be significant if a sequential Bonferroni test for $k \geq 2$ multiple tests of model residuals was applied (Rice 1989).

Potential effect of a HFC upon male fitness

To provide wider context to any HFC, an estimate of the inbreeding load can be derived using (1) an estimate of identity disequilibrium [g_2] and the basic descriptors of the HFC itself: (2) the mean [H] and (3) variance [$\sigma^2(H)$] of the estimate of MLH, (4) the regression slope ($\beta_{W,H}$) and (5) the coefficient of determination ($r^2_{W,H}$) of the relationship between MLH and the logarithm of the fitness measure (see Szulkin et al. 2010 for details). The potential impact of inbreeding upon the reproductive success of male black rhinoceros was estimated using the equations provided by Szulkin et al. (2010) that calculate the following:

(1) the squared correlation between the number of offspring and inbreeding ($r^2_{W,f}$),

$$r^2_{W,f} = (r^2_{W,H} / g_2) \times (\sigma^2(H) / H^2),$$

(2) the squared correlation between heterozygosity and inbreeding ($r^2_{H,f}$)

$$r^2_{H,f} = r^2_{W,H} / r^2_{W,f}, \text{ and}$$

(3) the potential inbreeding load ($\beta_{W,f}$)

$$\beta_{W,f} = \beta_{W,H} / \beta_{f,H},$$

where $\beta_{f,H}$ is $[-H g_2 (1-f)] / [\sigma^2(H)]$.

Note that the inbreeding load is the slope of the regression of the (logarithm of) fitness trait on inbreeding and thus requires an estimate of the inbreeding coefficient (f). For black rhinoceros, we assumed a negligible average inbreeding coefficient (*i.e*. f=0), which generates a reasonable estimate of the inbreeding load $\beta_{W,f}$ unless there is particularly high inbreeding (Szulkin et al. 2010).

It is difficult to determine whether any inbreeding depression is the result of a few major mutations (*i.e*. lethals or semilethals) or a consequence of many mutations that have rather smaller detrimental effects. Thus the inbreeding load $\beta_{W,f}$ is typically referred to as an estimate of the number of lethal equivalents (LEs) that represent the reduction in fitness due to deleterious alleles that are exposed in inbred individuals per gamete (Keller & Waller 2002); doubling $\beta_{W,f}$ thus estimates the number of LEs per diploid individual.

For male rhinos, the HFCs based on MLH and the logarithms of standardised number of offspring (ln[OFFs]) and standardised home range size (ln[HOMs]) were: ln(OFFs)=-1.885+0.473, p=0.001, R^2=0.499 and ln(HOMs)=-1.960+0.267, p=0.004, R^2=0.433. Hence, there is a strong correlation between MLH and inbreeding ($r^2_{H,f}$=0.401) and variation in inbreeding apparently explains all ($r^2_{W,f}$=1.245) of the variance in males offspring production, with the high r^2 reflecting the error associated with the parameters used to derive $r^2_{W,f}$ (P. David, pers. comm.). The inbreeding load ($\beta_{W,f}$) for male offspring production is -8.06, which represents 18 lethal equivalents per diploid individual. There was a comparably strong effect of variation in inbreeding upon male home range size ($r^2_{W,f}$=1.082) the lead to an estimated inbreeding load of $\beta_{W,f}$=-4.55 and a concomitant estimate of 9 lethal equivalents per diploid individual.

Given that the inbreeding load ($\beta_{W,f}$) is the decline in fitness with inbreeding (f) (Keller & Waller 2002; Szulkin et al. 2010), the potentially deleterious effect of mating between relatives upon fitness traits can be illustrated. For example, reproduction between half-sibs and first cousins produces offspring with inbreeding coefficients of f=0.125 and f=0.0625 respectively. Such inbred males are predicted to experience a reduction in home range size of ~0.57-0.28 km^2 (*i.e*. 4.55x0.125 and 4.55x0.0625) and a reduction in reproductive success of between 1 and "0.5" calves. Of course, the actual impact would depend upon the background genetic characteristics (heterozygosity) of the animals within other populations.

Analysis of potential female HFC

Because of the qualitative relationship between IR and OFF for females we examined the potential HFC effect for females in more detail. No HFC was detected using all data (OFF=0.962-0.524IR, p=0.415, R^2=0.028). Rerunning the model selection procedure by excluding the "outlier" females with low heterozygosity (high IR of >0.1 in Fig. 1 main text) and high numbers of offspring (n=4 and 5) returns a final model with age as the sole, best explanatory variable for both IR and MLH. GLMs that incorporate an interaction between age and heterozygosity to predict offspring number are not significant (p>0.05 for both IR and MLH). Examining the reduced data set with heterozygosity as the single predictor of offspring numbers returns a significant HFC for when IR us used as the estimator of genetic diversity (OFF=0.634-1.778IR, p=0.033, R^2=0.211) but not with MLH (OFF=0.055-0.130MLH, p=0.251, R^2=0.060).

An analysis of a separate black rhino data (Garnier et al. 2001) set also failed to uncover a significant HFC in females (Supporting Information Appendix S2 Table 3). Thus, selective removal of data reveals a potential HFC that should be monitored, but the effect at present is weak as almost all females get to breed.

Supporting Information Table 1. Information about ten microsatellite loci (originally characterised by Brown et al. 1999; Cunningham et al. 1999) used to genotype black rhinoceros in there Kenyan reserves. Motif, longest published stretch of uninterrupted repeats; Dye, 5' fluorophore (Applied Bioystems) used for to label primers; T_a, PCR annealing temperature (ºC); $MgCl_2$, magnesium chloride concentration in PCR; Size, size range (in base pairs) of alleles; N_a, number of alleles.

Locus	BR17	DB5	DB1	DB66	BR4	BR6	DB52	DB23	DB44	DB14
Motif	$(GT)_{18}$	$(CA)_{13}$	$(CA)_{14}$	$(CA)_{16}$	$(CA)_{19}$	$(CA)_{15}$	$(CA)_{21}$	$(CA)_{12}$	$(CA)_{16}$	$(CA)_{13}$
Dye	PET	PET	NED	VIC	VIC	6-FAM	VIC	6-FAM	PET	6-FAM
T_a (ºC)	59	59	59	57	46	50	63	55	64	60
$MgCl_2$ (mM)	2.5	2	2	2	2	2	2	1.5	2	2.5
Size (bp)	127-137	187-209	118-130	182-208	117-147	139-145	209-225	174-185	172-192	283-289
N_a	6	10	6	8	13	4	9	4	6	3

Supporting Information Table 2. Sample sizes and mean levels of genetic diversity (with standard errors in parentheses) for three populations of black rhinoceros in Kenya. *N*, total number of animals sampled; N_m, number of male samples; N_f, number of female samples; N_a, number of alleles; A_r, allelic richness; IR, internal relatedness separately for males and females and for the entire population; MLH, multilocus heterozygosity for males and females and for the entire population.

						IR			MLH		
Population	***N***	N_m	N_f	N_a	A_r	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
Lewa	39	17	22	4.56 (0.50)	3.88 (0.43)	-0.047 (0.067)	-0.121 (0.046)	-0.089 (0.039)	0.671 (0.041)	0.710 (0.029)	0.693 (0.024)
Mugie	27	14	13	5.44 (0.58)	4.52 (0.35)	-0.040 (0.053)	-0.031 (0.062)	-0.036 (0.038)	0.759 (0.041)	0.762 (0.047)	0.761 (0.029)
Pejeta	41	18	23	5.22 (0.60)	4.38 (0.44)	-0.041 (0.049)	-0.043 (0.041)	-0.041 (0.031)	0.731 (0.036)	0.735 (0.033)	0.737 (0.024)
Total	**107**	**49**	**58**	**5.07 (0.32)**	**4.26 (0.23)**	**-0.043 (0.032)**	**-0.065 (0.027)**	**-0.057 (0.021)**	**0.718 (0.023)**	**0.731 (0.019)**	**0.730 (0.015)**

Supporting Information Appendix S2

Re-analysis of Garnier et al.'s (2001) black rhinoceros data for an effect of HFC

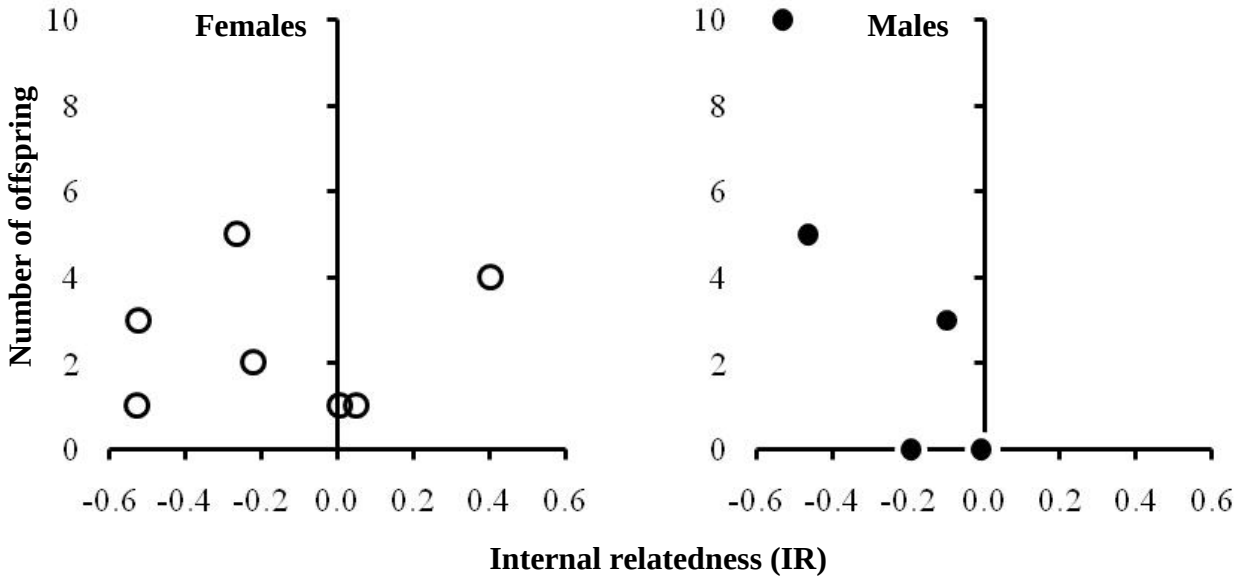
Garnier et al. (2001) provided an analysis of the mating system of a community of 35 black rhinoceros (17 males, 15 females, three of unknown sex and 19 offspring) in the Save Valley Conservancy (20°E, 31°S) in Zimbabwe. The animals were monitored over a four year period (August 1995 and August 1999) and exhibit substantial variance in reproductive success in males. There was no attempt to estimate a HFC in the study by Garnier et al. (2001). Using their genotype data (the same panel of ten microsatellite loci that were used in our study; Brown et al. 1999; Cunningham et al. 1999) and the results of the parentage analyses, we first calculated MLH and IR using IRMacroN4 (www.zoo.cam.ac.uk/zoostaff/meg/amos.htm#ComputerPrograms) (Amos et al. 2001) and then ran GLMs in *R* (R Development Core Team 2010) that used these estimators of heterozygosity as predictors of the numbers of offspring produced. One male was excluded from the analysis as he was one of the offspring that had matured sufficiently to sire just one offspring. No data on home range size or age were available for these animals.

Garnier et al.'s (2001) data reveal the same pattern reported for our 3 Kenyan reserves. Heterozygosity (both IR and MLH) are both significant predictors of the number of offspring produced by male rhinoceros, explaining about 60-70% of the variance between males (Supporting Information Appendix S2 Table 3). Heterozygosity has no significant effect upon offspring production by female black rhinos (Supporting Information Appendix S2 Table 3, Fig. 1).

Supporting Information Table 3. Results of GLMs that examine the ability of heterozygosity (IR or MLH) to explain variation in the number of offspring (OFF) produced by male and female black rhinoceros from the Save Valley Conservancy, Zimbabwe. R^2 is the proportion of variation (explained deviance) explained by the final model (Zuur et al. 2009). Data are taken from Garnier et al. (2001).

		intercept	HET	R^2
Females	IR	0.923***	0.260	0.018
	MLH	1.217	-0.046	0.021
Males	IR	-0.856	-5.772***	0.714
	MLH	-7.494**	1.049**	0.620

*p<0.05, **p<0.01, ***p<0.001



Supporting Information Figure 1. Variation in the numbers of offspring produced by male and female black rhinoceros at Save Valley Conservancy (20°E, 31°S) in Zimbabwe as a function of internal relatedness (a measure of heterozygosity, with more heterozygous individuals having lower values of IR). Data taken from Garnier et al. (2001).

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