Journal of Zoology

ZSL LET'S WORK FOR WILDLIFE

Journal of Zoology. Print ISSN 0952-8369

Starting small: long-term consequences in a managed large-mammal population

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Keywords
genetic analysis; rhinoceros; population growth; wildlife management; founder effect; population viability; genetic diversity; *Diceros bicornis*.

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Editor: Jean-Nicolas Volff

Received 14 December 2017; revised 18 April 2018; accepted 20 April 2018

doi:10.1111/jzo.12572

Abstract

Small populations are vulnerable to founder effects, stochastic demographic events and inbreeding depression. These factors raise the extinction probability of small populations, and thus effective management plans for endangered species have become essential. The black rhinoceros (*Diceros bicornis*) survived a historic global population crash and is currently facing renewed threat through poaching, population fragmentation and isolation. South Africa is home to approximately 36% of the world's remaining black rhinos; however, this population is fragmented across parks and reserves, and many of these small populations remain geographically and genetically isolated. The historic population crash, subsequent fragmentation and relentless poaching pressure together threaten the viability of the black rhino population within South Africa. This study employed a practical, non-invasive sampling protocol and genetic analyses to assess the growth and performance of a small founder population in a fenced reserve. We used these data and recorded life-history data to compare the population growth and vital rates between the first and second 10-year periods since founding. We report a decrease in population growth rate and male survival in the second 10-year period (0.06; 0.89) compared to the first (0.09; 0.97). Genetic diversity (H_{EXP} = 0.405) was low when compared to larger black rhino populations, and average pairwise relatedness was high (0.193). These results suggest that negative genetic consequences and the beginning of density-dependant growth regulation may be present in this population. This highlights the importance of establishing an evaluation framework for the assessment of small populations of long-lived mammals in order to inform metapopulation management.

Introduction

Small, isolated populations are extremely vulnerable to stochastic demographic events, genetic drift and inbreeding depression. Genetic drift can result in a loss of genetic diversity with each generation in small populations by chance, and inbreeding depression encompasses the negative effects seen in a population, particularly affecting fitness-related traits, which arise as a result of mating between close relatives (Crnokrak & Roff, 1999). Populations founded with very few individuals typically also start with substantially reduced genetic variation purely as a result of the small subset of animals. The combination of founder effects, genetic drift and increased inbreeding together raise the extinction probability of small populations (Keller & Waller, 2002; Frankham, 2005), and as such, the implementation of effective monitoring and management plans for fragmented populations of endangered species have become, in many cases, crucial for their survival.

The black rhinoceros, *Diceros bicornis*, hereafter black rhino, was widespread across Africa, with numbers estimated upwards of 100 000 animals as recently as the 1960s (Knight, 2016). Subsequently, human activities such as hunting, poaching and land clearance decimated the black rhino population to a low of 2410 animals across the continent in 1995 (Knight, Balfour & Emslie, 2013). Today, black rhinos are classified as 'Critically endangered' on the IUCN Red List of Threatened Species (Emslie, 2012), with the majority of the remaining 5000 animals occurring in four range states: South Africa, Namibia, Kenya and Zimbabwe. Within South Africa, black rhinos were virtually extinct by the mid-1900s, with fewer than 100 animals remaining (Hall-Martin, 1979). Targeted conservation measures increased the South African population to 1893 by 2015, representing approximately 36% of the world's remaining black rhinos (Knight, 2016).

The national black rhino population is, however, fragmented across a combination of national parks, provincial parks, contractual parks/custodian agreements and private reserves, and many of these small populations remain geographically and genetically isolated (Knight *et al.*, 2013). Within the Black Rhino Biodiversity Management Plan of South Africa (Knight *et al.*, 2013), biological management includes manipulating sex ratios and promoting genetic health in order to maximize

population growth. Realizing these goals requires a working knowledge of population size, sex ratios, fecundity and survival (Akçakaya, 2002; Law, Fike & Lent, 2013), and may be strongly influenced by cryptic breeding patterns and relatedness. Evaluating the population performance of large mammals often relies on aerial techniques or mark–recapture methods using known individuals (Khaemba *et al.*, 2001). Despite their size, black rhinos are difficult to census using traditional methods due to their elusive nature and preference for dense vegetation. Genetic samples obtained from dung samples can be used to provide some of the required population-level information and vital rates, such as sex ratios, genetic diversity and mating structure, which may be essential for effective management (Schwartz, Luikart & Waples, 2007; Beja-Pereira *et al.*, 2009). Non-invasive genetic sampling is regularly undertaken for monitoring and research studies of elusive and endangered species including elephants (Flagstad *et al.*, 2012) and bears (De Barba *et al.*, 2010). This approach uses unique molecular 'tags' extracted from field samples in lieu of physical tags to identify individuals.

Previous studies of black rhinos have utilized non-invasive genetic sampling to investigate mating skew (Garnier, Bruford & Goossens, 2001) and genetic differences between populations and ecotypes (Harley *et al.*, 2005; Muya *et al.*, 2011). For this study, we apply this technique as a population assessment tool where topography, vegetation and resource limitations have impeded routine assessment of the population. Our study undertook to utilize a practical, non-invasive genetic sampling protocol as a monitoring tool for black rhinos, and used it to evaluate the performance of an isolated black rhino population of conservation concern, where sampling constraints exist and total population size is unknown. We evaluated the population performance in the first 10 years since founding using available observation data, and compared it to the second 10-year period using non-invasive genetic sampling. More specifically, our objectives were: (1) to investigate any sex bias in sampling frequency, particularly in relation to using samples collected at midden sites where territoriality may bias sampling effort; (2) to determine a current minimum population size; (3) to calculate and compare vital rates in the two 10-year periods after the initial population founding; (4) determine the level of genetic diversity and relatedness in the population. Finally, we discuss the utility of this method for evaluating other small populations of black rhino in the broader metapopulation.

Materials and methods

Study area and population history

The study area is approximately 145 km^2 within a fenced South African National Parks (SANParks) reserve, with mountainous terrain and a combination of grassland, forest and thicket vegetation; further location and reserve details are not given for security purposes. Black rhinos were introduced into the area in 1995 with a single bull who died the same year. Seven black rhinos (four males, three females) were introduced between 1996 and 1997, and one male was removed during this period. An additional bull was introduced in 2003. Life-history data regarding introductions, births, deaths and removals were recorded from 1996 to 2006. Biobank samples (tissue or blood) were available for 11 individuals; DNA was extracted from these samples as references.

Sample collection

A non-invasive dung sampling protocol was undertaken in 2016 to establish a minimum population size estimate and population vital rates. The design of the protocol was influenced by constraints imposed by both the terrain and vegetation type which largely restricted sampling to roads and well-established game trails. All roads within the study area were driven once and all dung samples detected on or near the road were inspected. Accessible trails leading to or from water points were walked from the nearest road, and fresh spoor was tracked until a fresh dung sample was found or the spoor was lost. Only fresh dung samples (less than 24 h old) were collected and classified into four categories based on the estimated age of the sample (0-6, 6-12, 12–18 and 18–24-h old). The GPS location, placement (midden, isolated) and state (scattered or intact) of each sample was recorded. 'Midden' was defined as a large collective dung pile, typically found on a main road or pathway. Samples were classified into this category if they were located on a midden (centrally or peripherally), or within visual proximity (<10 m) of a midden with recent activity. 'Isolated' was defined as a single dung deposit not within visual distance of a midden with recent activity. 'Scattered' was defined as dung that had been purposefully scattered and scraped such that it was no longer present in defined boluses. 'Intact' was defined as dung that was not scattered.

Genotyping and individual identification

Approximately, 1–1.5 g of the outer layer of a single dung bolus was collected into a 50 mL tube of silica gel beads. DNA extraction was performed with a QIAmp Fast Stool Mini Kit (Qiagen), with slight protocol modification. Samples were incubated with approximately 10 × InhibEx buffer, vortexed vigorously and incubated with agitation for two hours at 40°C. Samples were vortexed halfway through incubation time, and again after incubation, until homogenized. Multilocus genotypes were generated using 11 microsatellite markers (Brown & Houlden, 1999; Cunningham, Harley & O'Ryan, 1999), and sexed using marker ARZF (Peppin *et al.*, 2009). Amplification details are described in Appendix S1. Any samples that failed to amplify or produced uncertain genotypes for a locus were repeated until a reliable genotype was obtained. All loci were re-amplified in 50% of the samples for quality control, consensus genotypes were generated for all samples (repeats: minimum = 1, maximum = 5, mean = 2.1), and genotype error rate was calculated following the method of Broquet & Petit (2004).

Unique multilocus genotypes were identified using the package 'alleleMatch' (Galpern *et al.*, 2012) in the R v3.3 (R Development Core Team, 2013). AlleleMatch uses a dynamic TreeCutting algorithm that utilizes a combination of mismatch and allele frequency data to determine probable multilocus genotype matches (Galpern *et al.*, 2012). For this analysis, the accepted level of mismatch between genotypes classified

as the same individual was set at two alleles. This is appropriate for a population with potentially high levels of relatedness and inbreeding. All samples that matched an identified unique genotype under this criterion were manually reviewed. Where mismatches were found, geographic and demographic data collection was interrogated for additional clarity. MicroChecker v2.2.3 (van Oosterhout *et al.*, 2004) was used to test for scoring errors or null alleles at each locus. The probability of a pair of individuals sharing a multilocus genotype in this dataset (PID: unrelated individuals; PID_{sib}: full siblings) was calculated using Cervus v3.0.7 (Kalinowski, Taper & Marshall, 2007). Mean expected and observed heterozygosities (H_{EXP}, H_{OBS}; Frankham, Ballou & Briscoe, 2002) were calculated, and deviation from Hardy–Weinberg Equilibrium (HWE) was tested using the package 'adegenet' (Jombart, 2008) in R. Linkage disequilibrium and inbreeding (F_{IS}) statistics were calculated using the package 'genepop' (Rousset, 2008) in R. Significance levels were adjusted using the Bonferroni correction for multiple tests. Average pairwise relatedness for the study population was calculated using the triadic likelihood estimator (Wang, 2007) using the package 'related' (Pew *et al.*, 2015) in R.

Investigating sex-specific sampling effects

Our sampling protocol was biased to roads and trails where middens are more common (Skinner & Chimimba, 2005). This sampling regime may therefore be biased to a particular sex or age group, if there is unequal utilization of middens. To investigate these potential sex-specific effects, all samples that were successfully genotyped were pooled by sex. We used Fisher's Exact test to test for significant differences in the proportion of samples in different location types (midden or isolated) between males and females.

Extracting and estimating vital rates

Individual observation data were available for the first 10 years ($T1$) after the population was founded. This included age, sex, mother-calf pairs, births, deaths, introductions and removals. We calculated the annual population growth rate from the observation data using an exponential population growth model adapted from (Caughley, 1977). We used the equation $r = \ln(N_{t+1}) - \ln(N_t - N_{r,t\to t+1} + N_{i,t\to t+1}) + \ln(\varepsilon_t)$, where N_{t+1} is the population estimate for the subsequent year; Nt is the population estimate for the current year, $N_{r,t\to t+1}$ is the number of rhino removed during the current year, $N_{i,t\to t+1}$ is the number of rhino introduced in the current year, and where εt is a multiplicative log-normal error. Birth sex ratios and sex-specific survival rates were also calculated from the observation data.

For the second 10-year period ($T2$), sex-specific survival rates were estimated using a Leslie matrix model (Leslie, 1945) that we transcribed into sex-specific deterministic equations using 1-year age-classes. We used the age- and sex-structure at the end of $T1$ together with the fecundity schedule derived from the birth sex ratios, $T1$ sex-specific survival rates, the 2016 genetic population size and sex estimates, an age at first parturition (AFP) of 7.54 years and an inter-calving interval of 2.27 years (Freeman *et al.*, 2014b) as input variables. We modelled both males and females assuming parity in sex ratios at birth. Input for number of males in age-class zero (i.e. number of males born) thus came from the female model. This allowed us to derive point estimates of sex-specific survival rates for T2 that would as closely as possible mimic the observed age- and sex-structure and population size estimates in both 2006 and 2016 using a least sum-of-squares approach and the SOLVER add-in in Microsoft Excel (2016). The approach is equivalent to a maximum likelihood approach, but we did not estimate standard errors due to likely sample effects that our small dataset may impose. The Leslie matrix solution estimates lambda as an intrinsic population growth rate, which we converted to an annual exponential growth rate.

Results

Sampling and marking behaviour

Sixty-nine dung samples were collected over a 10-day period, with a total distance of 391.1 km covered. This averaged to an overall effort of approximately 5.7 km/sample. DNA was extracted from all 69 samples; 64 samples yielded amplifiable black rhino DNA. Mean DNA concentration was 85.7 ng/μL; however, this metric is a combination of bacterial and rhino DNA of varying proportions per sample. DNA concentration was not significantly different between the sample age categories (Kruskal–Wallis rank sum test, $P > 0.05$) or state in which the dung was found (Kruskal–Wallis rank sum test, $P > 0.05$). Of the 64 working samples, 41 were females and 23 were males. A significantly higher proportion of female samples were found on or near middens, compared to male samples (Fisher's Exact, $P < 0.05$). No significant difference was found between the proportion of male and female samples on middens that were scattered (Fisher's Exact, $P > 0.05$).

Genotyping and individual identification

Multilocus genotypes were obtained for 64 dung samples and 11 biobank tissue/blood samples. Sample amplification success rates for dung and biobank samples were 92.8 and 100% respectively. Genotyping error rate (proportion of alleles incorrectly typed across two amplifications) was 0.05. AlleleMatch identified 36 individuals from the dung samples using the clustering algorithm based on allele frequencies and a mismatch parameter of two alleles. All mismatches and subsequent assignments were reviewed manually. Two male samples assigned as most likely belonging to a single individual were retained as two individuals based on geographic distance between sampling events and sampling data. Six female samples assigned to a single individual were separated into two individuals based on demographic and sampling data collected (such as physical dung size and placement). This resulted in a final population size of 38 black rhinos, comprised of 15 males and 23 females. The maximum number of dung samples collected from one individual was 5, and mean number of samples per individual was 1.68 (female: 1.78, male: 1.53). There

was no significant difference between the mean number of dung samples collected per individual between males and females (Kruskal–Wallis Rank sum test: $\chi^2 = 0.22$, $P > 0.05$).

Population statistics and relatedness

We determined a minimum population estimate of 38 black rhinos in 2016 with a density estimate of 0.27 rhino per km^2. The sex distribution within the estimated population was 15 males and 23 females, resulting in a sex ratio of 1:1.5 (male to female). The null hypothesis of sex parity was not rejected (Exact binomial test, $P > 0.05$). Unique genotypes were classified as individuals and genetic data assessment and statistics were calculated at the population level. Micro-Checker showed no evidence of scoring errors, allelic dropout or null alleles at any loci (data not shown). PID and PID_{sib} were 1.1×10^{-4} and 1.1×10^{-2} respectively. There was no significant deviation from Hardy–Weinberg Equilibrium. Linkage disequilibrium results showed DB49 and DB52 to be in linkage after correction for multiple testing ($P < 0.05$). As such, DB49 was excluded from further analyses. The number of alleles per locus ranged from 2 to 5 (mean 2.4), and the mean expected and observed heterozygosities (H_{EXP}, H_{OBS}) were 0.405 and 0.454 respectively (Table 1). Average F_{IS} across all loci was −0.088. Average pairwise relatedness in the population was calculated at 0.193.

Extracting and estimating vital rates

Initial black rhino introductions comprised three females (two of breeding age), and four males (three of breeding age), with an additional male introduced after 7 years. During this time, a single adult male died and 15 calves were born, with a calf sex ratio of 1:0.75 (male to female). The proportion of male and female calves born was not significantly different (Pearson's $\chi^2 = 0.173$, $P > 0.05$). The genetic estimates (population size = 38, 15 males, 23 females) were used as the input variables for population size and sex-structure at the end of *T*2. Sex-specific survival rates, annual growth rates and sex ratios for *T*1 and *T*2 can be seen in Table 2.

Discussion

This work evaluated the genetic health and population performance of a small black rhino population 20 years after founding.

Table 2 Black rhino population vital rates over 20 years since founding

Time Period	Sx, f [a]	Sx, m [b]	*R* [c]	Sex ratio	*P* [d]
*T*1	1.000	0.969	0.091	1:0.82 (2006)	0.725
*T*2	0.971	0.898	0.060	1:1.5 (2016)	0.108

[a]Survival rate, female.
[b]Survival rate, male.
[c]Annual exponential growth rate.
[d]*P* values for the Pearson's χ^2 test for significantly different proportions of males and females.

Our results showed a reduction in population performance in the second 10-year period after founding compared to the first, with reduced growth rate, decreasing male survival, low genetic diversity and high levels of relatedness. These results suggest that founder effects, genetic drift, inbreeding and density-dependent regulation may be adversely affecting the population, and management interventions may be required to offset long-term impacts.

Due to challenging topography, thick vegetation and a finite sampling period, dung samples were mostly collected from middens on or nearby roads and trails. Female samples included a significantly higher proportion of midden samples than male samples. We were not able to investigate sex bias in scattered versus intact due to the small sample size of dung in these categories. Freeman *et al.* (2014a) showed scraping behaviour (the process of dragging feet through and scattering dung) in black rhinos to be correlated with animal age and proximity to vegetation for both sexes. Taken together, these findings suggest that the potential female bias identified in our sampling protocol is most likely to result in the omission of sub-adult or young adult male rhino, as younger males would be less likely to utilize middens and scatter dung (Freeman *et al.*, 2014a).

Our genetic estimate of 38 black rhino individuals in 2016 represents a minimum population count and produces a population density estimate of 0.27 rhino/km^2, which falls within the range of other small founder populations (0.1–0.7 rhino/km^2; (Hitchins & Anderson, 1983; Conway & Goodman, 1989; Ferreira & Greaver, 2016)). The population growth rate decreased in the second 10-year period suggesting the onset of density-dependant growth regulation. Density-dependant growth regulation in large-mammal populations may manifest through increased female age at first parturition or inter-calving

Table 1 Genetic statistics for the study population

Locus	Alleles	Range	H_{OBS}	H_{EXP}	Prop Typed	HWE *P*	F_{IS}
BR4	3	129–135	0.210	0.191	1.00	1.00	−0.09
BR6	2	133–155	0.378	0.339	0.97	1.00	−0.103
BR17	2	123–133	0.579	0.499	1.00	0.50	−0.148
DB1	2	119–125	0.632	0.494	1.00	0.19	−0.265
DB5	2	189–207	0.486	0.491	0.97	1.00	0.023
DB14	2	282–288	0.250	0.388	0.95	0.05	0.369
DB23	1	179–179	0.000	0.000	1.00	1.00	0.000
DB44	3	174–178	0.605	0.521	1.00	0.57	−0.148
DB52	2	218–220	0.667	0.464	0.79	0.05	−0.422
DB66	5	187–207	0.737	0.666	1.00	0.68	−0.093

intervals, or decreased immature or adult survival (Eberhardt, 2002). In the case of black rhino, these processes most likely occur as a result of socially mediated cues rather than resource limitation (Ferreira *et al.*, 2011; Law *et al.*, 2013), particularly in small reserves with higher rates of association (Linklater & Swaisgood, 2008). Recorded fighting mortalities in *T*2 and decreased male survival rates suggest that reduced adult male survival may be a contributing factor. The *T*2 sex ratio of the population was 1:1.5 (male to female), which was more female-biased than expected, although not statistically significant.

In addition to ecological vital rates, measures of genetic diversity and relatedness are extremely useful metrics that contribute significantly to the understanding of population viability. The genetic diversity seen in the study population is lower than has been found in other large black rhino populations. For example, the Etosha black rhino source population showed a mean H_{EXP} of 0.52 compared to 0.405 in this study population (van Coeverden de Groot *et al.*, 2011). The mean number of alleles per locus in Etosha was 4.89 (van Coeverden de Groot *et al.*, 2011), more than double that seen in this population (2.4). Harley *et al.* (2005) showed expected heterozygosity levels of 0.51 for black rhinos across multiple populations, albeit with a small sample size for each, as well as a mean number of alleles per locus of four. Together, this suggests that a substantial amount of diversity has been lost in the study population relative to the source population, as a result of the small founder numbers and/or genetic drift and inbreeding. In addition, average relatedness in the population is high at 0.193, close to that expected for half-siblings (0.250). Black rhinos display varying levels of reproductive success (Garnier *et al.*, 2001; Cain *et al.*, 2014), which has been correlated with genetic diversity and home range size in males (Cain *et al.*, 2014). This suggests that inbreeding effects could occur in isolated black rhino populations, and with a relatedness estimate this high, there is cause for concern and careful monitoring of this population in the future.

Finally, we acknowledge the limitations of a rapid sampling strategy as was performed here. A more repetitive sampling effort would support additional capture–mark–recapture (CMR) analyses for overall population estimates, if financial and logistic resources were sufficient. CMR analysis can be used to predict total population size using non-invasive genetic samples in certain cases (Marucco *et al.*, 2011); however, our sampling protocol sought to maximize area coverage at the expense of repeat sampling of individuals which limited our ability to meet CMR assumptions.

We consider the dung sampling protocol established here and the resultant population metrics to provide a practical, cost-effective means for informing a population monitoring framework for the management of small black rhino populations. The annual population growth rate of 6% seen here exceeds the 5% target proposed by the South African Black Rhino Management Plan (Knight *et al.*, 2013) and is comparable with growth rates seen in other small black rhino populations (Ferreira & Greaver, 2016). If this population is to be managed as part of a greater metapopulation, this is the ideal time to consider the removal and/or exchange of individuals. As such, establishing an evaluation framework based on these metrics that can be used for the comparative assessment of all small, managed populations would enable superior decision-making and metapopulation management going forward.

Acknowledgements

We thank Dr Angela Gaylard for project facilitation, Dr Dave Zimmerman for biobank assistance, Dr Markus Hofmeyr for supportive collaboration and the South African Rhino Management Group for the life-history data. This study was funded by a Rufford Foundation Small Grant for Conservation Research and the National Research Foundation (NRF), South Africa. NLR was funded by the NRF and the University of Mpumalanga, South Africa.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. DNA amplification.