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Continued decline in genetic diversity among wild cheetahs (*Acinonyx jubatus*) without further loss of semen quality

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ABSTRACT

As a well-studied felid with limited genetic diversity, the cheetah (*Acinonyx jubatus*) has shaped much of the scientific debate surrounding inbreeding depression. The species survived a population bottleneck ~12,000 years ago and was extirpated from >75% of its historical range in the last century. Modern cheetahs produce poor-quality semen, a presumed manifestation of inbreeding depression. Within Felidae, a positive association between genetic diversity and semen quality is well supported by pedigree data and inter-species comparisons. However, this relationship has never been examined among individual cheetahs. Furthermore, whether ongoing population declines are exacerbating inbreeding depression in wild or captive cheetah populations is unknown. Using 12 microsatellite markers, we evaluated the relationship between heterozygosity and reproductive traits among wild ($n = 54$) and captive ($n = 43$) male cheetahs born from 1976–2007. We tested the hypotheses that genetic diversity has declined over the last ~30 years and is positively correlated with semen quality/breeding success in the cheetah. Findings revealed that genetic diversity has decreased in the wild, but not captive, population. Unexpectedly, heterozygosity was *lower* in proven versus unproven breeders and did not correlate with semen quality. A small proportion of all males (<10%) produced relatively high quality ejaculates, with sperm traits similar to those of non-inbred felid species. These data suggest a more complex relationship between inbreeding and male cheetah reproductive traits than previously appreciated. Intensive management of captive cheetahs appears to be minimizing inbreeding, whereas the continued erosion of genetic diversity in wild males is of conservation concern.

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1. Introduction

Inbreeding is linked to negative fitness consequences across a diversity of mammal, bird, fish, reptile, amphibian, insect, and plant species in the wild (Allentoft and O'Brien, 2010; Frankham et al., 2002; Keller and Waller, 2002). These negative effects are most profound in traits closely linked to reproductive success, including seminal quality and fecundity (Frankham et al., 2002). Species-level genetic diversity is correlated with semen quality among 20 mammals (Fitzpatrick and Evans, 2009), and analogous correlations have been documented at the individual level (i.e., within species) in the Iberian lynx (*Lynx pardinus*; (Ruiz-Lopez et al., 2012), Mexican gray wolf (*Canis lupus baileyi*; (Asa et al., 2007)), and Mohor gazelle (*Gazella dama mhorr*; (Ruiz-Lopez et al., 2012)). Within Felidae, the link between genetic diversity and male reproductive traits is well established. A single generation of inbreeding reduces semen quality in the domestic cat (*Felis catus*; (Neubauer et al., 2004)) and leopard cat (*Prionailurus bengalensis*; (Wildt, 1994)), while free-ranging inbred lions (*Panthera leo*) produce higher proportions of malformed spermatozoa and have fewer seminiferous tubules compared to non-inbred counterparts (Wildt et al., 1987). Consistent with this relationship, semen quality is relatively high among felid species with greater genetic diversity, including the ocelot (*Leopardus pardalis*), jaguar (*Panthera onca*), and African leopard (*Panthera pardus pardus*; (Pukazhenthi et al., 2006b; Swanson et al., 1995)).

Although some natural populations have persisted over long periods with limited genetic diversity (Reed, 2010), most studies support a general relationship between inbreeding and population decline/

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extirpation (Keller and Waller, 2002). In particular, the Florida panther (*Puma concolor coryi*) provides a compelling example of the consequences of extreme inbreeding. Compared to other puma subspecies, the Florida panther is highly inbred, with a population size of <100 individuals (Johnson et al., 2010; Roelke et al., 1993). Males experience severe reproductive defects, including an increased incidence of cryptorchidism, drastically reduced semen and testicular volumes, impaired sperm motility, and very high percentages (>90%) of structurally-abnormal spermatozoa (Mansfield and Land, 2002; Roelke et al., 1993), which are known to be incapable of fertilization (Howard et al., 1993). Conversely, introgression of DNA from eight Texas pumas (*Puma concolor stanleyana*) increased heterozygosity in the Florida population and resulted in fewer reproductive defects and greater offspring survival (Johnson et al., 2010). Aside from the Florida panther, the cheetah (*Acinonyx jubatus*) is perhaps the most thoroughly-studied wildlife model of inbreeding depression. The cheetah's lack of genetic diversity was originally detected by allozyme analysis and the ability of unrelated conspecifics to accept reciprocal skin grafts (O'Brien et al., 1983). This finding was subsequently confirmed by six additional measures of genomic variation (O'Brien, 1994), a lack of diversity in MHC class II-DRB alleles (Castro-Prieto et al., 2011), and the whole-genome sequencing of Namibian and Tanzanian cheetahs (Dobrynin et al., 2015). The cheetah's lack of genetic diversity is attributed to a severe population bottleneck that occurred ~12,000 years ago (Driscoll et al., 2002; O'Brien et al., 1985), from which the entire extant species is derived (Charruau et al., 2011). Intriguingly, recent genome sequencing suggests that a second ancient bottleneck occurred >100,000 years ago, coincident with the migration of cheetahs into Africa (Dobrynin et al., 2015). Importantly, the cheetah is the only modern felid species that lacks a non-inbred population – a fact that not only limits conservation options, but also complicates understanding the consequences of reduced genetic diversity (O'Brien and Johnson, 2005).

There has been substantial interest in understanding how genetic monomorphism influences health and reproduction in the cheetah, particularly because nearly all individuals studied to date consistently produce poor-quality semen (Crosier et al., 2007; Terrell et al., 2010; Wildt et al., 1983). Cheetahs maintained in zoological collections often fail to reproduce (Marker et al., 2014) and are susceptible to infectious disease (Munson, 1993) and birth defects (O'Brien et al., 1985). Initially, these issues were attributed to the species' lack of genetic diversity (O'Brien et al., 1985), but there is no evidence of impaired reproductive success, increased disease susceptibility, or high incidences of birth defects in wild cheetah populations (Caro and Laurenson, 1994; Castro-Prieto et al., 2011; Laurenson et al., 1995; Munson et al., 2004). Furthermore, although poor semen quality in the cheetah is presumed to have resulted from the ancient bottleneck (O'Brien et al., 1987), there has been no effort to empirically test this relationship. Therefore, while the cheetah is often cited for its extreme lack of genetic diversity, the manifestations of inbreeding are not entirely understood.

Although extensively debated (Caro and Laurenson, 1994; Laurenson et al., 1995; May, 1995; Merola, 1994; O'Brien, 1994), the question of inbreeding depression in the cheetah remains relevant because wild populations continue to decline (Durant et al., 2008). Over the last century, the cheetah was extirpated from >75% of its historical range, resulting in the geographic isolation of the southern African (*Acinonyx jubatus jubatus*) and east African (*Acinonyx jubatus raineyi*) subspecies (O'Brien et al., 1987; Ray et al., 2005). Whether these modern demographic changes have resulted in significant loss of genetic diversity is unknown. Since the early 1980s, captive cheetah populations in North America and Europe have been managed through cooperative breeding programs, with the goals of conserving rare genetic lineages and maintaining 90% of extant genetic diversity for the next 100 years (Association of Zoos and Aquariums, 2014). Given the cheetah's precarious status in the wild, the recent sequencing of its genome (Dobrynin et al., 2015), and the extensive efforts to create captive 'insurance' populations, it is an opportune time to evaluate the relationship between genetic diversity and reproductive traits in this species. We have a unique opportunity to test this relationship because our research group has collected DNA samples and/or reproductive data from >200 southern African cheetahs over the past 30 years. Additionally, these samples and records can provide insight into temporal changes in genetic diversity over several decades of population decline. In this study, our goal was to use archived DNA samples and paired reproductive data to better understand inbreeding depression in modern cheetahs. We predicted that genetic diversity of the southern African cheetah had eroded over the past 30 years, given the species' demographic declines in the wild and poor reproductive success in captivity. We further hypothesized that modern inbreeding (i.e., detected by microsatellite markers) would negatively affect reproductive traits in male cheetahs, specifically testis volume, sperm quantity and quality, and offspring production/survivorship.

2. Methods

2.1. Study populations

Our reproductive dataset included nearly 400 semen collections from wild ($n = 116$) and captive ($n = 99$) southern African cheetahs born from 1976–2007. Wild animals were captured (for reasons other than semen collection) throughout Namibia, excluding regions where the species is rare or absent (i.e., coastal areas, Kalahari Desert, and the Caprivi). The study area is arid to semi-arid, encompassing grassland and savanna, with ~400 mm rainfall per year. We identified 118 individuals in our dataset for which archived DNA samples also were available. These samples had been previously extracted from blood or tissue (using either a commercial kit (Qiagen; Valencia, CA) or standard phenol-chloroform procedure) and subsequently stored at −80 °C. After excluding DNA samples that failed to amplify ($n = 21$, see below), our genetic dataset represented 97 cheetahs, including those that were wild-born ($n = 54$) or captive-born in North America ($n = 27$), Europe ($n = 6$), or South Africa ($n = 10$). The latter group was of South African stock, but all other captive-born cheetahs were descendants of Namibian animals. Wild-born and captive-born populations are subsequently referred to as wild and captive, respectively. Importantly, these designations are based on population of origin (i.e., place of birth) and not whether the animals were subsequently housed in captivity. Wild cheetahs were either released into the wild (after semen collection) or transferred permanently to captive institutions. Captive individuals were born at accredited zoological institutions (North America and Europe) or breeding centers (South Africa). Birth years were obtained from the International Cheetah Studbook (Marker et al., 2014), except for five wild males for which this information was not recorded. Mean age at death for deceased males in our dataset was 12.0 ± 0.4 years, which is typical for a cheetah (Marker et al., 2014). Our dataset included seven suspected sibling groups ($n = 17$ wild cheetahs) and eight known sibling groups ($n = 18$ captive cheetahs). Required permits were obtained from the Namibian Ministry of Environment, and all animal procedures were approved by the Smithsonian Institutional Animal Care and Use Committee.

2.2. Microsatellite genotyping

We amplified 12 previously-described microsatellite markers (FCA8, FCA42, FCA85, FCA96, FCA97, FCA126, FCA214, FCA247, FCA298, FCA310, FCA441, FCA559) (Marker et al., 2008; Menotti-Raymond et al., 1999) using an Applied Biosystems® GeneAmp® 9700 Thermal Cycler and a 'touchdown' protocol (Marker et al., 2008). All loci were unlinked or >20 cm apart in the domestic cat (and therefore assumed to be unlinked in the cheetah), except for one pair (FCA85/FCA96) that was separated by 12 cm (Marker et al., 2008). These markers are unlikely to reflect the cheetah's bottleneck(s) ≥12,000 years ago because the present level of microsatellite diversity has likely accumulated over

the past 2928–10,716 years (Driscoll et al., 2002). Amplifications were performed in individual 10 μl reaction volumes that included 1× PCR Gold Buffer, 800 μM dNTPs, 2 mM $MgCl_2$, 0.4 μM dye-labeled M13, 0.03 μM M13 forward primer, 0.4 μM reverse microsatellite primer, 0.4 units of AmpliTaq Gold®, and 10 ng of genomic DNA. Allele sizes were estimated using a GeneScan™ 500 LIZ™ internal size standard and ABI 3730 Genetic Analyzer (Marker et al., 2008). Reagents were obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Microsatellites were genotyped using GeneMarker V1.85 software (Soft Genetics, State College, PA), and samples in which <9 loci amplified were excluded from analyses.

2.3. Reproductive traits

Semen was collected and analyzed using a standardized electroejaculation technique over the entire 30-year study period, as described previously (Pukazhenthi et al., 2006b; Wildt et al., 1983). Reproductive assessments of each individual included some or all of the following: testes volume, seminal volume, percentage of motile spermatozoa (%M), sperm forward progression (FP; scale, 0–5; 5 = fastest), sperm concentration, and percentage of structurally-normal spermatozoa. These sperm metrics are correlated with fertilization success in the cheetah (Donoghue et al., 1992; Howard et al., 1992). Sperm morphology and motility were assessed by phase-contrast microscopy, and a sperm motility index (SMI) was calculated as the average of %M and FP × 20. Six cheetahs in our dataset were excluded from reproductive analyses because they either produced sperm-free semen ($n = 1$), were part of a contraceptive study ($n = 1$), or were <2 years old (and likely sexually immature; $n = 4$) at the time of semen collection. The remainder of cheetahs were 2.5–10 years of age at the time of semen collection(s), a range considered sexually mature and not senescent (Crosier et al., 2007). Because reproductive data were absent or incomplete for some males, final sample sizes varied among reproductive metrics for both wild ($n = 43$–49) and captive ($n = 31$–42) cheetahs. Multiple ejaculates ($n = 2$–9) were collected for some wild ($n = 17$) and captive ($n = 23$) males. In these cases, average values were used for statistical analyses.

We estimated fecundity of wild (i.e., wild-born) and captive cheetahs housed in zoological institutions using studbook data (Marker et al., 2014). These estimates included breeding success (i.e., proven versus unproven), number of litters with at least one live cub (surviving >30 days), number of litters with at least one dead cub (surviving ≤30 days), and the absolute numbers and overall ratio of live/dead offspring (Marker et al., 2014). Individuals were excluded from these analyses if they were housed in Namibia (where captive breeding is restricted) or were alive at the time of the analysis (mean age = 10.0 ± 0.4 years) with possible subsequent breeding opportunities. These exclusions resulted in smaller sample sizes ($n = 15$ wild, $n = 38$ captive); therefore, we pooled wild and captive individuals for comparison of proven ($n = 6$ wild, $n = 20$ captive) versus unproven ($n = 9$ wild, $n = 18$ captive) breeders.

To determine how cheetah ejaculate quality compared to other felids (with varying levels of genetic diversity), we compared our database of cheetah ($n = 197$) sperm traits to an extensive database of 21 other species or subspecies, derived from our previous publications (Pukazhenthi et al., 2006b; Wildt et al., 1987) and unpublished observations (sample sizes provided in Fig. 1). We followed IUCN designations for species/subspecies, except that we grouped pumas geographically: Florida (*Puma concolor coryi*), other North America (*Puma concolor cougar*), and South America (*Puma concolor concolor*, *Puma concolor cabrerae*, *Puma concolor puma*, and *Puma concolor capricornensis*). Methods of semen collection and evaluation were identical to those described above.

2.4. Statistical analyses

Analyses were performed using R software (R Development Core Team, 2008) unless otherwise noted. Population-level (i.e., averaged across loci) estimates of genetic diversity were calculated using the *hierfstat* package and included *gene diversity* (i.e., expected heterozygosity), *observed heterozygosity*, *inbreeding coefficient*, and *allelic richness* (Goudet, 2014). Although differences among these metrics can provide insight into population dynamics, the latter three are included as supplemental data to avoid redundancy. Individual-level estimates of genetic diversity were calculated using the *Rhh* package and included *standardized heterozygosity* and *homozygosity by locus* (Alho et al., 2010). Standardized heterozygosity accounts for differences in microsatellite panels among individuals (Coltman et al., 1999), in this case due to differences in amplification success. Homozygosity by locus is a measure of internal relatedness that accounts for rare alleles (Aparicio et al., 2006) and is included as supplemental data to avoid redundancy.

We tested for evidence of inbreeding by calculating heterozygosity-heterozygosity (H-H) correlations for standardized heterozygosity (hereafter referred to simply as heterozygosity) (Balloux et al., 2004). Specifically, we divided the loci into two random groups, calculated heterozygosity estimates for each group, and obtained the mean correlation between the groups ($n = 10{,}000$ iterations). If inbreeding is present, these correlations should be significant (Alho et al., 2010).

We used Microchecker version 2.2.3 to test for scoring errors and null alleles (van Oosterhout et al., 2005) and ARLEQUIN v3.5 to test for deviations from Hardy-Weinberg and linkage equilibrium (Excoffier and Lischer, 2010). Boxplots were generated using the ggplot2 package

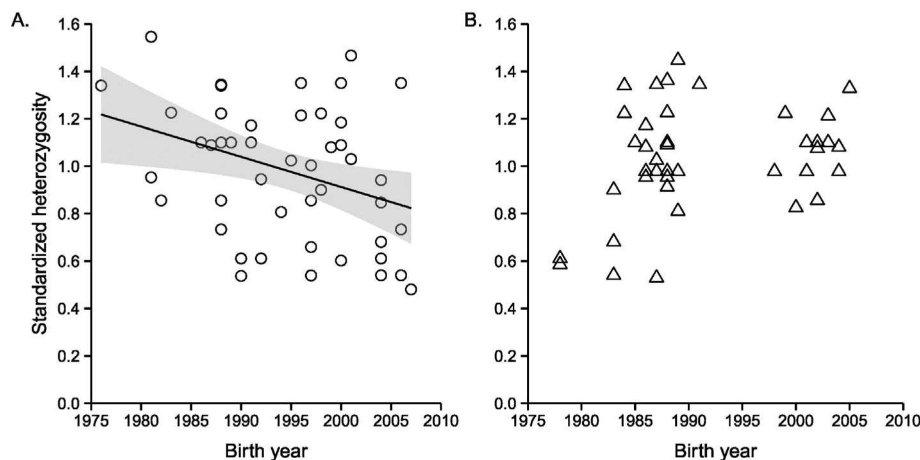

Fig. 1. Standardized heterozygosity relative to birth year among (A) wild and (B) captive cheetahs ($n = 49$, $r = -0.35$, $P = 0.015$ and $n = 43$, $r = 0.27$, $P = 0.077$, respectively). Shading represents the 95% confidence interval for the significant correlation.

(Wickham, 2009). We used a Student's t-test to compare genetic diversity estimates between wild and captive cheetah populations and between proven versus unproven breeders. The same approach was used to compare reproductive metrics between these groups. Pearson correlations were used to examine the relationship between birth year and heterozygosity separately for each population (i.e., wild versus captive). We also used Pearson correlations to test the relationship between heterozygosity and each reproductive metric separately for each population. To address potentially confounding sibling relationships within the dataset, comparisons of genetic diversity were repeated after omitting a randomly-chosen individual from each sibling pair. Values are reported as means ± standard errors unless otherwise indicated. Results were considered significant at the $P < 0.05$ level. A Holm-Bonferroni correction (Holm, 1979) was applied to each set of comparisons that included multiple measures of semen quality.

3. Results

3.1. Microsatellite genotyping

Across all individuals ($n = 118$), we observed 72 alleles in 12 microsatellites (3–9 alleles per locus). To reduce the chance of including unreliable data, only samples in which ≥9 loci successfully amplified ($n = 97$, representing all observed alleles) were included in subsequent analyses. The percentage of heterozygous samples per locus ranged from 42–84% (mean ± SD = 68% ± 12), with 80 to 100% amplification success. There was no evidence of deviation from Hardy-Weinberg equilibrium (HWE) in either the wild or captive population. Only one pair of loci (FCA08 and FCA310) was found to be in linkage disequilibrium ($P = 0.0002$) in the captive population only. These loci are located on separate chromosomes (A1 and C2, respectively) in the domestic cat and are assumed to be unlinked in the cheetah.

3.2. Genetic diversity and semen quality in wild versus captive cheetahs

There were no differences in any measure of genetic diversity between wild ($n = 54$) and captive ($n = 43$) cheetahs (Table 1, Appendix A), even when siblings were removed from the dataset (Appendix B). Despite the similarity in genetic diversity estimates, we detected a significant H-H correlation (i.e., evidence of inbreeding) in the wild population ($r = 0.30$, $P = 0.003$), but not in captive individuals ($P = 0.171$).

Across the 93 males for which both genetic and reproductive data were available, testes volume ranged from 7.0–22.0 cm^3 (mean ± SE = 13.0 ± 3.3 cm^3), and total spermatozoa per ejaculate from 0.2×10^6–289.0×10^6 cells ($55.5 \times 10^6 \pm 62.1 \times 10^6$ cells). Sperm motility index and the percentage of structurally-normal cells ranged from 40–80% (68 ± 8%) and 1–58% (22 ± 11%), respectively. For the 40 males in our dataset with multiple semen collections, average coefficients of variation were 13% for testes volume, 209% for total spermatozoa, 12% for sperm motility, and 42% for normal sperm morphology. For all comparisons described below, results did not change when analyses were repeated using maximum values instead of averages.

No differences were observed between wild and captive populations with respect to testes volume, total spermatozoa/ejaculate, or sperm motility (Table 1). The percentage of structurally-normal spermatozoa was greater ($t = -2.01$, $P = 0.0498$) in captive-born (25 ± 2%) versus wild-caught individuals (19 ± 1%). However, this difference became non-significant ($P = 0.199$) after applying a Holm-Bonferroni correction to control for family-wise error.

3.3. Genetic diversity and semen quality in proven versus unproven breeders

Studbook analysis revealed that 27% ($n = 26$) of males in our genetic dataset were proven breeders (i.e., produced offspring). The total number of live offspring per male ranged from 0 (i.e., dead offspring only) to 43 cubs, and represented from 1–12 litters. Dead offspring per male ranged from 0 to 19 cubs and represented from 1–8 litters. An additional 40% ($n = 39$) of males in our genetic dataset had no offspring recorded because: 1) they were released into the wild; 2) they were housed in Namibia (where captive breeding is restricted); or 3) breeding records were not maintained. Furthermore, 6% ($n = 5$) of males were alive at the time of analysis and might yet produce offspring. The remaining 28% ($n = 27$) of males were presumed to have had breeding opportunities, yet failed to produce offspring (i.e., unproven breeders).

Unexpectedly, gene diversity and heterozygosity were *lower* in proven versus unproven breeders ($t = -3.34$, $P = 0.007$ and $t = -2.65$, $P = 0.011$, respectively; Table 2). The same pattern was observed among other estimates of genetic diversity (Appendix C), even when siblings were removed from the dataset (Appendix D). Semen quality did not differ between proven and unproven breeders (Table 2).

3.4. Temporal patterns of genetic diversity

Heterozygosity declined over time among wild cheetahs born from 1976–2007 ($r = -0.35$, $P = 0.015$, Fig. 1A). In contrast, genetic diversity tended to increase in the captive population during the same period ($r = 0.27$, $P = 0.077$, Fig. 1B). The same patterns were observed with other metrics of genetic diversity (Appendix E).

3.5. Relationships between genetic diversity and reproductive traits

Unexpectedly, heterozygosity was not correlated with any measure of semen quality for either the wild or captive population ($-0.23 \leq r \leq 0.11$, $P \geq 0.243$; Fig. 2). Homozygosity by locus also was unrelated to semen quality ($-0.19 \leq r \leq 0.22$, $P \geq 0.220$; Appendix F). Within all proven breeders, heterozygosity did not correlate with numbers of live offspring ($r = 0.14$, $P = 0.487$), live litters ($r = 0.13$, $P = 0.518$), dead offspring ($r = -0.12$, $P = 0.568$), dead litters ($r = -0.15$, $P = 0.460$), or the percentage of dead offspring ($r = 0.029$; $P = 0.886$).

3.6. Variation in cheetah semen quality relative to other felids

Analysis of the overall semen collection database of 22 felid taxa revealed substantial variation in sperm production and cellular morphology within and across species/subspecies (Fig. 3). As expected, cheetahs ($n = 197$, including animals without DNA samples) produced relatively small numbers of spermatozoa and low percentages of structurally-normal cells compared to other large-sized felids. However, a relatively large number of outlying values (i.e., above the 75th percentile) were

Table 1
Genetic diversity and average semen quality of wild ($n = 54$) versus captive ($n = 43$) cheetahs.

Metric	Wild	Captive	t	P
Gene diversity	0.68 ± 0.03	0.70 ± 0.04	0.41	0.693
Standardized heterozygosity	0.98 ± 0.04	1.03 ± 0.03	−1.08	0.281
Combined testes volume (cm^3)	13.4 ± 0.5	12.5 ± 0.4	1.33	0.188
Total spermatozoa (10^6 per ejaculate)	61.0 ± 10.6	49.8 ± 8.3	0.83	0.407
Sperm motility index	68 ± 1.2	67 ± 1.3	0.46	0.649
Structurally-normal spermatozoa (%)	19 ± 1%	25 ± 2%	−2.01	0.050[a]

[a] $P = 0.199$ after Holm-Bonferroni correction.

Table 2
Genetic diversity and average semen quality of proven ($n = 26$[a]) versus unproven ($n = 27$[b]) breeders.

Metric	Proven	Unproven	t	P
Gene diversity	0.65 ± 0.04	0.75 ± 0.03	−3.34	0.007
Standardized heterozygosity	0.95 ± 0.05	1.11 ± 0.04	−2.65	0.011
Combined testes volume (cm^3)	12.7 ± 0.6	13.8 ± 0.6	−1.29	0.203
Total spermatozoa (10^6 per ejaculate)	57.2 ± 13.2	32.6 ± 7.3	1.63	0.112
Sperm motility index	67.5 ± 1.7	67.2 ± 2.0	0.10	0.920
Structurally-normal spermatozoa (%)	24.3 ± 3.1	23.5 ± 2.5	0.19	0.852

[a] Includes $n = 6$ wild males and $n = 20$ captive males.
[b] Includes $n = 9$ wild males and $n = 18$ captive males.

observed with respect to sperm production in cheetahs. Specifically, 9.6% of males ($n = 9$ wild, $n = 10$ captive) produced $\geq 100 \times 10^6$ spermatozoa per ejaculate (Fig. 3). Notably, these numbers were greater than the corresponding median values for nearly all large-sized felid species (Fig. 3). Furthermore, 4.6% of males ($n = 1$ wild, $n = 8$ captive) ejaculated proportions of structurally-normal spermatozoa (range, 40–59) comparable to other felid species considered to have 'normal' quality semen (i.e., ≥40% structurally-normal spermatozoa (Pukazhenthi et al., 2006b)). Approximately half of the individuals in each of these 'high-quality' semen groups (10 of 19 and 5 of 9, respectively) were represented in our genetic analyses. Testes volume and sperm motility data were not included in this comparison due to inadequate sample sizes for several of the species.

4. Discussion

This study was the first to investigate temporal patterns of genetic diversity in the cheetah and to examine the corresponding relationship with male reproductive traits. Our study yielded three major findings. First, genetic diversity has steadily declined among wild-born, male cheetahs from Namibia since the late 1970s. These animals represent the largest remaining, free-ranging cheetah population and likely the world's greatest resource for ensuring the species' survival (Durant et al., 2008). Second, in contrast to wild cheetahs, genetic diversity was maintained within the captive-born population. This latter finding suggests that the intensive genetic management of cheetahs in zoological institutions, which began in the early 1980s, has been largely successful in avoiding further losses in heterozygosity. Third, we found no relationship between modern inbreeding and semen quality in wild cheetahs. This finding contrasts with the well-established relationship between genetic diversity and reproductive health in felids.

While the cheetah continues to decline in the wild (Ray et al., 2005), a preponderance of evidence reveals the importance of maintaining extant genetic diversity in small or declining populations (Frankham et al., 2002). Experimental inbreeding/outbreeding has revealed the negative fitness consequences of reduced heterozygosity in felids (Mansfield and

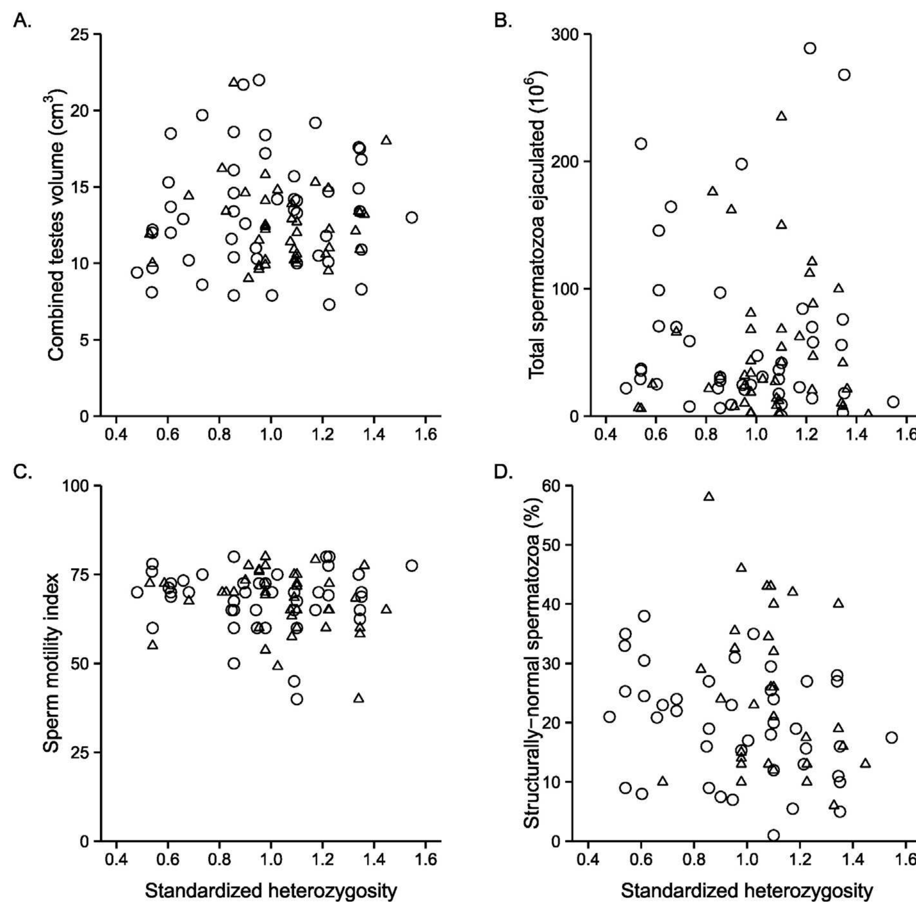

Fig. 2. Standardized heterozygosity of wild (circles; $n = 43$–49) versus captive (triangles; $n = 31$–42) cheetahs relative to average (A) testes volume, (B) total spermatozoa ejaculated, (C) sperm motility, and (D) sperm morphology.

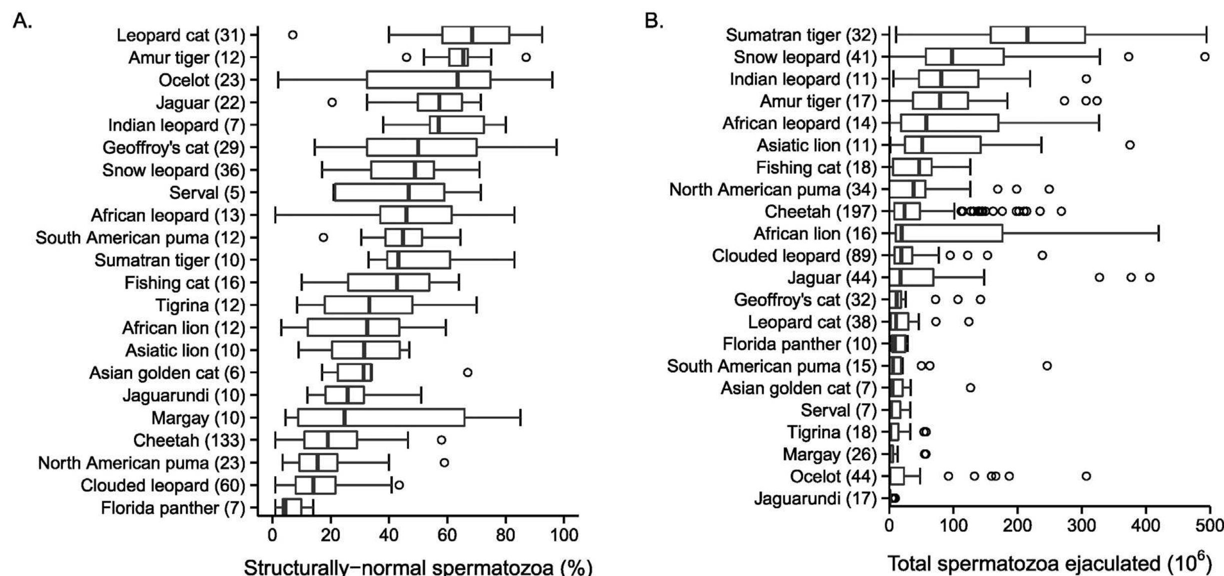

Fig. 3. Sperm (A) production and (B) morphology for 22 felid species or subspecies. Boxes represent 25th and 75th percentiles, error bars correspond to 1.5× the inter-quartile range, and open circles indicate outlying data. Values in parentheses represent the number of males in each group. One outlying data point for the Sumatran tiger is omitted from panel B due to scale. Figure is modified and updated from Pukazhenthi et al. (2006a, 2006b).

Land, 2002; Neubauer et al., 2004; Trinkel et al., 2011) and numerous correlative studies support this relationship (reviewed in Wildt et al., 2010). Our present findings suggest that careful, science-based population management of captive cheetahs over the last 30 years has minimized losses in genetic diversity. This finding is in contrast to the steady decline of heterozygosity among wild cheetahs and highlights the potential role of ex situ conservation programs in ensuring the long-term sustainability of wildlife populations. Indeed, such programs have markedly reduced extinction probabilities for a growing number of species, including (but not limited to) the black-footed ferret (*Mustela nigripes*; Wildt et al., 2016), golden lion tamarin (*Leontopithecus rosalia*; Kierulff et al., 2012), California condor (*Gymnogyps californianus*; Sarchet, 2015), and Houston toad (*Anaxyrus houstonensis*; Forstner and Crump, 2011). Moreover, the role of ex situ, 'insurance' populations in species conservation is likely to grow in the future, as anthropogenic pressures continue to exacerbate habitat loss and disease threats (Pritchard et al., 2012).

A major challenge to definitively understanding inbreeding consequences in the cheetah is the lack of a reference non-inbred population (O'Brien and Johnson, 2005). We were surprised to discover that recent loss of genetic diversity has not further compromised semen quality, and that heterozygosity was greater in 'unproven' males versus those that had produced offspring. This latter finding may indicate that reproductively-successful males belong to genetic lineages that are overrepresented in the population. In other words, genetic lineages that confer high fecundity might naturally dominate a small population. Alternately, a lack of mate choice among cheetahs managed in captivity could have confounded fecundity estimates by artificially reducing reproductive success in certain males. Finally, it was possible that our molecular markers did not accurately estimate heterozygosity in these individuals. Although models suggest that robust heterozygosity-fitness correlations require a large number of molecular markers, a smaller suite of microsatellites can accurately reflect genetic diversity in highly inbred populations, such as the cheetah (Balloux et al., 2004). Indeed, we detected a significant H-H correlation (a signal of inbreeding) in the wild, but not the captive population. This is consistent with the idea that genetic management efforts are minimizing inbreeding in the captive population. Furthermore, we observed a loss of heterozygosity in wild cheetahs over time, which is consistent with parallel observations of population declines (Durant et al., 2008; Ray et al., 2005).

We considered two alternative explanations for the observed lack of correlation between heterozygosity and semen quality. First, environmental factors might have confounded this relationship. Specifically, inbreeding depression in the cheetah may only be manifested under adverse environmental conditions or intense competition, as observed in the fruit fly (*Drosophila melanogaster*; Yun and Agrawal, 2014), red flour beetle (*Tribolium castaneum*; Pray et al., 1994), house mouse (*Mus domesticus*; Meagher et al., 2000), song sparrow (*Melospiza melodia*; Keller et al., 1994), and two Galapagos finch species (*Geospiza scandens* and *Geospiza fortis*; Keller et al., 2002). Second, and more likely, deleterious alleles regulating spermatogenesis may have become fixed in the global cheetah population during the ancient bottleneck(s), resulting in consistently poor semen quality irrespective of subsequent inbreeding (Frankham et al., 2002).

The high proportion (≥40%) of malformed spermatozoa ubiquitous among modern cheetahs supports the idea that deleterious alleles for spermatogenesis are fixed in the global population. It is important to note that sperm morphology was more consistent than the reported coefficient of variation (42%) would imply, since we reported percentages of *normal* spermatozoa and these values are typically very low (e.g., a male producing 5% and 10% normal ejaculates would have a CV of 47%). Still, our findings demonstrate that the cheetah exhibits considerable variation in seminal traits, with some individuals producing higher-quality semen comparable to non-inbred felid species. In particular, we observed a 100-fold difference in total spermatozoa/ejaculate across the males in our dataset. Although we did not find a relationship between semen quality and breeding success, our dataset did not account for differences in female fertility or behavioral compatibilities. Thus, the question of whether exceptionally high semen quality confers a reproductive advantage in cheetahs remains unanswered, and the factors contributing to this phenotype remain to be elucidated. Variation in cheetah semen quality is unrelated to age, season, or captivity (Crosier et al., 2007). Furthermore, semen quality can vary considerably among males housed within a single enclosure (Crosier et al., 2007; Terrell et al., 2010). Therefore, the question remains – why do some cheetahs produce substantially higher quality ejaculates than others?

It is possible that rare alleles are responsible for the relatively high measures of semen quality observed in a small percentage (≤10%) of our study animals. This possibility underscores the importance of conserving uncommon lineages in breeding programs. Three decades of fundamental research in cheetah reproductive physiology has provided

new tools for managing ex situ populations (reviewed in (Wildt et al., 2010)). These include novel approaches to animal husbandry (Wildt et al., 2012), assisted reproductive technologies (Donoghue et al., 1992; Howard et al., 1992), and genome resource banking (Wildt et al., 2010). A major challenge for the practical use of genome resource banks is the high cryo-sensitivity of oocytes and embryos (Pukazhenthi et al., 2006a), which are particularly important for maintaining mitochondrial and X-linked gene diversity. Intriguingly, recent sequencing of the cheetah genome revealed a high prevalence of mutations in *AKAP4* (an X-linked gene involved in sperm motility; (Dobrynin et al., 2015)), highlighting the importance of conserving diversity in sex-linked genes. In circumventing natural selection, assisted reproductive technologies may contribute to the loss of functional alleles for *AKAP4* and other genes linked to fertility/fecundity. Therefore, our findings serve as a reminder that successful management of insurance populations consists of more than the often-cited goal of preserving 90% gene diversity for the next century (Association of Zoos and Aquariums, 2014). Rather, it is necessary to also ensure that fitness-linked alleles persist in these populations, a goal that has become more feasible with modern advances in high-throughput and whole-genome sequencing.

With the recent sequencing of the cheetah genome (Dobrynin et al., 2015), this species presents exciting opportunities to assess the relative importance of heterozygosity versus rare alleles in the conservation of inbred populations. Yet, this flagship species continues to decline in the wild, and its future is uncertain. Our study reveals that the world's largest remaining cheetah population has lost genetic diversity at an alarming rate over the past 30 years. By contrast, the relatively stable level of heterozygosity in captive individuals suggests that intensive ex situ management is working, at least to minimize further inbreeding. Questions remain about if and how the continued erosion of genetic diversity in wild cheetahs will accelerate the species decline in nature. Is the species approaching a genetic threshold for sudden increases in infertility and/or disease susceptibility, as observed in highly inbred populations of puma (Roelke et al., 1993), lion (Wildt et al., 1987), and lynx (Ruiz-Lopez et al., 2012)? Can the long-term cryopreservation of gametes be used to recover lost gene diversity in the contemporary wild population, as recently achieved in the black-footed ferret (Wildt et al., 2016)? While habitat protection is essential to the ultimate survival of wildlife species, careful genetic management has the potential to contribute to the fight against extinction. Through a strategic blend of ex situ and in situ conservation efforts, we believe it is possible to stem the ongoing erosion of genetic diversity in the cheetah and other endangered wildlife species.

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Appendix A. Supplementary data

Additional metrics of genetic diversity are included for wild and captive cheetahs (Appendix A) and proven versus unproven breeders (Appendix C). Comparisons with sibling males omitted are provided for both sets of groups (Appendices B and D, respectively). Plots of homozygosity by locus versus birth year (Appendix E) and reproductive traits (Appendix F) are also included for wild and captive cheetahs. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.biocon.2016.05.034.

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