Relationship of inbreeding with sperm quality and reproductive success in Mexican gray wolves

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Abstract

The ultimate goal of the Mexican gray wolf *Canis lupus baileyi* captive management program is reintroduction of healthy individuals into wild habitats. To this end, zoo population managers work to provide not only for the physical well-being but also for the genetic health of these animals. However, the very limited genetic founder base, exacerbated by breeding within three distinct lineages, resulted in very high coefficients of inbreeding. Because support for measurable levels of inbreeding depression in the captive wolf population, as defined by reductions in common fitness measures such as juvenile survival or reproductive success, has been weak, we investigated the potential effects on male reproductive capacity. We analyzed semen samples from wolves from all three lineages and compared them with samples from subsequent lineage crosses and from generic gray wolves. We not only found a significant effect of inbreeding on sperm quality but we related both inbreeding and sperm quality to reproductive success. Samples from male offspring of lineage crosses, with inbreeding coefficients of zero were similar in quality to those from generic gray wolves. However, samples from a limited number of offspring from back-crosses were of extremely poor quality. Although it is reassuring that sperm quality was so much improved in male offspring of lineage crosses, the concomitant reduction in inbreeding coefficient does not eliminate the potentially deleterious alleles. Our results demonstrate that sperm quality is an important indicator of fertility and reproductive success in Mexican wolves. In addition, our data lend further support to the presence of inbreeding depression in this taxon.

Introduction

While still debated within many parts of the conservation community, the management of animal populations in captivity remains an important and often necessary component of endangered species recovery. The ultimate goal of most endangered species captive management programs includes the reintroduction of healthy individuals to wild habitats. To help achieve this goal, zoo population managers work to secure not only the physical well-being but also the genetic health of these animals. Specifically, the animals should be as similar as possible to their wild-born counterparts: the product of mating between healthy, unrelated animals so that concerns about the detrimental effects of inbreeding can be reduced or eliminated completely. Genetic health is not a concern solely for captive population management, because inbreeding may also affect survival of animals in the wild (Jiménez *et al.*, 1994; Keller *et al.*, 1994).

The conservation history of the Mexican gray wolf *Canis lupus baileyi*, listed in 1976 by the US Fish and Wildlife Service as an endangered subspecies of the gray or timber wolf, provides an excellent example of the successful use of scientific analysis in captive population management, as well as the practical difficulties of conserving such critically endangered species. The original captive population dates back to the late 1970s when six wolves were captured from Chihuahua and Durango in México and placed in zoos in the US. A decade later, only four of these original founders – just three males and a lone female – had offspring in the captive population. Since its inception, the population now known as the McBride lineage (first called the Certified lineage) has been carefully managed using the Association of Zoos and Aquariums (AZA) Species Survival Plan (SSP) methodology for maximum retention of the original genetic variation collected from the wild in these four animals.

Having only one female founder, all the descendents would be related as at least half-siblings and rates of inbreeding would quickly begin to increase, which would

have serious consequences for the long-term viability of the McBride lineage. However, two other populations of purported Mexican wolves existed in captivity: the Ghost Ranch lineage in Arizona and the Aragón lineage in México City. While each of these lineages was itself founded by a very small number of individuals (most likely a single pair each), and therefore had become highly inbred, integrating the two additional lineages into the SSP populations would reduce both the frequency of inbreeding and its potential consequences. Population genetics theory indicates that, even if two individuals are themselves highly inbred but unrelated to each other, their offspring will not be inbred and, most likely, more healthy than their inbred counterparts. The case of the highly inbred Florida panther *Felis concolor coryi* and its 'genetic rescue' using unrelated cougars from Texas is an outstanding case in point (Hedrick, 1995).

Following a detailed study of the molecular genetic relationships between McBride three of the four lineage founders, Hedrick *et al.* (1997) concluded that two were likely related, which reduced the official number of founders from four to three. Given these data, a number of matings once thought to be between unrelated individuals were subsequently identified as mother–son pairings. This finding made the need to evaluate the impacts of inbreeding on the health of the McBride lineage more urgent.

Inbreeding depression and its role in the conservation of endangered species has long been the subject of detailed study and emphatic debate (see Hedrick & Miller, 1992; Hedrick & Kalinowski, 2000, for reviews). Statistical analysis of the relationship between the level of individual inbreeding (measured by the inbreeding coefficient, f, for each animal) and specific fitness traits, such as survival of animals to a given age, is the standard method to evaluate the severity of inbreeding depression in populations for which complete pedigree information is available. Kalinowski, Hedrick & Miller (1999) used this methodology to study the McBride lineage and found no demonstrable evidence for inbreeding depression affecting litter size or survival of Mexican wolf pups to 180 days. However, a more recent analysis (Fredrickson & Hedrick, 2002) has demonstrated inbreeding depression for body size in captive Mexican wolves.

While fitness traits such as offspring survival or body size may be among the easiest parameters to measure in an analysis of inbreeding depression (e.g. Ralls, Brugger & Ballou, 1979; Lacy, Alak & Walsh, 1996; Coltman, Bowen, & Wright, 1998), the impact of this phenomenon can be manifested in many other aspects of biological function. For example, male cheetahs and Florida panthers with a low genetic variation have very poor sperm quality (Wildt *et al.*, 1987; Barone *et al.*, 1994), and sperm quality differs between two populations of lions, being poorer in the one more geographically isolated and presumed more highly inbred than the other. However, these studies compared genetic variability and semen quality only at the population level, and the relationship between extent of inbreeding and degree of sperm abnormality was not analyzed. Only a study of Cuvier's gazelles has demonstrated a direct relationship between inbreeding and sperm quality (Roldan *et al.*, 1998), but it did not relate sperm quality to individual male fertility.

We analyzed levels of inbreeding in Mexican wolves relative to two primary indicators of sperm quality: motility and morphology, and we compared those parameters with reproductive success, that is; the production of young, as a measure of fertility. We also compared sperm quality of Mexican and generic gray wolves.

Methods

The Mexican wolves were housed at the Wild Canid Survival and Research Center (WCSRC), Eureka, MO; the Minnesota Zoo, Apple Valley, MN; Detroit Zoo, Detroit, MI; Albuquerque Biological Park, Albuquerque, NM; Point Defiance Zoo, Tacoma, WA; Sevilleta Wolf Management Facility, Soccoro, NM; and San Juan de Aragón and Chapultepec Zoos, México City, México. The generic gray wolves were all housed at the Wildlife Science Center, Forest Lake, MN. Husbandry practices were similar for the Mexican wolves at the US facilities, because they are all part of a cooperative breeding program supervised by the US Fish and Wildlife Service and the AZA Mexican Wolf SSP. The SSP publishes a Mexican wolf husbandry manual and offers keeper workshops at WCSRC to train those who care for the animals in the program to standardize practices. All wolves were housed outdoors with access to den boxes or in some locations to indoor heated areas.

We collected semen samples during breeding seasons from 1992 to 2005. Males were selected for semen collection and cryopreservation by the US Fish and Wildlife Service and the AZA Mexican Wolf SSP. To control for possible lower semen quality early and late in the breeding season, only samples collected during the last week of January through the end of February were used in statistical analyses. Semen was collected under general anesthesia using ketamine hydrochloride (Ketaset, Boehringer Ingelheim Vetmedica Inc., St Joseph, MO, USA) and xylazine (Rompun, Bayer Corp., Shawnee Mission, KS, USA), with anesthesia maintained at some institutions by isoflurane (Isoflo, Abbott Laboratories, Chicago, IL, USA). The urinary bladder was flushed with sterile saline before stimulation with a Model 12 electroejaculator (G & S Instrument Co., Duncan, TX, USA) using a rectal probe (PT Electronics, Boring, OR, USA) with three linear electrodes, placed ventrally. Stimulation was increased slowly until the hindlimbs extended, returned to zero and repeated rhythmically, with an *c*. 5 s cycle, at gradually increasing voltage until ejaculation. Semen samples were immediately examined at $\times 200$ and estimates were made of per cent motile sperm and status (i.e. quality and vigor of movement). Sperm morphology was assessed after eosin–nigrosin staining.

We used an analysis of variance (NOSS2000, Kaysville, UT, USA) to compare the percentages of sperm cells that were motile and percentages that had normal morphology among the Mexican wolf lineages (Aragón, Ghost Ranch, McBride), generic gray wolves and Mexican wolf lineage

Table 1 Number of Mexican *Canis lupus baileyi* and generic gray wolf males sampled and the range of inbreeding coefficients per lineage for Mexican wolves

	Number of males	Inbreeding coefficients (range)
Mexican gray wolves (total)	55	
McBride	20	0.125–0.25
Ghost Ranch	7	0.5–0.6094
Aragón	3	0.25–0.3125
McBride × Ghost Ranch	7	0
McBride × Aragón	8	0
Tri-lineage	7	0.0527–0.1782
McBride × (McBride × Ghost Ranch)	2	0.1113
McBride × (McBride × Aragón)	1	0.1171
Generic gray wolves (total)	13	

Figure 1 Comparison of mean ($\pm$ SE) percentages of sperm motility and normal morphology for all pure-lineage Mexican wolves *Canis lupus baileyi* ($n = 30$), lineage-crossed Mexican ($n = 15$, excludes tri-lineage and back-crossed males) and all generic gray wolves ($n = 13$).

crosses. We evaluated significant differences with the Bonferroni multiple comparison test. Inbreeding coefficients for Mexican wolves were compared with percentages of motile sperm and of sperm with normal morphology with Kendall's τ. For this analysis, we used the value of the semen sample with the highest percentage of normal sperm for each male. We chose this value because sperm morphology, as well as other measures of sperm quality, can vary during the breeding season (i.e. early and late in the season, sperm are likely to be of lower quality) and with interval since the last ejaculation (sperm age and lose quality as they are held in the vas deferens). Thus, the samples of lower quality could represent artifacts that did not accurately reflect his potential fertility.

Reproductive success could be assessed only for a subset of 28 males, because not all 55 were recommended by the SSP to breed and so were not placed with a female before collection or by the time the analyses were performed. A partial Mantel with permutation test (Program Mantel, version 4.0, Casgrain, 2004) was used to compare sperm morphology and inbreeding coefficients with reproductive success, that is, birth of pups to the pair.

Figure 2 Percentage of sperm with normal morphology in samples from 55 Mexican wolves *Canis lupus baileyi* relative to their inbreeding coefficients. M, McBride lineage; G, Ghost Ranch; A, Aragón; X, lineage crosses; BX, lineage back-crosses. The total number of symbols is < 55 because some wolves had the same scores, and so those data points are superimposed. Outliers indicated by arrows are discussed in the text.

Results

Mexican wolf inbreeding coefficient was related to two standard measures of semen quality: cell morphology and motility. We found a highly significant negative correlation between inbreeding coefficient of Mexican wolves and percentages of normal sperm cells in their ejaculates (Kendall's $\tau = -0.3002$, $P = 0.002$). There was also a significant correlation between inbreeding and percentages of motile cells (Kendall's $\tau = -0.2379$, $P = 0.025$).

In comparing these sperm parameters among the three lineages, the lineage crosses and generic gray wolves (number of males per category is presented in Table 1), we found a significant difference for percentage of motile cells ($F = 3.05$, $P = 0.024$) and for cells with normal morphology ($F = 4.01$, $P = 0.006$) among wolf categories (Fig. 1). *Post hoc*
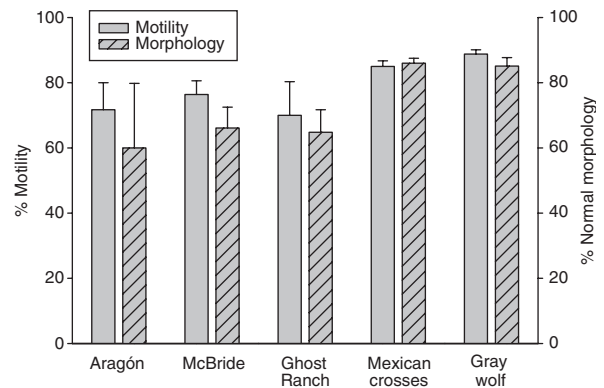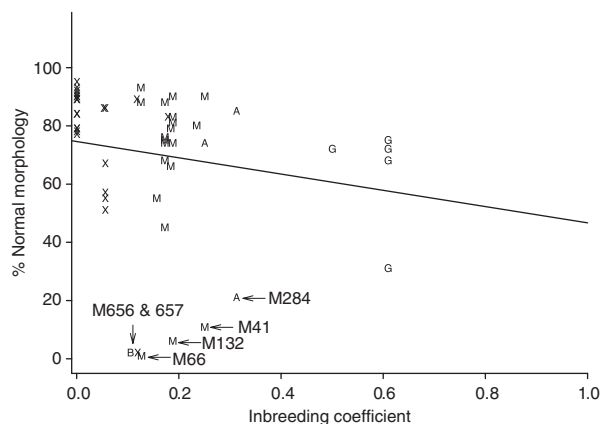
analysis showed a lower percentage of motile sperm per ejaculate in the Ghost Ranch lineage than in generic gray wolves. For percentage of cells with normal morphology, *post hoc* analysis revealed significantly lower percentages of sperm with normal morphology from McBride and Ghost Ranch males compared with the lineage crosses or generic gray wolves. Figures 2 and 3 illustrate the distribution of results for individual wolves for percentages of morphology and motility, respectively, relative to the level of inbreeding.

In examining results for individual males, the poorest samples for morphology were found in the McBride lineage, with four of 20 values (representing the best score for each male from multiple semen collections) below 50% normal (male 66: 1%, male 132: 6%, male 41: 10% and male 546:
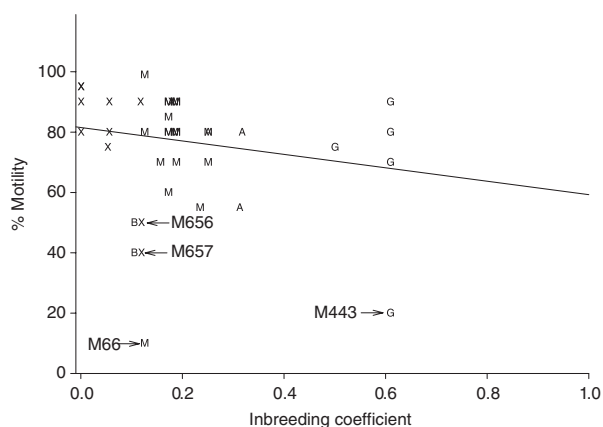
Figure 3 Percentage of motile sperm in samples from 55 Mexican wolves *Canis lupus baileyi* relative to their inbreeding coefficients. M, McBride lineage; G, Ghost Ranch; A, Aragón; X, lineage crosses; BX, lineage back-crosses. The total number of circles is <55 because some wolves had the same scores, and so those data points are superimposed. Outliers indicated by arrows are discussed in the text.

45%). One of seven Ghost Ranch and one of three Aragón males had values below 50% normal (male 427: 31% and male 284: 21%, respectively). The abnormalities were more severe in three of the McBride males; two had sperm that were microcephalic (tiny sperm heads indicating probable absence of chromosomes) with coiled tails, and the other had sperm with detached heads. The fourth (45% normal) had sperm with abnormal acrosomes. The Ghost Ranch male (31% normal) and Aragón male (21% normal) with the lowest morphology values had sperm with proximal droplets. In contrast, none of the normal sperm morphology values for generic gray wolves or lineage cross males were below 50% (lowest 60 and 77%, respectively). Two of three back-crossed males (MxMG, males 656 and 657) had particularly poor sperm, each with only 2% normal morphology. These two males, full siblings, were not paired with females before the end of our study, and so did not have an opportunity to reproduce. Although their sperm morphology scores were next to the lowest, their motility scores (40 and 50%, Fig. 3) were considerably higher than those of males 66 and 143.

The poor morphology and motility scores for McBride male 66 (Figs 2 and 3) may have been due to his advanced age (10 years) at the time of semen collection, because he had reproduced successfully when younger. The reasons for poor sperm morphology for McBride males 41 and 132 (Fig. 2) are not apparent. Male 41 had poor sperm quality when collected at 5 and 8 years of age. Similarly, male 132 had poor sperm when collected at 6 years of age. Neither male reproduced when placed with a female.

Abnormalities were primarily detached heads and coiled tails. The samples with especially low percentages of sperm with normal morphology did not always have very low percentages of motile sperm as well. Motility for the samples with normal morphology below 50% had motility ranging

from 10 to 80%. However, there was a significant correlation between motility and morphology ($r = 0.54$, $P < 0.001$)

For McBride lineage males, nine of 13 (69%) paired with females reproduced; for Ghost Ranch males, zero of two (0%); for Aragón males, one of three (33%); and for the lineage crosses, six of six (100%) reproduced successfully. There was a highly significant relationship between sperm morphology and reproductive success as well as inbreeding coefficient (Mantel r statistic $= 0.175$, $P = 0.009$), indicating that sperm quality can affect fertility.

Discussion

These results build upon the work of Fredrickson & Hedrick (2002) by providing important data to enhance our understanding of the role that inbreeding plays in the fitness of Mexican wolves. High levels of inbreeding were inversely correlated with two of the major indicators of semen quality, percentages of motile sperm and of sperm with normal morphology, with the effect on morphology being stronger. In addition, analyses of samples from pure lineage males showed differences relative to Mexican wolf lineage crosses and to generic gray wolves. Both McBride and Ghost Ranch males had significantly lower percentages of normal sperm than did either the Mexican wolf lineage crosses or the generic gray wolves (Fig. 1), but the very low number of samples from Aragón males may have prevented detection of a difference for that lineage. However, only the highly inbred Ghost Ranch-lineage males had significantly lower percentages of motile sperm than did the generic wolves. This considerable reduction in sperm quality among Ghost Ranch males is consistent with the findings of Hedrick, Lee & Parker (2000), who found an important Class II gene in the major histocompatibility complex, thought to be vital for genetically based pathogen recognition and resistance in mammals, to be monomorphic in this lineage. These authors also found microsatellite variation to be low in this same lineage. Both these data and our findings are readily explained by high rates of inbreeding leading to significant declines in heterozygosity

Our results are also the first to relate inbreeding to sperm quality for canids, although reduced heterozygosity has been correlated with poorer sperm quality in Cuvier's gazelles *Gazella cuvieri* (Roldan *et al.*, 1998), wild rabbits *Oryctolagus cuniculus* (Gage *et al.*, 2006), lions *Panthera leo* (Wildt *et al.*, 1987) and domestic cats (Pukazhenthi, Wildt & Howard, 2001). Thus, reduced sperm quality may not be an unusual sequela of inbreeding and may explain reduced rates of reproduction in some populations. In particular, severely restricted populations or those that have undergone bottlenecks may be particularly vulnerable.

The level of inbreeding was associated not only with poor semen quality (motility and morphology), but both variables were significantly correlated with reproductive success, indicating that the level of defective sperm observed could depress fertility. Sperm structure can affect parameters such as velocity and direction of movement (midpiece and tail), as well as ability to penetrate an ovum (acrosome), which has

been related to fertility in other species (e.g. Gomendio, Cassinello & Roldan, 2000; Malo *et al.*, 2005). Similarly, sperm with poor motility have been associated with reduced fertility [red deer *Cervus elaphus hispanicus* (Malo *et al.*, 2005)], perhaps by reducing the number of sperm that reach the site of fertilization. Previous studies have found only weak evidence of inbreeding depression in Mexican wolves (Kalinowski *et al.*, 1999; Fredrickson & Hedrick, 2002). Similar research on Fennoscandic wolves (Laikre & Ryman, 1991) shows some support for reduced juvenile body weight resulting from inbreeding among genes of Swedish origin, with stronger evidence for reduced productivity and longevity through inbreeding among genes of Finnish origin. In the Fennoscandic example, the mechanisms mediating the effects were not studied.

A highly inbred captive population of Finnish gray wolves *Canis lupus* had low reproductive rates and reduced longevity (Laikre & Ryman, 1991), but the mechanisms mediating the effects were not studied. Similarly, Vila *et al.*, 2003; Liberg *et al.*, 2005, studied a highly inbred population of free-ranging Scandinavian gray wolves, but sperm parameters were not measured.

Establishing a minimum level of semen quality for fertility in any species is difficult if not impossible, and so we related semen quality to a male's ability to produce offspring, that is, his reproductive success. Even without measuring direct effects on fertility, it is very likely that males with lower than 10% normal sperm are functionally infertile. However, for males with values between 10 and 60%, fertility may be only of relatively lower probability. In a study of domestic dogs, fertility of males with >60% normal sperm was 61% (14 of 23 females inseminated conceived), whereas, for those with <60% normal sperm the conception rate was only 13% (Oettlé, 1993). As for the Mexican wolves in this study, sperm head abnormalities were also the most common problems reported for the subfertile male dogs. Nöthling, Gerstenberg & Volkmann (1997), however, found that sperm motility was a better predictor of fertility in domestic dogs, although proximal cytoplasmic droplets were also implicated. Proximal droplets were a common abnormality in Mexican wolves as well, although motility was not as important a factor. An important parameter not measured in this study was fertility of females, with the result that assessments of male reproductive success were conservative, because they could have been depressed due to sub-fertile female partners (e.g. Amann, 2005).

Although the generic gray wolves were housed at a different facility and not required to follow the Mexican wolf husbandry guidelines, the very close similarity on measures of sperm motility and morphology between them and the Mexican wolf lineage crosses suggests that any possible husbandry differences were not important. The Mexican wolf lineage crosses were all housed at facilities that also held pure lineage males included in the analyses, which provides further evidence of the relative unimportance of location and details of management. Similarly, although the anesthetic protocol at the facility housing generic wolves differed slightly from that used by most of the zoos, the lack of a difference in semen quality parameters between generic wolves and Mexican wolf lineage crosses indicates that anesthesia was not the variable responsible for the lower sperm quality in the pure-lineage males.

After the three lineages were certified and breeding between them began in 1995, sperm quality of the resulting male offspring improved. However, the reappearance of high percentages of severely deformed sperm cells in subsequent back-crosses is of concern. While we have only a small sample of individuals from which to draw conclusions, this observation is likely to have a demonstrable genetic basis. One plausible hypothesis to explain this observation predicts that a significantly detrimental recessive allele is almost certainly covered in the heterozygous state in the parent produced by the McBride × Ghost Ranch lineage cross. The allele may also be heterozygous in the McBride male parent, although it may perhaps be homozygous in this inbred individual. Given this genetic background, the back-cross then exposes the deleterious variant in the progeny, leading to lower sperm quality compared to those F_1 males produced directly by crosses between lineages. If the hypothesis were to be confirmed, it would be particularly interesting in the sense that the observed reduction in sperm quality may be caused by homozygosity at just a few genetic loci.

An alternative, and more complex, interpretation of this observation relies on the knowledge that the fitness benefit derived from crossing lineages is often lost in the production of F_2 progeny, as co-adapted gene complexes in the original parental populations, left undisturbed in the first round of F_1 chromosomal recombination, are disrupted in this second bout of recombination. This interpretation must rely on a rather rapid rate of co-adaptation, but may nevertheless be worthy of consideration. Such a hypothesis is complicated further by considering the presence of epistatic interactions between loci that collectively underlie the genetic basis of individual phenotype, and how this mechanism works in concert with the genetic process of inbreeding. A more detailed treatment of this topic is presented by Lynch (1991). In order to better distinguish between these two alternatives, a much larger sample of materials derived from individuals produced through hybridization and back-crossing would be necessary.

It is reassuring that sperm quality is so much improved in male offspring of lineage crosses, but the concomitant reduction in inbreeding coefficient does not eliminate the potentially deleterious alleles. Thus, back-crosses in particular may continue to have poor sperm quality. Continued monitoring of semen parameters in the population may reveal the extent and severity of the effect. It would also be interesting to examine semen samples from generic gray wolves or other canids that experienced severe population bottlenecks, for example, the wolves of Isle Royale in Lake Superior.

Although it has long been established that inbreeding could affect reproduction, only more recently have specific

reproductive parameters such as sperm quality been measured relative to inbreeding (e.g. *G. cuvieri* Roldan *et al.*, 1998; *Gazella dorcas*, *G. cuvieri*, *Gazella dama* Gomendio *et al.*, 2000; rabbit *O. cuniculus* Gage *et al.*, 2006). The relationship between poor sperm quality and level of inbreeding in such diverse taxa suggests that it might be more widespread.

Acknowledgements

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