

The effects of inbreeding on sperm morphometry of captive-bred endangered mammals

M. Lawrence, G. Mastromonaco, K. Goodrowe, R.M. Santymire, W. Waddell, and A.I. Schulte-Hostedde

Abstract: Captive breeding is used for the conservation of endangered species, but inbreeding can result when a small number of founders are used to establish populations. Inbreeding can reduce the proportion of normal sperm in an ejaculate, but may also have effects on sperm size and shape (morphometry). We investigated the effects of inbreeding on sperm morphometry of black-footed ferrets (*Mustela nigripes* (Audubon and Bachman, 1851)) and red wolves (*Canis rufus* Audubon and Bachman, 1851) from captive breeding programs to determine if more inbred males produced sperm of poor quality (bulky head, small midpiece, short tail). We measured sperm head length, head width, midpiece length, midpiece width, and tail length on 10 sperm from each male of both species. A negative relationship between variation in sperm tail length and inbreeding coefficient (f) was found in black-footed ferret, suggesting that more inbred individuals will have reduced genetic and phenotypic variation. Analyses indicated a negative relationship between sperm head width and f and a positive relationship between sperm tail length and f in red wolf, suggesting that more inbred male red wolves could have faster sperm. These results indicate that inbreeding affects functionally important aspects of sperm morphometry, but that these effects may not be entirely negative.

Key words: sperm morphometry, conservation, captive breeding, zoo, phenotypic variation, red wolf, *Canis rufus*, black-footed ferret, *Mustela nigripes*.

Résumé : L'élevage en captivité est utilisé pour la conservation d'espèces en voie de disparition, mais une consanguinité peut se produire quand un petit nombre d'individus fondateurs sont utilisés pour établir des populations. Si la consanguinité peut réduire la proportion de spermatozoïdes normaux dans un éjaculat, elle peut également avoir des effets sur leur taille et leur forme (morphométrie). Nous avons examiné les effets de la consanguinité sur la morphométrie des spermatozoïdes de putois d'Amérique (*Mustela nigripes* (Audubon et Bachman, 1851)) et de loups roux (*Canis rufus* Audubon et Bachman, 1851) issus de programmes d'élevage en captivité afin de déterminer si les mâles plus consanguins produisent des spermatozoïdes de piètre qualité (tête massive, petite pièce intermédiaire, flagelle court). Nous avons mesuré la longueur et la largeur de la tête, la longueur et la largeur de la pièce intermédiaire et la longueur du flagelle de 10 spermatozoïdes de chaque mâle des deux espèces. Une relation négative entre la variation de la longueur du flagelle et le coefficient de consanguinité (f) a été notée pour les putois d'Amérique, qui donne à penser que les individus plus consanguins présentent moins de variation génétique et phénotypique. Des analyses indiquent une relation négative entre la largeur de la tête des spermatozoïdes et f et une relation positive entre la longueur du flagelle et f chez les loups roux, donnant à penser que les spermatozoïdes des mâles plus consanguins de cette espèce pourraient être plus rapides. Ces résultats indiquent que la consanguinité a une incidence sur des aspects fonctionnellement importants de la morphométrie des spermatozoïdes, mais que ces effets pourraient ne pas être complètement négatifs. [Traduit par la Rédaction]

Mots-clés : morphométrie des spermatozoïdes, conservation, élevage en captivité, zoo, variation phénotypique, loup roux, *Canis rufus*, putois d'Amérique, *Mustela nigripes*.

Introduction

Captive breeding programs are widely used to support endangered species conservation, and have successfully rescued numerous species from extinction (Snyder et al. 1996; Dobson and Lyles 2000; Frankham 2008; Hedrick and Fredrickson 2008; Williams and Hoffman 2009). The goal of many conservation breeding programs is to maintain genetic diversity, reduce inbreeding, and reintroduce animals to the wild (Bryant et al. 1999; Dobson and Lyles 2000; Asa et al. 2007; Hedrick and Fredrickson 2008). Typically, species brought into these programs are at risk of extinction and have few individuals remaining in the wild, which can lead to inbreeding and low genetic diversity (Snyder et al. 1996; Bryant

et al. 1999; Roldan and Gomendio 2009). Captive breeding programs, with their detailed pedigrees, also offer opportunities to test evolutionary hypotheses such as those related to inbreeding.

Inbreeding depression, the reduction in fitness experienced by offspring from related parents, is inevitable when relatively large populations are propagated from a small number of founders (Bryant et al. 1999; Hedrick and Kalinowski 2000; Lynch and O'Hely 2001; Keller and Waller 2002; Roldan and Gomendio 2009). High levels of inbreeding result in a loss of genetic diversity, low fertility rates, and the expression of recessive deleterious alleles that can lead to abnormalities and death (Keller and Waller 2002; Charlesworth and Willis 2009). In captive populations where stud-

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books are maintained, it is possible to calculate the coefficient of inbreeding (f), which is the probability that two alleles will be identical by descent (Keller and Waller 2002; Walling et al. 2011). Inbreeding depression can compromise reproductive output in a number of ways including the following: decreased juvenile survival, decreased egg production, decreased hatching rates, reduced ejaculate volume, and decreased proportions of normal and motile sperm cells (Crnokrak and Roff 1999; Keller and Waller 2002; Asa et al. 2007; Fitzpatrick and Evans 2009). Here, we focus on how inbreeding depression could compromise efforts to breed and reintroduce sustainable populations by negatively impacting male fertility. For example, the number of offspring born in a laboratory population of inbred male fruit flies (*Drosophila simulans* Sturtevant, 1919) was significantly less than the number of offspring born to outbred males, and outbred males attracted mates more quickly than inbred males (Okada et al. 2011). Wild red deer (*Cervus elaphus* L., 1758) had decreased lifetime breeding success compared with less inbred males (Slate et al. 2000). Captive Mexican wolves (*Canis lupus baileyi* Nelson and Goldman, 1929) with a high f had smaller proportions of motile and morphologically normal sperm than those with a lower f (Asa et al. 2007). This reduction in sperm quality was related to reduced reproductive success, indicating that depressed sperm quality affects fertility (Asa et al. 2007). Finally, low heterozygosity in endangered species has been linked to increased proportions of abnormal and immotile sperm (Fitzpatrick and Evans 2009). This relationship was not found in species that were not at risk (Fitzpatrick and Evans 2009).

Variability in sperm size and shape can have a significant effect on sperm swimming speed and fertilization success through changes in head size and shape, size of the midpiece, and length of the tail (Malo et al. 2006; Humphries et al. 2008; Tourmente et al. 2011; Simmons and Fitzpatrick 2012; Ramon et al. 2013; Simpson et al. 2014). Head elongation has a positive impact on sperm swimming speed because of the reduction in drag associated with longer, thinner heads (Malo et al. 2006; Tourmente et al. 2011). Additionally, Ramon et al. (2013) showed that male red deer with high proportions of sperm with small, elongated heads had increased fertility relative to males with sperm with small, wide heads. The sperm midpiece contains mitochondria, which produces energy used for sperm movement (Anderson and Dixson 2002; Firman and Simmons 2010). Species whose sperm were subject to a greater degree of sperm competition had increased midpiece volume, potentially containing greater mitochondrial loading and fuelling faster sperm (Anderson and Dixson 2002; Anderson et al. 2005). Greater midpiece lengths were also a predictor of faster sperm swimming velocity in house mice (*Mus musculus* L., 1758) (Firman and Simmons 2010). Tail length is also important to sperm swimming speed, but the relationship between tail length and swimming speed has been inconsistent (Humphries et al. 2008; Simpson et al. 2014). Some studies have revealed a positive relationship between sperm velocity and sperm length, indicating that longer sperm may move faster and be more successful at fertilizing a female ovum (Gomendio and Roldan 1991; Tourmente et al. 2011). Simpson et al. (2014) found a negative relationship between tail length and swimming speed in one internal fertilizer and no relationship between length and swimming speed in two others. Overall, we predict that because inbreeding depression can affect other sperm traits (Asa et al. 2007; Fitzpatrick and Evans 2009), then sperm morphometry may also be negatively affected by inbreeding. If this is the case, then more inbred males should have sperm traits that negatively affect sperm swimming speed such as less hydrodynamic heads (Malo et al. 2006; Tourmente et al. 2011; Ramon et al. 2013) and smaller midpieces (Anderson and Dixson 2002).

Our objectives for the current study were to evaluate the impacts of inbreeding, measured using inbreeding coefficient, in captive-bred animals on sperm size and shape (sperm morphometry). We chose to focus only on the effects of inbreeding on sperm

morphometry because the effects of inbreeding on other aspects of sperm morphology (proportions of normal, motile sperm) have previously been investigated (Asa et al. 2007; Fitzpatrick and Evans 2009). Sperm size and shape are generally not considered in captive breeding, but can affect fertility success and ultimately fitness. We examined two species that have been extensively bred in captivity, the black-footed ferret (*Mustela nigripes*) and the red wolf (*Canis rufus*). The black-footed ferret was once believed to be extinct in the wild, but a small population was discovered in Meteetse, Wyoming, in the 1980s (Dobson and Lyles 2000). The black-footed ferret captive breeding program was launched with 18 founders, but after a severe population bottleneck from disease, only 25% of the founder's genes are represented in the population as of 2000 (Dobson and Lyles 2000). Red wolves have been bred in captivity since the 1970s when their wild population reached critically low levels (Hedrick and Fredrickson 2008). Fourteen red wolves were captured from the wild and are founders of the current population, which includes approximately 200 captive and 80 wild wolves (Hedrick and Fredrickson 2008; Hinton et al. 2013). Captive breeding programs produce animals with varying levels of inbreeding, as they try to maximize genetic diversity despite often having few founders. Both black-footed ferrets and red wolves have similar population histories (low number of founders), and so we expect variation in inbreeding coefficients among the individuals in these populations. These populations should therefore be experiencing some inbreeding depression (Dobson and Lyles 2000; Hedrick and Fredrickson 2008; Hinton et al. 2013). Thus, we predicted that increased levels of inbreeding would result in a less hydrodynamic head shape (Malo et al. 2006; Tourmente et al. 2011; Ramon et al. 2013), a smaller midpiece (Anderson and Dixson 2002; Firman and Simmons 2010), and a shorter tail (Gomendio and Roldan 1991; Tourmente et al. 2011). Inbreeding can also lead to reduced genetic diversity, which could result in diminished phenotypic diversity (Keller and Waller 2002). Because of this, we also predicted that more inbred males would have reduced phenotypic variation in sperm morphometry.

Materials and methods

Semen collection

Black-footed ferret

Approval was received for the collection of black-footed ferret semen samples from the Lincoln Park Zoo Research Committee. Semen was collected by electro-ejaculation while the animals were under anesthesia (Howard et al. 1991; Wolf et al. 2000). A total of 32 black-footed ferrets were included in the study. Samples from five black-footed ferrets were acquired from the population held at the Toronto Zoo (Scarborough, Ontario, Canada). Smears were prepared from a varying volume of fresh sperm samples mixed with phosphate-buffered solution (PBS) on a warmed slide (37 °C). Slides were allowed to air-dry before they were fixed and stained (below). Samples from 27 black-footed ferrets were acquired from the United States Fish and Wildlife Service's National Black-Footed Ferret Conservation Center (Carr, Colorado, USA). Samples were from ferrets born in 2004 or later. Smears were prepared from sperm samples fixed in 0.3% glutaraldehyde (Santymire et al. 2006) that were air-dried before they were fixed and stained using methods described below. There was no difference in sperm-trait measurements between the samples acquired from the Toronto Zoo and samples acquired from the United States Fish and Wildlife Service's National Black-Footed Ferret Conservation Center (Head length, $p = 0.313$; head width, $p = 0.401$; midpiece length, $p = 0.685$; midpiece width, $p = 0.938$; tail length, $p = 0.166$).

Red wolf

Protocols for red wolf semen collection were approved by the Red Wolf Species Survival Plan and the Point Defiance Zoo and

Table 1. Descriptive statistics for sperm morphometric data and inbreeding coefficient (f) of red wolves (*Canis rufus*) and black-footed ferrets (*Musteles nigripes*).

	Red wolf ($n = 35$)					Black-footed ferret ($n = 32$)				
	Mean	SD	CV _b	CV _w	Range	Mean	SD	CV _b	CV _w	Range
f	0.04	0.03	NA	NA	0–0.135	0.12	0.009	NA	NA	0.107–0.148
Head length	5.69	0.22	3.8	4.5	5.28–6.13	6.88	0.21	3.1	4.8	6.37–7.34
Head width	3.81	0.14	3.5	4.8	3.52–4.05	5.46	0.15	2.8	4.8	5.04–5.73
Head area	17.3	0.99	5.7	7.1	15.45–19.71	30.5	1.41	4.6	7.7	29.86–33.30
Head perimeter	15.7	0.46	2.93	3.8	14.74–16.70	20.4	0.49	2.4	3.8	19.09–21.46
Head elongation	1.49	0.06	4.0	5.5	1.39–1.59	1.26	0.04	3.2	4.8	1.20–1.37
HL/TL	0.10	0.008	8.0	5.5	0.09–0.13	0.10	0.003	3.3	5.1	0.09–0.11
Midpiece length	10.2	0.26	2.6	3.1	9.54–10.68	13.0	0.17	1.3	2.8	12.73–13.30
Midpiece width	1.02	0.04	4.3	6.7	0.94–1.10	1.11	0.06	5.6	7.8	0.98–1.24
Midpiece volume	8.32	0.74	8.9	14.2	7.02–9.57	12.6	1.45	11.5	16.0	9.85–15.83
MPL/TL	0.19	0.008	4.2	4.5	0.17–0.20	0.20	0.004	2.0	3.05	0.19–0.20
Tail length	54.7	2.87	5.2	3.6	47.36–60.67	66.5	0.75	1.1	1.7	64.75–67.96
Total length	60.4	2.78	4.6	3.3	53.29–66.23	73.4	0.79	1.1	1.6	71.81–75.30

Note: All sperm traits are in micrometres (μm) except head area (μm^2), midpiece volume (μm^3), head length/tail length (HL/TL) ratio, and midpiece length/tail length (MPL/TL) ratio. Mean, standard deviation (SD), coefficient of variation between individuals (CV_b), and mean within individual coefficient of variation (CV_w) are included in the table. NA, not applicable.

Aquarium Animal Welfare Committee. Semen was collected by electro-ejaculation while the animals were under anesthesia using methods outlined in Goodrowe et al. (1998). Samples from 35 red wolves were obtained from the collection of cryopreserved red wolf semen samples stored at the Point Defiance Zoo and Aquarium (Tacoma, Washington, USA) and the Toronto Zoo. Cryopreserved samples from these collections originated from males housed at a variety of facilities housed throughout the United States. A combination of samples from early in the breeding program through to more recently living animals were included. Samples were thawed, washed in PBS, and smears were prepared and air-dried before they were fixed and stained (below). Only frozen-thawed samples were used because access to an adequate number of fresh samples was not possible due to the seasonal nature of sperm production in red wolves.

Slide preparation

Each smear was fixed and stained using a Spermac staining kit (FertiPro N.V., Beernem, Belgium). Each slide was mounted with a permanent cover slip using Permount (Fisher Scientific, Fair Lawn, New Jersey, USA).

Measurements

Ten haphazardly selected, normal sperm with an intact head, midpiece, and tail from each individual in the study were photographed with a DIC filter at 1000 \times magnification (oil immersion) using a Leica DFC 450 camera (Leica, Heerbrugg, Germany) mounted on a Leica DM5500B microscope (Leica, Wetzlar, Germany). Ten sperm per male were measured because there is little variation within ejaculates/males and across males in sperm morphology and measuring small numbers of sperm (up to 5) has been shown to capture the majority of the morphometric variation in sperm morphology across males (Calhim et al. 2007, 2011; Pitnick et al. 2009). Head length (μm), head width (μm), head perimeter (μm), head area (μm^2), midpiece length (μm), midpiece width (μm), and tail length (μm) on each sperm was measured three times using Leica LAS version 4.0.0 measurement software; the mean value was used for each trait in subsequent analyses.

Calculated measurements

Sperm midpiece volume was calculated as in Anderson et al. 2005. The ratio of head length to head width was calculated to determine head elongation (Malo et al. 2006). Total length was calculated by adding head length and tail length. Variation of sperm size within each of the individuals was assessed using the coefficient of variation (CV).

Inbreeding coefficient (f)

Genetic variation is not routinely examined for animals in captive breeding programs, as such inbreeding coefficients (f), calculated using pedigrees, were used to determine relatedness among animals in this study. Both of the endangered species that we examined are under captive breeding management by the United States Department of Fish and Wildlife with pedigrees developed based on studbooks maintained for the entirety of the breeding program (Waddell 2008; Marinari 2014). For both species, individual f values were calculated using the Single Population Analysis and Record Keeping System (SPARKS) version 1.52 (ISIS 2004).

Statistical analysis

All analyses were conducted using R version 2.15.3 (R Core Team 2013). Using linear mixed-effects models, run with the lme4 package (Bates et al. 2013), we tested for relationships between measured traits (head length, head width, midpiece length, midpiece width, tail length), calculated traits (midpiece length/tail length (MPL/TL) ratio, head elongation, midpiece volume, total length), and f , using individual identity as a random effect. Significance was determined using confidence intervals. Repeatability among individuals was calculated according to Lessells and Boag (1987) and Whitlock and Schluter (2009). Values close to 0 indicate high intraindividual variation (Whitlock and Schluter 2009). Head length and elongation measures in red wolves, and MPL/TL ratio, midpiece volume, and total length in black-footed ferrets, were log-transformed to adhere to normal distribution. Linear regression was used to evaluate relationships between f and CV for all measured and calculated morphometric values.

We used principal component analyses (PCA) with log-transformed measurements and CV in both species using head length, head width, midpiece length, midpiece width, and tail length to investigate changes in sperm size, shape, and variation. Principal components were retained based on Kaiser Criteria (Kaiser 1960). Using linear regression, we tested for relationships between f and retained principal components.

Results

Black-footed ferret

Descriptive statistics for sperm morphometric variables and f are found in Table 1. There was no relationship between f and sperm component sizes for head length, head width, head area, head elongation, midpiece length, midpiece width, midpiece volume, MPL/TL ratio, tail length, and total length (Table 2). Males with a higher f had a lower CV in sperm tail length ($F_{1,30} = 5.912$,

Table 2. The relationship between inbreeding coefficient (f) and sperm traits in red wolves (*Canus rufus*) and black-footed ferrets (*Mustela nigripes*) in a linear mixed-effects model.

	Effect	Estimate	SE	t	Confident intervals		Repeatability	
					Lower	Upper		
Red wolf								
Head length	f	-0.18	0.21	-0.85	-0.611	0.268	0.366	
Head width		-1.52	0.73	-2.10	-3.054	-0.148	0.259	
Head area		-10.0	5.37	-1.87	-20.32	1.090	0.314	
Head perimeter		-3.68	2.55	-1.44	-9.192	1.362	0.303	
Head elongation		0.22	0.21	1.04	-0.213	0.636	0.253	
HL/TL		-0.10	0.04	-2.25	-0.184	-0.013	0.609	
Midpiece length		0.99	1.50	0.66	-1.896	3.969	0.349	
Midpiece width		-0.05	0.26	-0.21	-0.522	0.486	0.241	
Midpiece volume		0.11	4.34	0.03	-9.189	7.725	0.225	
MPL/TL		-0.13	0.04	-2.93	-0.209	-0.045	0.400	
Tail length		42.7	14.7	2.91	13.27	71.11	0.608	
Total length		41.7	14.2	2.95	12.25	69.59	0.582	
Black-footed ferret								
Head length		f	2.47	4.50	0.55	-6.745	11.13	0.219
Head width	2.25		3.20	0.70	-4.159	9.072	0.176	
Head area	44.6		29.1	1.53	-13.67	99.34	0.178	
Head perimeter	15.7		10.1	1.56	-5.583	35.87	0.196	
Head elongation	-0.01		0.79	-0.14	-0.179	1.472	0.204	
HL/TL	0.08		0.07	1.10	-0.057	0.219	0.220	
Midpiece length	0.13		0.28	0.48	-0.455	0.680	0.090	
Midpiece width	0.69		1.31	0.53	-1.856	3.210	0.277	
Midpiece volume	1.59		2.48	0.64	-3.762	6.219	0.271	
MPL/TL	0.05		0.39	1.38	-0.254	1.260	0.185	
Tail length	-27.0		15.3	-1.76	-57.76	0.070	0.203	
Total length	-0.33		0.22	-1.49	-0.763	0.086	0.203	

Note: Significance was determined using bootstrapped confidence intervals; those that do not cross 0 are significant. Confidence intervals for significant models are set in boldface type. Among individual repeatability is also shown. HL/TL is head length/tail length ratio and MPL/TL is midpiece length/tail length ratio.

$p = 0.021$) (Fig. 1). There was no relationship between f and variation in sperm components with the exception of tail length (head length, $p = 0.78$; head width, $p = 0.22$; midpiece length, $p = 0.19$; midpiece width, $p = 0.35$).

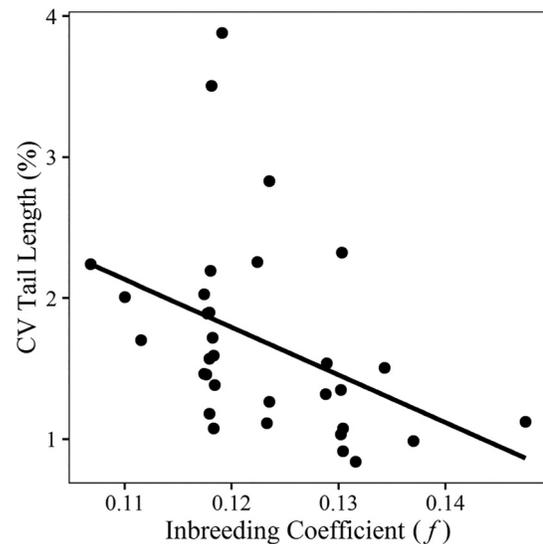
The log PC1 and log PC2 both met the Kaiser Criteria from the PCA using log-transformed sperm-trait sizes (Table 3; Kaiser 1960). There was no relationship between f and log PC1 ($F_{[1,30]} = 0.8032$, $p = 0.38$) or between f and log PC2 ($F_{[1,30]} = 1.047$, $p = 0.31$).

The CV PC1 and CV PC2 both met the Kaiser Criteria from the PCA using CV of sperm traits (Table 3; Kaiser 1960). We considered PC2 valuable because midpiece length contributed strongly and was nonsignificant in CV PC1. Subsequent principal components were dropped. There was a nonsignificant negative trend between CV PC1 and f ($F_{[1,30]} = 3.29$, $p = 0.08$). There was no relationship between CV PC2 and f ($F_{[1,30]} = 1.632$, $p = 0.21$).

Red wolf

Descriptive statistics for sperm morphometric variables and f are found in Table 1. Males with higher f had narrower head widths (Table 2, Fig. 2A). More inbred males had a smaller head length/tail length (HL/TL) ratio (Table 2, Fig. 2B). More inbred males had longer tails (Table 2, Fig. 2C). This is in contrast to the MPL/TL ratio, which decreased in males with a higher f (Table 2, Fig. 2D). The relationships between head width, tail length, MPL/TL ratio, and f all remained significant when the two individuals with a much higher f ($F = 0.09$ and 0.13 , respectively) were removed from the analysis. There was no relationship between f and head length, midpiece length, midpiece width, midpiece volume, or head elongation in red wolves (Table 2). CV of all sperm traits was not related to f in red wolves (head length, $p = 0.114$; head width, $p = 0.70$; midpiece length, $p = 0.22$; midpiece width, $p = 0.26$; tail length, $p = 0.29$).

The log PC1 generated using log-transformed sperm-trait sizes was retained because it met the Kaiser Criteria (Table 3; Kaiser

Fig. 1. Relationship between inbreeding coefficient and coefficient of variation (CV) in tail length for 32 black-footed ferrets (*Mustela nigripes*).

1960); subsequent principal components were not retained. The factor loadings of log PC1 reflected variation in sperm shape rather than sperm size, because some traits were positively related to log PC1 (midpiece length and tail length), whereas others were negatively related to log PC1 (head length, head width, and midpiece width) (Fig. 2E). A linear regression indicated a positive relationship between log PC1 and f ($F_{[1,33]} = 4.485$, $p = 0.042$) (Fig. 2E).

Table 3. Factor loadings and percent variance for PC1 (red wolf (*Canis rufus*) and black-footed ferret (*Mustela nigripes*)) and PC2 (red wolf only).

	Red wolf (n = 35)				Black-footed ferret (n = 32)	
	log PC1	log PC2	CV PC1	CV PC2	log PC1	CV PC1
Head length	-0.590	-0.336	-0.331	-0.572	0.6	0.498
Head width	-0.336	-0.408	-0.625	-0.223	0.488	0.610
Midpiece length	0.366	-0.694	-0.356	-0.710	0.357	0.238
Midpiece width	-0.327	-0.338	0.567	-0.270	0.521	0.391
Tail length	0.546	-0.353	0.227	-0.216	—	0.412
Percent variance	41.5	23.3	35.0	23.6	37.8	41.3

Note: Values are for four principal component (PC) analyses that were conducted using log-transformed sperm morphometric traits and coefficient variation (CV) for sperm traits in red wolves and black-footed ferrets.

The CV PC1 generated using CV for five measured sperm traits met the Kaiser Criteria (Table 3; Kaiser 1960); subsequent principal components were not retained. Variation in midpiece width increased, whereas variation in head length, head width, and midpiece length decreased (Table 3). The CV PC1 did not relate to f in red wolves ($F_{[1,33]} = 0.058$, $p = 0.81$).

Discussion

Analysis of red wolf sperm revealed that the most inbred males in this sample had sperm with narrower heads. Previous studies have shown that sperm with small, elongated heads swim faster and are more successful at fertilizing ova (Malo et al. 2006; Tourmente et al. 2011; Ramon et al. 2013). However, we found no relationship between head elongation and f , and a PCA revealed that head length was decreasing more than head width in more inbred males, indicating that the sperm heads of more inbred red wolves may not be more hydrodynamic. Despite this, more inbred males had smaller sperm heads, which could increase swimming speed and provide a fertilization advantage (Ramon et al. 2013). We also found that midpiece length and tail length increased in more inbred males, potentially producing sperm with greater forward propulsion. Previous studies have shown that sperm with longer tails are able to swim faster (Gomendio and Roldan 1991; Malo et al. 2006; Tourmente et al. 2011). However, Humphries et al. (2008) and Simpson et al. (2014) suggested that evidence for the positive relationship between tail length and speed is inconsistent. Simpson et al. (2014) found a negative relationship between sperm length and swimming speed in Emus (*Dromaius novaehollandiae* (Latham, 1790)), but no relationship between sperm length and swimming speed in two other internal fertilizers (humans (*Homo sapiens* L., 1758) and guppies (*Poecilia reticulata* Peters, 1859)), suggesting that sperm with longer tails may not swim faster in all species. Finally, more inbred male red wolves in our sample had a smaller HL/TL ratio. In a study across 226 mammals, Tourmente et al. (2011) found that as the HL/TL ratio decreased, straight-line velocity of sperm also decreased. In this study, more inbred males had a smaller HL/TL ratio, indicating that more inbred males may have slower swimming sperm, which is contradictory to some of our other results. Overall, these results suggest that more inbred male red wolves have sperm with some traits that have the potential to provide them with a fertilization advantage. However, species-specific investigation into the implications of changes in sperm size and shape in relation to levels of inbreeding to sperm swimming speed are needed to provide a clearer picture of the consequences of changes in sperm morphometry.

In black-footed ferrets, there was no relationship between f and sperm size or shape. Black-footed ferrets had a relatively small range (0.11–0.14) in f , which may have limited our ability to detect any significant effects of inbreeding on sperm morphometry. Although the red wolves in this study had a low mean f (0.04), we were able to detect significant effects on sperm morphometry

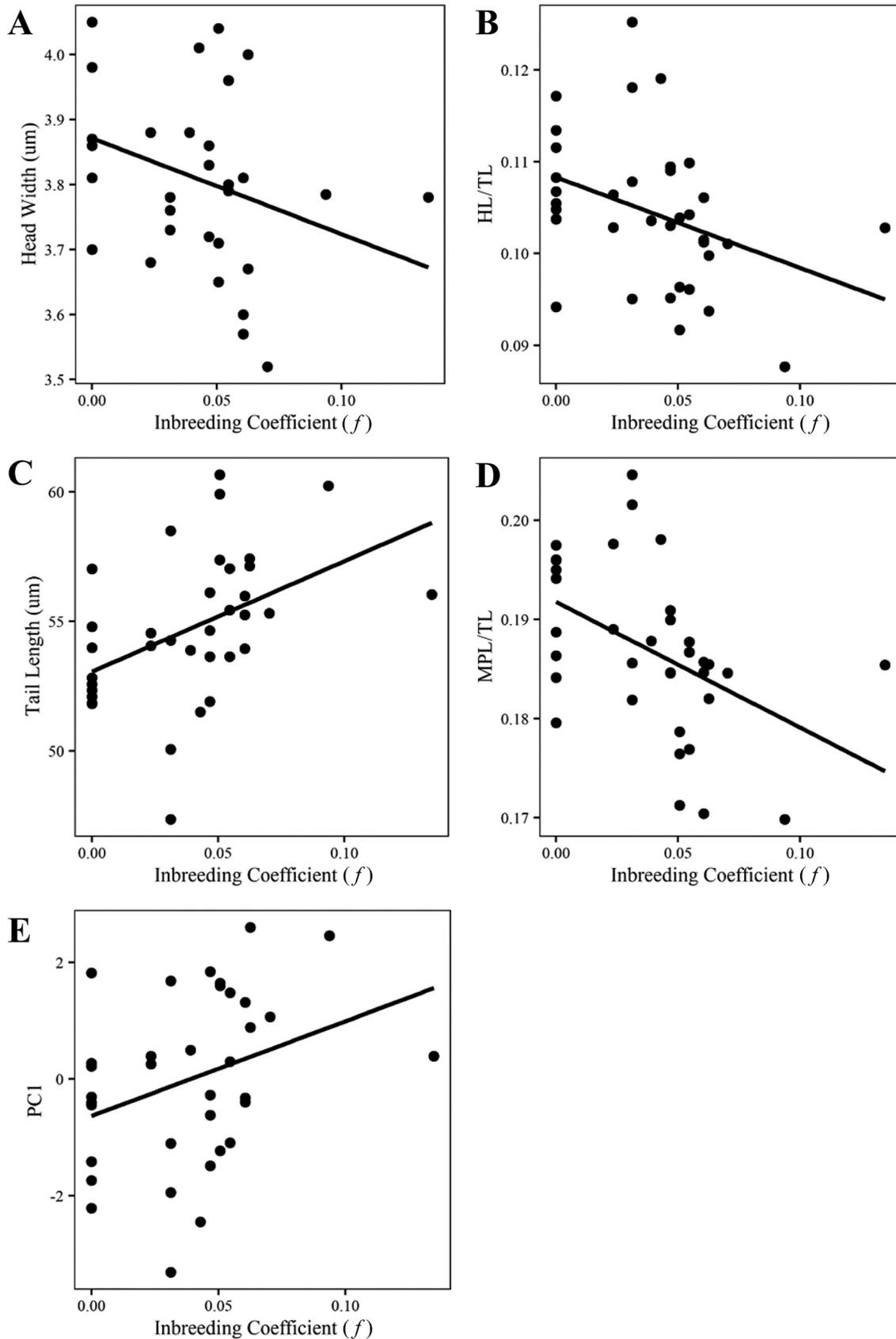
consistent with enhanced sperm performance, perhaps due to the much larger range in f (0–0.135).

Red wolves in our study had a low mean degree of inbreeding, and sperm traits that could be associated with increased sperm swimming speed and improved fertility, which was contrary to our predictions. Although inbreeding depression is capable of producing many negative fitness effects, outbreeding depression also negatively affects reproductive fitness (Lynch 1997; Lehnert et al. 2014). Outbreeding depression occurs when offspring of genetically different parents have reduced fitness, usually because breeding from outside, unrelated populations introduces intermediate phenotypes that are maladaptive (Lynch 1997; Escobar et al. 2008; Robinson et al. 2009; Lehnert et al. 2014). For example, offspring survival is highest in outbred greater horseshoe bats (*Rhinolophus ferrumequinum* (Schreber, 1774)) and Arabian oryx (*Oryx leucoryx* (Pallas, 1777)) (Marshall and Spalton 2000; Rossiter et al. 2001). It has been predicted that an optimal level of inbreeding exists (Escobar et al. 2008; Robinson et al. 2009), suggesting that some level of inbreeding may be adaptive. The red wolves in our study may have experienced positive consequences of relatively low levels of inbreeding. Optimal levels of inbreeding have been identified in the Common Lizard (*Lacerta vivipara* Jacquin, 1787) and the Arabian oryx (Marshall and Spalton 2000; Richard et al. 2009). Both species experienced decreased juvenile survival at high and low levels of inbreeding and increased juvenile survival at moderate levels of inbreeding (Marshall and Spalton 2000; Richard et al. 2009). Reproductive success in Common Lizards followed a similar pattern (Richard et al. 2009). Currently, there is no direct evidence in the literature indicating that red wolves may gain fitness benefits from low to moderate levels of inbreeding. In the wild, red wolves are monogamous (Sparkman et al. 2011) and would likely have low natural levels of inbreeding. However, there is some evidence of wolves (Ethiopian wolf (*Canis simensis* Rüppell, 1840) and red wolf) inbreeding in the wild when opportunities for dispersal were limited or populations were small, leaving open the possibility that outbreeding depression could occur in wolves (Sillero-Zubiri et al. 1996; Liberg et al. 2005). It is likely that red wolves engaged in some degree of inbreeding while in the wild, as their populations decreased to small numbers prior to the inception of the red wolf captive breeding program (Hedrick and Fredrickson 2008). Identifying optimal levels of inbreeding in captive-bred or reintroduced species could be beneficial to their management, as not all species will benefit from minimizing inbreeding as much as possible.

We also found relationships between variation in sperm traits and inbreeding. Black-footed ferrets with higher f values had less variable tail length. Additionally, these data showed a trend towards decreasing phenotypic variation in head length, head width, midpiece width, and tail length in more inbred males. These findings support our prediction that variation in sperm traits will decrease with increased levels of inbreeding, due to the suspected loss of genetic diversity, which likely occurred with inbreeding (Keller and Waller 2002). In contrast, we found no evidence that inbreeding affected variation in sperm-trait sizes in red wolves; this could again be attributed to the relatively low level of inbreeding in the red wolves of our study.

Sperm traits in both red wolves and black-footed ferrets tended to be more variable than in wild animals. For example, red wolves had an intraspecific tail length variation (CV) of 3.6%, whereas black-footed ferrets had 1.7% variation in tail length. In comparison, yellow-pine chipmunks (*Tamias amoenus* J.A. Allen, 1890) had an intraspecific sperm tail length variation (CV) of 2.2% (Schulte-Hostedde and Millar 2004), Northern Watersnakes (*Nerodia sipedon* (L., 1758)) had a sperm length variation of 0.9% (Schulte-Hostedde and Montgomerie 2006), and sperm length variation in passerine birds ranged from 0.75% to 3.5% in males with varying degrees of extrapair paternity (Kleven et al. 2008). Tail length variation in the red wolf was high relative to variation

Fig. 2. Relationship between inbreeding coefficient and (A) mean sperm head width, (B) head length/tail length (HL/TL) ratio, (C) mean sperm tail length, (D) midpiece length/tail length (MPL/TL) ratio, and (E) principal component 1 (PC1) scores for log-transformed sperm traits in 35 red wolves (*Canis rufus*).



found in wild animals. More inbred male black-footed ferrets had less variable tail length, but when compared with other species, mean variation in tail length is intermediate, not low as might be expected if loss of genetic variation has occurred as predicted (Keller and Waller 2002). Higher levels of sperm-trait variation found in red wolves and black-footed ferrets could be attributed to relaxed selection on fitness traits sometimes experienced by animals managed in captivity (Araki et al. 2008; Christie et al. 2012). Notably, variation in sperm head dimensions in both species (4.8% in head width and 7.8% in head area of black-footed ferrets) was similar to variation in sperm head traits found in domestic animals. For example, in commercial bulls, sperm head length variation was 3.5%, whereas sperm head area was 8.5% (Gravance et al. 1996); in domestic llamas (*Lama glama* (L., 1758)), variation in sperm head length, width, and area was 3.83%, 2.78%, and 2.75%, respectively (Casaretto et al. 2012). Red wolves and black-footed ferrets have both been propagated in managed captive breeding programs (Dobson and Lyles 2000; Hedrick and Fredrickson 2008; Hinton et al. 2013). When animals are managed outside of their natural habitats, natural selection is likely relaxed, and traits that would not be successful in the wild may be retained, which can result in greater levels of variation in some traits, such as sperm traits (Araki et al. 2008; Christie et al. 2012). For example, captive breeding programs generally manage populations to minimize kinship between breeding pairs (Schulte-Hostedde and Mastromonaco 2015), rather than selecting for specific traits, such as sperm morphometry. In addition, black-footed ferrets and red wolves are polygynous (Livieri 2007) and monogamous (Sparkman et al. 2011), respectively; thus, the risk of sperm competition and strength of selection on sperm traits are weak (Birkhead 1998; Calhim et al. 2007; Pitnick et al. 2009). This results in high sperm-trait variation in species with little or no sperm competition, relative to variation in species that experience high levels of sperm competition (Calhim et al. 2007; Pitnick et al. 2009). Sperm variation may be relatively high in red wolves and black-footed ferrets because selection is being relaxed in two ways, through (1) low or absent levels of sperm competition and (2) relaxed selection from breeding in captivity. Both species that we have examined have undergone genetic bottlenecks, with expected declines in genetic diversity (Kalinowski et al. 1999; Wisely et al. 2002). Whether this has a role to play in our results is unclear. Previous work has shown little effects of inbreeding in captive populations associated with these bottlenecks on traits such as litter size and juvenile survival (Kalinowski et al. 1999; Wisely et al. 2002). Nonetheless, this study and a number of others have shown that changes with the potential to affect fitness can occur in captivity, sometimes very quickly (Araki et al. 2008; Christie et al. 2012). In this case, we have shown that inbreeding in captivity affected aspects of sperm morphometry, including size and size variation. Morphology, behaviour, and other characteristics can also vary in captivity for a number of reasons including inbreeding depression and adaptation to captivity (O'Regan and Kitchener 2005; Araki et al. 2008; Christie et al. 2012). Small changes in traits which affect fitness that have been overlooked by captive population managers could compromise the fitness of reintroduced animals and undermine the success of conservation breeding programs (O'Regan and Kitchener 2005). Increasing research into the effects of captive breeding on traits often overlooked when managing captive populations could provide an opportunity to improve captive management and ultimately the success of endangered species breeding programs. In addition, detailed pedigrees kept by zoos can be a rich resource for testing evolutionary hypotheses, and further work should continue to explore this.

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