

Inbreeding and inbreeding depression in endangered red wolves (*Canis rufus*)

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Abstract

In natural populations, the expression and severity of inbreeding depression can vary widely across taxa. Describing processes that influence the extent of inbreeding and inbreeding depression aid in our understanding of the evolutionary history of mating systems such as cooperative breeding and nonrandom mate selection. Such findings also help shape wildlife conservation theory because inbreeding depression reduces the viability of small populations. We evaluated the extent of inbreeding and inbreeding depression in a small, re-introduced population of red wolves (*Canis rufus*) in North Carolina. Since red wolves were first re-introduced in 1987, pedigree inbreeding coefficients (f) increased considerably and almost every wild born wolf was inbred (average $f = 0.154$ and max $f = 0.383$). The large inbreeding coefficients were due to both background relatedness associated with few founders and numerous close relative matings. Inbreeding depression was most evident for adult body size and generally absent for direct fitness measures such as reproductive success and survival; no lethal equivalents ($LE = 0.00$) were detected in juvenile survival. The lack of strong inbreeding depression in direct measures of fitness could be due to a founder effect or because there were no outbred individuals for comparison. Our results highlight the variable expression of inbreeding depression across traits and the need to measure a number of different traits when evaluating inbreeding depression in a wild population.

Keywords: body size, *Canis rufus*, inbreeding, inbreeding depression, pedigree

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Introduction

Inbreeding depression, the reduction in offspring fitness caused by mating among close relatives (Allendorf & Luikart 2007), is widespread in small, wild populations and is a major concern in conservation biology because it can directly affect population persistence (Crnokrak & Roff 1999; Keller & Waller 2002). The negative effects of inbreeding depression in wild populations are well documented in a diversity of taxa, from insects (Saccheri *et al.* 1998; Franke & Fischer 2013), fish (Ala-Honkola *et al.* 2009; Naish *et al.* 2013) and birds (Keller 1998;

Townsend *et al.* 2009; Grueber *et al.* 2010), to small (Gage *et al.* 2006; Nielsen *et al.* 2012) and large mammals (Coltman *et al.* 1999; Dunn *et al.* 2011; Walling *et al.* 2011). Harmful effects of inbreeding are attributed to an increase in genomewide homozygosity resulting in the expression of deleterious recessive alleles (dominance hypothesis) and/or loss of heterozygous advantage (overdominance hypothesis; Charlesworth & Willis 2009). There is evidence to support both processes, but expression of deleterious alleles appears to be the most common cause of inbreeding depression (Charlesworth & Charlesworth 1999; Keller & Waller 2002).

Recessive mutations will only cause inbreeding depression if they occur at gene(s) affecting fitness and result in a lower fitness than the general population

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(Allendorf & Luikart 2007). A population may, by chance, have few deleterious alleles at adaptive loci because of founder effects or genetic drift (Lacy *et al.* 1996; Keller & Waller 2002). When this happens, the expression and severity of inbreeding depression may vary or escape notice. Lacy *et al.* (1996) found that inbred lines of mice (*Peromyscus* spp.) exhibited reduced fitness at different traits and varying levels of severity as a consequence of random founder effects. Genetic purging, the removal of deleterious alleles through natural selection, can also influence the expression and severity of inbreeding depression (Lacy & Ballou 1998). However, in theory, no population is invulnerable to the deleterious effects of inbreeding, making it a major concern for endangered species management (Lacy 1997; Saccheri *et al.* 1998; Crnokrak & Roff 1999; O'Grady *et al.* 2006).

A complete understanding of the consequences of inbreeding in wild populations requires robust and direct measures of relatedness, and careful, long-term measures of reproductive success and survivorship (Pemberton 2004; Szulkin *et al.* 2007). Heterozygosity values calculated from multilocus genotype data have been used to evaluate inbreeding depression in wild populations but are not ideal because they do not directly measure inbreeding (Pemberton 2008; Szulkin *et al.* 2010; Taylor *et al.* 2010; Grueber *et al.* 2011b). Multigenerational pedigrees which map relatedness of breeding individuals are preferred, but such studies are generally rare as pedigrees are uncommon and long-term life history data on wild populations is often lacking. Therefore, species, such as the red wolf (*Canis rufus*), for which inbreeding and fitness data are available serve as model organisms because they reveal the influence of inbreeding and inbreeding depression in wild populations (Keller 1998).

Red wolves are critically endangered canids endemic to the south-eastern United States (Phillips & Parker 1988; Nowak 2002; Hinton *et al.* 2013). Although once abundant throughout the southeast, persecution and habitat loss confined red wolves to Louisiana and Texas where they suffered from high levels of parasitism and hybridization with coyotes (*Canis latrans*; Paradiso & Nowak 1972; Custer & Pence 1981; Phillips *et al.* 2003). The threat of extinction *in situ* led the United States Fish and Wildlife Service (USFWS) to bring the remaining individuals into captivity in the mid to late 1970s and establish a captive breeding programme, after which red wolves were declared extinct from the wild in 1980. Fourteen individuals eventually became the founders of all present day red wolves, although only 12 are represented genetically in the current population (Riley & McBride 1975; Phillips & Parker 1988; Phillips *et al.* 2003; USFWS 2013). Starting in 1987, red

wolves were re-introduced to Alligator River National Wildlife Refuge in north-eastern North Carolina, where the population has grown since re-introduction (Phillips *et al.* 2003; Hinton *et al.* 2013). The USFWS Red Wolf Recovery Program has maintained detailed records, including reproductive histories, birth dates, causes of death, pack composition and a population-wide pedigree.

In the captive red wolf population, increased levels of inbreeding are correlated with decreased litter size, but lethal equivalents are near zero suggesting minimal inbreeding depression has occurred relative to other inbred canids (Kalinowski *et al.* 1999; Rabon & Waddell 2010). Current management procedures include deliberately pairing captive red wolves to reduce inbreeding and maximize genetic diversity (Waddell & Long 2013), thus, the results of inbreeding depression studies from captive wolves may not reflect the potentially high levels of inbreeding found in the wild population where wolves are free to choose mates. For instance, wild Scandinavian grey wolves (*Canis lupus*) have large inbreeding coefficients that are correlated with decreased pup survival (Liberg *et al.* 2005). This result is consistent with other captive and wild wolf populations where clear associations exist between inbreeding and blindness, reduced reproductive success, decreased litter size, reduced sperm quality and congenital bone deformities (Laikre & Ryman 1991; Laikre *et al.* 1993; Asa *et al.* 2007; Räikkönen *et al.* 2009).

Wolves may be able to avoid the deleterious effects of inbreeding depression by choosing unrelated individuals as mates, a behaviour that has been documented in a number of wild wolf populations (Sillero-Zubiri *et al.* 1996; Smith *et al.* 1997). Re-introduced Yellowstone grey wolves nearly completely avoid inbreeding despite a small founding population (vonHoldt *et al.* 2007). There is evidence of inbreeding avoidance in wild red wolves as well (Sparkman *et al.* 2012a), but because the wild red wolf population is small and isolated, inbreeding may be unavoidable if background levels of relatedness are high. Given potential problems associated with inbreeding depression, an assessment of inbreeding and associated fitness costs in the wild red wolf population is warranted. More broadly, the red wolf pedigree and long-term data provide a rare opportunity to evaluate inbreeding and inbreeding depression in a long-lived carnivore, and contribute to our understanding of the patterns and effects of inbreeding in wild populations. Our objectives were to evaluate (i) the degree to which inbreeding has increased since red wolf re-introductions, (ii) the number of lethal equivalents (a standardized measure of inbreeding depression) and (iii) the effect of inbreeding on fitness-related traits.

Methods

Study population

We used 23 years of data collected from the re-introduced wild red wolf population. Red wolf re-introduction efforts began in 1987 with the release of four adult wolf pairs at Alligator River National Wildlife Refuge (ARNWR) in north-eastern North Carolina (Phillips *et al.* 2003; Hinton *et al.* 2013). From October 1987 to November 1994, an additional 60 wolves were intermittently released to bolster the new population; wolves were released either as pairs, sibling groups or family groups (A. B. Beyer, personal communication). By 1994, the wild population was self-sustaining via wild births, although occasional cross-fostering of captive born pups into wild litters continues to the present. Since the original re-introductions, the recovery area has grown to encompass 1.7 million acres throughout five counties (Dare, Tyrrell, Hyde, Beaufort, and Washington), and the red wolf population has increased to about 100 individuals (USFWS 2013).

USFWS biologists closely monitor red wolf reproduction, mortality, home range and pair affiliation with biweekly aerial flights and radio telemetry (Phillips *et al.* 2003; USFWS 2013). Each year's juveniles are target trapped and fitted with radio-collars; adults are recaptured when radio-collars need to be replacement. Wolves are captured with soft-catch, off-setting foothold traps, during which USFWS biologists take genetic samples and record morphological measurements and overall health. When a radio-collar mortality signal is detected, biologists attempt to collect the wolf and assess cause of death. USFWS biologists also search out denning red wolf pairs to determine litter size, implant transponders and take genetic samples from pups each spring.

Due to coyote range expansion eastward into the recovery area, coyote-red wolf hybridization was first documented in 1993 (Phillips *et al.* 2003). Hybridization is considered a major threat to red wolf recovery and prompted development of an adaptive management strategy to prevent further introgression of coyote genetic material into the wild red wolf population (Kelly *et al.* 1999; Stoskopf *et al.* 2005; Rabon *et al.* 2013). Under the adaptive management plan, a genetic based maximum-likelihood approach was designed to identify hybrids and assign red wolf ancestry (see Miller *et al.* 2003 for genetic classification details); animals considered to be $\geq 87.5\%$ red wolf were allowed to remain in the wild population (Stoskopf *et al.* 2005). We followed the USFWS criteria and treated all animals determined to be at least 87.5% red wolf as part of the wild red wolf population. Part of the adaptive management plan also

included sterilizing coyote and hybrid mates, so some red wolves had sterile mates for parts of their reproductive years, which we accounted for in the analyses (see Reproductive success).

Pedigree

The red wolf pedigree was previously constructed from extensive field data and verified with genetic analyses (Adams 2006). Briefly, red wolves were genotyped at 18 microsatellite loci; multilocus genotypes were used to confirm parentage determined from field data and assign parentage to individuals with unknown pedigrees (Miller *et al.* 2003; Adams 2006). Parentage could be successfully assigned at the 95% confidence level 95% of the time when one parent was known (~14% of cases) and 88% of the time when neither parent was known (~27% of cases); in most cases (~59%), both parents were identified through field information and verified via genetic methods (see Adams 2006 for details). All known red wolves were included in pedigree construction and calculation of inbreeding coefficients; per cent red wolf ancestry was determined after parentage assignment for management purposes and to characterize hybridization events in the population (Miller *et al.* 2003; Adams 2006). In the pedigree, 90% of all ancestry is known. The pedigree includes 764 wild born red wolves; of these, at least one parent is known for 738 wolves, both the dam and sire are known for 685 wolves, and all four grandparents are known for 635 wolves. The pedigree spans almost seven generations and is maintained in the program SPARKS (ISIS 2011). Inbreeding coefficients were derived from PMx software (Lacy *et al.* 2011); the pedigree inbreeding coefficient (f) was the probability that two copies of an allele were identical by descent; an individual was inbred if $f > 0$.

To assess pedigree complexity and visualize the potential inbreeding loops within the population, we plotted the lineage of the first wild born breeding red wolf (studbook id = 10344) and her mate (studbook id = 10392) through time with R package kinship2 (Therneau *et al.* 2014). The pair was representative of the entire pedigree in that their offspring encompassed the spread of inbreeding coefficients observed. Non-breeding offspring were excluded from the plot for simplicity. We determined whether the average f of wild born litters increased over time using linear regression.

Lethal equivalents. We estimated the number of lethal equivalents (LE) per haploid genome (β) for red wolf survival to 18 months (S_{18}) following Kalinowski & Hedrick's (1998) maximum-likelihood method. Lethal equivalents are a standardized measure of the effect size of inbreeding depression in a population (Morton *et al.*

1956) and defined as the number of deleterious alleles in a haploid genome whose cumulative effect is equivalent to 1 LE (Allendorf & Luikart 2007).

Inbreeding depression analyses

Fitness is defined as the average number of offspring an individual contributes to the next generation and is calculated as the product of reproductive success and survivorship (Falconer 1960; Allendorf & Luikart 2007). Thus, to determine if red wolf fitness was influenced by inbreeding, we investigated whether parental or individual inbreeding coefficients predicted: lifetime number of litters (LNL), the average number of litters a wolf had per reproductive year (ANL), litter size, probability of becoming a breeder, adult survival, juvenile survival and adult body size. To avoid underestimating inbreeding depression, we only included animals in analyses if they were wild born in the recovery area and all four grandparents were known. We originally included red wolf ancestry (0 = introgressed ancestry, 1 = 100% red wolf) as an explanatory variable in our analyses because individuals with coyote ancestry could have experienced heterosis and suffered less from inbreeding depression (Grant *et al.* 2003). Alternatively, introgression could have caused outbreeding depression and reduced individual fitness (reviewed in Edmands 2007). However, we removed ancestry from all final models except those evaluating body sizes because it was not an important predictor of fitness, substantially decreased sample sizes, and removing it did not qualitatively change results. Unless otherwise reported, models encompassed fitness data collected from 1989 to 2012; specific data constraints for each fitness variable are discussed in detail below.

Reproductive success. We estimated LNL and ANL by the number of litters an individual produced rather than the total number of offspring, because until 1999 dens were not consistently sampled and pups were not counted; instead breeding pairs and the presence or absence of litters were noted. To determine the effect of inbreeding on LNL and ANL, we ran generalized linear mixed effect models (GLMM) using the R package lme4 (Bates & Maechler 2010) with a log-link function and Poisson distribution. Only individuals that lived to reproductive age (18 months), had known death dates, or were suspected dead from field signs were included in the LNL models ($n = 168$); all wolves that lived to reproductive age were included in ANL ($n = 201$). For the models with LNL and ANL as response variables, explanatory variables were f , years reproductively available (LNL only), years holding a territory (ANL only), sex, dam f , sire f , dam age, sire age and presence of

helpers at birth (yes or no) as fixed factors; litter ID (identifier for the litter in which the focal animal was born) and year born were random factors. We included random factors to control for nonindependence between litter mates and variation in year born. Parental f and age were used to test if there was a parental effect on offspring reproductive success. The variable 'years reproductively available' was included in all LNL models to account for years red wolves were paired with sterile mates and thus were unable to reproduce irrespective of inbreeding; years reproductively available was calculated based on the number of years a wolf was reproductively available minus the number of years paired with a sterile mate. Presence of helpers in a pack has been shown to affect red wolf reproductive success and was therefore included in models (Sparkman *et al.* 2011, 2012b). We defined the presence of helpers at birth as the incidence of nonbreeding pack members that participated in pup rearing.

We ran GLMMs with a logit-link function and binomial error distribution to determine whether inbreeding affected the probability that a wolf became a breeder. We defined breeder status as 1 if a red wolf had at least 1 litter in its lifetime or 0 if it never bred. With breeder status as the response variable, the fixed and random explanatory variables were the same as LNL and ANL and only included individuals that lived to reproductive age (18 months) and had known or suspected death dates ($n = 168$). We reran LNL, ANL and probability of breeding models to evaluate whether inbreeding depression differed when using a data set that only included individuals born 2001 onward, the time frame where the most inbred litters were born and litters were monitored more closely than previous years for management purposes.

We evaluated models with litter size as the response variable because inbreeding in the captive population was correlated with reduced litter size (Rabon & Waddell 2010). We only included litters where all pups were given transponders before becoming mobile, usually within approximately 2 weeks of parturition ($n = 105$; A. B. Beyer, personal communication). Although this removed litters from the early years of the programme before dens were sampled and pups were fitted with transponders, it assured the most accurate litter counts available. We used GLMMs with a log-link function and Poisson distribution and the following explanatory variables: f of the litter, dam f , sire f , dam age, sire age and year born as fixed factors and pair ID as a random factor. Each breeding pair was given a unique identity that was used instead of litter ID because pair ID accounted for different litters with the same parents. We excluded 4 litters that each had multiple sires and therefore lacked a single f value.

Survival. To examine the effect of inbreeding on survival, we ran Cox proportional hazard mixed effect models with the R package *coxme* (Therneau 2013) with adult and juvenile survival as response variables; we defined juvenile survival as living to 18 months. Cox proportional hazard models estimate a baseline hazard function where the null expectation is equal to 1, meaning that parameter estimates >1 increase the hazard of dying while estimates <1 increase the probability of survival. Cox models are useful because you can include individuals that outlive or are removed from the population during the specified survival time frame [censored]. We censored individuals that were alive at the end of each survival period while individuals that died or were suspected dead with high confidence were uncensored; for both survival periods, the terminal event was death, where age at time of death was calculated in days. We also included individuals that died due to anthropogenic or management causes but censored them, such that 0 = a censored individual (survived, removed from the population or died due to anthropogenic causes) and 1 = an individual that died during the specified time window (adult survival: 0 = 178, 1 = 104, juvenile survival: 0 = 237, 1 = 36). Known causes of death included anthropogenic incidents (gunshots and vehicular accidents), management actions (trapping, injury and removal) and natural events (disease and interspecific conflict). Explanatory variables for both model sets included: f , sex, dam f , sire f , dam age, sire age and territory (yes or no if a holder; adult survival only), as fixed factors and litter ID and year born as random factors. There were seven outlier sire f values (sire $f > 0.30$), thus we ran survival models with and without the outliers to evaluate model sensitivity. We also reran adult and juvenile survival models, like reproductive models, with a data set truncated to only include individuals born 2001 onward.

Body size. We tested if inbreeding influenced body size because physical size can affect behaviours important to fitness, such as an individual's ability to secure a mate, effectively hunt, or hold a territory. To create a single measure of overall body size, we implemented a principle components analysis (PCA) with measurements of body length, hind-foot length, shoulder height, ear size and tail length. PC1 encompassed 62% of the total variance, after which there was a precipitous drop in the variance explained by PC2–PC5. All morphometric variables were positively associated with each other, and based on individual loadings, each variable was important in PC1 (Table S1, Supporting information). Thus, PC1 effectively represented overall body size and was used as the response variable in models to evaluate the effect of inbreeding on red wolf body size (Fig. S1,

Supporting information). We used linear mixed effect models with a Gaussian error distribution to evaluate the effect of inbreeding on body size. Explanatory variables were f , sex, ancestry (0 = introgressed ancestry (any coyote ancestry), 1 = 100% red wolf), dam f , sire f , dam age, and sire age as fixed factors, and pair ID and year born as random factors; sex was included in every model to account for sexual dimorphism. Only measurements taken from fully grown wolves (>10 months old) were used ($n = 128$); if individuals were captured multiple times as adults, we averaged their measurements.

We evaluated GLMM reproductive success and Cox proportional hazard adult survival models with PC1 as an explanatory variable to evaluate the relationship between body size and fitness, similarly to methods in Sparkman *et al.* (2011). We also evaluated if PC1 predicted the probability of holding a territory, an important component of annual reproductive success (Table 1). GLMMs with a logit-link function and binomial error distribution were run with territory (0 = never held a territory, 1 = held a territory for at least one breeding season) as the response variable, fixed explanatory variables included PC1, sex, dam age, sire age, and an interaction between PC1 and sex, and litter ID as a random variable.

Model selection. Initial data exploration for all analyses followed Zuur *et al.* (2010), where collinearity, independence, heterogeneity, interactions, normality and the influence of outliers were examined for each model set. Individual f was correlated with parental inbreeding coefficients, and parental ages were correlated with each other, thus these variables were not included in the same models (Table S2, Supporting information). We also confirmed that year of birth was not a confounding variable or directly correlated with fitness variables. All models were ranked with AIC_c and AIC_c

Table 1 Known relationships for red wolf (*Canis rufus*) breeding pairs resulting in offspring with inbreeding coefficients (f) >0.19

Relationship category	Breeding pairs
1st cousin	7*
Half uncle/niece	2
Uncle/niece	4
Aunt/nephew	2
Half-sibling	1
Full-sibling	5

*In three of the 1st cousin breeding pairs, one mate had full-sibling parents and one cousin pair was 1st cousins from both parents.

weight (w_i ; Burnham & Anderson 2002) and validated by examining residuals and fitted values as suggested by Zuur *et al.* (2009). We averaged models encompassing 95% of $AIC_c w_i$ using the natural-average method (Burnham & Anderson 2002) in R package MuMIn (Barton 2009). We standardized input variables in R package arm (Gelman *et al.* 2009) to rank explanatory variables and directly compare the effect size of model-averaged coefficients (Grueber *et al.* 2011a). Model averaging was used because it takes model selection uncertainty into account and provides methods to evaluate the relative importance of each variable. Relative importance was calculated by summing $AIC_c w_i$ across all models where a variable occurs in the final model set. Larger values indicate that a variable is more important relative to other variables in explaining variance in the response variable (Burnham & Anderson 2002). We report model-averaged coefficients, unconditional standard errors which incorporate model selection uncertainty and relative variable importance.

Results

All but the first wild born red wolf (studbook id = 10344) had inbreeding coefficients >0 (mean $f = 0.154$, range 0–0.383; Fig. 1). Out of all wild breeding pairs, fourteen had litters with $f \geq 0.25$, producing 102 highly inbred wolves with inbreeding coefficients above the equivalent of sibling–sibling/parent–offspring matings ($n = 31$ litters). The most inbred individuals ($f > 0.28$) were from litters born 2001 to 2012, more than half of which were from 2008 to 2012. A large per cent

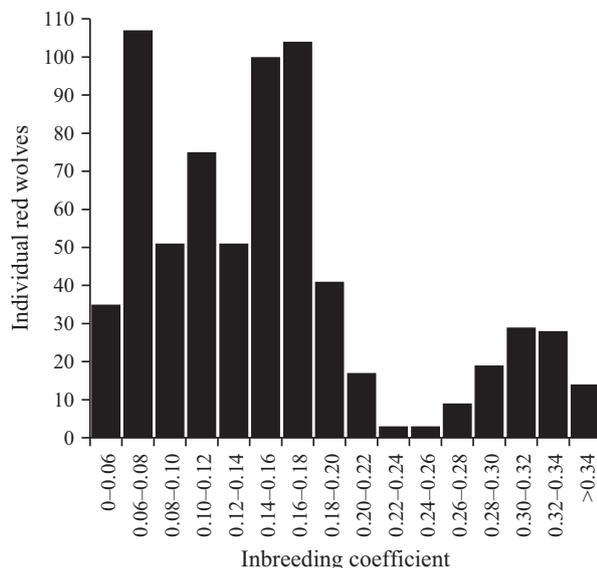


Fig. 1 Distribution of inbreeding coefficients for wild born red wolves (*Canis rufus*) of known parentage ($N = 685$).

of the population (85.1%) was either low to moderately inbred at $0 < f < 0.125$ ($N = 290$ from 86 litters) or had high levels of inbreeding at $0.125 \leq f < 0.25$ ($N = 293$ from 67 litters). There was no difference between average male ($f = 0.154$) and female ($f = 0.156$) inbreeding coefficients ($t = 1.65$, d.f. = 673, $P = 0.47$). Since re-introductions, the average inbreeding coefficients of litters increased significantly from 0.031 in 1988 to 0.169 in 2012 ($F = 82.78$, d.f. = 23, $P < 0.001$; Fig. 2). We estimated β (the number of LE per haploid genome) on juvenile survival to 18 months as 0.00.

The ancestry plot of red wolf 10344 and her mate, 10392, revealed a complex pedigree with numerous inbreeding loops (Fig. 3). Although 10344 was the only wild born wolf with $f = 0.00$ (her parents were unrelated), the kinship between the pair was 0.102; this was likely due to background levels of relatedness in captive breeding prior to re-introductions (the pair shared the same maternal grandmother as well as 10392's parents were half aunt/half nephew). There were also a number of matings among close relatives resulting in the highest inbreeding coefficients observed (Table 1).

Inbreeding depression varied by trait; body size was strongly affected by inbreeding, whereas reproductive and survival traits were only minimally affected by inbreeding. In body size models, individual f was negatively associated with overall size. This relationship was strongly supported given that 95% confidence intervals

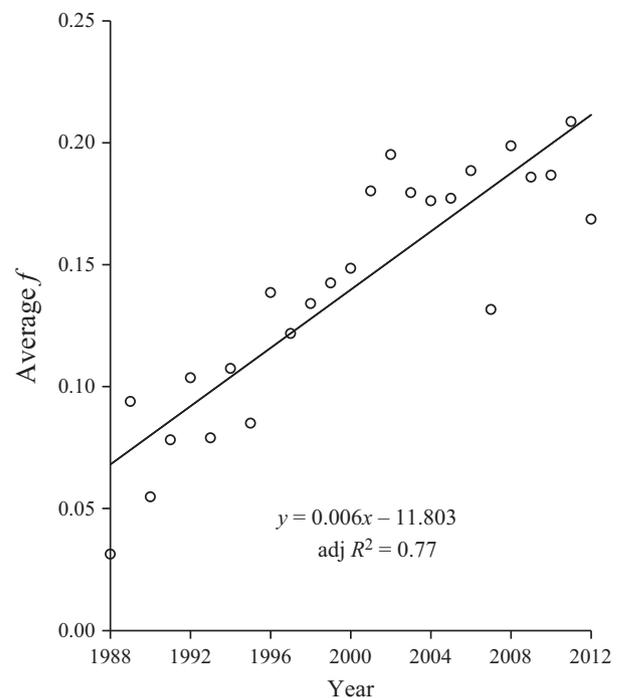


Fig. 2 Average yearly inbreeding coefficients (f) for wild born red wolf (*Canis rufus*) litters ($n = 182$) since 1988.

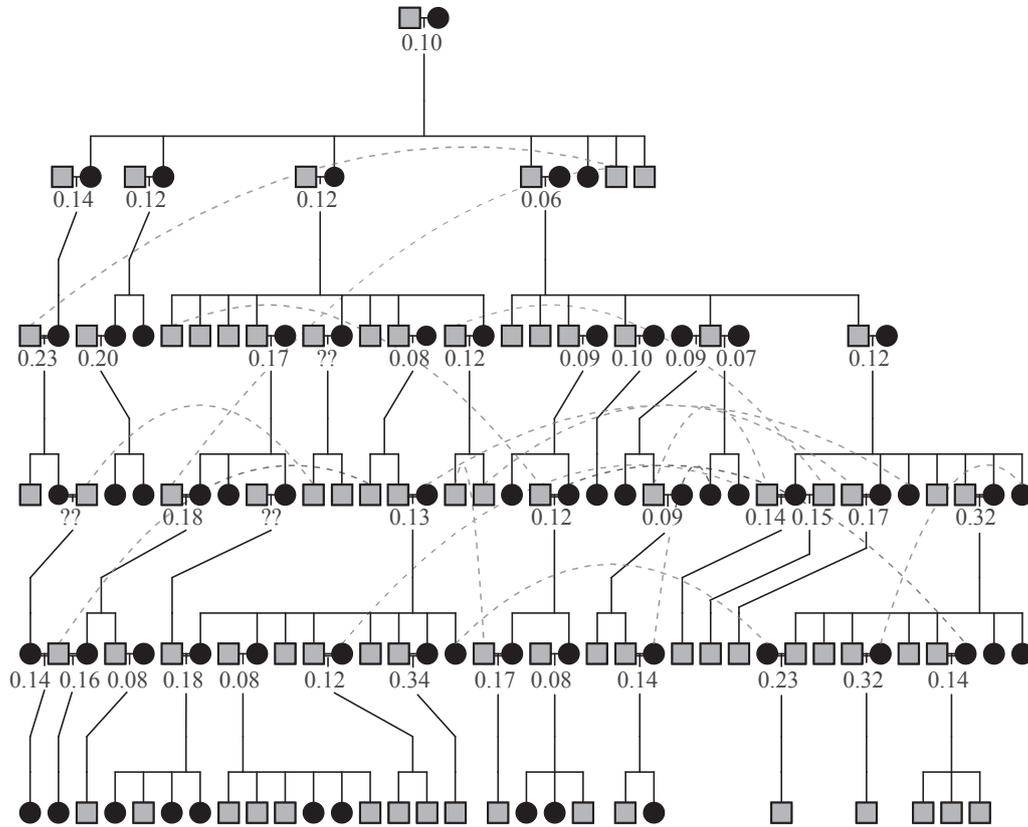


Fig. 3 Pedigree plot of one of the first wild born red wolf (*Canis rufus*) breeding pairs; circles denote females, squares denote males, and the dashed line connects the same individual present multiple times throughout the pedigree, first as offspring and later as a breeder. The kinship of each pair (and resulting inbreeding coefficient of their offspring) is displayed below the pair. ?? is indicative of unknown parents or grandparents. Nonbreeding offspring were excluded for simplicity.

did not overlap zero, and f was highly ranked in the average model set (Table 2). Red wolf ancestry, parental ages and parental f values were not influential in body size models (Table 2). We found body size did not affect the fitness measures we evaluated, similarly to Sparkman *et al.* (2011), but body size was positively associated with the probability of holding a territory (Table S3, Fig. S2, Supporting information).

Inbreeding depression was less evident in reproductive success, although in ANL GLMMs individual f and dam f had high relative importance and negatively affected ANL (Table 2). Sire f was also negatively associated with ANL but had low relative importance. GLMMs evaluating the probability of becoming a breeder were similar to ANL in that individual f and dam f adversely affected the probability of breeding, although both were relatively less important than they were in ANL models (Table 2). Sire f was positively correlated with the probability of breeding but was the lowest ranked variable in the models. Sire f , dam f and individual f all negatively influenced LNL but had the lowest relative importance in LNL models (Table 2). The

final GLMM set for litter size encompassed the null model, and thus provided little support for inbreeding depression. Model inferences for all models of reproductive success, with data truncated to wolves born 2001 onward, were comparable to the full data models.

No inbreeding depression was observed in adult or juvenile survival (Table 3); the final Cox proportional hazard model set evaluating juvenile survival encompassed the null model, and individual f and dam f had little relative importance in either survival period. However, for adult Cox proportional hazard models, sire f was negatively associated with hazard (Table 3), such that an individual with an average inbred sire (sire $f = 0.154$) was 2.932 [$\exp(6.984 \times 0.154)$] times more likely to survive compared with an individual with an outbred sire. This was a strong relationship, where the 95% confidence interval of sire f did not overlap zero. When we tested model sensitivity by removing the seven most inbred sires (sire $f > 0.30$), sire f was no longer an important factor in survival, suggesting the relationship was driven solely by the outlier sires (Table 3). Similarly to reproductive success, adult survival analyses

Table 2 Parameter estimates (β), unconditional standard error (SE), 95% confidence limits (CL), and relative importance (RI) of variables in the final averaged models evaluating lifetime number of litters (LNL), annual number of litters (ANL), the probability of becoming a breeder, litter size, and body size in endangered wild red wolf (*Canis rufus*); 95% confidence limits not overlapping 0 are in bold

Dependent variable	A priori models	Explanatory variable	β	SE	CL	RI		
LNL	28	Years reproductively available	2.17	0.19	1.79, 2.54	1.00		
		Helper	-0.40	0.21	-0.81, 0.02	1.00		
		Dam age	0.40	0.15	0.10, 0.70	1.00		
		Sire age	-0.36	0.18	-0.70, -0.02	0.52		
		Sex (m)	-0.22	0.15	-0.52, 0.07	0.32		
		Sire <i>f</i>	-0.16	0.18	-0.50, 0.19	0.24		
		Dam <i>f</i>	-0.12	0.15	-0.41, 0.16	0.23		
		<i>f</i>	-0.05	0.20	-0.44, 0.34	0.20		
ANL	30	Years with a territory	0.37	0.07	0.24, 0.50	1.00		
		Helper	-0.15	0.09	-0.33, 0.03	0.49		
		<i>f</i>	-0.07	0.07	-0.21, 0.07	0.41		
		Dam <i>f</i>	-0.07	0.07	-0.21, 0.07	0.40		
		Dam age	0.09	0.07	-0.04, 0.22	0.37		
		Sire <i>f</i>	-0.06	0.07	-0.19, 0.07	0.19		
		Sire age	0.03	0.07	-0.11, 0.17	0.16		
		Sex (m)	-0.03	0.06	-0.16, 0.09	0.09		
Probability of breeding	28	Years reproductively available	3.05	0.48	2.12, 3.99	1.00		
		Helper	-0.75	0.55	-1.82, 0.33	1.00		
		Dam age	0.36	0.40	-0.43, 1.16	0.28		
		Sex (m)	-0.42	0.40	-1.20, 0.35	0.27		
		<i>f</i>	-0.11	0.41	-0.91, 0.68	0.23		
		Dam <i>f</i>	-0.13	0.45	-1.00, 0.74	0.21		
		Sire age	-0.06	0.42	-0.88, 0.75	0.19		
		Sire <i>f</i>	0.07	0.39	-0.70, 0.84	0.15		
Litters*	27	Dam age	-0.20	0.11	-0.42, 0.02	0.60		
		litter <i>f</i>	-0.12	0.13	-0.38, 0.13	0.27		
		Dam <i>f</i>	0.01	0.12	-0.22, 0.24	0.19		
		Sire age	-0.01	0.11	-0.24, 0.21	0.18		
		Sire <i>f</i>	-0.05	0.13	-0.30, 0.20	0.15		
		Year born	-0.06	0.12	-0.29, 0.16	0.09		
		Body size	18	Sex (m)	2.26	0.21	1.86, 2.67	1.00
				<i>f</i>	-0.98	0.36	-1.69, -0.28	0.87
Ancestry	-0.40			0.34	-1.06, 0.26	0.24		
Dam age	-0.33			0.27	-0.86, 0.20	0.19		
Sire age	-0.09			0.28	-0.64, 0.46	0.09		
Sire <i>f</i>	-0.29			0.29	-0.86, 0.28	0.02		
Dam <i>f</i>	-0.14	0.37	-0.86, 0.57	0.02				

*Denotes response variable where final model set encompassing 95% of AIC weight included the null model.

with full data were qualitatively similar to models run with data truncated to wolves born 2001 onward; when sires with $f > 0.30$ were removed, dam f increased in relative importance from 0.08 to 0.22 but 95% confidence intervals still overlapped zero. All final juvenile model sets based on truncated data encompassed the null model.

Other variables important to predicting reproductive success and survival included parental age, years of reproductive availability, years with a territory, presence of helpers and sex. Longer-lived red wolves, red wolves with a territory, and individuals with older

dams had higher LNL, ANL, a greater probability of breeding, and increased survival. In contrast, sire age, and presence of helpers negatively affected reproductive traits and survival except for ANL and adult survival models (Tables S4–S10, Supporting information).

Discussion

The deleterious effects of inbreeding are a serious consideration for small wildlife populations of conservation concern (Keller & Waller 2002; O'Grady *et al.* 2006; Wright *et al.* 2007). In red wolves, inbreeding has

Table 3 Cox proportional hazard results from models evaluating adult and juvenile survival (survival to 18 months) in endangered wild red wolf (*Canis rufus*). Effect size, unconditional standard error (SE), 95% confidence limits (CL) and relative importance (RI) of variables are reported from the final averaged models; 95% confidence limits not overlapping 0 are in bold and (–) indicates a variable was not in the final average model set. Effect size refers to the influence a parameter has on the proportional survival hazard where positive parameter estimates increase the hazard of dying while negative estimates increase survival

	Explanatory variable	All data				Sire <i>f</i> values >0.30 removed			
		Effect	SE	95% CL	RI	Effect	SE	95% CL	RI
Adult survival	Territory	–2.70	0.37	–3.42, –1.98	1.00	–2.72	0.37	–3.45, –1.99	1.00
	Sire <i>f</i>	–6.98	3.05	–12.96, –1.01	0.83	–1.55	4.71	–10.78, 7.68	0.14
	Sire age	–0.12	0.10	–0.30, 0.07	0.41	–0.12	0.09	–0.30, 0.07	0.30
	<i>f</i>	–2.87	2.20	–7.17, 1.43	0.08	–1.62	2.25	–6.04, 2.79	0.18
	Sex (m)	0.22	0.25	–0.27, 0.72	0.02	0.25	0.25	–0.24, 0.75	0.12
	Dam age	—	—	—	—	0.06	0.09	–0.12, 0.23	0.09
	Dam <i>f</i>	—	—	—	—	1.70	3.51	–5.18, 8.57	0.08
Juvenile survival*	Sire <i>f</i>	–6.49	6.56	–19.34, 6.36	0.42	–0.21	7.44	–14.79, 14.37	0.11
	Sire age	0.00	0.20	–0.39, 0.39	0.38	–0.02	0.18	–0.37, 0.33	0.33
	Sex (m)	0.01	0.39	–0.75, 0.77	0.31	0.00	0.38	–0.75, 0.74	0.20
	<i>f</i>	–0.35	4.09	–8.36, 7.65	0.14	0.83	4.24	–7.48, 9.15	0.25
	Dam <i>f</i>	3.20	5.06	–6.71, 13.11	0.06	3.82	4.96	–5.90, 13.53	0.12
	Dam age	0.00	0.14	–0.28, 0.28	0.05	0.04	0.14	–0.23, 0.32	0.12

*Denotes response variable where final model set encompassing 95% of AIC weight included the null model.

increased substantially since re-introductions in 1987, resulting in a population that is almost completely inbred. The observed level of inbreeding appeared to be the result of both high background levels of relatedness and a number of matings among close relatives (Fig 3, Table 1). Compared with other wild populations, red wolf inbreeding coefficients are high (Table 4). The mean red wolf *f* value of 0.154 is greater than half-sibling matings, and although other wild populations have individuals with high *f* values, few have a documented population mean as high as wild red wolves (Table 4). The wild mean *f* value was also much higher than the captive red wolf population mean of 0.076 (Waddell & Long 2013). Similarly, the percentage of inbred wild red wolves (99%) is greater than other reported wild populations (Table 4). Although cooperative breeding species, like wolves, often have mechanisms to avoid inbreeding (Pusey & Wolf 1996; Fadao *et al.* 2000; Jamieson *et al.* 2009; Sparkman *et al.* 2012a), inbreeding values have significantly increased through time, a result attributable to a small number of founders (*n* = 12) and a single population with no possibility of gene flow from other wild red wolves.

In contrast, the number of lethal equivalents (β = 0.00) detected for juvenile survival in red wolves was much lower than other captive and wild populations (Ralls *et al.* 1988; Kruuk *et al.* 2002; Liberg *et al.* 2005; O’Grady *et al.* 2006; Dunn *et al.* 2011). For example, the average number of haploid LE for juvenile survival is 2.3 in 38 captive species (Ralls *et al.* 1988) and 1.2 in six wild species (O’Grady *et al.* 2006); haploid LE

as high as 12.1 have been documented in wild pronghorns (*Antilocapra americana*; Dunn *et al.* 2011). Yet, there are also examples of inbred populations with few LE, such as red-cockaded woodpeckers (*Picoides borealis*) which suffer from inbreeding depression in both reproductive and survival traits but have haploid LE = 0.37 for first year survival (Daniels & Walters 2000; O’Grady *et al.* 2006). Our results are consistent with Kalinowski *et al.* (1999) who found few LE for captive red wolf survival to 180 days and estimated the number of LE in 13 founders to be near zero.

Fitness consequences associated with inbreeding varied by trait where inbreeding depression was strongest for body size such that more inbred individuals were smaller. Conversely, no inbreeding depression was detected in reproductive and survival measures, a finding consistent with our observed values of zero for lethal equivalents. The lack of inbreeding depression in reproductive and survival traits was surprising because inbreeding depression is generally strongest for direct fitness traits, which are under greater selective pressure and exhibit more directional dominance [where dominant alleles affect a trait in the same direction, resulting in a difference in means between heterozygous and homozygous phenotypes (Barton & Keightley 2002)] than morphometric measures (Crnokrak & Roff 1995; Roff 1998; De Rose & Roff 1999). Red wolf body size did not influence fitness directly but it did increase the probability of having a territory, which is important for securing reproductive opportunities, suggesting that smaller body size influences fitness indirectly by reduc-

Table 4 Species, inbreeding coefficient (f), mean f , per cent of population inbred (% inbred), the fitness consequences of inbreeding, and the population status (wild/captive) from studies with pedigrees that evaluated the effects of inbreeding (see Keller & Waller 2002 for older research). Table focused on wild populations but included captive Mexican grey wolves for comparison

Species	f	Mean f	% Inbred	Fitness consequences	Captive/Wild	Author
Mexican wolf	0–0.608	—	—	Reduced litter size	Captive	Hedrick & Fredrickson (2008)
Scandinavian wolf	0–0.410	—	92.0%	Decreased pup survival, litter size	Wild	Liberg <i>et al.</i> (2005)
Red wolf	0–0.383	0.154	99.0%	Reduced body size	Wild	Present study
Bighorn sheep	0–0.314	0.042	25.0%	Decreased survival of female lambs	Wild	Rioux-Paquette <i>et al.</i> (2010)
African wild dogs	0–0.281	0.074	37.5%	Shorter lifespans	Wild	Spiering <i>et al.</i> (2011)
Stewart Island robin	0–0.250	0.070	—	Little inbreeding depression found	Wild	Laws <i>et al.</i> (2010)
Pronghorn	0–0.250	0.026	22.0%	Decreased fawn survival to weaning, birth mass, foot length and condition	Wild	Dunn <i>et al.</i> (2011)
Red deer	0–0.250	0.007/0.013	22%/42%	Decreased birth weight and first year survival	Wild	Walling <i>et al.</i> (2011)
Collared flycatchers	0–0.250	0.002	1.0%	Reduced hatching, fledging, juvenile survival, recruitment, and juvenile skeletal size	Wild	Kruuk <i>et al.</i> (2002)
Great tit	0–0.250	0.004	3.0%	Reduced hatching, fledging, recruitment, production of grand offspring	Wild	Szulkin <i>et al.</i> (2007)
Meerkats	0–0.130	0.078	44.0%	Decreased pup mass at emergence, hind-foot length, growth, juvenile survival	Wild	Nielsen <i>et al.</i> (2012)
Yellowstone grey wolf	<0–0.084	0.000	3 related matings	None observed	Wild	vonHoldt <i>et al.</i> (2007)

ing the probability of becoming a territory holder. A reduction in body size associated with inbreeding has also been detected in other wolf species, including Mexican (*C. lupus baileyi*) and Nordic grey wolves (Laike 1999; Fredrickson & Hedrick 2002). Additional studies have documented similar correlations between inbreeding depression and body size or skeletal measures in noncanid species, which may have indirect effects on sexual selection, intraspecific competition, survival, or fecundity (Fredrickson & Hedrick 2002; Kruuk *et al.* 2002; Wisely *et al.* 2008; Bolund *et al.* 2010; Dunn *et al.* 2011; Nielsen *et al.* 2012; Naish *et al.* 2013). Inbreeding may affect morphology more than previously thought (Wright *et al.* 2007) and may represent a cost-effective way of measuring the effects of inbreeding *in situ*, although see Ibáñez *et al.* (2011) who found no inbreeding depression in morphology, suggesting the large variation observed in inbreeding effects may make it difficult to generalize a trait's response.

Other traits that influenced red wolf fitness included parental age and years reproductively available. The influence of parental age was most evident in LNL

models, where individuals with older dams and younger sires had higher LNL. Generally, reproductive success decreases with maternal age (Rabon 2014), but older females have more parental experience (Mech 2000), and in some mammals have heavier offspring to compensate for smaller litters (Ericsson *et al.* 2001), both of which could increase offspring fitness (Curio 1983). The only fitness measures that were negatively associated with dam age in red wolves were litter size and adult body size, but confidence limits overlapped zero for both traits thereby limiting our ability to make inference. Sire age varied more by trait and was not as relatively important as dam age, possibly reflecting different reproductive strategies between sexes (Weimerskirch *et al.* 2000; Miller *et al.* 2003).

The number of years a red wolf was reproductively available also increased LNL and the probability of breeding. While this is an intuitive relationship (the more years an individual is able to breed the higher their reproductive success), it also demonstrates the negative impact that sterile coyote placeholders may have on red wolf reproductive success. Habitat conver-

sion and fragmentation, combined with animal translocations have increased rates of hybridization across animal taxa (Rhymer & Simberloff 1996; Allendorf *et al.* 2001), and as demonstrated with red wolves, managers face a challenge of maintaining reproductive output while preventing introgression (Miller *et al.* 2003; Allendorf & Luikart 2007). The use of sterile placeholder mates has been a successful management technique to reduce coyote hybridization and introgression with red wolves (Stoskopf *et al.* 2005; Rabon *et al.* 2013), but it also reduces the years an individual is reproductively available. Interestingly, red wolf ancestry had little influence on body size or fitness, suggesting coyote introgression did not strongly influence fitness. However, future work focused on coyote introgression is needed to fully understand the influence of hybridization on wild red wolves.

There are several potential reasons for the lack of lethal equivalents and inbreeding depression observed in traits other than body size. Genetic purging could have reduced the genetic load in red wolves such that deleterious alleles directly associated with fitness were purged, whereas alleles indirectly associated with fitness such as body size, persisted in the population (Crnokrak & Roff 1995; Lacy & Ballou 1998). Although genetic purging may be effective at removing deleterious alleles in some inbred populations (Ballou 1997; Reed *et al.* 2003), in general, genetic drift is a stronger force than purging selection in small populations such as red wolves (Hedrick & Kalinowski 2000). A founder effect may be a more likely explanation for the pattern of inbreeding depression we detected. Random sampling of alleles in founder lineages affects the severity of inbreeding depression in inbred mice (Lacy *et al.* 1996) and white pigs (Rodríguez *et al.* 1998) where inbreeding depression was attributed to a few deleterious alleles, which were not carried by all founders. This may be true for red wolves given that 13 founders had few LEs, and no LEs were detected for captive juvenile viability (Kalinowski *et al.* 1999). If all red wolf founders lacked deleterious recessive alleles at genes affecting fecundity as well as survival, then we may continue to see minimal inbreeding depression at these fitness traits. However, standard errors and confidence limits for effect sizes of individual and parental f values were large, especially in survival models (Table 3). This could indicate there is a lack of statistical power to detect inbreeding depression. For instance, in Cox juvenile survival models, the upper range of the 95% confidence limits for dam f (-6.71, 13.11) and individual f (-8.36, 7.65) encompassed some of the more dramatic inbreeding depression values reported in the literature.

The absence of significant inbreeding depression in reproductive success and survival may also have been

caused by the lack of outbred individuals for comparison. In captive Mexican wolves, minimal inbreeding depression was detected until individuals from three unrelated lineages bred and the resulting offspring had higher fitness than the inbred parental lineages (Fredrickson *et al.* 2007; Hedrick & Fredrickson 2008); inbreeding depression could not be detected without outbred individuals because there was too little variation in f . The lack of noninbred red wolves in our study may have masked the most detrimental effects of inbreeding depression. Alternatively, the most detrimental effects of inbreeding depression may not yet be detectable because all of the highest f values (>0.28) are from red wolf litters born 2001 to 2012, more than half of which were born after 2008. Although model inferences for all fitness variables were similar between the full and restricted data set, it is possible that fitness consequences from highly inbred wolves born recently may be detected once complete life history data are collected.

Hedrick & Kalinowski (2000) suggest that the true effects of inbreeding are generally greater than those observed, not less. This may be true for wild red wolves, and given that inbreeding will likely continue to increase, inbreeding depression is a continued concern for red wolf viability. Population management aimed at reducing inbreeding and inbreeding depression is needed. Common genetic management techniques, such as genetic rescue, have been successful with Florida panthers (Johnson *et al.* 2010), Mexican wolves (Fredrickson *et al.* 2007) and bighorn sheep (Hogg *et al.* 2006), and could be applied by introducing more distantly related individuals through cross-fostering wolves from the managed captive breeding programme into wild litters. There have been 21 cross-fostering events since 2002; in each instance, the captive born cross-fostered pups were less inbred (mean $f = 0.074$) and had lower mean kinship values (MK = 0.095) than the wild born averages (mean $f = 0.154$, MK = 0.160). Future management practices could increase cross-fostering or release captive born juveniles with the aim of reducing overall inbreeding and mean kinship in the wild population.

Multigenerational pedigrees are rare in wild populations, (see Table 4), but can provide unique insights into processes that influence inbreeding. For example, in a population of highly social African wild dogs (*Lycaon pictus*), high levels of inbreeding were attributable to a single pack (Spiering *et al.* 2011). This contrasts with results from meerkats (*Suricata suricatta*) where inbreeding was not the result of a few inbred family groups but was influenced by social dominance and was ubiquitous throughout the population (Nielsen *et al.* 2012). Red wolves live in social family groups, and similarly

to meerkats, inbreeding was spread throughout the population. However, meerkats may tolerate low levels of inbreeding because the benefits of securing a breeding opportunity, even if with a related mate, outweigh the cost of inbreeding depression (Nielsen *et al.* 2012), unlike red wolves where inbreeding was likely the result of few founders and a closed population; an inherent problem facing any extremely small or endangered population.

The pervasiveness of inbreeding in wild populations is well recognized (Keller & Waller 2002), but factors influencing the extent of inbreeding depression are still being evaluated. Our results demonstrate that inbreeding depression varies substantially by trait, highlighting the need to evaluate a number of different fitness parameters/traits when examining inbreeding depression. While inbreeding significantly reduced red wolf body size, its influence on direct measures of red wolf fitness appears to be weak. With continued monitoring and pedigree construction in wild red wolves, the efficacy of genetic purging and prevalence of founder effects can be evaluated as individuals continue to become more inbred.

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Data accessibility

All fitness data were collected in the Red Wolf Recovery Area, a region encompassing 17 million acres throughout five counties (Dare, Tyrrell, Hyde, Beaufort, and Washington) in North Carolina, where the USFWS Red Wolf Recovery Program archives the wild red wolf life history and fitness data. The red wolf pedigree is maintained by the Red Wolf Species Survival Plan Coordinator at the Point Defiance Zoo in Tacoma, Washington. Contact the USFWS Red Wolf Recovery Program assistant coordinator for data inquires.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Results from a Principle Components Analysis used to create a body size measure for red wolves (*Canis rufus*); the first principle component (PC) encompasses a large portion of overall variance and was used as a response variable in models.

Table S2 Pearson correlation coefficients between all numerical explanatory variables, including individual inbreeding coefficients (f), parental inbreeding coefficients (dam f , sire f), parental ages (dam age, sire age), years reproductively available (ry), and territory holders (territory) used in red wolf (*Canis rufus*) fitness models; no variables with a correlation >0.4 were used in the same model, except global models which were included to assess model fit.

Table S3 Parameter estimates (β), unconditional standard error (SE), 95% confidence limits (CL), and relative importance (RI) of variables in the final averaged models evaluating the probability of having a territory in wild red wolves (*Canis rufus*); 95% confidence limits not overlapping 0 are in bold.

Table S4 Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models

evaluating lifetime number of litters (LNL) in endangered red wolves (*Canis rufus*).

Table S5 Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models evaluating annual number of litters (ANL) in endangered red wolves (*Canis rufus*).

Table S6 Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models evaluating the probability of breeding in endangered red wolves (*Canis rufus*).

Table S7 Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models evaluating litter size in endangered red wolves (*Canis rufus*).

Table S8 Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models evaluating body size in endangered red wolves (*Canis rufus*).

Table S9 Cox proportional hazard model parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models evaluating adult survival in endangered red wolves (*Canis rufus*).

Table S10 Cox proportional hazard model parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (w_i) for all models evaluating the probability of juvenile survival (18 months) in endangered red wolves (*Canis rufus*).

Fig. S1 Principle components analysis of body length, hind foot length, shoulder height, ear size and tail length showing how PC1 encompassed overall body size, as demonstrated by the separation of male (blue M) and female (red F) adult red wolves (*Canis rufus*).

Fig. S2 The effect adult red wolf (*Canis rufus*) body size had on the probability of holding a territory for at least one breeding season (1; 0=never held a territory).