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Effects of Genetic Captive-Breeding Protocols on Sperm Quality and Fertility in the White-Footed Mouse[1]

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ABSTRACT

?2 **Mice (*Peromyscus leucopus noveboracensis*) from a captive-**
?3 **breeding program were used to test the effects of three genetic**
?4 **breeding protocols (minimizing mean kinship [MK], random breeding, and selection for docility [DOC]) and inbreeding levels on sperm traits and fertility. Earlier, in generation 8, one DOC replicate went extinct because of poor reproductive success. By generation 10, spermatozoa from DOC mice had more acrosome and midpiece abnormalities, which were shown to be strong determinants of fertility, as well as lower sperm production and resistance to osmotic stress. In addition, determinants of fertility, including male and female components, were assessed in a comprehensive manner. Results showed that the probability (*P*) of siring litters is determined by sperm number, sperm viability, and midpiece and acrosome abnormalities; that the *P* of siring one versus two litters is determined by tail abnormalities; and that the total number of offspring is influenced by female size and proportion of normal sperm, showing the relative importance of different sperm traits on fertility. On average, males with 20% normal sperm sired one pup per litter, and males with 70% normal sperm sired eight pups per litter. Interestingly, the proportion of normal sperm was affected by docility but not by inbreeding at relatively low**
?5 **levels. However, inbreeding depression in terms of sperm motility was detected. In the MK group, inbreeding depression not only affected sperm motility but also fertility: An increase in the coefficient of inbreeding (*f*) of 0.03 reduced sperm motility by 30% and translated into an offspring reduction of three pups**
?6 **in second litters. A genetic load of 48 fecundity equivalents was calculated.**

conservation breeding, docility, genetic adaptation to captivity, inbreeding depression, mean kinship, Peromyscus leucopus, *reproductive success*

INTRODUCTION

Ex situ conservation breeding programs of captive populations help species, originally either highly endangered or no longer found in their natural habitats, to be successfully reintroduced into the wild. Such programs have already saved several species from extinction. The examples of the California ?7
condor, Guam rail, European bison, Pere's David's deer, Przewalski's horse, and black-footed ferret are notable [1]. ?8
However, whereas many other species have been reintroduced after captive-breeding programs, the overall success of these reintroductions is still low [2]. The reasons for this low success are multiple. One possible explanation that needs to be tested experimentally is that captive-breeding programs cause genetic changes that negatively impact fitness-related traits after animals have been returned to their natural habitats [3, 4].

Captive-breeding programs cause three types of genetic effects: genetic drift, inbreeding, and genetic adaptation to captivity. Genetic drift adds random variation, and it becomes larger as populations decline in size. Inbreeding depression (i.e., the decrease in fitness brought about by inbreeding) is thought to result mainly from the expression of mildly deleterious mutations in homozygotes [5] and is a serious threat to captive and natural populations [6, 7] because of its effects on juvenile survival, size, and life span [8–11]. Inbreeding is difficult to avoid in captive-breeding programs due to low numbers of founders, small population sizes, and logistic constraints regarding the translocation of animals for breeding [12].

Genetic adaptation to captivity can be defined as the change in the genetic makeup of a population that occurs when the selection forces change from those imposed by the natural environment to those imposed by the captive environment. Artificial selection for docility is expected to be a common cause of genetic adaptation to captivity in captive-breeding programs, because more docile animals tend to breed more than the average. This happens either because more aggressive or stressed animals are more likely to be injured, die from trauma, or reproduce poorly in the confined spaces of captivity or because animal managers might favor animals that are easier to handle. The efficiency of selection decreases with decreasing population size [13], and because captive populations are generally small, genetic adaptation to captivity may not play a significant role in captive-breeding programs [14]. Currently, however, no estimations are available regarding the strength of artificial selection for docility in captivity.

Understanding the impact of inbreeding on fecundity is central both to evolutionary biology [15] and to conservation biology [16]. During the past two decades, growing evidence

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has indicated the effects of inbreeding on male reproductive quality in mammals. Inbred dogs and cats experience reduced reproductive performance, and effects of inbreeding on sperm quality have been shown to be responsible [17–19]. Other studies with inbred or endangered populations of wild carnivores (e.g., lions [20], cheetahs [21, 22], and Florida panthers [23]) have shown poor male reproductive quality.

Classical studies pointed out the deleterious effects of inbreeding on sperm quality [24]. In studies with captive populations of endangered species, the Sri Lankan leopard (*Panthera pardus kotiya*) presented a high frequency of sperm abnormalities [25], and the clouded leopard (*Neofelis nebulosa*) presented severe spermatogenic dysfunction [26]. These captive populations are small, and they have descended from few founders. However, those studies did not report level of inbreeding. Two different studies at the population level, one in natural populations of lions [20] and another in captive populations of three species of gazelles (*Gazella dama mhorr, Gazella dorcas neglecta,* and *Gazella cuvieri*) [27–29], found similar results, with a negative correlation between sperm quality and the average inbreeding levels of the population or species. Only one study in *G. cuvieri* has shown a direct negative relationship between coefficient of inbreeding (f range, 0.04–0.24) and sperm quality at the individual level [30]. Another study in captive populations of Mexican wolf [31] found a similar result, although in this case, the comparison involved pooling subjects from different captive populations (f range, 0–0.6). Regarding sperm production, negative [32] and positive [33] relationships between inbreeding and sperm production have been reported.

These studies suggest that in captive-breeding programs, inbreeding brings about negative effects on sperm quality. However, in most of these studies, the effects of inbreeding were not quantified, and artificial selection for docility or other forms of adaptation to captivity were not considered as possible factors affecting those results. Furthermore, in multigenerational captive-breeding programs, the levels of inbreeding will often be correlated with the levels of selection for docility and unintentional adaptation to captivity. Thus, it remains to be substantiated whether the decline in sperm traits during captive-breeding programs results from inbreeding effects or from artificial selection for docility or other traits favored under captive conditions.

Captive-breeding programs for endangered species typically use a mating protocol that minimizes mean kinship (MK) in the population [34, 35]. The effectiveness of the MK protocol in terms of maintaining heterozygosity and allelic diversity and minimizing inbreeding has been shown theoretically through simulations [34, 36, 37] and experimentally in *Drosophila* sp. [38]. However, the effects of following such a protocol to maintain genetic diversity have not been tested empirically on the vertebrate species that conservation breeding programs often target.

In the present study, we used a population of a promiscuous wild species (white-footed mice, *Peromyscus leucopus noveboracensis*), kept for 10 generations in captivity, to address the effects of three different genetic captive-breeding protocols (minimizing MK, selection for docility [DOC], and random breeding with accumulated inbreeding [RAN]) on sperm traits and fertility. Given that one line in the DOC protocol went extinct in generation 8, our expectation was that selection for docility might bring about more deleterious effects on fertility than the RAN and MK protocols. Opportunistically, we also tested the effect of inbreeding levels on sperm traits and fertility. Lastly, we tested the effects of sperm traits on fertility.

MATERIALS AND METHODS

Mice

In October 2001, fifty none white-footed mice were trapped at Volo Bog State Natural Area (Lake County, IL, USA). After quarantine and disease testing, mice were brought into a captive environment at the Chicago Brookfield Zoo research facilities to set up the founder population. Sampling theory estimates the amount of heterozygosity that can be retained from a wild to a founder population [39]. On the basis of this theory, it is assumed that no more than 20 individuals are required to establish a captive population [40, 41], because they are expected to represent, on average, $1 - 1/2N$, or 97.5%, of the wild genetic diversity and, thus, nearly all wild additive genetic variance (for details, see [42]). Nineteen pairings were set up, of which 12 produced litters. The most productive 10 pairs gave birth to more than 240 individuals in 5–10 litters each. These progeny were randomly allocated to six experimental groups, representing two replicates for each of three breeding protocols (20 pairs per protocol and replicate) and subsequently bred in captivity for 10 generations. The three breeding protocols were as follows: 1) MK protocol, which minimized mean kinship (maximizes gene diversity) by pairing the males and the females with the lowest average kinships to the rest of the population; 2) DOC protocol, in which artificial selection for docility was practiced by pairing the males and female with the lowest scores for gnawing and flipping behaviors; and 3) RAN protocol, in which individuals were assigned to pairings in a random manner. The MK protocol followed the genetic management strategy used by many zoos. The DOC protocol aimed to mimic the kinds of purposeful or inadvertent selections for docility that often occur in captive-breeding programs. Two replicate populations were used per protocol. The six populations have never interbred. The data presented in this paper correspond to the 10th generation of captive breeding. The animal care protocols and experiments described here complied with all current laws and were approved by the Animal Care and Use Committee of the Chicago Zoological Society.

Experimental Breeding Protocols

In the overall experiment, after nine generations in captivity, the accumulation of inbreeding was, on average, higher in DOC mice ($f = 0.18$), lower in RAN mice ($f = 0.14$), and lowest in MK mice ($f = 0.12$). Within protocols, however, differences occurred in the accumulated levels of inbreeding between the two replicates. For the present study, only one replicate per protocol was selected in a design aimed at disentangling the effects of inbreeding and artificial selection for docility. For the MK protocol, the replicate was selected randomly (MK2, $f = 0.11$). For the DOC protocol, we could only use one replicate (DOC1, $f = 0.13$), because the DOC2 population had died out at generation 8 because of poor reproductive success. For the RAN protocol, we selected the replicate that had accumulated more inbreeding (RAN2, $f = 0.15$) to avoid the DOC group being divergent in both inbreeding and selection for docility, potentially confounding these effects. For simplicity, hereafter the replicate number of the protocol will be omitted: MK represents minimal selection for docility and minimal inbreeding ($f = 0.11$), RAN represents minimal selection for docility and maximum inbreeding ($f = 0.15$), and DOC represents maximal selection for docility and intermediate inbreeding ($f = 0.13$). This design allows us to disentangle, in part, the effects of inbreeding and selection for docility.

Sample Size

Twenty adult males from each breeding protocol ($n = 60$) were paired with females from the same replicate and protocol for a period of up to 70 days. Males were separated from the females after they had produced two litters or when they had been paired for 70 days. Mean age at death was 204 days (SD, 19.9 days; range, 153–241 days), and mean weight was 25 g (SD, 3.7 g; range, 19.2–40.5 g). This design results in conservative comparisons of fertility between protocols, because males that are unable to sire litters stay paired for more time than males that sire two litters in a short period. For this reason, MK mice were, on average, 23–24 days younger than RAN and DOC mice when killed (Table 1), because MK mice were faster at siring litters. However, age had no effect on any of the sperm quality traits analyzed ($P > 0.05$).

After being separating from the female, every male was left in an individual cage for at least 7 days before it was killed. This was done to allow the replenishment of sperm reserves and to minimize the female presence- and copulation-induced effects on sperm reserves. By doing this, we aimed to further standardize the conditions for males before sperm collection and to minimize residual variance in sperm numbers.

TABLE 1. Descriptive statistics by protocol (mean ± SEM), variance explained for each trait by protocol after ANOVAs, *P* values, and summary of Tukey post-hoc tests and significant pairwise comparisons for significant models.

Variables	MK (n = 20)	RAN (n = 20)	DOC (n = 20)	R^2 (%)	*P*	Tukey post-hoc[b]
F	0.11 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	88	<0.001	R > D > M
Age (days)	188.0 ± 20.6	212.0 ± 13.5	211.0 ± 15.8	37	<0.001	R = D > M
Body length (mm)	100.0 ± 3.1	96.5 ± 3.1	97.4 ± 3.5	23	0.012	M > R = D
Body weight (g)	26.61 ± 4.95	24.93 ± 2.12	23.38 ± 2.97	12	0.021	M > D
Kinship	0.12 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	89	<0.001	R > D > M
Testes weight (g)	0.51 ± 0.14	0.58 ± 0.16	0.46 ± 0.09	15	0.017	M = R > D
Sperm numbers ($\times 10^6$ sperm/ml)	141 ± 71.1	165 ± 81.6	101 ± 25.6	13	0.008	M = R > D
Sperm motility (%)	77.2 ± 14.1	71.5 ± 15.7	72.0 ± 16.4	8	0.237	—
Sperm osmotic resistance (%)	45.0 ± 15.0	40.0 ± 13.5	28.0 ± 13.7	42	0.001	M = R > D
Sperm viability (%)	60.6 ± 12.4	63.5 ± 11.73	59.4 ± 10.8	1	0.901	—
Sperm acrosome integrity (%)	84.1 ± 7.47	84.2 ± 8.736	85.9 ± 4.8	2	0.697	—
Normal sperm morphology (%)	38.9 ± 18.3	43.9 ± 20.4	36.1 ± 18.0	3	0.633	—
Head abnormality (%)	1.0 ± 0.33	0.2 ± 0.08	0.8 ± 0.36	7	0.129	—
Acrosome abnormality (%)	0 ± 0	0 ± 0	0.2 ± 0.07	20	0.002	M = R < D
Midpiece abnormality (%)	3.7 ± 0.68	2.7 ± 0.56	5.85 ± 1.13	11	0.031	R < D
Bent midpiece (%)	5.8 ± 1.21	6.1 ± 1.23	6.63 ± 0.69	0	0.858	—
Swollen midpiece (%)	45.4 ± 3.84	44.1 ± 4.85	42.7 ± 3.60	0	0.894	—
Tail abnormality (%)	5.0 ± 1.09	2.9 ± 0.37	5.93 ± 0.38	7	0.114	—
Mated female body length (mm)	99.9 ± 0.89	99.4 ± 1.03	100.9 ± 0.87	3	0.527	—

[a] Values are presented as the mean ± SEM. Variance is explained for each trait by protocol after ANOVA.
[b] D, DOC; M, MK; R, RAN.

Spermatozoa Collection

After males were killed, testes were extracted from the abdominal cavity. Testes weight was obtained by adding left and right testis weight after separating the epididymides. The dissected cauda epididymides and vasa deferentia were stored in 0.5 ml of modified Tyrode-Hepes buffer (mT; 300 mOsm/kg, pH 7.4) covered with paraffin oil in a Petri dish at 37°C. Connective tissue, fat, and blood vessel remains were separated under a binocular microscope. The cauda epididymides and vasa deferentia were then transferred to another Petri dish with 0.5 ml of clean mT. To extract the sperm, the cauda epididymides were punctured with a sterile needle for 90 sec and gently handled to help the contents out of the tubules and vasa deferentia. Samples were allowed to rest for 5 min before removal of the remaining tissue. Then, sperm was transferred to a 1.5-ml Eppendorf tube resting in a warm bath at 37°C.

Sperm Trait Measures

After spermatozoa collection, sperm traits were measured (Supplemental Table S1; all Supplemental Data are available online at www.biolreprod.org). Previous research has shown that sperm number [43], motility [44, 45], viability [46, 47], as well as morphology and acrosome integrity (%NAR) [45, 48, 49] are associated with fertility. Osmotic resistance has been shown to be affected by the genetic background of mice [50, 51]. In addition, sperm motility is substantially more sensitive to osmotic stress compared to the sensitivity of other sperm parameters [52]. Thus, we included a sperm osmotic resistance test [53] to detect changes in membrane resistance caused by altered spermatogenesis or sperm maturation.

Total sperm number. Total sperm number was calculated as the volume of sperm medium (0.5 ml of mT media) multiplied by sperm concentration (sperm/ml) as assessed with a hemocytometer. This gives an estimate of sperm production to supplement measures of testes size.

Sperm motility. Sperm motility was subjectively assessed with a phase-contrast microscope at 37°C using a slide warmer. The percentage of motile spermatozoa (0–100% using increments of 5%) was recorded.

Sperm viability, morphology, and %NAR. Sperm viability, morphology, and %NAR were assessed under a bright field in 200 spermatozoa from smears stained with eosin-nigrosin and mounted in DPX (Fisher Scientific). Regarding sperm morphology, samples were classified according to morphological abnormalities of the head (acrosome and abnormal heads), midpiece (bent, swollen, or other abnormalities), and tail. The proportion of normal spermatozoa was calculated as the proportion of sperm not presenting abnormalities in any of these components (Supplemental Fig. S1).

Sperm osmotic resistance. Sperm osmotic resistance was quantified as the value for sperm motility reached after an osmotic stress test. In this test, an aliquot of the sperm sample was added to a hyperosmotic medium (500 mOsm/kg) at 37°C, and 5 min later, distilled water was added to the sperm sample to restore isosmotic conditions. Sperm motility was reassessed after 5 min.

Genetic Load

To calculate the average effect of accumulated detrimental genes per individual on fertility fitness, and to put this measure onto the same scale of fitness as that used for effects of inbreeding on survival [54], we calculated fecundity equivalents [55, 56]. For the present study, the number of fecundity equivalents per diploid genome was calculated as twice the slope ($2B$) of the regression of the natural log (ln) of relative fecundity (N/N_{max}) on the coefficient of inbreeding (f) using the following formula:

$$\ln \frac{N}{N_{\max}} = -A - Bf$$

where N is fecundity measured as the number of progeny produced, A is the intercept at zero inbreeding, and B is the number of fecundity equivalents per haploid genome. (For more details, see Supplemental Text).

Statistical Analyses

To test for confounding associations, we first explored whether sperm traits were associated with age or body weight. We found no effects of age on either testes size or the sperm quality traits analyzed ($P > 0.2$, n = 60) except for osmotic resistance, in which a marginally significant effect was observed ($r = -0.25$, $P = 0.05$, n = 59). As expected, body weight had a significant effect on testes weight ($r = 0.42$, $P < 0.001$, n = 60).

The ANOVA and Levene tests were used to examine for differences between protocols in the mean and variance of the traits analyzed. Multiple ANOVAs reporting Wilks lambda were used to test for multivariate significant differences between protocols in sperm quality traits and, more specifically, sperm morphological abnormalities.

The effect of different sperm traits on fertility was tested while controlling for possible confounding factors, such as allometry, age, inbreeding, and female effects. The effect of the breeding protocol on the probability of siring offspring (0–1) was assessed through chi-square tests. After extinction of one DOC replicate in generation 8, the a priori expectation was that the DOC protocol would have a negative effect in the remaining replicate. To understand the sperm determinants of fertility, we used a three-step approach. Using logistic regression analyses and fitting protocol as a categorical predictor, we first tested which sperm traits determine siring litter success (0 vs. 1). Second, we tested which sperm traits influence the probability of having one versus two litters. Third, we explored which factors determine total number of offspring using generalized linear models. Final models were obtained by stepwise deletion of nonsignificant terms. Given the a priori expectation of the negative relationship between inbreeding levels and sperm traits, we used one-tailed *t*-test to examine for inbreeding depression.

All analyses were conducted in STATISTICA v. 6.0 for Windows [57].

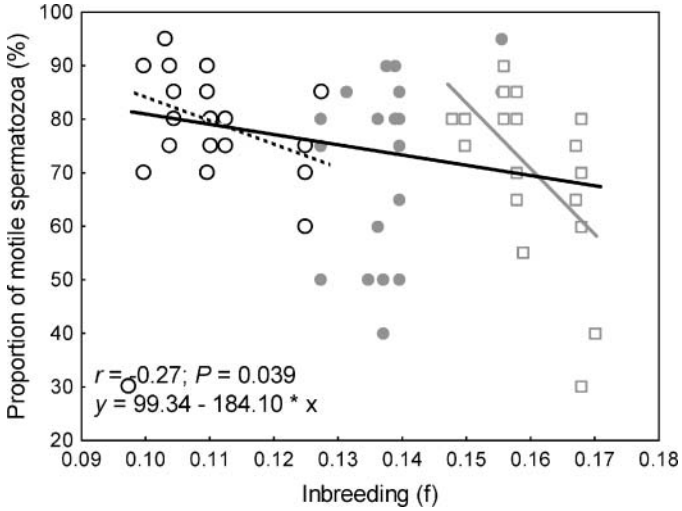

FIG. 1. Relationship between the coefficient of inbreeding (*f*) and the sperm motility in *Peromyscus leucopus* (n = 59). Overall linear regression (black line) is presented. Gray circles represent DOC mice (linear regression not shown, $P > 0.1$), empty circles represent MK mice (linear regression: $y = 128.45 - 442.05x$; $r = -0.44$, $P = 0.069$), and gray squares represent RAN mice (linear regression: $y = 266.72 - 1225.0x$; $r = -0.57$, $P = 0.009$).

RESULTS

Effects of Captive-Breeding Protocols and Inbreeding on Testes Size and Sperm Production

Compared with RAN or DOC males, MK males were larger and heavier (Table 1). RAN males had significantly larger testes than DOC males, but not significantly larger than MK males (Table 1). Two generalized linear models including testes size and sperm numbers as the response variables, protocol as a categorical predictor, and body weight and age as continuous predictors rendered two significant maximal models (not shown) that in turn rendered two even more significant minimal adequate models after deletion of the only nonsignificant term: age (testes size: $F_{3,56} = 7.15$, $P < 0.001$, $R^2 = 0.28$; sperm numbers: $F_{3,56} = 7.85$, $P < 0.001$, $R^2 = 0.30$). A significant effect of breeding protocol on testes size ($F_{2,56} = 3.87$, $P = 0.026$, $R^2 = 0.10$) and sperm numbers ($F_{2,56} = 3.79$, $P = 0.028$, $R^2 = 0.095$) was found, accounting in both cases for the significant effect of body weight on sperm production variables ($P = 0.001$ in both models). The males from the more inbred group (RAN) had significantly larger testes and more sperm than DOC males, but not significantly more than MK males.

We also tested the within-protocol effect of inbreeding levels, conducting regression models including testes size as the response variable and body size and *f* as the predictors with stepwise backward deletion. In the model conducted for the MK group, but not in the models for the other two groups, inbreeding had a significant positive effect ($F_{1,18} = 5.57$, $P = 0.029$, $R^2 = 0.24$, $\beta \pm SE = 0.49 \pm 0.21$) on testes size. This shows that sperm production is positively associated with inbreeding both between and within groups.

Effect of Captive-Breeding Protocols and Inbreeding on Sperm Quality and Fertility

An overall main multivariate effect of protocol on sperm quality was found (Wilks lambda = 0.65, $F_{10,106} = 2.51$, $P = 0.009$). MK and RAN mice presented higher sperm osmotic resistance than DOC mice ($F_{2,57} = 7.69$, $P = 0.001$). No univariate effects were detected on the other sperm quality traits considered. However, the overall effect of breeding protocol on sperm morphology was also significant (Wilks lambda = 0.59, $F_{12,104} = 2.61$, $P = 0.004$). More specifically, MK and RAN mice had fewer acrosome and midpiece abnormalities than DOC mice ($F_{2,57} = 6.91$, $P = 0.002$ and $F_{2,57} = 3.68$, $P = 0.031$, respectively) (Table 1).

Regarding inbreeding, a significant negative relationship between *f* and osmotic resistance was found within the RAN protocol ($r = -0.59$, $P = 0.006$, $y = 215.9 - 1103x$), which was the protocol with the highest levels of inbreeding (*f* range, 0.145–0.17), but not in MK or DOC protocol (*f* range, 0.095–0.125 and 0.125–0.155, respectively). After pooling males from the three protocols, we tested for an effect of inbreeding by conducting simple regressions for the remaining sperm quality traits. After the removal of a strongly influential outlier (Cook distance, 0.41), a negative relationship was found between *f* and sperm motility ($R^2 = 0.07$, $P = 0.039$) (Fig. 1). This relationship arises from an increased number of animals with low sperm motility at higher levels of inbreeding. The negative effect of *f* on motility was observable within the two protocols with the highest variation in *f* (RAN and MK), but not in the DOC protocol, which had little variation in *f* (Fig. 1).

We then tested whether this inbreeding depression in terms of sperm motility translated into reductions in overall fertility or in first or second litters of RAN or MK protocols. We found an effect of inbreeding on fertility in second litters for the MK protocol, apparently driven by the impact of inbreeding on sperm motility (Fig. 2). The same effect was not observed in the first litters, perhaps because of the lower variance ($F_{69,80} = 1.95$, $P < 0.01$) in and mean ($t_{85} = 4.42$, $P < 0.001$) number of pups born in first litters (first litters, 4.1 ± 1.3; second litters, 5.3 ± 1.7). The genetic load was 48.6 fecundity equivalents, as

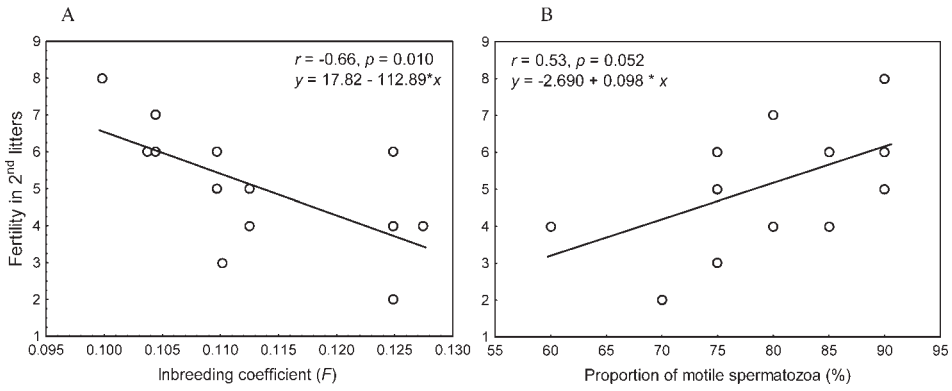

FIG. 2. Linear regressions of fertility on inbreeding levels (**A**) of the sire and sperm motility (**B**) in second litters of the MK group. Only individuals that sired at least one offspring are considered. For both graphs, n = 14; note one and two overlapping data points on **A** and **B**, respectively.

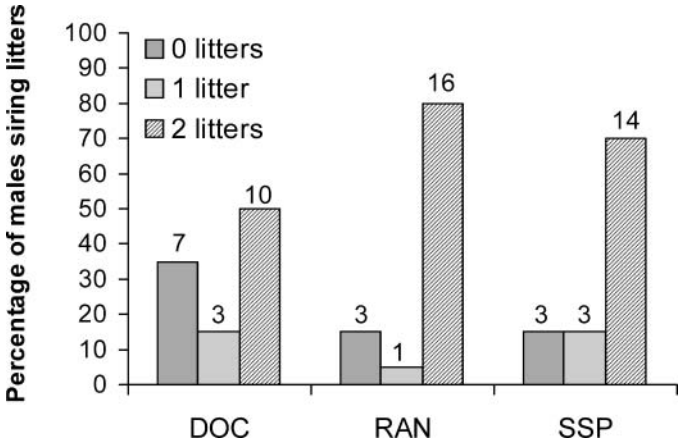

FIG. 3. Differences between breeding protocols in the proportion of males siring zero, one, or two litters after 70 days paired with a female. Numbers indicate total number of males per group (n = 60).

calculated from twice the slope of the regression of fecundity on f for the sire (f_{sire}; $r = -0.63$, $P = 0.017$, $y = 4.311 - 24.295x$). No effect of f for the dam (f_{dam}) on fecundity was detected ($P > 0.1$), although the trend was also negative ($r = -0.45$).

Given the extinction of one of the DOC replicates in generation 8, we tested whether DOC males, after pairing, failed to produce offspring more frequently than RAN or SSP. This was, in fact, the case. RAN and SSP pairings failed to produce offspring less than half the number of times that DOC pairings did (12% vs. 35%, $\chi^2_1 = 4.01$, $P = 0.045$). No differences were found between SSP and RAN mice or between first and second litters ($P > 0.05$). Figure 3 shows a clear trend toward RAN and MK being more productive than DOC, which had a lower proportion of males siring two litters and a higher proportion of males siring zero litters.

Determinants of Male Fertility

Effect of sperm traits on probability of siring litters. Only sperm viability and proportion of normal sperm were found to determine the likelihood of siring offspring. Males siring offspring had significantly higher sperm viability (63% vs. 54%, $F_{1,57} = 5.51$, $P = 0.02$) and percentage normal sperm (43% vs. 27%, $F_{1,57} = 7.02$, $P = 0.01$) than those not siring litters (Fig. 4A). A close inspection showed that the sperm normality variables responsible for this effect were midpiece and acrosome abnormalities (Table 2); note that DOC mice had significantly lower values for three of these variables. We then compared these sperm traits for males siring zero versus males siring two litters. The differences increased even more (results not shown), and another sperm morphology variable (swollen midpiece abnormality) also showed significant differences ($P < 0.001$) between males that sired either zero or two litters. Males that sired zero litters had a mean of 25% normal sperm, whereas those that sired one or two litters had a mean of 45% normal sperm ($F_{2,57} = 3.76$, $P = 0.029$) (Fig. 4C).

Effect of sperm traits on probability of siring one or two litters. After including all sperm traits in the model and protocol, the logistic model showed a significant effect on the likelihood of siring one or two litters only for the proportion of tail abnormalities (log-likelihood = −18.47, $\chi^2_1 = 4.87$, $P = 0.027$). Males siring one litter had 9% ± 2.7% sperm with abnormal tails as compared to 4% ± 0.7% in males that sired two litters (Fig. 4B).

Effect of sperm traits on number of offspring. Failures to breed could have been the result of behavioral or other factors, whereas among pairs that did breed, number of offspring produced might reflect greater effects of sperm quality. Excluding those males with no offspring, an ANOVA showed no significant differences ($F_{2,37} = 0.479$, $P = 0.62$) between protocols in the total number of offspring. Hence, males from the three protocols were pooled to conduct a multiple-regression model including as predictors sperm numbers, five sperm quality traits (% motile, % viable, % normal spermatozoa, %NAR, and osmotic resistance), dam body length, age,

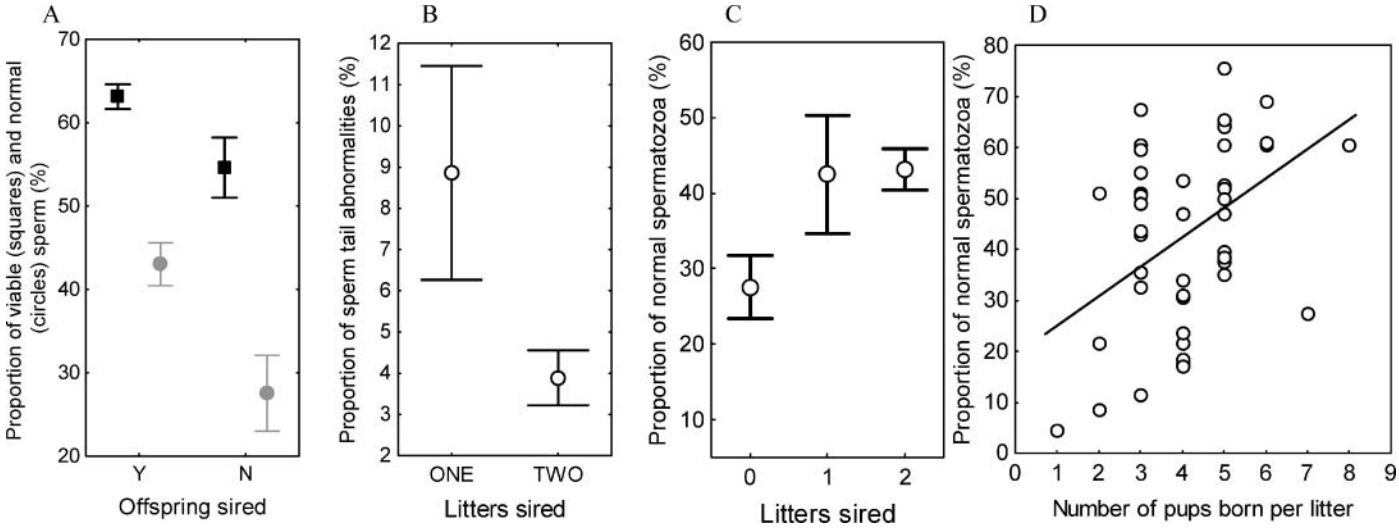

FIG. 4. **A**) Mean differences in the proportion of viable and normal sperm ($F_{1,57} = 5.51$, $P = 0.022$ and $F_{1,57} = 7.02$, $P = 0.01$, respectively) between males that were able (Y) or unable (N) to sire offspring (n = 58). **B**) Mean difference in the proportion of tail abnormalities ($F_{1,45} = 6.12$, $P = 0.017$) between males that sired one or two litters (n = 46). **C** and **D**) Relationship between mean percentage normal spermatozoa and number of litters (**C**) and between mean percentage normal spermatozoa and number of pups born per first litter obtained (**D**; n = 60). Axes are reversed for illustration purposes. Linear-regression equation in **D** shows regression of number of offspring on percentage normal sperm ($y = 2.7501 + 0.0315x$). All analyses were conducted in males after 70 days paired with a female. Error bars indicate 95% SEM.

TABLE 2. Logistic regression models (binomial distribution and logit link function) showing the effects of sperm traits that significantly influenced the probability of siring offspring fitted independently in different models.[a]

Parameter	*df*	Log-likelihood	χ^2	*P*
Sperm numbers	1	−30.6058	7.212	0.00724
Sperm viability (%)	1	−33.2089	12.418	0.00043
Midpiece abnormality	1	−32.0917	10.183	0.00142
Acrosome abnormality	1	−29.1142	4.228	0.03975

[a] Best model including the four predictors (AIC = 49.95; log-likelihood ratio = 19.65; $P = 0.0006$).

and both f_{sire} and f_{dam}. The reduced model ($R^2 = 0.56$, $F_{3,35} = 15.43$, $P < 0.001$) only retained three significant variables and showed that dam size was the most important determinant of total number of offspring ($R^2 = 0.30$, $P < 0.001$), followed by percentage normal spermatozoa ($R^2 = 0.22$, $P < 0.002$) and, lastly, by %NAR ($R^2 = 0.05$, $P < 0.049$). A male having 20% normal sperm would, on average, sire one pup per litter, and a male having around 70% normal sperm would, on average, sire eight pups (Fig. 4D). Another model breaking down sperm morphology into its basic components showed that the morphology variable swollen midpiece entirely accounted for the effect on percentage normal spermatozoa, whereas the other components had nonsignificant negative effects. No effect of sperm numbers or testes size was detected, suggesting that sperm quality can be a more important determinant of fertility than sperm production. A generalized linear model including breeding protocol as a covariate rendered identical results. Two more models were conducted for first and second litters separately and produced similar results (models not shown).

DISCUSSION

To our knowledge, the present study is the first time that the accumulated effects of artificial selection for an aspect of domestication (docility) and inbreeding have been considered jointly. Importantly, in the overall breeding study (Lacy et al., unpublished data), one of the two replicates selected for docility had gone extinct in generation 8 because of ?37 increasingly poor reproductive success. To determine if genetic effects negatively affecting sperm traits were responsible for this extinction, we explored the sperm determinants of fertility, and we compared fertility and sperm traits between the remaining docility replicate and the two other breeding protocols. Genetic drift among and between replicates might have reduced our ability to document the effects of inbreeding or selection; however, we were able to document important differential effects in the breeding protocols. The DOC males performed worse than both RAN males (with the highest accumulated inbreeding levels) and MK males, suggesting that selection for docility has more deleterious effects than inbreeding at the *f* levels considered. This may result from the fact that selection for docility would be expected to have cumulative effects on fitness if an association between the two exists, whereas inbreeding would be expected to show lower association with fitness if inbreeding accumulation is slow enough for purging to operate.

Artificial selection for docility negatively impacted two sperm morphology traits, acrosome and midpiece abnormalities, which together with sperm viability determine the probability of siring litters. This shows that selection for docility can impact fertility through an effect on the proportion of normal sperm, and it suggests a likely pathway through which the docility replicate went extinct in generation 8. We also show that the proportion of tail abnormalities predicts a second component of fertility, the probability of siring a second litter, and that a third component, total number of offspring, is predicted by the proportion of normal sperm. This hierarchical analysis showing specific contributions of sperm quality parameters to different fertility components strongly emphasizes the multivariate nature of male fertility, explaining, in part, why the sperm determinants of fertility have remained so elusive [58]. Interestingly, we found that dam body size explained a considerable amount of the variance in number of offspring, and including this factor in the model improved our ability to quantify male effects more precisely. In agreement ?38 with previous research [45], proportion of normal sperm was the main sperm trait influencing offspring number, stressing the generality of this pattern for mammals. The joint inclusion of sperm production and sperm quality traits in the models allowed us to quantify the effect of each sperm trait independently. Under the assumption that sperm numbers reflect the amount of sperm inseminated, our results suggest that quality is more important than quantity in noncompetitive scenarios.

On top of the increased acrosome and midpiece abnormalities, DOC males also had lower sperm production than RAN males, suggesting that artificial selection for docility can have a stronger negative impact than inbreeding on testes size and sperm production. One possibility is that selection for docility indirectly selects for low testosterone levels [59] and, hence, for smaller testes size [60]. Other effects in the DOC protocol ?39 further suggest this possibility. Indeed, the decreased resistance to osmotic stress detected in DOC males could be the result of alterations in spermatogenesis or sperm maturation in the epididymides that reduce the ability of the sperm cell to regulate volume changes [61]. Relatively high androgen levels of dihydrotestosterone (synthesized from testosterone) are known to play a major role in sperm maturation [62–64], which in turn influences osmotic resistance [65]. So, reduced dihydrotestosterone at the site of sperm maturation could have altered osmotic resistance in the DOC males and, potentially, have an impact on fertility [66]. Our undetected effect of protocol on sperm viability and detected effect on sperm morphology also fit this explanation, because spermatozoa remain viable in a low androgen environment [62] whereas sperm morphology parameters tend to be impacted by low androgen levels [63].

Could behavioral selection for docility, however, have indirectly selected for lower testosterone levels? Several lines of evidence suggest this could have been the case [67]. First, testosterone is known to affect body size [68, 69] and testes size [60], and DOC mice suffered a reduction in both body and testes size. Second, testosterone is positively associated with mobility [70] and physical activity [71], which are proxies for the flipping behavior selected against in the DOC protocol. This testosterone-driven effect would also explain the lack of differences between RAN and MK males, none of which are expected to suffer a reduction in testosterone levels.

Selection for docility has negative effects on sperm quality traits that impact the probability of siring litters. This should cause us to avoid in conservation breeding programs not only intentional selection for behavioral traits that we think will improve performance in captivity but also unintentional favoring of more docile animals.

Insights regarding the effects of inbreeding were gained by comparing sperm and fertility traits between protocols with different accumulated inbreeding levels and by analyzing the effect of increasing *f* levels on sperm parameters and fertility within groups. Although the DOC protocol did perform more poorly than the other two protocols, no differences were found

in the average levels of sperm quality or fertility reached by MK and RAN males.

The negative effect of inbreeding of sperm motility suggests that nuclear genes are predominantly determining this sperm trait, because we would not expect to detect inbreeding depression in terms of traits encoded by the mitochondrial genome, which is haploid and, therefore, unaffected by inbreeding. This agrees with previous research showing nuclear encoded structures as the main determinants of motility [72]. However, the negative effect of inbreeding was driven by an increased variation in motility with increasing inbreeding, showing that males with higher levels of inbreeding have an increased probability of presenting low sperm motility. Within protocols, this pattern was also followed by RAN and MK mice. This decrease in sperm motility translated into decreased fertility in MK second litters. Surprisingly a 0.03 change in f translated into a 30% reduction in sperm motility (60–90%) and resulted in a second-litter size difference of three offspring. The reported genetic load of 48 fecundity equivalents for MK suggests a high number of genes affecting sperm quality, with even relatively small differences in f (and, thus, differences at a small percentage of loci) resulting in insignificant effects. Again, this would not be a surprise considering that fertility is a multidimensional trait determined by multiple sperm variables, all of which are targets of mutation. It also suggests a larger number of deleterious recessive alleles. Our inability to detect a significant effect on fecundity in the MK protocol, but not in the DOC and RAN protocols, may have been the result of deleterious alleles not yet having been purged from the MK lines (i.e., the more outbred populations with high levels of heterozygosity as opposed to the populations in the other protocols).

Because we found a similar but not significant trend in first litters, one possible explanation is that inbreeding depression was not detected because of a lack of statistical power. Another, more plausible explanation, however, is that inbreeding load increases with age [73], enhancing the chances of detecting inbreeding depression in second litters. This agrees with the age-specific inbreeding depression in terms of male mating success previously shown for *Drosophila* sp. [74]. The lack of an effect of f_{dam} on fecundity suggests a lower genetic load for fecundity in females than in males, and it supports previous research in a butterfly that also showed male-biased inbreeding depression [55]. As suggested by those authors, this pattern could be explained by the fact that fertility selection per sperm is expected to be weaker than per egg.

The fact that we have uncovered, at the low levels of f considered in the present study, inbreeding depression only in terms of sperm motility is interesting. Sperm motility is expected to be affected by sperm and seminal traits more than the other variables considered here and, hence, to be potentially determined by a higher number of genes (from sperm morphology, biochemistry, or energetics to seminal parameters). In light of these results, we propose that sperm motility suffers higher mutational load than other sperm traits. This should be expected in that if a low inbreeding level only slightly affects individual sperm traits, the possibility of detecting that effect would less than the possibility for a variable that is cumulatively affected by multiple such traits.

Lastly, the positive relationship between inbreeding and sperm production found between protocols (i.e., the RAN protocol presenting high sperm production) and within protocols could be the result of a compensatory effect between sperm quality and production, as already proposed to explain increased sperm output in individuals with increased abnormal sperm [33, 75]. Previous research in oldfield mice (*Peromyscus polionotus*) showed the same pattern, with a transient increase in testes size for low levels of f that is eventually reversed at the higher inbreeding levels [32]. However, we failed to detect a negative correlation between proportion of normal sperm and absolute/relative testes size (results not shown) at the f levels considered, so this question deserves further attention.

Although not as deleterious for reproductive fitness as docility selection, inbreeding also has a negative effect. Sperm quality and fertility decreased with increasing inbreeding in the most outbred protocol after just 10 generations of a captive-breeding program. Several reasons could explain why the effects reported here for low inbreeding levels likely are an underestimate of the negative effects of inbreeding on the main determinants of fertility. First, captivity masks inbreeding effects on male mating success [76], probably because of the absence of male-male competition for mating, which magnifies inbreeding depression [77]. In the present study, males were given 70 days to mate with the female. Second, in captive-breeding programs of vulnerable or endangered species, the founders might already be inbred at a level higher than that tested in the present study and, thus, be vulnerable to more detrimental effects. Lastly, random breeding likely is not being achieved, because logistic constraints in translocating breeding individuals among zoos can generate higher inbreeding levels than expected with more complete mixing.

Overall, our results have shown that 1) different fertility components are affected by different sperm traits; 2) the DOC protocol has the highest negative impact on fertility, and the driver for the extinction of one line of this protocol in generation 8 is likely to have been decreased sperm quality; 3) this effect is mainly mediated by sperm morphology traits impacting the probability of siring litters; 4) use of the RAN protocol versus use of the MK protocol makes no difference in the average reproductive fitness after 10 generations; 5) negative effects of inbreeding are found within protocols; and 6) inbreeding translates into decreased fecundity, at least within the MK protocol, by reducing the size of second litters.

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