

# Inbreeding depression in ring-tailed lemurs (*Lemur catta*): genetic diversity predicts parasitism, immunocompetence, and survivorship

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**Abstract** The consequences of inbreeding have been well studied in a variety of taxa, revealing that inbreeding has major negative impacts in numerous species, both in captivity and in the wild; however, as trans-generational health data are difficult to obtain for long-lived, free-ranging species, similar analyses are generally lacking for nonhuman primates. Here, we examined the long-term effects of inbreeding on numerous health estimates in a captive colony of ring-tailed lemurs (*Lemur catta*), housed under semi-natural conditions. This vulnerable strepsirrhine primate is endemic to Madagascar, a threatened hotspot of biodiversity; consequently, this captive population represents an important surrogate. Despite significant attention to maintaining the genetic diversity of captive animals, breeding colonies invariably suffer from various degrees of inbreeding. We used neutral heterozygosity as an estimate of inbreeding and showed that our results reflect genome-

wide inbreeding, rather than local genetic effects. In particular, we found that genetic diversity affects several fitness correlates, including the prevalence and burden of *Cuterebra* parasites and a third ( $N = 6$ ) of the blood parameters analyzed, some of which reflect immunocompetence. As a final validation of inbreeding depression in this captive colony, we showed that, compared to outbred individuals, inbred lemurs were more likely to die earlier from diseases. Through these analyses, we highlight the importance of monitoring genetic variation in captive animals—a key objective for conservation geneticists—and provide insight into the potential negative consequences faced by small or isolated populations in the wild.

**Keywords** Heterozygosity · Inbreeding depression · Strepsirrhine primate · Parasitism · Blood parameters · Captive breeding

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## Introduction

Maintaining genetic diversity is a key concern of conservation biologists (Frankham 1995, 2003), as genetic diversity is required for populations to adapt to environmental changes. At the individual level, loss of genetic diversity is linked to fitness reduction, most likely through inbreeding. Mating between related individuals leads to inbreeding depression in virtually all species studied thus far, both in captivity and in the wild (Wright 1977; Charlesworth and Charlesworth 1987; Frankham 1995; Lynch and Walsh 1998; Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Keller and Waller 2002). Inbreeding depression reflects the deleterious consequences of increased homozygosity at loci that affect fitness (Charlesworth and Charlesworth 1999), either by

permitting the expression of recessive deleterious alleles or by reducing heterozygote advantage (Charlesworth and Charlesworth 1987). Whereas the causes and consequences of inbreeding have been well studied in a variety of taxa, similar analyses are generally lacking for nonhuman primates (however see Charpentier et al. 2007). Therefore, in this study, we analyze the effects of inbreeding on the health and longevity of a captive population of ring-tailed lemurs (*Lemur catta*), housed under semi-natural conditions.

The study of inbreeding depression requires both the use of genetic analyses, necessary to detect inbred individuals, and extensive data on life-history traits. Because inbreeding reduces heterozygosity (Hartl and Clark 1997), neutral heterozygosity calculated from microsatellite data is often used as an estimate of inbreeding. Numerous examples of correlations between heterozygosity and fitness are available in the literature (see the meta-analysis in Reed and Frankham 2003). The lack of comparable data in primates, however, owes to two main factors. First, primates are characterized by long generation times, limiting the feasibility of multigenerational studies. Second, long-term life-history data are often difficult to retrieve from wild populations. These constraints are especially limiting for species that live in closed environments, such as tropical forests, which represent the habitat of about 90% of primate species (Mittermeier and Cheney 1987). Long-term data on captive or semi-free-ranging populations therefore greatly facilitate addressing questions about inbreeding effects in primates.

Ring-tailed lemurs are vulnerable strepsirrhine primates endemic to Madagascar. From a conservation perspective, they are a prime example of a population in peril, owing primarily to habitat loss, through deforestation and human encroachment, and secondarily to hunting (Green and Sussman 1990; Du Puy and Moat 1998; Lehman and Wright 2000; Goodman and Raselimanana 2003; Lehman 2006). From an ecological perspective, they represent a fragmented population that could become increasingly vulnerable through loss of genetic diversity and decreased gene flow (Frankham 1995, 2003). Despite significant attention devoted to maintaining the genetic diversity of captive primates, breeding colonies invariably suffer from various degrees of inbreeding (for review on captive primates, see Lacy et al. 1993). Studies of inbreeding depression in strepsirrhine primates are limited to that of Noble et al. (1990). These researchers showed that the percent survivorship per litter was negatively correlated to the offspring's inbreeding coefficient in black-and-white ruffed lemurs (*Varecia variegata variegata*), but not in red-ruffed lemurs (*V. v. rubra*), highlighting different tolerance to inbreeding across species, maybe owing to various breeding histories (Noble et al. 1990). For instance, the

red-ruffed lemurs could have purged their genetic load by repeated consanguineous mating such that inbreeding depression could no longer be detectable (however, see Keller and Waller (2002) for a discussion on the purge of deleterious alleles).

We conducted our study on a colony of ring-tailed lemurs established at Duke University in 1966. Detailed life history and health records of these animals have been maintained since the inception of the colony. This population therefore presents a unique opportunity to examine the consequences of inbreeding depression on a large panel of health parameters. We investigate several fitness traits or correlates of the lemurs, including the prevalence, burden, and richness of their parasites, as well as numerous blood parameters, weight, and survival of the animals. Because inbreeding affects the whole genome, we expected several of these parameters to correlate with genetic diversity in this population.

## Methods

### Study population

The subjects of this study were 73 ring-tailed lemurs (37 females, 36 males), including living and deceased individuals, born between 1971 and 2006. All were members of the captive colony housed at the Duke Lemur Center (DLC) in Durham, North Carolina. Life-history and health records (see below) have been maintained for the many DLC animals since the establishment of the colony in 1966; however, a detailed monitoring system for the medical records database (MedARKs, Medical Record Keeping system, software; ISIS) was implemented in 1994. Our study on health therefore includes only those 64 individuals that were closely monitored from 1 January 1994 to 30 November 2006; however, for the survival analysis (see below), we use dates of birth and death from all 73 subjects during the entire study period (1971–2006). These animals ranged in age from 0.19 to 20.01 years, calculated as age at death or at the end of the study. Based on endocrine correlates and age at first conception or sirring, we consider captive animals to be reproductively mature at about 2 years (Drea 2007), although in the wild, males and females typically mate for the first time at about 2.5–3 years of age (Sussman 1991). For simplicity, we refer to all animals under the age of 2 years as juveniles. For some of the animals, data were available only during the juvenile period (29.7%), only during adulthood (29.7%), or during both life stages (40.6%).

Most of the subjects were housed socially in semi-free-ranging groups that occupied forested enclosures

(ranging in size from 1.5 to 10.9 ha), each with access to indoor, heated rooms or thermostatically controlled nest boxes. Beginning in 1996, the social groups were kept in their heated indoor areas (110–246 feet<sup>2</sup>) during inclement weather (less than 40°F). Typically, these indoor areas are connected to an outdoor run (278–347 feet<sup>2</sup>) enclosed by chain-link fencing and containing branch supports. Other individuals were routinely housed with fewer conspecifics and in smaller enclosures, comparable in size to the outdoor runs. These enclosures are delineated by a combination of solid walls and chain-link fencing, and are covered and heated during winter months; otherwise, like free-ranging animals, these subjects are exposed to the elements. Throughout the period of study, a given subject may have experienced different social housing conditions. For the analyses (see below), we defined three classes of housing experiences, including: (1) most of the animal's lifespan spent as part of a semi-free-ranging social group (hereafter 'free-range' condition); (2) most of the time spent in a small enclosure with few or even no conspecifics (hereafter 'enclosure' condition); and (3) approximately equal time spent free-ranging and in enclosures (hereafter 'mixed' condition).

The subjects were fed daily rations of a commercially available primate diet (Purina® Monkey Diet 5038, PMI Nutrition International, Inc., Brentwood, MO 63144, USA), supplemented with fresh fruits and vegetables. When the animals were free ranging, they additionally complemented their diet with food foraged from the forest. Water was always freely available. The animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

#### Life-history variables and weight measurements

Ring-tailed lemurs are strictly seasonal breeders that show some degree of estrus synchrony (Jolly 1966). As they are polyestrous, cycling up to three times per breeding season (Evans and Goy 1968; Drea 2007), their birthing season shows roughly three peaks. Ring-tailed lemurs in nature typically give birth to singletons, but twinning is relatively common, particularly in captivity. Life history records therefore include date of birth, litter size (i.e., singleton or twin), and age at death.

In addition to life history variables, birth weight (recorded when the individual was not older than 4 days) and repeated weight measurements were available for most of the subjects. These included 954 weight measurements for 34 of the female subjects and 740 weight measurements for 30 of the male subjects.

#### Health records

Health data were recorded on a routine basis (i.e., as part of annual physicals), when illnesses were suspected, or whenever animals were captured or immobilized for research, and included the following parasitological examinations and blood analyses.

#### Parasites

First, the animals were checked seasonally during the summer and fall months for warbles, i.e., the larvae of the fly *Cuterebra* sp., and records were taken on warble prevalence and burden. Second, fecal examinations were conducted and involved both direct microscopic and flotation methods, although the latter predominated. Fecal examinations revealed the presence of the following gastrointestinal parasites: trichomonas, strongyles, strongyloides, trichuris, enterobius, and entamoeba. Species were not specified for the different parasite types. Strongyles and strongyloides were the two types of parasites most often detected. Six classes of burden were defined by the veterinary staff, representing incremental ranges of parasites counted. For instance, burden level 1 included 1–10 parasites, burden level 2 included 11–20 parasites, and so on, until burden level 6, which represented more than 50 parasites.

#### Blood parameters

Blood analyses provided information on over 60 parameters, of which we identified 18, *a priori*, that might be relevant to a study of inbreeding depression. The 60 parameters were part of a comprehensive complete blood count (CBC) and serum chemistry panel and the 18 parameters studied were selected because they provided a general screen of major organ and immune system function. These 18 parameters are listed in Table A (supplementary material).

#### Genetic analyses

Blood or tissue samples were available for all 73 subjects. We performed DNA extractions from whole blood, buffy coat, or tissue using a DNA miniprep kit (Sigma) and genotyped the subjects at 10–15 microsatellite loci developed for *L. catta* (6 loci: Pastorini et al. 2005) or bamboo lemur (*Hapalemur griseus*, 9 loci: Sommer et al. 2002). One locus was tetra-nucleotidic (45HDZ052) and all 14 others were di-nucleotidic. Two supplementary loci were

tested (Sommer et al. 2002), but not included in our analysis because they were monomorphic.

The polymerase chain reaction (PCR) mixture followed Pastorini et al. (2005). Briefly, we amplified approximately 10–100 ng template DNA in 20  $\mu$ l reactions, using 0.06 M Tris, 0.015 M  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 mM  $\text{MgCl}_2$ , 0.78 M DMSO, 0.025 mM each dNTP, 1 mM each primer, and 0.5 U Ampli-Taq Gold DNA polymerase (Roche). PCRs were carried out on an Eppendorf Mastercycler. We amplified samples for 35 cycles, with denaturing at 95°C for 30 s, primer annealing at 49–55°C for 60 s, and extension at 72°C for 60 s, followed by a final extension for 5 min at 72°C. We changed the annealing temperature, as necessary, to optimize the PCR conditions for individual loci (Table B, supplementary material). We analyzed all microsatellites using an Applied Biosystems 3730xl DNA analyzer and GeneMarker software (version 1.4) to identify the different alleles and determine their size. The test for deviation from Hardy-Weinberg (HW) expectations revealed that one locus was more heterozygous than expected (CERVUS version 2.0; Marshall et al. 1998; Slate et al. 2000; Table B, supplementary material), often the case in small populations (Balloux 2004). One other locus, however, showed a strong deficit in heterozygosity (Lc 10; Table B, supplementary material), suggesting the presence of null alleles. We therefore excluded this locus from the calculation of mean heterozygosity (see below).

#### Evaluating genetic diversity and the contribution of each genetic locus

Because we could not obtain a complete pedigree of the lemur population, we first used mean heterozygosity ( $H_o$ ) per individual as an estimate of inbreeding. We calculated  $H_o$  as the number of heterozygous loci divided by the number of genotyped loci (from 9 to 14; mean loci typed per individual  $\pm$  s.e.m =  $13.85 \pm 0.08$ ). We repeated these analyses using internal relatedness as a second estimate of neutral heterozygosity (Amos et al. 2001); however, because we obtained consistent results for both estimates, we present data only on  $H_o$ .

$H_o$  will affect phenotypes either because it reflects genome-wide inbreeding or because one or more microsatellite loci are physically linked to functional loci. In the former case, the correlation between  $H_o$  and fitness should be equivalent across all neutral microsatellite markers. In the latter case, the relationship between  $H_o$  and fitness will depend mainly on  $H_o$  at a single locus (or at a few loci). To investigate whether there were locus-specific effects on fitness, we examined the correlation between  $H_o$  in two data sets, each containing half of the microsatellites selected randomly from the 14 loci analyzed. We repeated

this analysis 100 times using SPLUS 2000. A significant “Ho–Ho correlation” indicates that our estimate of genetic diversity reflects inbreeding; the higher the correlation, the more precisely  $H_o$  reflects inbreeding in the population (Balloux et al. 2004; Slate et al. 2004).

Lastly, to examine if the mean genetic diversity in the population changed over time, we divided our period of study into decades and calculated a mean  $H_o$  for infants born in those decades. We plotted these means against time.

#### Statistical analyses and co-variables considered

We used generalized linear models (SAS version 9, GENMOD or GLM procedure) for all the analyses, except those involving survival. The error structure and link function were defined for each model according to the residual distribution of the response variable and following use of exploratory models (see below). Table A (supplementary material) summarizes the models used, along with the co-variables considered. Although we present all significant findings, we focus our attention on the effects of heterozygosity on fitness.

For our GLM analyses, we used mean values per individual. We analyzed both juveniles and adults separately. In all the following analyses, we considered the sex and housing condition (free-range, enclosure, and mixed) of the subjects. Although ring-tailed lemurs are unequivocally female dominant (Jolly 1966), social status within the sexes can be nonlinear, non-transitive, and highly variable, particularly among males (Pereira 1995). Therefore, we did not consider dominance rank as a supplementary variable. In the following sections, we outline the specific analyses used for each data set.

#### Parasites

As an estimate of *Cuterebra* prevalence, we used the number of times larvae were found in a given time period (offset, see below). As an estimate of *Cuterebra* burden, we used the total number of larvae found per infected individual. In both cases, because the dependent variable was a count with a variance superior to the mean, we used a negative binomial distribution of the residuals (GENMOD procedure) to model the relationship between  $H_o$  and parasitism. Because the animals were not observed for the same amount of time, we included the total duration of observations per individual (from start to end date of this study) as an offset in the regressions (summarized in Table A, supplementary material).

We analyzed the gastrointestinal parasitic species richness present in feces using both examination methods (direct and flotation). In addition to the co-variables described above, we included the total number of fecal examinations performed per individual. Because the dependent variable was a count with a variance equal or close to the mean, we used a Poisson distribution of the residuals (GENMOD procedure) to model the relationship between richness in species of gastrointestinal parasites and genetic diversity.

We further examined the prevalence of the two most common species of gastrointestinal parasites, in relation to  $H_o$ , using a binomial distribution of the data (GENMOD procedure). We used the number of responses,  $N$ , as the number of positive parasitological exams in which either strongyles or strongyloides had been detected.  $N$  was modeled as a binomial random variable for each combination of explanatory variables. For this analysis, the binomial parameter for the number of trials was the total number of parasitological exams performed per individual, and the binomial probability was the probability of a response.

Lastly, only strongyloides occurred with sufficient frequency to allow an examination of burden in relation to  $H_o$ . This measure involved a dependent variable occurring in six classes, so we could not use a mean per individual; instead, we used repeated measures. As each data point corresponded to a given date, in addition to the co-variables described above, we included both the exact age of the individual and the season in which the data had been collected. For the latter, we differentiated winter months (October–March) from summer months (April–September). We used repeated measures (with the individual's identity as a repeated factor), with a multinomial distribution of the residuals (GENMOD procedure) to model potential effects of  $H_o$  on strongyloides burden.

#### *Blood parameters*

Blood analyses had been performed on 17 juveniles only, limiting to adults our investigation of the relation between  $H_o$  and the mean value of each of the 18 blood parameters. For these analyses, we removed extreme values to avoid overweighting outliers. These extreme values were selected according to the distribution of the data. When the residuals were normally distributed, as was typically the case, we used a Gaussian distribution of the residuals (GLM procedure). When they were not normally distributed, as was the case for alkaline phosphatase, eosinophil count, and platelet count, we applied a log-transformation of the explanatory variable (Table A, supplementary material). The residuals for aspartate aminotransferase and monocyte

counts were still not normally distributed after various transformations, so we divided the given variable to explain into three classes (high, medium, and low levels) to conduct a multinomial analysis (Table A, supplementary material).

#### *Weight*

In a first analysis of the relation between weight and  $H_o$ , we treated the repeated measurements as a mixed longitudinal and cross-sectional sample. For each sex and following Moses et al. (1992), we used locally weighted least squares regression (SAS version 9, LOESS procedure,  $f = 0.5$ , 10 iterations) to fit curves to the data. This method produces an estimated average value for each age without assuming an underlying form for the curve. We computed residuals for each data point contributing to the curves of body mass as the natural logarithm of the ratio of the observed value to the average value given by the lowest curve for that age (Moses et al. 1992). Because residuals were normally distributed, we examined the mean of an individual's residuals by considering a Gaussian distribution.

A second analysis of weight and  $H_o$  involved birth weight. Because the residuals were normally distributed, we used a Gaussian distribution. In addition to the explanatory variables described above, we included infant age (in days 0–4), birth month, litter size (singleton vs. twin), and mean weight of the mother (as calculated above).

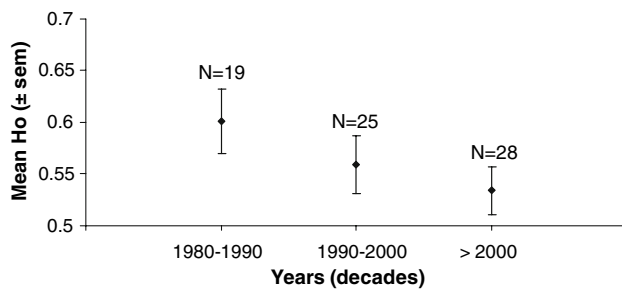
#### *Survival*

To account for the bias introduced by animals that were alive at the end of the data collection period, we used Kaplan–Meier survival analyses to examine the age at death. We used Wilcoxon tests to examine the effect of  $H_o$  on survival distribution. We treated sex as a stratum (SAS version 9, LIFETEST procedure). We verified that any effect was also independent of housing condition.

## **Results**

### *Heterozygosity*

For our study population,  $H_o$  ranged from 0.21 to 0.86 (mean  $\pm$  sem:  $0.559 \pm 0.016$ ). We obtained a significant correlation between the two microsatellite data sets in 31% of simulated cases. The number of significant  $P$ -values exceeded the 0.05 level (binomial test:  $P < 0.001$ ). The



**Fig. 1** Mean Ho of newborn *L. catta* at the DLC, separated by decade of birth

average correlation coefficient over 100 simulations was  $r = 0.17$ . Although this correlation coefficient is modest, this result indicates that our 14 markers are representative of genome-wide heterozygosity. Within this *L. catta* population, mating between relatives (such as parent and offspring) was known to occur; however, such close inbreeding was also counterbalanced by the introduction of unrelated individuals (from other facilities) that contributed to the ‘renewal’ of the genetic pool. Consequently, this population represents reasonable variation in Ho, ranging from quite inbred to outbred individuals. Over time, however, we observed a marginally significant trend that reflected a decrease in the mean Ho of new-born individuals (Spearman correlation,  $N = 72$ ;  $r = -0.22$ ;  $P = 0.06$ ; for this analysis, we removed one individual born before 1980; Fig. 1), suggesting the gradual erosion of genetic diversity.

As suggested by Balloux et al. (2004), under the present conditions involving a range of highly inbred to outbred individuals, Ho could be a powerful enough predictor of inbreeding to detect inbreeding depression. Nevertheless, as further validation of this measure, whenever we detected an effect of Ho, we repeated the analyses, dropping one locus at a time from the calculation of genetic diversity (see also

Hoffman et al. 2004). In most cases (88.5%), the effects remained significant (data not shown). The few times the effect disappeared ( $P > 0.10$ ), the direction of the effect nevertheless remained constant, suggesting a decrease in the power of calculating Ho (i.e., the fewer loci, the worse the estimation of inbreeding through neutral heterozygosity). Given that these results remained qualitatively consistent with our prior results, despite a potential decrease in statistical power, we conclude that our measure of genetic diversity is a good estimate of genome-wide inbreeding.

## Parasitological examinations

### *Cuterebra* larvae

We found that the prevalence of parasites in ring-tailed lemurs was influenced by Ho, but that the direction of the effect depended on the age class analyzed. In adults, as anticipated, we found that more homozygous individuals were more susceptible to infection by warbles (Table 1). Moreover, once infected, the parasite burden was greater in more homozygous adults than in more heterozygous adults (Table 1). By contrast, and rather curiously, more homozygous juveniles were less susceptible to warbles than were their more heterozygous peers (Table 1). Similarly, more homozygous juveniles also tended to be less heavily infected, but this finding was only marginally significant (Table 1). No other variable was significantly related to either prevalence or burden of *Cuterebra* parasites in adults or juveniles.

### Gastrointestinal parasites

The richness of parasites, as determined by either direct or flotation methods, was not correlated with Ho, either in

**Table 1** Relationships between parasitism and heterozygosity

	Age class	Sample size	Estimate	$\chi^2$	$P$
<i>Cuterebra</i>					
Prevalence	Adults	45	-21.52	$\chi^2_1 = 11.40$	0.0007
	Juveniles	41	3.07	$\chi^2_1 = 5.68$	0.017
Burden	Adults	30	-19.42	$\chi^2_1 = 11.72$	0.0006
	Juveniles	28	2.30	$\chi^2_1 = 3.46$	0.063
<i>Gastrointestinal parasites</i>					
Richness	Adults	40	-0.19	$\chi^2_1 = 0.03$	0.86
	Juveniles	28	-1.04	$\chi^2_1 = 0.37$	0.55
Prevalence (strongyles)	Adults	40	-0.16	$\chi^2_1 = 0.01$	0.91
	Juveniles	27	5.92	$\chi^2_1 = 3.99$	0.046
Prevalence (strongyloides)	Adults	40	0.22	$\chi^2_1 = 0.07$	0.80
	Juveniles	27	2.71	$\chi^2_1 = 1.75$	0.19
Burden (strongyloides)	All	170 (39)	0.63	$\chi^2_1 = 0.22$	0.64

The direction of the effect is given by the sign of the estimate (+: positive correlation; -: negative correlation)

juveniles or in adults (Table 1); however, the total number of fecal examinations significantly predicted parasitic richness in both adults ( $\chi^2 = 5.78, P = 0.016$ ) and juveniles ( $\chi^2 = 9.95, P = 0.002$ ), i.e., the more examinations performed, the more species detected. Similarly, the prevalence of strongyles or strongyloides, as revealed by the flotation method alone, was not affected by Ho in adults. Nevertheless, we observed another unexpected, positive correlation between Ho and the prevalence of strongyles in juveniles (Table 1). Similar to the effects we found for *Cuterebra* larvae, more homozygous juveniles were less susceptible than were their heterozygous peers to certain gastrointestinal parasites. Additionally, we found an effect of sex on parasitism in juveniles, with the prevalence of strongyles being greater in males than in females ( $\chi^2 = 4.17, P = 0.04$ ). Lastly, we did not detect any effect of Ho on strongyloides burden in any lemurs; nevertheless, we found a negative relationship between age and strongyloides burden, in that younger animals were more heavily infected with strongyloides than were older animals ( $\chi^2 = 6.95, P = 0.008$ ).

Blood parameters

Table 2 summarizes the effects of Ho or interactions between Ho and sex on the 18 blood parameters we analyzed. Of these 18 parameters, six correlated with individual heterozygosity. First, we found that the interaction between Ho and the sex of the individual influenced

the amount of blood urea nitrogen (BUN); however, when we ran separate analyses for each sex, we found no effect. Presumably, the two sexes show divergent patterns, but the relationship between BUN and heterozygosity within either sex was too slight to be detected independently. Second, we found that both blood albumin and total protein occurred in higher concentrations in more homozygous individuals than in more heterozygous individuals (Table 2). The two major proteins in the blood are globulin, for which we did not find an effect independently, and albumin. Consequently, the effect of Ho on total proteins may principally reflect an effect on albumin. Lastly, we observed an increase in neutrophil count and a decrease in lymphocyte count in more homozygous individuals. These combined results were expected because neutrophil and lymphocyte counts were highly correlated ( $r = 0.94$ ); however, the ratio of neutrophils to lymphocytes increased in the more homozygous individuals (Table 2). We found no relationship between Ho and any of the remaining 12 parameters.

Of the 18 parameters analyzed, we further found a relationship between the sex of the individual and its blood values of aspartate aminotransferase ( $\chi^2 = 4.63, P = 0.032$ ), eosinophil count ( $F_{1,31} = 13.24, P = 0.001$ ), and hematocrit ( $F_{1,35} = 5.63, P = 0.023$ ): females showed higher values of aspartate and eosinophil count than did males, but the reverse was observed for hematocrit values. Lastly, the housing condition of animals was related to both their neutrophil ( $F_{1,35} = 4.66, P = 0.017$ ) and lymphocyte ( $F_{1,35} = 5.01, P = 0.013$ ) counts, as well as to

**Table 2** Relationships between blood parameters and heterozygosity

Blood parameters	Sample size	Variable	$F/\chi^2$	$P$	Direction
Blood urea nitrogen (BUN)	37	Ho * sex	$F_{1,35} = 5.17$	0.030	Divergent effect between sexes
Albumin	37	Ho	$F_{1,35} = 5.00$	0.032	More homozygous have higher albumin
Total protein	37	Ho	$F_{1,35} = 4.90$	0.034	More homozygous have higher total protein
Segmented neutrophils	37	Ho	$F_{1,35} = 4.34$	0.045	More homozygous have more neutrophils
Lymphocytes	37	Ho	$F_{1,35} = 4.23$	0.048	More homozygous have fewer lymphocytes
Ratio Seg. Neutro/Lympho	37	Ho	$F_{1,35} = 4.90$	0.034	More homozygous have increased ratio
Eosinophils	33	Ho	$F_{1,31} = 0.18$	0.67	No effects throughout
Monocytes	37	Ho	$\chi^2_1 = 0.02$	0.88	
White blood cells	37	Ho	$F_{1,35} = 1.31$	0.26	
Globulin	37	Ho	$F_{1,35} = 0.08$	0.78	
Hematocrit	37	Ho	$F_{1,35} = 0.02$	0.89	
Platelet count	33	Ho	$F_{1,31} = 0.32$	0.58	
Creatinine	37	Ho	$F_{1,35} = 0.25$	0.62	
Alanine aminostransferase	37	Ho	$F_{1,35} = 0.30$	0.59	
Aspartate aminostransferase	36	Ho	$\chi^2_1 = 2.58$	0.11	
Alkaline phosphatase	37	Ho	$F_{1,35} = 0.26$	0.62	
Bilirubine	37	Ho	$F_{1,35} = 1.25$	0.27	
Gamma glutamyltransferase	36	Ho	$F_{1,34} = 0.10$	0.76	

their neutrophil-to-lymphocyte ratio ( $F_{1,35} = 4.33$ ,  $P = 0.021$ ): compared to animals in the free-range condition, animals in enclosures showed decreased neutrophils, elevated lymphocytes, and a decreased ratio of neutrophils to lymphocytes.

### Weight

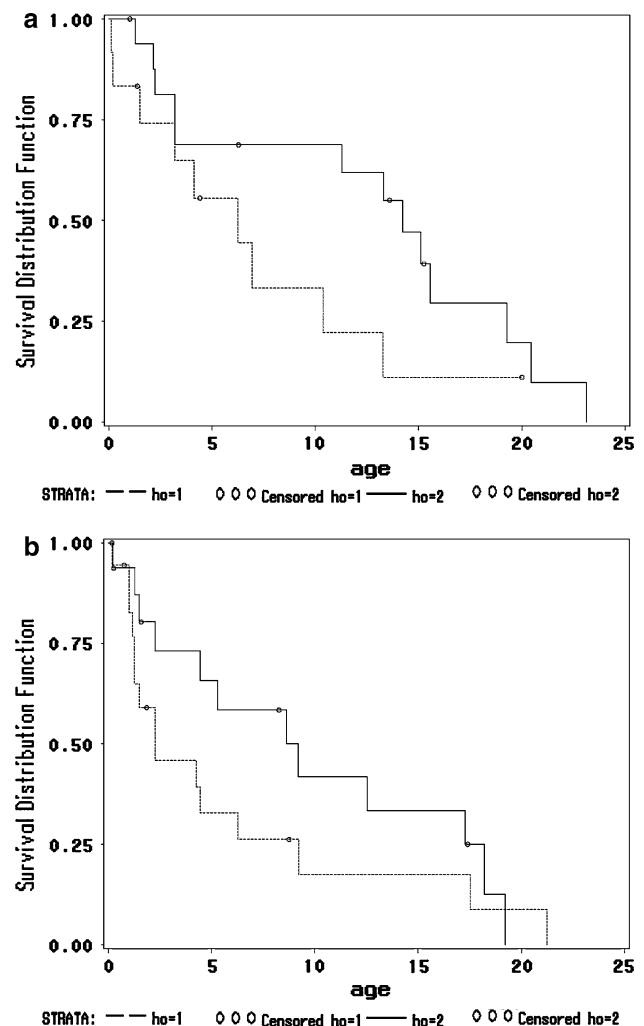
We did not detect any effect of Ho on either mean weight ( $N = 64$ ,  $F_{1,62} = 1.21$ ,  $P = 0.28$ ) or weight at birth ( $N = 24$ ,  $F_{1,22} = 0.65$ ,  $P = 0.43$ ). Unsurprisingly, an infant's birth weight was positively correlated with the number of days (until 4) that had elapsed since birth ( $F_{1,22} = 6.69$ ,  $P = 0.018$ ), as well as with its mother's weight ( $F_{1,22} = 9.01$ ,  $P = 0.007$ ).

### Survival

When we considered all individuals in this study, including those that died from various causes (including disease, falls, or bite wounds), we found a marginal effect of Ho on the probability of survival ( $N = 73$ ,  $\chi^2_1 = 3.13$ ,  $P = 0.077$ ), suggesting that lower genetic diversity tended to decrease the likelihood of survival. When we excluded all deaths from falls or bite wounds and considered only individuals that died from diseases, this trend was confirmed: more homozygous lemurs were significantly less likely to survive compared to more heterozygous lemurs ( $N = 64$ ,  $\chi^2_1 = 6.31$ ,  $P = 0.012$ ). This effect was consistent across the sexes (Fig. 2).

### Discussion

The captive population of ring-tailed lemurs examined in the present study showed significant variation in their genetic diversity, as determined by mean heterozygosity, ranging from individuals that were highly inbred to individuals that were quite outbred. This population therefore proved to be ideal for examining the relationship between genetic variation and several fitness traits or correlates, and serves as an important surrogate for estimating the potential effects of loss in genetic diversity to small or fragmented populations in the wild. Here, we have shown that loss in genetic diversity through inbreeding can negatively impact the health of ring-tailed lemurs through a combined effect on parasitism and blood parameters, including some relating to immunocompetence. More profoundly, these factors ultimately contributed to the decreased survivorship of inbred animals. These findings highlight the importance of maintaining gene flow in



**Fig. 2** Failure time graphs for death as a function of age for (a) males and (b) females, according to Ho. For presentation purposes, individuals were separated in two classes: homozygous individuals ('Ho = 1': Ho < mean Ho) and heterozygous individuals ('Ho = 2': Ho > mean Ho). *Open squares* represent censored cases. *Dashed line*: probability of survival in homozygous individuals; *solid line*: probability of survival in heterozygous individuals

captive and wild populations alike, and have particular relevance to conservation efforts, given the vulnerable status of this species.

We found that more homozygous adult lemurs were more susceptible to and more heavily infected by *Cutebrea* larvae than were more heterozygous adults. Similar results have been shown for parasitism in *Drosophila* (Luong et al. 2007). In that study, inbred flies apparently were unable to sustain defensive behavior against parasitic mites, owing to their compromised physiological competence, and those flies consequently suffered a greater parasite burden than did outbred flies. Although mites are classified as ectoparasites, similar factors may have contributed to warble infestation: inbred adult lemurs may



have been more vulnerable to *Cuterebra* infestation because their immunocompetence was compromised.

By contrast with our findings in adults, both estimates of warble infestation (prevalence and burden), as well as the prevalence of strongyles, were negatively influenced in juveniles by heterozygosity (i.e., more homozygous youngsters were less infected). These results, albeit surprising, are nonetheless consistent across several measures and therefore suggest a real phenomenon. As yet, we have no clear explanation for these findings; however, we suspect that they may relate to the development of immunocompetence and to the stressors experienced by immature animals. Notably, infant lemurs show elevated fecal glucocorticoid concentrations (fGC) compared to adults (A. Starling, C. Fitzpatrick, E. S. Scordato, and C. M. Drea, unpublished data). In a preliminary analysis using mean fGC for a subset of juveniles ( $N = 12$ ), we found that more heterozygous juveniles tended to have higher fecal glucocorticoid concentrations than did more homozygous juveniles ( $P = 0.10$ )—a trend that disappeared in adulthood. Thus, outbred juveniles may experience more stressors (social or environmental) and greater parasitism than do inbred juveniles. Further study will be needed to evaluate the relationship between age, glucocorticoid concentrations, parasitism, and heterozygosity.

It is also possible that lemurs face tradeoffs throughout their lifetime that could explain why more heterozygous juveniles appear to be more compromised only at the beginning of their development. Clearly, age alone can significantly predict the occurrence of certain gastrointestinal parasites (e.g. strongyloides). Alternately, behavioral ontogeny may contribute to the differential age patterns observed. For instance, more heterozygous juveniles might be more prone to exploration and hence suffer from increased susceptibility to infectious agents (see also Nunn and Tae-Won Dokey 2006). Consistent with the relationships we found between housing conditions and certain blood parameters, social context and behavioral interaction can have a profound effect on an animal's well being. Similar social factors might contribute to explain the sex differences we observed in certain blood parameters in this female-dominant species. Whatever the explanation for the juvenile pattern, it is clear that more heterozygous adults overcome this early setback.

Of the blood parameters we selected for study, a third were affected by neutral heterozygosity. We found that, on average, more homozygous individuals had higher concentrations of total protein, specifically albumin, in their blood. The most common cause of increased albumin concentration in blood is dehydration, a condition that results in hemoconcentration (Willard and Tvedten 2004). The variation in quantity of total protein in plasma or serum that we observed between more homozygous versus

more heterozygous animals was mild and unlikely to be clinically apparent; nevertheless, the generally higher values seen in inbred individuals may reflect a tendency for these animals to be more hemoconcentrated than their outbred counterparts. Anything that causes an animal to decrease fluid intake (e.g. illness, limited access to water, etc.) or increase fluid loss (e.g. increased environmental temperatures, greater exertion, increased metabolic rate, fever, etc.) can result in dehydration (Willard and Tvedten 2004). It would appear that inbred individuals may have been slightly, but chronically, dehydrated, presumably as a consequence of chronic maladies and repeated fevers or diarrhea, rather than as a consequence of environmental challenges.

We also found several significant relationships between genetic diversity and white blood cell parameters, in the form of higher neutrophil counts and lower lymphocyte counts in more homozygous animals compared to more heterozygous animals. White blood cells are cells of the immune system that defend the body both against infectious disease agents and foreign materials (Willard and Tvedten 2004). Neutrophil numbers increase in response to inflammation, infection, and stress (Morrowtesch et al. 1993; Honess et al. 2005; Sapolsky 2005). Lymphocytes perform a number of immunologic functions, including the production of antibodies and the mounting of cytotoxic defenses against tumor cells or intracellular pathogens. Persistent and high increases in lymphocyte number usually signify strong immune stimulation from chronic infection or immune-mediated disease, whereas decreases most commonly occur in response to stress and increased glucocorticoid concentrations (Willard and Tvedten 2004). A pattern of increased neutrophils in the presence of decreased lymphocytes is consistent with an increase in glucocorticoid exposure or stress (Willard and Tvedten 2004). Indeed, an increase in the ratio of neutrophils to lymphocytes, as we found among inbred lemurs, suggests reduced immune system function and has been linked to increased stress in primates (Kim et al. 2005; see also Sapolsky 2005). Davis et al. (1991) report that increases in the neutrophil-to-lymphocyte ratio are due, at least partially, to the destruction of lymphocytes in the cortex of the thymus and the extension of neutrophil half-life. Animals in the free-range condition (compared to those in the enclosure condition) resembled inbred animals along these measures, suggesting that social interaction introduces significant stressors into the daily lives of lemurs.

Lastly, and most critically, we observed that more homozygous lemurs died at a younger age, particularly from diseases, than did more heterozygous lemurs. This result provides the strongest evidence that genetic diversity affects fitness. Reduced viability due to inbreeding has been shown in several studies, most of which dealt with

juvenile survival (Ralls and Ballou 1983, 1988; Lacy et al. 1993; Coulson et al. 1998, 1999; see however: Laikre and Ryman 1991; Laikre et al. 1997; Cassinello 2005). Here, we show that inbreeding in *L. catta* affects survivorship at any age and in both sexes.

In this study, we showed that several fitness traits or correlates were influenced by genetic diversity. Moreover, our results did not change qualitatively after dropping one locus at a time from the calculation of genetic diversity. Given these two sets of strong evidence, we conclude that neutral heterozygosity is a good estimate of genome-wide inbreeding and that the effects we observed likely owe to inbreeding depression. Nevertheless, in several recent empirical and theoretical studies, researchers have questioned the validity of using multilocus heterozygosity as an estimate of inbreeding because, in some cases, heterozygosity is found to be weakly correlated with inbreeding (Coltman and Slate 2003; Balloux et al. 2004; Pemberton 2004; Slate et al. 2004). Although these authors have raised important concerns, their criticisms did not apply here. Our study population presented an ideal situation for estimating inbreeding through the use of neutral heterozygosity because (1) the population was relatively small, in terms of the number of reproducing individuals, (2) reproduction occurred between closely related individuals, and (3) gene flow was limited to the arrival of genetically distant animals transferred from other facilities. These factors combined to produce significant variation in heterozygosity.

Inbreeding is most likely to occur in small populations and may contribute to the further decline and eventual extinction of threatened species (Gilpin and Soulé 1986; Saccheri et al. 1998; Hedrick 2001; O'Grady et al. 2006). Monitoring genetic variation and measuring the occurrence of inbreeding in threatened populations has become a key objective for conservation geneticists (Keller and Waller 2002; Garner et al. 2005; Goossens et al. 2006). A major problem faced by captive-breeding programs at the inception of a colony involves the founder effect, i.e., deleterious effects on the captive population derived from few founder individuals and the consequent loss of genetic diversity (Gompper et al. 1997). An emphasis for institutions that maintain captive-breeding programs is to maximize population heterozygosity by carefully selecting breeding individuals according to their genetic variability, so that the presence of individuals homozygous for deleterious alleles is minimized (Foose and Ballou 1988). Despite these efforts, genetic diversity can erode with time (see Fig. 2). We suspect that the effects we document in captivity would parallel situations in the wild whenever gene flow is compromised.

Madagascar has been classified as a 'biodiversity hotspot' (Mittermeier et al. 1998; Myers et al. 2000): it is an island in peril that is facing a dramatic, imminent, and overall decrease in its biodiversity. Owing to a decline in area of

occupancy, extent of occurrence, and quality of habitat, *L. catta* is classified in the 2006 IUCN red book as 'vulnerable' and is facing a high risk of extinction in the wild in the medium-term future. The population of *L. catta* studied here represents a surrogate to the wild populations living in Madagascar. Whereas the former is isolated by the availability of newly introduced breeders, the latter are isolated by forest fragmentation due to deforestation and hunting pressures. Although the exact illnesses and parasites that affect captive lemurs may differ from those encountered in the wild, many of the processes affecting susceptibility should be the same. Thus, the findings generated from this study have both practical and broad theoretical applications.

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