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Heterozygosity–Fitness Correlations Reveal Inbreeding Depression in Neonatal Body Size in a Critically Endangered Rock Iguana

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ABSTRACT

Inbreeding depression, though challenging to identify in nature, may play an important role in regulating the dynamics of small and isolated populations. Conversely, greater expression of genetic load can enhance opportunities for natural selection. Conditional expression concentrates these opportunities for selection and may lead to failure of detection. This study investigates the possibility for age-dependent expression of inbreeding depression in a critically endangered population of rock iguanas, *Cyclura nubila caymanensis*. We employ heterozygote-fitness correlations to examine the contributions of individual genetic factors to body size, a fitness-related trait. Nonsignificant reductions in homozygosity (up to 7%) were detected between neonates and individuals surviving past their first year, which may reflect natural absorption of inbreeding effects by this small, fecund population. The majority of variation in neonate body size was attributed to maternal or environmental effects (i.e., clutch identity and incubation length); however, heterozygosity across 22 microsatellite loci also contributed significantly and positively to model predictions. Conversely, effects of heterozygosity on fitness were not detectable when adults were examined, suggesting that inbreeding depression in body size may be age dependent in this taxon. Overall, these findings emphasize the importance of taking holistic, cross-generational approaches to genetic monitoring of endangered populations.

Subject areas: Conservation genetics and biodiversity
Keywords: inbreeding depression, age dependent, reptiles, island population

Small, isolated populations are subject to stochastic loss of genetic diversity and recurrent inbreeding because selection operates less efficiently and opportunities for mating are restricted. This can lead to inbreeding depression, the reduction in fitness of relatively inbred individuals, over the short term, and reduced adaptive potential over the long term (Charlesworth and Charlesworth 1987). Conversely, increased expression of load due to inbreeding exposes a greater proportion of load to natural selection, such that the mean effects of deleterious alleles segregating in historically small populations may be weak (Keller and Waller 2002). Recent reviews provide mixed support for the predictive power of population size as an indicator of genetic load (Díez-del-Molino et al. 2018) or adaptive potential (Hoffmann et al. 2017), with inbreeding depression varying widely among species (Frankham et al. 2017). Factors such as species-specific life-history traits (Romiguier et al. 2014) and ancient population bottlenecks (Rasmussen et al. 2011) have gained increasing

attention as determinants of genetic viability, underscoring the need for more empirical studies spanning diverse threatened taxa.

Inbreeding depression is challenging to identify in nature. This is in part due to the paucity of sufficiently detailed and quality-controlled pedigrees available in most studies of wild populations (Keller and Waller 2002; Pemberton 2008). Another factor that could obscure symptoms of inbreeding depression is conditional expression, whereby fitness reductions are magnified in stressful environments but are largely masked under other conditions (Keller et al. 2002; Armbruster and Reed 2005; Fox and Reed 2011). Inbreeding depression may also be influenced by factors such as age or sex. For example, pronounced effects of inbreeding depression on performance and survival during early life stages have been documented in mammals (Rijks et al. 2008; Cohas et al. 2009; Brambilla et al. 2015; Huisman et al. 2016), birds (Keller et al. 2002; Szulkin et al. 2007; Olano-Marin et al. 2011; Bichet et al. 2019), and marine organisms (Pujolar et al. 2006). Inbreeding effects associated with specific life stages can also disproportionately affect one sex; for example, genetic components underlying reproductive senescence (de Boer et al. 2018) and exacerbated disease progression with age (Benton et al. 2018; Queirós and Vicente 2018) have been found in females, but not males. Thus, when testing for signatures of inbreeding depression in a natural population, it is important to consider the potentially implicit bias of sampling from a single cohort.

One tool that has been extensively employed to study inbreeding depression in wild populations is the heterozygosity–fitness correlation (HFC), a marker-based estimate of the association between genetic diversity and phenotypic fitness. The method invokes multilocus heterozygosity (MLH) calculated over a panel of neutral marker loci to approximate variation in genome-wide heterozygosity (H_{GW}) among individuals. The utility of HFCs is based on the prediction that excess homozygosity generated by inbreeding unmasks recessive deleterious alleles and causes additive declines in individual fitness (Charlesworth and Charlesworth 1987). In studies of natural populations suffering from inbreeding depression, MLH has been shown to correlate positively with fitness traits (Bensch et al. 2006; Cohas et al. 2009; Hoffman et al. 2014; Velando et al. 2015) as well as phenotypic traits with substantive effects on fitness (Forstmeier et al. 2012; Ruiz-López et al. 2012; Brommer et al. 2015). Although criticized for their low statistical power and large sample size requirements (Balloux et al. 2004; Pemberton 2004; Slate et al. 2004; Miller and Coltman 2014), when used with care HFCs can serve as valuable tools for the detection of recent or ongoing inbreeding depression in natural populations (Szulkin et al. 2010).

In this study, we investigate hypothesized patterns of covariance between heterozygosity and age-dependent fitness traits in a critically endangered population of Caribbean rock iguanas (genus *Cyclura*). *Cyclura* includes some of the most endangered lizards in the world, with most taxa severely reduced from historical population sizes and ranges due to human activity (Alberts 2000; Lemm and Alberts 2012). Despite an inferred large role for overwater dispersal in facilitating gene flow between iguana isolates in the Caribbean (Censky et al. 1998; Knapp 2005), fine-scale genetic differentiation among *Cyclura* populations separated by broad water channels suggests that realized gene flow is often lower than expected (Colosimo et al. 2014). Although recent molecular investigations into a *Cyclura* mating system confirmed the incidence of multiple paternity in ~38% of clutches (Moss et al. 2019), which could mitigate inbreeding risk in a small population (Cornell and Tregenza 2007; Germain et al. 2018), mate choice with respect to inbreeding and relatedness did not appear to deviate from random. Thus, population size reductions are unlikely to be met with a strong behavioral response. Rather, a companion study found pronounced dispersal among neonates and juvenile iguanas on Little Cayman, suggesting that passive inbreeding avoidance strategies may be under strong selection in pre-reproductive life stages (Moss et al. in revision).

Our focal taxon, *Cyclura nubila caymanensis*, occurs exclusively on Little Cayman and Cayman Brac, two small islands situated ≥ 100 km from neighboring Grand Cayman, Cuba, and Jamaica. This study employs molecular approaches to examine the demography of the population of Little Cayman (28.5 km^2), which supports between 1200 and 1500 iguanas by minimum estimates (Goetz and Burton 2012) or between 2000 and 4100 by maximum estimates (Rivera-Milán et al. unpublished data). Although average fecundity in this taxon is relatively high (one clutch annually averaging 15 eggs; range: 7–25) and probably contributed to its colonization success, ongoing population declines driven by human development (Goetz and Burton 2012) could increase the risk of inbreeding. Because neonate iguanas are elusive, reductions in recruitment resulting from age-dependent effects of inbreeding may go undetected or be masked by overall high survivorship and fecundity among adults. We tested for inbreeding depression by assessing whether MLH in neonates was elevated relative to that in individuals that survived through their first year. We also examined the effects of inbreeding on individual fitness by employing body size as a morphological proxy. This trait has been correlated with critical aspects of early survivorship in lizards, including resource acquisition and predator avoidance (Clobert et al. 2000; Le Galliard et al. 2004). Body size is also an indicator of fitness among adult iguanas. In males, large size confers competitive ability and reproductive dominance (Alberts et al. 2002; Moss et al. 2019), and in females, it confers higher fecundity (Iverson et al. 2004 and references therein; Moss et al. in review). Thus, we test for significant HFCs in body size in both neonates and adults.

METHODS

Sampling

Tissue samples were obtained from unique individuals captured on Little Cayman in the summer of 2015. Complete protocols for capturing and processing adult, juvenile, and neonate iguanas are described elsewhere (Moss et al. 2019). Briefly, neonates were captured by hand as they emerged from pre-marked egg chambers enclosed with aluminum flashing, or were captured opportunistically with the aid of a noose. Sampling commenced at the beginning of the emergence season (August 1) such that all neonates in this study were sampled within weeks of emergence (age 0; n = 168). In addition, animals ≥1 year of age (age 1+; n = 120), which were distinguished on the criteria of body size and sampling month (for individuals measuring < 12 cm), were captured opportunistically between the months of May and September with the aid of a noose or net. All captured individuals were injected subdermally with HPT8 MiniChip passive integrative transponder tags to facilitate permanent identification and avoid replicate sampling (BioMark, Boise, ID). Small volumes of blood (0.5–1.0 mL) were drawn from the ventral caudal vein of each iguana and stored in cryovials containing a 1.5:1 ratio of blood buffer (100 mM Tris–HCl, 100 mM ethylene diamine tetraacetic acid, 10 mM NaCl, and 0.5% sodium dodecyl sulfate) to blood.

Standard morphometric measures from each animal, including snout–vent length (SVL), vent–tail length (VTL), head width (HW), and head length (HL), were measured with rulers and calipers, and animals were weighed individually in plastic or cloth bags with 60

g–10 kg spring scales (Pesola, Feusisberg, Switzerland). For individuals in the age 1+ cohort, additional measurements were recorded to quantify variation. These included length of tail regrowth, width of the jowls, and the height of the tallest crest spine on the neck, body, and tail. Although *Cyclura nubila* spp. are highly sexually dimorphic in adulthood (Lemm and Alberts 2012), immature animals are largely sexually monomorphic. In these cases, sex determination was made using cloacal probe protocols outlined by Dellinger and von Hegel (1990). In total, we captured 31 males, 70 females, and 19 animals of unknown sex in the age 1+ cohort and 72 males, 95 females, and 1 neonate of unknown sex in the age 0 cohort.

DNA Extraction and Amplification of Target Regions

DNA was extracted using a Maxwell 16 Nucleic Acid Extraction System along with Maxwell 16 Tissue DNA Purification Kits and protocols (Promega, Fitchburg, WI). Over 70 nuclear microsatellite markers developed for various *Cyclura* taxa (Malone et al. 2003; An et al. 2004; Rosas et al. 2008; Lau et al. 2009; Welch et al. 2011) were screened. Of these, 22 were identified as polymorphic in *C. nubila caymanensis* (Supplementary Table 1) and selected for individual genotyping. Target regions were amplified by touchdown polymerase chain reaction (PCR) protocols: initial denaturation at 94 °C for 5 min, 10 touchdown cycles (94 °C for 30 s, 10° above final annealing temperature for 30 s, and 72 °C for 30 s), 25 regular cycles (94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 30 or 45 s), and final elongation at 72 °C for 7 min (Don et al. 1991). Following visual confirmation by gel electrophoresis, PCR products were analyzed on ABI 3730 capillary sequencers (LIZ-500 Size Standard; Applied Biosystems) at Arizona State University Core Laboratories. Of 6358 potential genotypes, 6081 were successfully scored (96%).

Estimation of Genetic Diversity Indices

Individual microsatellite genotypes were scored visually using Peak Scanner version 1.0 (Applied Biosystems). In an effort to avoid replication bias imposed by whole-clutch sampling, only samples from the age 1+ cohort were analyzed to calculate genetic diversity metrics. Loci were first tested for deviations from Hardy–Weinberg equilibrium (HWE), rather than for heterozygote deficits, using GenePop on the Web (Rousset 2008). The same application was used to assess linkage disequilibrium (LD). Tests were performed with 10 000 dememorization steps, 1000 batches, and 10 000 iterations, and sequential Bonferroni corrections were applied to adjust critical significance levels for multiple tests (Holm 1979). Each locus was also tested for the presence of null alleles with Micro-Checker (Van Oosterhout et al. 2004).

To evaluate past demographic and contemporary levels of population genetic diversity, population genetic summary statistics, including observed heterozygosity (H_O), expected heterozygosity (H_E), and *F*-statistics were calculated in Genepop on the Web (Rousset 2008). In addition, allelic richness (A_R) as defined by El Mousadik and Petit (1996) was estimated with the R package PopGenReport (Adamack and Gruber 2014). To test for signatures of a recent population bottleneck (within the past 2 N_e to 4 N_e generations), deviations from mutation-drift equilibrium heterozygosity were examined under three mutation modes in the program BOTTLENECK v.1.2.02 (Piry et al. 1999). These included the infinite alleles model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM). The TPM was parameterized according to recommendations by Garza and Williamson (2001) for microsatellites, with an SMM proportion of 90% and a variance of 12. Sign and Wilcoxon tests were used to determine significance. Finally, to compute effective population size (N_e) following ancestral inbreeding and drift, we implemented a single-sample method based on bias-corrected LD (Hill 1981; Waples 2006; Waples and Do 2010) in NeEstimator v. 2 (Do et al. 2014). This metric was selected over other single-sample methods such as the heterozygote excess method (Pudovkin et al. 1996; Zhdanova and Pudovkin 2008), and the molecular coancestry method (Nomura 2008) because it accounts for small sample sizes and alleles with low frequencies, and because other methods are known to yield biased estimates of N_e (Do et al. 2014). A second, maximum likelihood estimate of N_e—Wang's (2009) sibship frequency (SF) estimate—was computed in the program COLONY (Jones and Wang 2010) from the frequency with which pairs of individuals taken at random from the cohort share sibship.

MLH Calculations

To investigate the pervasiveness and variance in inbreeding among distinct cohorts, MLH was estimated from individual microsatellite genotypes. Microsatellites are generally considered selectively neutral (Queller et al. 1993; Jarne and Lagoda 1996), and because their stepwise mode of mutation facilitates the maintenance of highly polymorphic loci, each individually conveys more information than bi-allelic markers such as single-nucleotide polymorphisms (SNPs; Vignal et al. 2002). This high mutability also makes microsatellites well-suited to detect HFCs that arise due to short-term processes, such as crosses between relatives or small population size (Tsitrone et al. 2001). Because this study utilizes whole clutches of offspring and because realized inbreeding coefficients do not always correspond with pedigree expectations (Forstmeier et al. 2012), the high allelic variation introduced by microsatellites may also be important for detecting variation among siblings.

MLH was estimated with three alternative estimators in the R package Rhh v. 1.0.2 (Alho et al. 2010). The first method, standardized heterozygosity (SH), calculates individual MLH divided by the average heterozygosity of the sample population (Coltman et al. 1999). Second, internal relatedness (IR) calculates the proportion of loci that are homozygous weighted on the basis of the frequency of alleles (Amos et al. 2001). Finally, homozygosity by locus (HL) calculates homozygosity weighted for individual loci on the basis of allelic number and evenness (Aparicio et al. 2006).

Test for Age-Dependent Selection

Tests for age-dependent selection were performed using a stratified sampling approach, sampling one hatchling per each unique clutch, to minimize bias resulting from lack of independence. Two sample *T*-tests were performed to test for statistically significant differences in mean MLH measures between age cohorts. The differences between means of traits before (age 0 cohort) and after (age 1+ cohort) selection, the directional selection differential, and the standardized selection differential, or selection intensity, were computed for each MLH measure following Kingsolver et al. (2001) and Falconer and Mackay (1996). Data were scaled to MLH distributions of the preselection sample, such that selection intensity represented the mean of the selected proportion in standard deviations (SD). These values were then used to quantify the proportion of the age 0 cohort selected, following Falconer and Mackay (1996; Appendix Table A). Stratified sampling of the preselection cohort and associated calculations were conducted for 1000 bootstrap iterations.

Computing Individual Fitness Scores

Suites of morphometric measures taken from individuals in the age 0 (Supplementary Table 2) and age 1+ (Supplementary Table 3) cohorts were considered jointly to assess variation in fitness. Because it would be inappropriate to compare body sizes between immature and fully grown individuals when assessing relative fitness, the 1+ cohort was subdivided into immature and mature individuals based on size calibration via size trajectory analysis (Moss et al. in revision). Females with SVL > 33 cm (n = 63) and males with SVL ≥ 39 cm (n = 22) were considered mature (n = 85). The number of yearlings (SVL < 20 cm; n = 23) and subadults (20 cm ≤ SVL ≤ 33 cm; n = 12) sampled was comparatively small, and because HFCs require robust sample sizes, only neonate and adult age classes were retained for HFC analysis. To account for sexual size dimorphism characteristic of adult iguanas, adult morphometric measures were independently transformed into Z-scores for each sex (Sokal and Rohlf 1995). Measures of VTL were eliminated from the suite of adult traits, as tail breakage and subsequent regrowth was documented frequently in iguanas older than 1 year (Supplementary Table 3).

Morphometric measures and their Z-scores appeared to be intercorrelated within both neonates (Supplementary Figure 1) and adults (Supplementary Figure 2). Thus, principal component analysis was used to compute individual scores from linear combinations of variables. Missing values were estimated via nonparametric multiple imputation, using the R package missMDA (Josse and Husson 2016). Only scores from principal components (PCs) with eigenvalues ≥ 1 were retained. These analyses yielded single PCs for both neonates and adults, which accounted for 46.5% and 68.8% of variance in body size, respectively. All morphometric measures loaded positively on their respective PC (Tables 2 and 3).

Heterozygosity–Fitness Correlations

The R package lme4 (Bates et al. 2015) was employed to fit linear mixed models of individual PC scores within age cohorts. Prior to performing HFC regressions, multiple factors were evaluated for effects on the intercept and slope of the relationship of interest. Among these were capture date and sex, as sex-specific differences often underlie age-dependent HFCs (Benton et al. 2018; de Boer et al. 2018; Queirós and Vicente 2018). Kinship was also taken into account, as large groups of siblings with equal F often share heritable similarities in phenotype despite underlying variation in MLH, and this can obscure relationships between MLH and fitness (Taylor et al. 2010). To detect such "sibship structures" in the data set, a likelihood-based pedigree reconstruction approach was implemented in the program COLONY (Jones and Wang 2010). Molecular pedigrees of neonates were carried over from a previous study (Moss et al. 2019; Supplementary Table 3), which leveraged field data on clutch identity to consider nest mates jointly when reconstructing parentage. Because all clutches analyzed in this study were sampled from nests over a single season, each clutch matched to a unique dam but some non-nest mates shared the same sire. No prior information on family structures was available for adults, and thus a single simulation was performed on all individuals. Fullsib cluster assignments were retained if the exclusion probability was >0.70. The largest cluster identified in the age 1+ cohort contained 4 individuals (Supplementary Table 4).

Random effects were selected for incorporation into HFC models using the function "ranova" in the R package lmerTest (Kuznetsova et al. 2017). Although clutch identity accounted for significant variation in PC1 × MLH in neonate models (SH: P = 0.018; SD = 1.11; IR: P = 0.021; SD = 1.088; HL: P = 0.018; SD = 1.106), dam identity (SH: P = 0.812; SD = 0.285; IR: P = 0.758; SD = 0.325; HL: P = 0.817; SD = 0.273), sire identity (SH: P = 1.00; SD = 3.57e-08; IR: P = 1.00; SD = 0; HL: P = 1; SD = 0), sex (SH: P = 0.152; SD = 0.253; IR: P = 0.160; SD = 0.247; HL: P = 0.176; SD = 0.243), and capture date (SH: P = 0.246; SD = 0.566; IR: P = 0.200; SD = 0.597; HL: P = 0.235; SD = 0.574) failed to explain significant additional variance after accounting for clutch. These terms were therefore removed from the model. No random terms tested for the adult model—fullsib cluster assignment (H: P = 0.169; SD = 1.748; IR: P = 0.177; SD = 1.719; HL: P = 0.148; SD = 1.769), sex (SH: P = 1; SD = 0; IR: P = 1; SD = 0; HL: P = 1; SD = 0), or capture date (SH: P = 0.536; SD = 1.436; IR: P = 0.562; SD = 0.686; HL: P = 0.521; SD = 0.733)—accounted for significant variance in PC1 × MLH among individuals. Thus, adult HFCs were run as simple linear regressions.

In addition to random terms, HFC models for neonates incorporated incubation length and clutch size as fixed effects. The former effect has been implicated in offspring body size in reptiles (Shine and Olsson 2003; Brown and Shine 2005), whereas the latter imposes tradeoffs on the amount of resources dams can invest in individual eggs and offspring (Brown and Shine 2009). Only 17 of 55 clutches possessed complete data on both clutch size (n = 21) and incubation length (n = 19), reducing the sample size for HFC computation from 168 to 117. A total of 16 multivariate models with up to three predictors of neonate PC1 were ranked in the R package MuMIn (Bartón 2015) based on corrected Akaike information criteria (Akaike 1974; Hurvich and Tsai 1989). If no model was ≥2 AIC_c units clear of the next best model, models comprising the top 2 AIC_c units were collectively considered the nominal "best." Marginal R^2 of fixed effects were calculated for each model with the MuMIn function "r.squaredGLMM."

Table 1. Summary statistics of three MLH measures, SH, IR, and HL within the age 0 and age 1+ cohorts

	Age 0 (pre-selection)	Age 1+ (post-selection)	Prop. significant t-tests	Selection intensity (proportion selected)
N	168	120		
SH	0.993 ± 0.186	1.018 ± 0.195	0.001	0.134 ± 0.06 (96–97%)
IR	0.101 ± 0.164	0.061 ± 0.163	0.125	−0.245 ± 0.063 (93–94%)
HL	0.402 ± 0.103	0.397 ± 0.109	0	−0.047 ± 0.057 (98–99%)

Mean hatchling measures were estimated within 1 SD by resampling of 55 unique clutches, and the proportion of 10 000 bootstrap samples possessing significant differences in means compared with the adult sample (−1.96 ≤ t-statistic ≥ 1.96) is reported. Average values of selection intensity (standardized selection differential) for SH, IR, and HL across 10 000 bootstrap samples are reported within 1 SD along with corresponding values for selected proportion, estimated from a table of selection intensities in Falconer and Mackay (1996; Appendix Table A), in parentheses.

Table 2. Factor loadings on the first principal component (PC1; eigenvalue = 2.33) of a principal components analysis of morphometric measures within the age 0 cohort

Morphometric	PC1 (46.5%)
Snout–vent length	0.562
Vent-tail length	0.468
Head width	0.410
Head length	0.375
Body mass	0.395

Table 3. Factor loadings on the first principal component (PC1; eigenvalue = 5.50) of a principal components analysis of morphometric measures within the age 1+ cohort

Morphometric	PC1 (68.8%)
Snout–vent length	0.346
Head width	0.324
Head length	0.340
Jowl width	0.379
Maximum crest spine height (neck)	0.356
Maximum crest spine height (body)	0.373
Maximum crest spine height (tail)	0.336
Body mass	0.371

Tests for General and Local Effects

Although global reductions in heterozygosity resulting from inbreeding are hypothesized to affect fitness negatively, HFCs at individual loci may be positive, negative, or null if they arise as artifacts of LD between neutral markers and genes affecting fitness (David and Jarne 1997). To discriminate between genome-wide, or "general" effects of inbreeding depression and "local" effects resulting from associations of single-locus heterozygosity with particular fitness traits, additional tests were needed. A modification of Cockerham and Weir's (1973) identity disequilibrium (ID) estimate, the g_2 statistic (David et al. 2007), was employed to evaluate the focal panel of loci for significant covariance in heterozygosity, which should occur if MLH is capturing variation in individual inbreeding. This test was carried out using the R (R Core Development Team 2017) package, inbreedR (Stoffel et al. 2016).

To test for significant local effects across marker loci, the equation presented by Szulkin et al. (2010) for comparing the goodness-of-fit of a simple linear regression to the goodness-of-fit of a multiple linear regression was employed (Equation 1). According to this test, an *F*-value greater than the critical value of *F* (based on degrees of freedom) suggests significant local effects. Following the recommendation of Szulkin et al. (2010) missing data were replaced with mean heterozygosity states for each locus to compare model fits between multiple and simple linear regression. Sequential Bonferroni corrections were applied to all single-locus regressions to account for multiple comparisons.

$$F = \frac{(resSS_1 - resSS_2)/(df_1 - df_2)}{resSS_2/df_2}$$

Equation 1: Szulkin et al.'s (2010) *F*-ratio calculation to compare the goodness-of-fit of a general effect and a local effect model to explain HFCs. $resSS_1$ and $resSS_2$ are the residual sum of squares estimated from models of simple linear regression and multiple linear regression, respectively, and df_1 and df_2 are degrees of freedom.

RESULTS

Genetic Diversity

After Bonferroni corrections, no significant deviations from HWE were detected at any of the 22 microsatellite loci amplified (Supplementary Table 1). Only one pairwise test for LD was significant ($P > 0.05$). No loci exhibited null allele frequencies exceeding 0.20.

Overall genetic diversity indices ($H_E = 0.495$, $H_O = 0.514$, and $A_R = 4.83$) and population-level inbreeding ($F_{IS} = -0.034$) imply heterozygote excess in the age 1+ cohort. The program BOTTLENECK also detected significant heterozygosity excess under the IAM using the Wilcoxon test ($P = 0.012$), but not the sign test ($P = 0.125$). Conversely, heterozygosity deficit was detected under both the SMM (sign $P = 0.012$; Wilcoxon $P = 0.002$) and the TPM (sign $P = 0.097$; Wilcoxon $P = 0.043$), the mutation modes of which may more closely resemble those of microsatellites than the IAM (Shriver et al. 1993). A normal, L-shaped distribution was produced by the program's graphical allele frequency distribution test. Contemporary N_e of the age 1+ cohort as estimated by the LD and SF methods was 157.6 (95% CI = 116–232.7; minimum allele frequency = 0.05) and 65 (95% CI = 4–96), respectively. Computed for the age 0 cohort, these estimates were smaller: $N_e = 71.8$; (95% CI = 63.9–80.7) and 40 (95% CI = 26–64), respectively.

Patterns of MLH and Age-Dependent Selection

Mean heterozygosity differed significantly between age classes in a maximum of 12.5% of bootstrap sampling iterations (Table 1). Nonsignificant reductions in homozygosity in the age 1+ cohort compared with the age 0 cohort were detected using each of the three measures (Figure 1). Selection intensity for heterozygosity was positive for all measures of MLH (IR and HL are interpreted with inverse signs, as these measure homozygosity) and ranged from 0.047 to 0.245. Extrapolating from these, the proportion of the preselection cohort that would need to be removed to arrive at mean MLH values observed in the post-selection cohort ranged from 1% to 7%, with the largest effect being observed for estimates of IR.

Heterozygosity–Fitness Correlations

Clutch identity explained a large proportion of the variance observed in neonate PC1 ($R^2_{conditional} = 0.63$) in the null model. Moreover, a significant positive correlation was detected between incubation length and mean PC1 score across 11 observed incubation lengths (mean = 72.6 ± 8.6 days; range = 58–88 days) spanning 166 neonates ($F_{(1,9)} = 10.06$; $P = 0.011$; Figure 2A). A similar regression on 10 observed clutch sizes (mean = 14.2 ± 3.3; range = 7–20 days) did not reveal a significant relationship with mean PC1 ($n = 130$ neonates; $F_{(1,8)} = 0.577$; $P = 0.469$; Figure 2B), although trends followed a trajectory of decreasing body size with increasing clutch size.

Estimates of individual MLH varied within clutches of neonates, and independent of other predictors, accounted for between 1.3% and 2% of variance in PC1 (Table 4). The nominal best models of neonate PC1 (AIC_C improvement of 4.88–6.04 relative to null) included incubation length ($R^2_{marginal} = 0.35$) and one of IR or HL as predictors.

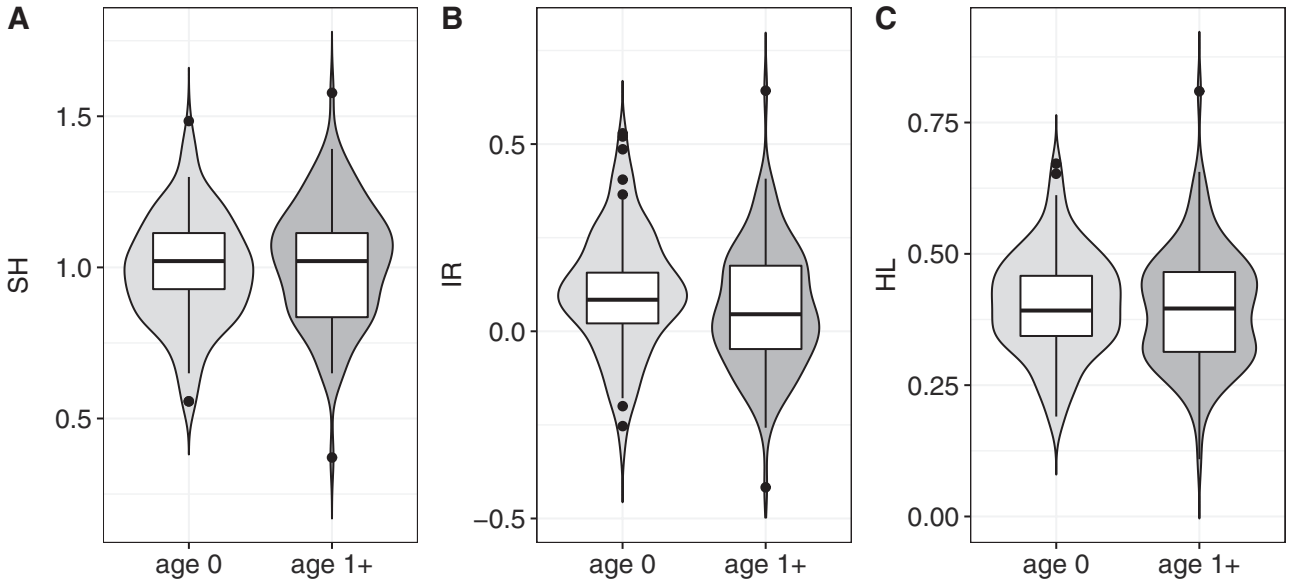

Figure 1. Distributions of (**A**) standardized heterozygosity (SH), (**B**) internal relatedness (IR), and (**C**) homozygosity by locus (HL) in the age 0 and age 1+ cohorts. Violin plots were generated using all samples from each age class (n = 168 and n = 120, respectively), and overlaid box plots summarize the distributions of independent samples for each age class (n = 55 and n = 120, respectively).

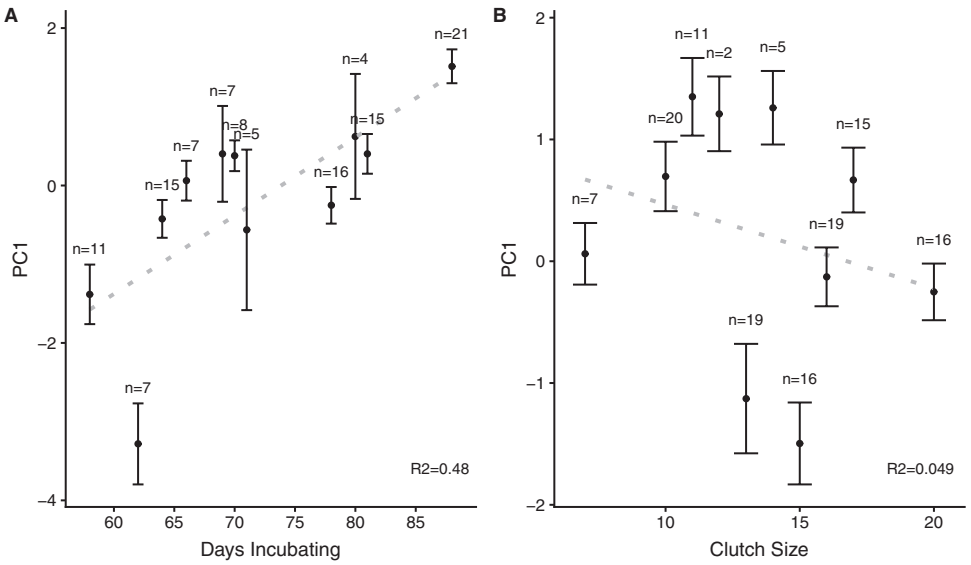

Figure 2. Mean individual scores obtained from a principal component analysis of neonate morphometric measures (PC1) for a given (**A**) incubation length (n = 11) and (**B**) clutch size (n = 10). Error bars represent the observed range (minimum and maximum) of principal component 1 (PC1) for a particular value. Sample sizes are reported above each respective error bar. The R^2 value of each regression is reported on the graph and is associated with the shown trend line.

In adults, MLH accounted for between 1.1% and 1.9% of variance in PC1. Although the relationships followed the expected positive trend, HFCs failed to reach significance (Table 5).

General and Local Effects of Markers

No single-locus effects were statistically significant after correcting for multiple comparisons. Significant local effects could not be invoked in either age class to explain HFCs (F-ratio << $F_{critical}$ for df = [21, 144]), suggesting a larger role for general effects. However, the g_2 statistic computed to assess the degree of identity disequilibrium was not statistically different from zero across the sample population as a whole (g_2 = −0.0004, SE = 0.0028, P = 0.544) or within the age 0 cohort (g_2 = −0.0015, SE = 0.0040, P = 0.663) or age 1+ cohort (g_2 = 0.0021, SE = 0.0046, P = 0.322). Thus, caution is required in invoking general effects as an overall explanation for HFCs.

Discussion

Inbreeding depression can have severe consequences for population persistence and may strike disproportionately under varying environmental conditions and within different ages and sexes. This study evaluates the role that inbreeding plays in regulating the population dynamics of the rock iguana, *C. nubila caymanensis*, on Little

Table 4. Results of linear mixed models for predicting principal component 1 (PC1) of neonate body size (n = 117)

Variable	Random effect SD		Fixed effect coefficients					df	Delta AICc	Weight	Marginal R^2
	Between clutch	Within clutch	inc.	cl. size	SH	IR	HL				
inc. + IR	**0.944**	**1.000**	**0.107**	—	—	**−1.560**	—	**5**	**−6.038**	**0.272**	**0.362**
inc. + HL	**0.952**	**1.009**	**0.104**	—	—	—	**−1.971**	**5**	**−4.875**	**0.152**	**0.356**
inc. + SH	0.966	1.007	0.106	—	1.108	—	—	5	−3.747	0.087	0.353
inc. + cl. size + IR	1.004	1.001	0.105	−0.054	—	−1.578	—	6	−3.697	0.085	0.363
IR	1.345	0.999	—	—	—	−1.467	—	4	−3.680	0.084	0.020
HL	1.332	1.008	—	—	—	—	−1.906	4	−2.967	0.059	0.013
inc. + cl. size + HL	1.012	1.011	0.103	−0.055	—	—	−2.003	6	−2.563	0.048	0.357
cl. size + IR	1.416	1.000	—	−0.075	—	−1.493	—	5	−2.432	0.045	0.036
SH	1.354	1.006	—	—	1.053	—	—	4	−1.712	0.031	0.014
cl. size + HL	1.403	1.009	—	−0.075	—	—	−1.942	5	−1.696	0.031	0.029
inc.	0.962	1.020	0.103	—	—	—	—	4	−1.685	0.031	0.345
inc. + cl. size + SH	1.026	1.009	0.105	−0.058	1.138	—	—	6	−1.507	0.028	0.354
cl. size + SH	1.425	1.008	—	−0.078	1.085	—	—	5	−0.512	0.017	0.029
Null Model	1.331	1.019	—	—	—	—	—	3	0.000	0.013	—
inc. + cl. size	1.028	1.021	0.102	−0.048	—	—	—	5	0.632	0.010	0.346
cl. size	1.406	1.020	—	−0.069	—	—	—	4	1.311	0.007	0.019

Clutch identity was specified as a random effect in each model, and SD are reported. In addition to three measures of multilocus heterozygosity (standardized heterozygosity [SH], internal relatedness [IR], and homozygosity by locus [HL]), incubation length (inc.) and clutch size (cl. size) were tested as fixed effects. Models are ranked by the nominal "best" AIC_c value.

Table 5. Summary results of linear models for predicting individual scores for the first principal component of body size in iguanas ≥1 year of age (standardized by sex; n = 85)

Predictor	Estimate	SE	R^2	P
SH	1.610	1.146	0.011	0.164
IR	−2.079	1.407	0.014	0.143
HL	−3.399	2.093	0.019	0.108

R^2 and P-values of correlations with three values of multilocus heterozygosity, including standardized heterozygosity (SH), internal relatedness (IR), and homozygosity by locus (HL), are reported.

Cayman. We observed heterozygote excess among individuals 1 year and older; however, directional selection for MLH was negligible. Indeed, an absence of significant age-dependent genetic variation suggests that recruitment is largely unbiased with respect to inbreeding level. Despite this, we detected significant positive effects of heterozygosity on neonate body size after accounting for clutch-level effects (e.g., clutch identity and incubation length). This relationship appeared the most pronounced among hatchlings experiencing the shortest and longest incubation times. In contrast, significant HFCs were not observed among individuals that survived through their first year, suggesting that inbreeding depression in body size may be age and environment dependent, but not sex dependent, in this critically endangered taxon.

The historical and contemporary demography of Little Cayman's iguana population inferred from a single sample of individuals aged 1 or older is consistent with a genetic bottleneck. Overall genetic diversity indices (H_E = 0.495, H_O = 0.514, and A_R = 4.83) were comparable to estimates obtained from related taxa (*C. cychlura cychlura*: H_E = 0.58–0.62, H_O = 0.45–0.56, and A_R = 3.04–3.84; Colosimo et al. 2014; *C. cychlura inornata*: H_E = 0.13–0.46 and A_R = 1.31–2.09; Aplasca et al. 2016; *C. ricordi*: H_E = 0.65–0.71 and H_O = 0.56–0.62; Carreras-De León et al. 2019; *C. carinata*: H_E = 0.25–0.62 and H_O = 0.22–0.63; Welch et al. 2017; *C. pinguis*: H_E = 0.59 and H_O = 0.50; Mitchell et al. 2011) and population-level inbreeding was small and, if anything, negative (F_{IS} = −0.034), reflecting heterozygote excess in the age 1+ cohort. Although we failed to detect mode shifts in the allele frequency distribution, indicative of a recent bottleneck, and detected heterozygosity deficit rather than excess under two mutation mode models regarded as most closely resembling that of microsatellites (SMM and TPM), loci evolving under strict SMM do not always produce detectable signatures of heterozygosity excess (Cornuet and Luikart 1996). Under the IAM, a Wilcoxon test did find significant heterozygosity excess. Although this contrasting piece of evidence supports a recent bottleneck, it does not exclude the possibility that a historical bottleneck was followed by rapid population expansion. Such a scenario may have produced the observed signature of heterozygosity deficit detected by the other models. Although LD and SF methods yielded slightly different estimates of N_e for this population, extremely large N:N_e ratios by either estimation are indicative of a severe historical bottleneck, and reduced N_e among neonates may be consistent with ongoing genetic erosion. Such conditions probably amplify the risk of genetic drift and inbreeding for existing and future generations of iguanas.

Although our power to detect differences in MLH between age classes had a low effect size, the direction of age-dependent variation follows expectations that selection should remove highly inbred individuals from the population prior to their achieving one year of age. Mean hatchling MLH was consistently lower than mean adult MLH according to all three measures. Selection intensity computed from IR implied that 6–7% of neonates would need to be removed from the preselection cohort to arrive at the mean levels of heterozygosity detected in individuals one year or older. Although IR has been found to outperform other accepted molecular measures for quantifying individual inbreeding (Forstmeier et al. 2012), selection intensities computed from SH (3–4%) and HL (1–2%) imply that differences may be negligible. Losses on this order attributed to genetic factors likely comprise only a small fraction of total attrition, given that the life history of *Cyclura* predicts high fecundity and

high juvenile mortality. Indeed, a study of *Cyclura carinata* on Little Water Cay (Berk 2013) reported substantial purging of homozygous offspring (49.8–62.7%), and yet this population is extremely dense and healthy and has likely remained stable for hundreds of generations (Gerber 2004). Thus, at stable population densities, a highly fecund species should be capable of absorbing some inbreeding costs (Mills and Smouse 1994). Selective mortality may even be a natural dynamic that could enhance the effectiveness of selection at minimizing the genetic load in populations of small effective size.

Negative effects of inbreeding on early fitness were evident in this study, suggesting a mechanism for age-dependent selection. Consistent with a number of studies that have sought to disentangle sources of trait variation in neonate lizards (Uller et al. 2011; Noble et al. 2014; Martínez-Caballero et al. 2017), maternal and early environmental effects were the most influential factors shaping natal characters in our models. Clutch identity and incubation length explained substantial variance in body size at hatching, a phenotypic indicator of fitness in neonate iguanas (Clobert et al. 2000; Le Galliard et al. 2004). In concordance with this finding, a recent study of *C. nubila caymanensis* demonstrated that the probability of an egg failing to hatch or hatching prematurely is significantly predicted by phenology, nest depth, and surrounding nest densities (Moss et al. manuscript in preparation)—conditions that may have important consequences for early growth and survivorship (Warner and Shine 2007). Nevertheless, inclusion of individual MLH measures was found to significantly improve the fit of models constructed from clutch identity and incubation length alone. Inbreeding depression in birth weight and size has been documented in a number of mammal species (Slate and Pemberton 2002; Dunn et al. 2011; Walling et al. 2011; Hoffman et al. 2014; Huisman et al. 2016), but fewer studies have investigated these effects in neonate reptiles. Because hatchling iguanas are completely independent at hatching and subsist on residual stored yolk during their first days to weeks (Carey 1975; Christian 1986; Vogel et al. 1996; Levering and Perry 2003), effects of inbreeding on initial body size could impact survival. Inbreeding could also impose fitness consequences at a stage earlier than sampling was conducted. Indeed, a companion study by Moss et al. (2019) reported negative effects of parental relatedness on clutch hatching success in *C. nubila caymanensis*. Thus, future studies may investigate the feasibility of obtaining genetic samples from inviable or unhatched eggs.

In contrast to neonates, no significant HFCs were detected in adults. Weak (1–2% measurable fitness differences; Slate et al. 2004) and nonsignificant HFCs are common in the literature (76% of studies reviewed in Chapman et al. 2009) and were recently reported in a genetically depauperate iguana population (Judson et al. 2018). In practice, power to detect variation in fitness due to inbreeding may be improved through more robust sampling (Slate and Pemberton 2002) or by increasing genomic coverage (Miller et al. 2014). Meeting robust sampling requirements can present logistical and ethical challenges in studies of free-ranging endangered species, and it is possible that our small sample of sexually mature individuals imposed statistical limitations. However, decreasing HFCs with age have been reported in a number of taxa including mammals (Rijks et al. 2008; Cohas et al. 2009; Brambilla et al. 2015; Huisman et al. 2016), birds (Keller et al. 2002; Szulkin et al. 2007; Olano-Marin et al. 2011; Bichet et al. 2019), and marine organisms (Pujolar et al. 2006). One explanation is that as unfit genotypes are eliminated from aging cohorts and variance decreases, the relative advantage of heterozygosity should also deteriorate (Cohas et al. 2009). However, the absence of significant differences in MLH between age cohorts in this study refutes this simple explanation. Rather, it is possible that the survival of homozygotes is determined not on the basis of genome-wide rates (e.g., "quantity" of homozygosity across loci), but of allelic composition (e.g., the relative "quality" of homozygosity). Indeed, Soulsbury and Lebigre (2018) demonstrated how the selective mortality of low heterozygosity–low body mass individuals early in life reduces the fitness discrepancy between homozygotes and heterozygotes downstream. In fact, the authors reported *negative* HFCs among older cohorts, which they attributed to overrepresentation of low heterozygosity–high body mass individuals. Weiser et al. (2016) allude to this idea—that the average "quality" of inbred individuals improves with age—in a study showing reduced survival of offspring of close relatives but improved survival of offspring of highly inbred mothers. The authors reasoned that inheriting "proven" genotypes could confer fitness advantages. In theory, such dynamics could help to explain the general weakness of HFCs in nature.

Such effects need not set the precedent for HFCs in adult iguanas, however. Another study in this system uncovered a positive relationship between female MLH and fecundity (Moss et al. 2019), illustrating that inbreeding depression may persist in critical fitness traits well into adulthood. Similarly, Phillips et al. (2017) found that clutches of sea turtles sired by males with low MLH suffered reduced egg success when parental relatedness was also high. It is possible that inbreeding depression manifests only weakly in morphometric traits compared with life-history traits (Roff 1998; DeRose and Roff 1999; Wright et al. 2008). Moreover, owing to the particular life-history characteristics of this system—longevity, high annual fecundity, and overlapping generations—it is unavoidable that older age classes comprised many cohorts. Although our analysis attempted to partition out individuals that had yet to reach sexual maturity, we could not control for possible differences in growth rates among mature individuals that hatched during "good" or "bad" years. Similarly, because neonates sampled for this study all belong to a single cohort, it is possible that the significant HFCs detected should not be attributed to life-stage-specific stressors but to the ecological or climatic conditions specific to 2015. Hence, decoupling lifetime and cross-generational fitness consequences of inbreeding in this population will require more long-term research and possibly, greater depth of genomic sampling.

Although we did not find evidence for general or local inbreeding effects driving HFCs in this study, statistics calculated from small panels of loci under small effective population sizes should be interpreted cautiously. Although Miller and Coltman (2014) found that the magnitude of g_2 tends to be associated with the average effect sizes observed in HFC studies, HFCs often reach significance before ID does (Szulkin et al. 2010). Indeed, Kardos et al. (2014) demonstrated using computer simulations that for populations exhibiting low variance in individual inbreeding coefficients, significant identity disequilibrium is unlikely to arise in small panels of loci (<100 microsatellites or 1000 SNPs) even when significant HFCs are caused by inbreeding depression. Despite historical selection pressures, contemporary variance in inbreeding may be low, which could contribute to the small effect sizes detected in this study. Moreover, although significant single-locus effects were not found to underlie HFCs, these tests fail to account for the confluence of negative and positive effects of different allelic combinations within loci. Because the simultaneous action of general and local effects on a panel of loci could confound overall correlations of MLH with fitness, failure to detect ID or $F >> F_{critical}$ does not necessitate that effects of inbreeding depression on fitness traits be dismissed.

Because small populations face heightened risks of extinction when demographic, environmental, and genetic stochasticity are compounded (Gilpin and Soulé 1986; Reed and Frankham 2003; Fagan and Holmes 2006), genetic monitoring to detect early signatures of inbreeding depression is crucial for the management of threatened island taxa. The population examined in this study is small and isolated and likely experienced a severe bottleneck at some point in its history. Although highly effective genetic "purging" is often invoked to explain long-term population persistence under these demographic conditions (Templeton and Reed 1984; Lande 1988; Simberloff 1988; Robinson et al. 2018), a number of studies in natural populations have since demonstrated that populations subjected to ancestral inbreeding and serial bottlenecks are no less susceptible to severe inbreeding depression than outcrossed populations (Frankham 1998; Leberg and Firmin 2008; Kennedy et al. 2014). Under times of stress, further fitness reductions resulting from genetic factors could accelerate population decline. Qualitative observations of iguana densities on Little Cayman indicate that numbers have been declining gradually since around the 1970s (Townson 1980) and that this trend may have escalated in recent years due to increased vehicular traffic and the spread of feral mammals (Goetz and Burton 2012). Future intensified inbreeding accompanying these trends may not be readily detectable if signatures such as reduced recruitment are masked by the extreme longevity of adults (*Cyclura* live in excess of 50 years in some taxa; Iverson et al. 2004; Burton 2012) and overlapping generations. Thus, expanded genetic monitoring involving larger panels of neutral SNPs would be desirable to evaluate recent or ongoing inbreeding depression in *C. nubila caymanensis*.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

Morphometric data and microsatellite genotypes are available at Dryad https://doi.org/10.5061/dryad.8kprr4xj1

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