Inbreeding and adaptation to captivity depress the response to stress

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Lay Summary

We explored the stress hormone changes (glucocorticoids) during a captive breeding program. Stress affects reintroduction success and survival in the wild. Using 326 wild mice bred in captivity we show that generations in captivity and inbreeding reduced glucocorticoids. This shows the positive association of glucocorticoids and fitness in the wild.

Abstract

The success of reintroductions using captive-bred populations of wild species is potentially impacted by adaptations to non-natural captive environments. Little research has been done into how physiological traits change from wild to captive populations. We do not yet understand how glucocorticoid secretion patterns, a critical aspect of the stress response and other underlying life-history traits, change in the captive environment. Here we used 326 white-footed mice (Peromyscus leucopus) to test how the baseline concentrations of fecal glucocorticoid metabolites (fGM) change as this wild rodent adapts to captivity and becomes increasingly inbred over several generations. Breeding protocols did not influence FGM but showed a strong decrease with generations in captivity, an effect driven by both plastic and genetic effects. We also found that juvenile fGM concentrations strongly predict adult fGM concentrations. This allows intra-generation effects, such as habituation, to be transformed into inter-generation effects. Lastly, the relationship between inbreeding and baseline fGM concentrations suggests that the intensity of baseline adrenal activity and mounted stress response is positively associated with fitness. Also, because the relationship is significantly stronger for females than for males, the result gives us some insight into the sex-specific adaptive value of fGM concentrations.

Introduction

Captive breeding has allowed wild endangered species to be propagated and re-introduced successfully back into their natural habitats (Frankham, Ballou & Briscoe, 2002; Soorae, 2016). However, the overall success of reintroductions is still low (Balmford, 2000). This may happen in part because in captivity the effects of inbreeding, genetic drift or inadvertent selection on fitness-related traits lead to a departure from the value displayed by their wild ancestors (Frankham, 2008; Lacy, 1993). In fact, captivity poses strong selective forces that can generate both rapid and considerable shifts in phenotypic traits (Christie, Marine, French & Blouin, 2012; Frankham, 2008; Williams & Hoffman, 2009) as well as restructuring of genetic variance (Lacy, Malo, Alaks, 2018). Thus, slowing evolution in captivity seems necessary for successful reintroduction of captive-bred animals into their native habitats (Lacy, 2009).

Despite the fact that genetic adaptation to captivity has been long recognized as a conservation issue requiring research attention (Frankham & Loebel, 1992), we are only now starting to better understand its effects on different taxa (Williams & Hoffman, 2009) and on different traits. There has been research on life history (Price, 1972), behavioral (Lacy, Alaks & Walsh, 2013; McPhee, 2004) and morphological traits (Lacy et al., 2013; O'Regan & Kitchener, 2005). It is worth noting however that, except for model organisms such as *Drosophila* (Frankham, 2008) that are quite distantly related to the multitude of endangered vertebrate taxa focus of captive breeding programs, most of the research is not experimental and co-factors such as inbreeding are usually not controlled for (Lacy et al., 2013). Fewer efforts have been devoted to characterize physiological changes in captivity (Malo, Martinez-Pastor, Alaks, Dubach & Lacy, 2010).

Hormones are recognized as one of the drivers of life-history trade-offs (Ketterson & Nolan Jr, 1992; Zera & Harshman, 2001) and their response to captivity can affect fitness-related traits in ways that might be detrimental after reintroduction. The effect of multiple generations of captive breeding on physiological traits remains unexplored. Specifically, we lack understanding about how the hormones that underlie the response to stress might be affected. In captivity, one of the effects of the artificial environment on reproduction and survival can be chronic, continuous stress (Marti &

Armario, 1998). This can affect individuals, which respond plastically, or the captive population, which can respond to those selective forces.

Populations of many vertebrate species experiencing stressful conditions, show elevated glucocorticoid levels (for rodents see Boyle, de la Sancha, Pérez, Kabelik, 2021). Individual baseline glucocorticoid levels (rather than stress-induced) have been used as a general indicator of an organism's ability to respond to stress, but there is still debate about whether physiological responses to stress are positively or negatively associated with individual or population fitness (Dickens & Romero, 2013). Two main hypotheses have been proposed, the Cort-Fitness and the Cort-Adaptation hypotheses. The Cort-Fitness Hypothesis states that because chronic stress can create a negative energy balance or allostatic overload that can severely handicap individuals, glucocorticoid levels are negatively associated with fitness (McEwen, 1998; McEwen & Wingfield, 2003). Although there are many studies apparently supporting the negative association with fitness (Blas, Bortolotti, Tella, Baos & Marchant, 2007), there is heterogeneity in the sign of the relationship between glucocorticoids and fitness proxies. For example, several studies support the idea that baseline glucocorticoid levels positively influence reproductive and viability fitness (Cabezas, Blas, Marchant & Moreno, 2007; Comendant, Sinervo, Svensson & Wingfield, 2003; Meylan & Clobert, 2005), suggesting that glucocorticoid levels can be used as indicators of individual and population quality (Bonier, Martin, Moore & Wingfield, 2009; Dingemanse, Edelaar & Kempenaers, 2010). These later results have been taken as support of the Cort-Adaptation hypothesis, which states that the positive relationship between glucocorticoids and fitness is due to the hormone effects during the reproductive period (Bonier et al., 2009; Crossin, Trathan, Phillips, Gorman, Dawson, Sakamoto & Williams, 2012; Escribano-Avila, Pettorelli, Virgos, Lara-Romero, Lozano, Barja, Cuadra & Puerta, 2013). Overall, our knowledge about the effects of glucocorticoids on different biological functions remains limited, with the stress response only being one of many. The above hypotheses about the glucocorticoid-driven adrenal response can be confounded with general possibly seasonal and reproductive-related glucocorticoid elevation that has differing functions influencing, for instance, activity levels, vigilance, foraging, and interactions with other hormones. Captive populations offer a powerful tool to study this question as

they allow repeated sampling of individuals to control for seasonality-driven elevations glucocorticoid fluctuations.

Given that departures of baseline glucocorticoid levels in the captive population from the levels set by natural selection in wild populations can impact reproduction and survival —and hence reintroduction success, understanding how captivity impacts glucocorticoids and the stress axis in general concerns conservation biology. At the same time, from an evolutionary perspective, understanding how responsiveness to stressors evolves in captivity is relevant to our understanding of the forces generating phenotypic changes, including selection.

Our aim with this study was two-fold. First, we longitudinally measured fecal glucocorticoid metabolite concentrations (fGM), via non-invasive fecal hormone metabolite monitoring across populations and generations of a wild rodent species, the white-footed mice (*Peromyscus leucopus noveboracensis*), to investigate the effects of generations in captivity, breeding protocols, inbreeding, and genetic drift on general adrenal activity. We accounted statistically for a variety of potentially confounding individual and environmental factors that can influence fGM, such as habituation, seasonality, sex, and age. Second, given that inbreeding depression, due to the expression of deleterious alleles (Charlesworth, 2009), leads to a decrease in fitness-related traits, the relationship between the coefficient of inbreeding and a fitness–trait has been used to reveal the sign of the relationship between fitness and the trait (Ketola & Kotiaho, 2009; Ketola & Kotiaho, 2012; Mallet & Chippindale, 2011). Here we use for the first time the sign of the relationship between the coefficient of inbreeding (*f*) and stress response (baseline fGM) to test the relationship between fitness and the response to stress.

Materials and Methods

Mice, Breeding Protocols, and Replicates

In October 2001 fifty-one white-footed mice were trapped at Volo Bog State Natural Area, Lake County, IL, USA, using Sherman box traps baited with peanut butter. After quarantine and disease testing, mice were brought into a research facility at the Brookfield Zoo, Chicago Zoological Society, Brookfield, IL, USA, to create the founder population. Nineteen pairings were set up, of

which 12 produced litters. The most productive 10 pairs gave birth to > 240 individuals (in 5–10 litters each) that were randomly allocated to six experimental groups (3 breeding protocols x 2 replicates; 20 pairs maintained each generation per protocol and replicate) and that subsequently bred in captivity for 9 generations up to this study. The three breeding protocols were as follows: 1) The mean kinship, MK, protocol, in which mean kinship was minimized (and gene diversity maximized) by pairing males and females with the lowest average kinships to the rest of the population (Ballou & Lacy, 1995; Fernandez, Toro & Caballero, 2004); 2) The docility, DOC, protocol, in which artificial selection for docility was practiced by pairing males and females with the lowest scores for voluntary gnawing and flipping behaviors; and 3) The random, RAN, protocol, in which individuals were assigned to pairs in a random manner. The MK protocol represents the standard genetic management protocol followed for the breeding of many established zoo populations (Ballou, Lees, Faust, Long, Lynch, Bingaman & Foose, 2010). The DOC protocol aimed to mimic the kinds of purposeful or inadvertent selections for docility that often also occur in captive-breeding programs. The RAN protocol serves as a control with no intentional genetic management. Details of the breeding and husbandry protocols and a summary of the changes in reproduction and behaviour across generations are provided in (Lacy et al., 2013). The animal care protocols and experiments described here complied with all current laws and were approved by the Animal Care and Use Committee of the Chicago Zoological Society. A total of 326 mice (161 males and 165 females) from generations 1 (founder population consisting of wild-caught mice), 2, 3, 6 and 9 were included in the present study (Table 1). The original study planned monitoring fGM changes for 5 generations. The decrease in the fGM differences between generations 1, 2 and 3, led us to space the sampling of subsequent generations —allowing for 2 generations between samples — to maximize the probability of capturing meaningful fGM changes during later generations. By including a replicate for each breeding protocol, we can estimate and account for the effects of random genetic drift as a contributing factor to any divergence among experimental populations.

Long-term measurement of fGM, without the confounding effects of reproductive condition, was logistically incompatible with breeding. Given that we had to measure fGM in mice not used for breeding, we therefore could not directly test if fGM levels were associated with higher reproduction

by individual mice, or if there were maternal effects such that the fGM level of the dam influenced the fGM level in her offspring.

Inbreeding

We used the pedigree of the population to calculate the coefficients of inbreeding (*f*) for every individual. The MK breeding protocol is expected to reduce the rate of divergence from the original wild outbred population due to the combined effects of genetic drift, accumulated inbreeding, and selection. Thus, we predicted that this group would present higher glucocorticoid levels than the RAN and DOC groups that would be expected to adapt more rapidly to captivity. Also, as the MK group is expected to overall retain higher levels of additive genetic variation, we predicted it would present higher level of variance in fGM than RAN and DOC.

Sample Collection and Fecal Glucocorticoid Metabolite Extraction and Assay Analyses

Mice used in this study had fecal samples taken weekly (mean ± sd samples/mouse = 21.73± 10.9) when mice were about two months of age, and then continued for about 6 months at which time they were fully adult (mean ± sd age across all samples = 169 ± 36). We used fecal hormone metabolite extraction to reduce any potentially stressful impact caused by more intensive blood sampling protocols for longitudinal hormone monitoring. Samples were collected twice a week when handling them briefly during cage cleaning. They appeared to readily habituate to the weekly protocol. However, given that metabolites are deposited into the feces hours before the handling and collection of the feces, any short-term stress related to handling would not impact fGM measures. All samples were stored in zip-lock bags at -20°C until further processing.

The assay used for fGM analyses was physiologically and biologically validated (Touma & Palme, 2005) as detailed below, and has been shown to be sufficiently sensitive to detect biologically meaningful variation in glucocorticoid concentrations in other species of the same genus (Good, Khan & Lynch, 2003). Prior to extraction, samples were crushed and pulverized, since most samples were very dry and compact. Due to the relatively small size of individual samples (0.2-0.4 g) the entire sample was always used for extraction. To extract steroid metabolites, 0.5ml of 80% ethanol in

194 distilled water was added to the crushed and weighed sample in a 1.5 ml Eppendorf tube (e.g.,
195 Fanson, Wielebnowski, Shenk, Vashon, Squires & Lucas, 2010; Margulis, Atsalis, Bellem &
196 Wielebnowski, 2007). Capped tubes were vortexed for 5 minutes. Subsequently the tubes were
197 placed on a rotator overnight and then centrifuged for 15 min at 1500rpm. 250 µl of supernatant from
198 each sample tube was then transferred into 12x75mm polyethylene tubes and diluted with 750ul of
199 assay buffer. Extracts were stored at -20°C until assay analysis.
200 We used a commercially available corticosterone EIA assay (Arbor Assays, Ann Arbor, MI, USA,
201 catalogue #K014-H) to quantify fecal corticosterone and check for cross reactivity with other
202 glucocorticoids. This assay cross-reacts with corticosterone (100%), deoxycorticosterone (28.6%),
203 and progesterone (1.7%). All other cross-reactivities were less than 1%. Assay analyses were
204 performed according to kit instructions. Briefly, 100µl of standard, control, or sample were added to
205 each well, immediately followed by 50µl of conjugate and antibody. Plates were incubated for 2h
206 while shaking. After washing three times to remove unbound steroids, 200µl of substrate solution
207 was added to each well. The reaction was stopped after 1 hour. Plates were read with a single filter at
208 405nm using an optical density plate reader (Dynex MRX Revelation). All samples were assayed in
209 duplicate. Assay sensitivity was 27 pg/well.
210 The assay was biochemically validated for *Peromyscus* by demonstrating 1) parallelism
211 between serially diluted extracts and the standard curve, and 2) significant (>80%) recovery of
212 exogenous corticosterone added to fecal extracts. To monitor precision and reproducibility, low
213 (~70% binding) and high (~30% binding) quality control samples were run on each plate. Intra-assay
214 coefficients of variation were 7.5% and 11.4% (n=19) for low and high controls, respectively. The
215 inter-assay coefficients of variation were 12.4% and 9.8% (n=75), respectively. Data are expressed as
216 ng/g fecal weight.
217
218 *Physiological Assay Validation*
219 To ensure that the chosen corticosterone EIA assay detects biologically relevant changes in
220 adrenal activity, we conducted an adrenocorticotropic hormone (ACTH) challenge (for more details
221 see ESM: *S1. Physiological Assay Validation* and figure S1). Comparison of results from mice

receiving ACTH injections to control mice receiving saline injections showed that the corticosterone EIA assay appropriately reflected adrenal activity due to ACTH challenge. A 3 to 6-fold increase in fGM concentrations was measured between 4-7 hours after ACTH injection (figure S1).

Biological Assay Validation

We also collected pre- and post-trap samples from 44 (22 males and 22 females) wild-caught mice. Already deposited fecal samples were collected from the trap of each wild-caught mouse as it was removed from the trap. Traps were checked approximately every four hours (based on the measured lag-time as a consequence of ACTH challenges), so that corticosterone measured in the feces should reflect the levels of blood-circulating glucocorticoids prior to capture or "pre-trap" levels (Good et al., 2003). In addition, fecal samples were collected from each animal while they were being handled during removal from the trap and then twice a week for two subsequent weeks while the mice were already in captivity.

Remarkably, among the wild-caught mice about half showed significantly higher pre-trap fGM than after they were brought into captivity. The other half showed low corticosterone, both pre- and post-trapping. Of the mice that showed a marked decline in corticosterone, the decrease typically occurred within one or two days after capture. Overall, we observed considerable variation in corticosterone among mice in both pre-trap and permanent captive conditions (figure S2), thus further demonstrating that the corticosterone EIA assay did detect significant shifts in hormone levels that occurred in response to major events and changes in the environment.

Baseline fGM

We measured individual baseline fGM as the average concentration by individual, calculated from the monthly means. Given that all individuals had fGM measured during the same age interval (6 months), age is not expected to account for differences in baseline fGM between individuals. Initially, arithmetic and harmonic means were calculated, but given the high correlation between the monthly estimates of these two measures (r = 0.94-0.99, $p < 0.0001$) we only used arithmetic means.

As in other studies with rodents (Vuarin, Pillay, Schradin 2019), basal fGM showed repeatability, displaying smaller variation within than between individuals.

Habituation

Habituation is defined as a decrease in an individual's response intensity to a novel stressor as it becomes a familiar stressor due to repetitive experiences (Cyr & Romero, 2009). Individual time series data – not individual average values – were used to test for the effect of habituation to captivity. A significant negative effect of sample collection date on individual fGM was used to ascertain the presence of habituation. Two types of habituation responses were distinguished. First, a habituation response to the change from the wild to the captive environment was tested for mice born in the wild entering the captive breeding program; second, an intra-generational response to the captive environment that may take place over time for individuals born in captivity. This was tested for generations 2, 3, 6 and 9.

Sex and Generation Effects

Given that females of many mammalian species are known to have on average higher circulating serum and fecal glucocorticoid levels than males (Touma, Palme & Sachser, 2004; vom Saal, 1983), *sex* was also included in the analytical models used, and sex-specific models were examined. *Generation number* was also included as adaptation to captivity co-varies with number of generations in captivity and it is expected to influence FGM (Dickens, Earle & Romero, 2009; Millspaugh & Washburn, 2004). Generation was treated as a categorical variable with 5 levels: generation 1, 2, 3, 6 and 9.

Seasonality

In vertebrates, circulating serum glucocorticoid levels often vary seasonally (Harper & Austad, 2001; Millspaugh & Washburn, 2004; Romero, 2002). This seasonality should be considered when trying to explain variation in fGM. In our research facility, seasonal effects on hormone levels were likely minimized since mice were kept in an indoor lab without natural light, under constant

277 photoperiod (14L:10D) and at a room temperature maintained within about 1º C of 21º C. Despite
278 this, it is known that rodents housed in labs can be entrained by cues from slight variations in
279 temperature, humidity or scents carried in by technicians, and wild-caught mice might have a residual
280 endogenous circannual rhythm. We accounted for seasonality by including monthly variation in fGM
281 concentrations.
282
283 *Consistency of fGM concentrations*
284 We tested for a correlation between juvenile and adult fGM to examine whether juvenile
285 differences remain constant throughout life. This is important because, if paternal and maternal
286 behavior affects juvenile fGM and this difference remains constant, this can potentially generate non-
287 genetic inter-generational change in fGM.
288
289 *Inbreeding effects on baseline fGM*
290 We tested for an effect of individual coefficient of inbreeding (*f*) on individual baseline fGM.
291 This analysis could only be conducted for generations 6 and 9 since there was not sufficient variation
292 in *f* in other generations. Given that inbreeding tends to increase with generation and that breeding
293 protocol can also affect inbreeding levels, we initially constructed models testing for the relationship
294 between *f* and fGM controlling for generation and breeding protocol. We also used the sign of the
295 relationship between the trait of interest (baseline fGM) and the coefficient of inbreeding (Ketola &
296 Kotiaho, 2010) to inform us about the relationship between fGM (reflecting circulating serum
297 glucocorticoid levels) and fitness.
298
299 *Statistical Analysis*
300 Habituation within generation was tested by using all available measures for each individual
301 and conducting linear mixed effects models in R using *lmer* and *lme* functions and including
302 individual as a random effect. Habituation for mice born in the wild and transferred to captivity
303 (generation 1, founders) and for mice born in captivity was tested using the same model. Sex and

304 generation effects were also tested on the same model. We tested whether there were differences
305 between monthly measures of fGM within generation and protocol.
306 The impact of seasonality was investigated by including the effect of month on average fGM,
307 together with sex and generation in the same model, and alternatively including and excluding the
308 founder generation, as wild-caught mice might retain a seasonality effect that might not be seen in
309 captive-born mice. A factorial ANOVA model was employed to test for the effect of sex, generation,
310 and their respective interaction, on fGM. Since the first generation was not the result of the three
311 breeding protocols we applied to future generations, this first generation had to be excluded from the
312 analyses testing the effects of breeding protocol. Generation 9 did not have a replicate population for
313 the DOC protocol (as it went extinct in generation number 8; (Lacy et al., 2013). The associations
314 between fGM and morphological, behavioural and reproductive traits were all tested together in the
315 same models to account for between-trait correlations. The presence of inbreeding depression was
316 examined by regressing fGM on inbreeding levels (f) by generation. To gain insight into potential
317 sex-specific inbreeding effects separate regressions were then conducted for males and females.
318
319 **Results**
320 *Effect of Sex and Generation*
321 Figure 1 shows yearly variation in fGM levels by sex in the founder (wild) population and
322 the seasonal variation in fGM levels by generation and sex in the captive population. Accounting for
323 the effects of time in captivity and generation, the model shows that males had significantly lower
324 fGM than females ($\beta \pm se$ = -223.35 ± 59.50, t = -3.75, df = 322, p = 0.0002, figure S3). FGM
325 decreased as the number of generations in captivity increased ($\beta \pm se$ = -3812.55 ± 427.12, t = -8.92,
326 df = 322, $p < 0.0001$).
327
328 *Effect of Breeding Protocol and Replicates*
329 Figure 2 shows the raw data by breeding protocol and generation. We ran separate models for
330 each sex, nesting breeding protocol within generation. For females, when accounting for the
331 significant effect of generation (Mean and Variance in fGM, $F_{3,143}$ = 43.6, $p < 0.0001$, r^2 = 0.49;

$F_{3,143}$=8.4, $p < 0.0001$, $r^2 = 0.18$), there was no effect of breeding protocol on fGM ($p > 0.2$). Similarly, no significant differences among breeding protocols were observed in males. Results for replicate populations were not significant in any model either, suggesting no significant effect of random genetic drift among populations on baseline fGM. Independent models run per generation also confirmed there was no effect of protocol on the mean or variance of fGM.

Habituation

Variation in fGM is best explained by the model that included time in captivity (date), sex, generation, and the interactions between generation and sex and between date and generation (29% deviance; table 2). Specific results for this model show that, overall, mice show habituation to captivity, as illustrated by the negative association between time in captivity (date) and fGM ($\beta \pm se$ = -0.95 ± 0.078, t = -12.13, df = 6756, $p < 0.0001$). However, the significant effect of the interaction between time in captivity and generation ($\beta \pm se$ = 0.10 ± 0.01, t = 8.92, df = 6756, $p < 0.0001$) indicates that there is heterogeneity across generations (Table 2). Further generation-specific models show that the strength of the relationship to time in captivity changed with generation and even the sign in the last generation ($\beta \pm se$ by generation: G1, -1.10 ± 0.21; G2, -0.19 ± 0.32; G3, -0.70 ± 0.21; G6, -0.05 ± 0.10; G9, 0.26 ± 0.14). Thus, habituation effects are stronger in the first generation of the captive breeding program than later, when they weaken out, not being present after generation 3 (Figure 1).

Seasonality

To examine the effect of month (1-12) on fGM, a model including sex and generation as categorical predictors ($r^2 = 0.36$, $F_{26,1829} = 40.48$, $p < 0.001$) was used (this model excluded the founder generation).The results showed a statistically significant, but very small, effect of month ($F_{3,1829} = 2.00$, $p = 0.025$, figure S4), which was, respectively, one and two orders of magnitude smaller than the effects of sex and generation ($F_{1,1829} = 88.72$, $p < 0.0001$; $F_{3,1829} = 247.22$, $p <$ 0.0001, respectively). FGM remained stable from December to May, increased from June to August,

and decreased to reach a minimum in November. There was no sex by month interaction, showing that fGM fluctuated monthly in a similar way for males and females (figure S4).

Consistency of fGM concentrations within individuals

There was a strong correlation between juvenile and adult average fGM values ($r = 0.47$, $p < 0.001$, $n = 57$, figure S5), showing there are consistent baseline fGM differences between individuals that do not change with ontogeny.

Inbreeding effects on fGM

Significant variation in inbreeding levels within generation started to appear from generation 6, thus we present within generation results for generations 6 and 9. In generation 6, inbreeding levels negatively affected fGM ($F_{1,125} = 10.7$, $r^2 = 0.07$, $\beta = -0.31$, CI=-0.50, -0.12). This relationship, although significant in the overall model, remained only significant for the MK protocol (figure 3A), probably because variation in *f* and fGM was higher in MK than in DOC and RAN, providing higher statistical power to detect any inbreeding effects. In generation 9, *f* also showed a significant negative effect on average fGM ($F_{1,28} = 6.43$, $r^2 = 0.16$, $p = 0.017$, $\beta = -0.43$, CI=-0.78, -0.08, figure 3B) with similar effect size as in generation 6. Effect sizes were again stronger for the MK protocol, which had greater variance in *f*. Given that both sexes showed differences in fGM, we subsequently tested for potential effects of inbreeding depression on fGM for each sex separately in generations 6 and 9. Although both sexes showed a negative slope for the relationship between *f* and fGM, this association was significant only in females (G6, females: $r = -0.54$, $p = 0.0041$; males: $r = -0.35$, $p = 0.096$; G9, females: $r = -0.61$, $p = 0.035$; males: $r = -0.15$, $p = 0.64$).

Discussion

We examined how the physiological stress response, as measured by variation in average fGM over time, changes after a founder population of a small mammal species, the white footed mouse *Peromyscus leucopus*, has been removed from the wild and propagated in captivity for several generations. Baseline levels of corticosteroids might change in the captive environment because of

habituation by individuals over their lifetimes, evolution of lower stress response as animals adapt to the more benign captive environment, or depression of fitness traits as small captive populations become increasingly inbred. After potentially confounding factors such as sex, age and seasonality were accounted for statistically, we show that as the number of generations in captivity increased and mice became partly inbred, their baseline fGM decreased, suggesting a possible reduction in mouse responsiveness to stressors. This reduction across generations explained more of the variance in fGM than did the accumulated inbreeding, and it occurred across breeding protocols. The populations adapted to captivity by showing increased activity levels and increased reproduction across generations (Lacy et al., 2013). Thus, adaptation to captivity – which is by definition a reduction in the adaptation to wild habitats – was associated with lower baseline fGM levels.

There was also a significant negative effect of inbreeding on fGM across and within generations, indicating a positive association of fGM, and hence of corticosterone, with fitness in the wild. There is a difference between what leads to high fitness in the wild as compared to captivity. The expected relationship of inbreeding depression with "fitness traits" should be driven by the genetic architecture that evolved under natural selection in the wild. Thus, depression of a trait under inbreeding would indicate that the trait was likely important for fitness in the wild but might not be associated in the same way (or at all) with fitness in captivity.

We observed no effects of the other two sources of genetic change: random genetic drift (which would cause variation among replicates) and artificial selection on behavioral traits (breeding protocol). This has implications for the debates regarding the relationship between fitness and the glucocorticoid response to stress – as it appears that higher glucocorticoid levels may be adaptive in the wild, while reduced glucocorticoid levels may result from adaptation to captivity. This evolution to potentially lower stress responsiveness in captivity also has implications for conservation programs that might be considering releasing animals from long-term captive populations back into the wild environment.

The strong effect of generation could be augmented by habituation of individuals within each generation. Habituation occurs when an individual learns to perceive a repeated stressor as innocuous, reducing stress hormones secretion over time (Cyr & Romero, 2009; Dickens et al., 2009). Here, we

show that habituation plays a role in the reduction of baseline (average) fGM when mice were brought into captivity. This reduction in fGM over time occurred also in each of the first few captive generations but was absent in generation 6 and 9. FGM decreased between generations as well, indicating either a genetic effect or non-genetic transmission from parents to offspring. We cannot rule out that other potential factors generally known to also affect glucocorticoid concentrations, such as reduced seasonal responsiveness, exhaustion or desensitization are also be playing a role (Cyr & Romero, 2009) in this decrease.

In our study, baseline fGM early in life substantially predicted concentrations measured in adults, so that parental effects causing low fGM in offspring might be transmitted across generations. Given that habituation to captivity reduces stress response throughout their lives, this could lead to a non-genetic basis for the reductions accumulating across generations.

The relationship between fitness and baseline glucocorticoid levels remains controversial. Across taxa there are more examples of negative than positive or non-significant associations (Bonier et al., 2009). However, three underlying assumptions required to make inferences between phenotypic traits and overall fitness, are rarely fulfilled in empirical studies: 1) the repeatability of glucocorticoid measures over time, 2) repeatability of glucocorticoid-fitness relationship, or 3) the appropriateness of the fitness metrics. In our study we have tried to address these. First, our measure is calculated averaging monthly baseline fGM (up to 4 samples per month), across 6 months per individual. This allows us to reliably measure individual baseline fGM concentrations which have been shown to be reflective of circulating serum fGM levels. Second, by keeping mice under the experimental conditions of a constant environment, we minimize the environmentally driven 'noise' that so often reduces glucocorticoid-fitness repeatability measures in field studies. Third, regarding the appropriateness of the fitness metrics, most studies use a single indirect surrogate of either reproductive or viability fitness. The difficulty of inferring what phenotypic traits reflect higher fitness was shown in that levels of stereotypic behaviors in these experimental populations were positively associated with reproductive success (Lacy et al., 2013), opposite the intuitive prediction. Here we use an unbiased overall measure of the association between fitness and baseline fGM levels, inferred from the sign of the relationship between inbreeding and the trait.

Inbreeding depression —the decrease in fitness due to the expression of recessive deleterious alleles that occurs in homozygotes (Charlesworth & Charlesworth, 1987) – occurs in fitness-related traits (DeRose & Roff, 1999; Lynch & Walsh, 1998), and inbreeding has been observed to negatively impact many components of fitness in *Peromyscus* mice (Brewer, Lacy, Foster & Alaks, 1990; Lacy & Alaks, 2013; Lacy, Alaks & Walsh, 1996; Ryan, Lacy & Margulis, 2003). With this theoretical and empirical basis for inbreeding depression and fitness, various studies have used the relationship between inbreeding and phenotypic traits to infer the direction and strength of relationship between fitness and a phenotypic trait (Ketola & Kotiaho, 2010; Malo et al., 2010; Malo et al., 2017). Inbreeding depression in fitness traits is the evolutionary outcome of long-term selection, long before the captive breeding program started. Traits favored by selection over evolutionary time would be the ones reduced under inbreeding. The inbreeding depression that we observed in glucocorticoid secretion as measured by FGM therefore suggests that the relationship between baseline glucocorticoid levels and fitness is generally positive in this species. Thus, our results strongly support the idea that in the wild non-captive population of *Peromyscus leucopus,* selection favored individuals with higher baseline glucocorticoid levels, contradicting the Cort-fitness hypothesis (Bonier et al., 2009) and supporting the Cort-adaptation hypothesis.

The transition from a wild to a captive environment represents a dramatic example of environmental change that can have important fitness consequences (Araki, Cooper & Blouin, 2007). In captivity, increases in the stress response do not increase survival through reduced predation as they would in the wild, and could even decrease it if stress increases the probability of injury in captivity. Thus, the relationship between glucocorticoid secretion and some fitness metrics could be erased or reversed. However, this does not happen as otherwise the relationship between inbreeding and glucocorticoids would have been positive, and we detect inbreeding depression, a negative relationship with inbreeding. This shows that fGM, and hence corticosterone, was adaptive in the wild population. This is also supported by the evidence that the relationship between inbreeding and fGM remains unaltered (inbred individuals present weaker response to stress) after hundreds of generations of domestication (Kosowska, 1992; Kosowska & Zdrojewicz, 1989; Kosowska & Zdrojewicz, 1991a; Kosowska & Zdrojewicz, 1991b). A possible explanation is that is that there is a weak or no positive

selection for low glucocorticoid levels in captivity (or negative for high), or because the strength of the genetic architecture of fitness-related traits is such that a reversal cannot occur. This is supported by the fact that, across species, the traits that are impacted by inbreeding depression in domesticated and laboratory populations are the same that are impacted by inbreeding in their wild counterparts (Falconer & Mackay, 1996). Our results suggest that baseline adrenal activity and mounted stress response is positively associated with fitness. Further research will be needed to verify directly that the glucocorticoid levels are associated with reproductive success of individual mice.

Acknowledgements

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References

Araki H, Cooper B, Blouin MS. 2007. Genetic effects of captive breeding cause a rapid, cumulative fitness decline in the wild. *Science* 318: 100-103.

Ballou J, Lees C, Faust L, Long S, Lynch C, Bingaman L, Foose T. 2010. Demographic and genetic management of captive populations. In: Kleiman D, Thompson K and Baer C, eds. *Wild mammals in captivity: principles and techniques for zoo management.* . 2nd ed ed. Chicago: University of Chicago Press. 219–252.

Ballou JD, Lacy RC. 1995. Identifying genetically important individuals for management of genetic variation in pedigreed populations. In: Ballou JD, Gilpin M and Foose TJ, eds. *Population Management for Survival and Recovery: Analytical Methods and Strategies in Small Population Conservation*. New York: Columbia University Press. 76-111.

497 Balmford A. 2000. Priorities for captive breeding - which mammals should board the ark? In:
498 Entwistle A and Dunstone N, eds. *Priorities for Conservation of Mammalian Diversity*.
499 Cambridge: Cambridge University Press. 291-307.
500 Blas J, Bortolotti GR, Tella JL, Baos R, Marchant TA. 2007. Stress response during development
501 predicts fitness in a wild, long lived vertebrate. *Proceedings of the National Academy of*
502 *Sciences of the United States of America* 104: 8880-8884.
503 Bonier F, Martin PR, Moore IT, Wingfield JC. 2009. Do baseline glucocorticoids predict fitness?
504 *Trends in Ecology & Evolution* 24: 634-642.
505 Boyle SA, de la Sancha NU, Pérez P, Kabelik D. 2021. Small mammal glucocorticoid concentrations
506 vary with forest fragment size, trap type, and mammal taxa in the Interior Atlantic Forest.
507 *Scientific Reports* 11: 2111.
508 Brewer BA, Lacy RC, Foster ML, Alaks G. 1990. Inbreeding depression in insular and central
509 populations of Peromyscus mice. *Journal of Heredity* 81: 257-266.
510 Cabezas S, Blas J, Marchant TA, Moreno S. 2007. Physiological stress levels predict survival
511 probabilities in wild rabbits *Horm. Behav.* 51: 313-320.
512 Charlesworth B. 2009. Effective population size and patterns of molecular evolution and variation.
513 *Nature Reviews Genetics* 10: 195-205.
514 Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences.
515 *Annual Review in Ecology and Systematics* 18: 237-268.
516 Christie MR, Marine ML, French RA, Blouin MS. 2012. Genetic adaptation to captivity can occur in
517 a single generation. *Proceedings of the National Academy of Sciences of the United States of*
518 *America* 109: 238-242.
519 Comendant T, Sinervo B, Svensson EI, Wingfield J. 2003. Social competition, corticosterone and
520 survival in female lizard morphs. *Journal of Evolutionary Biology* 16: 948-955.
521 Crossin GT, Trathan PN, Phillips RA, Gorman KB, Dawson A, Sakamoto KQ, Williams TD. 2012.
522 Corticosterone Predicts Foraging Behavior and Parental Care in Macaroni Penguins.
523 *American Naturalist* 180: E31-E41.

524 Cyr NE, Romero LM. 2009. Identifying hormonal habituation in field studies of stress. *General and*
525 *Comparative Endocrinology* 161: 295-303.
526 DeRose MA, Roff DA. 1999. A comparison of inbreeding depression in life-history and
527 morphological traits in animals. *Evolution* 53: 1288-1292.
528 Dickens MJ, Earle KA, Romero LM. 2009. Initial transference of wild birds to captivity alters stress
529 physiology. *General and Comparative Endocrinology* 160: 76-83.
530 Dickens MJ, Romero LM. 2013. A consensus endocrine profile for chronically stressed wild animals
531 does not exist. *General and Comparative Endocrinology* 191: 177-189.
532 Dingemanse NJ, Edelaar P, Kempenaers B. 2010. Why is there variation in baseline glucocorticoid
533 levels? *Trends in Ecology & Evolution* 25: 261-262.
534 Escribano-Avila G, Pettorelli N, Virgos E, Lara-Romero C, Lozano J, Barja I, Cuadra FS, Puerta M.
535 2013. Testing Cort-Fitness and Cort-Adaptation hypotheses in a habitat suitability gradient
536 for roe deer. *Acta Oecologica-International Journal of Ecology* 53: 38-48.
537 Falconer DS, Mackay TFC. 1996. *Introduction to Quantitative Genetics*. Longman: Essex.
538 Fanson KV, Wielebnowski NC, Shenk TM, Vashon JH, Squires JR, Lucas JR. 2010. Patterns of
539 ovarian and luteal activity in captive and wild Canada lynx (Lynx canadensis). *General and*
540 *Comparative Endocrinology* 169: 217-224.
541 Fernandez J, Toro MA, Caballero A. 2004. Managing individuals' contributions to maximize the
542 allelic diversity maintained in small, conserved populations. *Conservation Biology* 18: 1358-
543 1367.
544 Frankham R. 2008. Genetic adaptation to captivity in species conservation programs. *Molecular*
545 *Ecology* 17: 325-333.
546 Frankham R, Ballou JD, Briscoe DA. 2002. *Introduction to Conservation Genetics*. Cambridge
547 University Press: Cambridge, UK.
548 Frankham R, Loebel DA. 1992. Modeling Problems in Conservation Genetics Using Captive
549 Drosophila Populations - Rapid Genetic Adaptation to Captivity. *Zoo Biology* 11: 333-342.

550 Good T, Khan MZ, Lynch JW. 2003. Biochemical and physiological validation of a corticosteroid
551 radioimmunoassay for plasma and fecal samples in oldfield mice (*Peromyscus polionotus*).
552 *Physiology & Behavior* 80: 405-411.
553 Harper JM, Austad SN. 2001. Effect of capture and season on fecal glucocorticoid levels in deer mice
554 (Peromyscus maniculatus) and red-backed voles (Clethrionomys gapperi). *General and*
555 *Comparative Endocrinology* 123: 337-344.
556 Ketola T, Kotiaho JS. 2009. Inbreeding, energy use and sexual signaling. *Evolutionary Ecology* 24:
557 761-772.
558 Ketola T, Kotiaho JS. 2010. Inbreeding, energy use and sexual signaling. *Evolutionary Ecology* 24:
559 761-772.
560 Ketola T, Kotiaho JS. 2012. Inbreeding depression in the effects of body mass on energy use.
561 *Biological Journal of the Linnean Society* 105: 309-317.
562 Ketterson ED, Nolan Jr V. 1992. Hormones and lifehistories: an integrative approach. *American*
563 *Naturalist* 140: S33-S62.
564 King CM. 1985. Interactions between woodland rodents and their predators. In Flowerdew JR, ed.
565 Symp. Zool. Soc. Lond. London, pp. 219-247.
566 Kosowska B. 1992. The effect of genetic-variability (degree of homozygosity) on serum levels of the
567 anterior-pituitary hormones prolactin, corticotropin, and growth-hormone in rats. *Biochemical*
568 *Genetics* 30: 581-589.
569 Kosowska B, Zdrojewicz Z. 1989. Adrenal-medulla hormone response (adrenaline, noradrenaline,
570 dopamine) to stress in rats of various inbreeding levels. *Journal of Animal Breeding and*
571 *Genetics* 106: 455-460.
572 Kosowska B, Zdrojewicz Z. 1991a. The role of genetic variability in the hormonal adaptative
573 mechanism ii. histometric changes in the width of glomerular and fascicular layers of adrenal
574 cortex in rats under the influence of inbreeding and stress. *Journal of Animal Breeding and*
575 *Genetics* 108: 156-160.
576 Kosowska BA, Zdrojewicz Z. 1991b. The role of genetic variability in the hormonal adaptative
577 mechanism i. the effect of various homozygosity level on the adrenal cortex hormone

concentrations corticosterone and aldosterone in the blood serum of rats exposed to two types of stress. *Journal of Animal Breeding and Genetics* 108: 148-153.

Lacy RC. 1993. Impacts of inbreeding in natural and captive populations of vertebrates: implications for conservation. *Perspectives in Biology and Medicine* 36: 480-496.

Lacy RC. 2009. Stopping evolution: Genetic management of captive populations. In: G. Amato RD, O.A. Ryder, and H.C. Rosenbaum, ed. *Conservation genetics in the age of genomics*. New York: Columbia University Press. Pages 58-81

Lacy RC, Alaks G. 2013. Effects of inbreeding on skeletal size and fluctuating asymmetry of Peromyscus polionotus mice. *Zoo Biology* 32: 125-133.

Lacy RC, Alaks G, Walsh A. 1996. Hierarchical analysis of inbreeding depression in Peromyscus polionotus. *Evolution* 50: 2187-2200.

Lacy RC, Alaks G, Walsh A. 2013. Evolution of Peromyscus leucopus mice in response to a captive environment. *PLoS ONE* 8: e72452.

Lacy RC, Malo AF, Alaks G. 2018. Maintenance of genetic variation in quantitative traits of a woodland rodent during generations of captive breeding. *Conservation Genetics* 19: 789-802.

Lynch M, Walsh B. 1998. Genetics and analysis of quantitative traits *Genetics and analysis of quantitative traits*.

Mallet MA, Chippindale AK. 2011. Inbreeding reveals stronger net selection on Drosophila melanogaster males: implications for mutation load and the fitness of sexual females. *Heredity* 106: 994-1002.

Malo AF, Martinez-Pastor F, Alaks G, Dubach J, Lacy RC. 2010. Effects of genetic captive-breeding protocols on sperm quality and fertility in the white-footed mouse. *Biology of Reproduction* 83: 540-548.

Malo AF, Martinez-Pastor F, Garcia-Gonzalez F, Garde J, Ballou JD, Lacy RC. 2017. A father effect explains sex-ratio bias. *Proc. R. Soc. B.* 284: 20171159.

Margulis SW, Atsalis S, Bellem A, Wielebnowski N. 2007. Assessment of reproductive behavior and hormonal cycles in geriatric western lowland gorillas. *Zoo Biology* 26: 117-139.

605 Marti O, Armario A. 1998. Anterior pituitary response to stress: Time-related changes and adaptation.
606 *International Journal of Developmental Neuroscience* 16: 241-260.
607 McEwen BS. 1998. Stress, adaptation, and disease: Allostasis and allostatic load *Annals of the New*
608 *York Academy of Sciences; Neuroimmunomodulation: Molecular aspects, integrative systems,*
609 *and clinical advances*. 33-44.
610 McEwen BS, Wingfield JC. 2003. The concept of allostasis in biology and biomedicine. *Hormones*
611 *and Behavior* 43: 2-15.
612 McPhee ME. 2004. Generations in captivity increases behavioral variance: considerations for captive
613 breeding and reintroduction programs. *Biological Conservation* 115: 71-77.
614 Meylan S, Clobert J. 2005. Is corticosterone-mediated phenotype development adaptive? - Maternal
615 corticosterone treatment enhances survival in male lizards. *Hormones and Behavior* 48: 44-
616 52.
617 Millspaugh JJ, Washburn BE. 2004. Use of fecal glucocorticold metabolite measures in conservation
618 biology research: considerations for application and interpretation. *General and Comparative*
619 *Endocrinology* 138: 189-199.
620 O'Regan HJ, Kitchener AC. 2005. The effects of captivity on the morphology of captive,
621 domesticated and feral mammals. *Mammal Review* 35: 215-230.
622 Price GR. 1972. Fisher's fundamental theorem made clear. *Annals of Human Genetics* 36: 129-&.
623 Romero LM. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living
624 vertebrates. *General and Comparative Endocrinology* 128: 1-24.
625 Ryan KK, Lacy RC, Margulis SW. 2003. Impacts of inbreeding on components of reproductive
626 success. In: Holt WV, Pickard AR, Rodger JC and Wildt DE, eds. *Reproductive Science and*
627 *Integrated Conservation*. Cambridge, UK: Cambridge University Press. 82-96.
628 Soorae PS. 2016. *Global Re-introduction Perspectives, 2016: Case-studies from Around the Globe*.
629 IUCN/SSC Re-introduction Specialist Group: Abu Dhabi, UAE.
630 Tokarz RR. 1987. Effects of corticosterone treatment on male aggressive behavior in a lizard (Anoiss
631 sagrei). *Hormones and Behavior* 21: 358-370.

Touma C, Palme R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation *Bird Hormones and Bird Migrations: Analyzing Hormones in Droppings and Egg Yolks and Assessing Adaptations in Long-Distance Migration*. 54-74.

Touma C, Palme R, Sachser N. 2004. Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. *Hormones and Behavior* 45: 10-22.

vom Saal FS. 1983. The interaction of circulating oestrogens and androgens in regulating mammalian sexual differentiation. *Hormones and behavior in higher vertebrates*. Berlin: Springer. 159-177.

Vuarin P, Pillay N, Schradin C. 2019. Elevated basal corticosterone levels increase disappearance risk of light but not heavy individuals in a long-term monitored rodent population. *Hormones and Behavior* 113: 93-102.

Williams SE, Hoffman EA. 2009. Minimizing genetic adaptation in captive breeding programs: A review. *Biological Conservation* 142: 2388-2400.

Zera AJ, Harshman LG. 2001. The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics* 32: 95-126.

FIGURES

Figure Legends

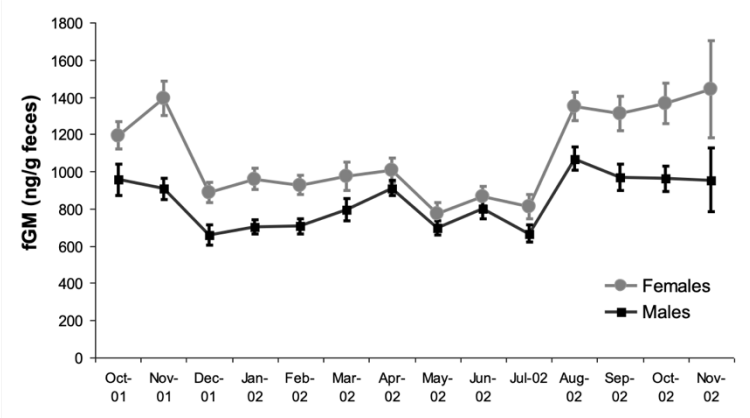
Figure 1. (A) Yearly variation in fGM levels by sex in the wild (founder) population (top left) and (B) seasonal variation in fGM levels by sex in the captive population (top right). (C) Seasonal variation in fGM levels by generation in females (bottom left) and (D) in males (bottom right). Error bars indicate standard errors.

Figure 2. Yearly and seasonal variation in mean annual fGM levels for the docility (DOC), random (RAN) and mean kinship (MK) protocol for males (A) and females (B).

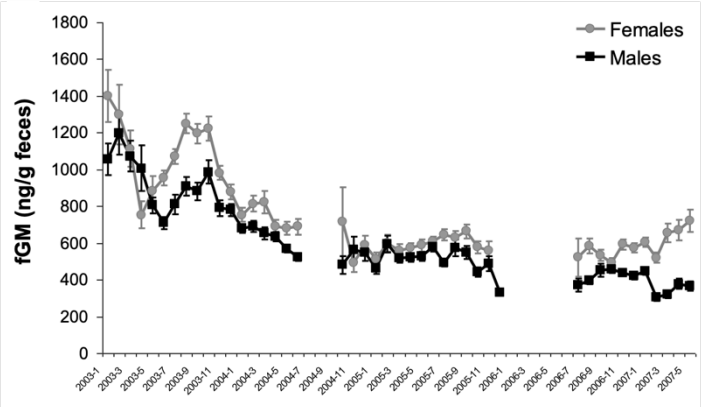
Figure 3. Inbreeding effects on mean annual fGM for MK protocol (A) generation 6 and (B) generation 9.

Figure 1.

A



B



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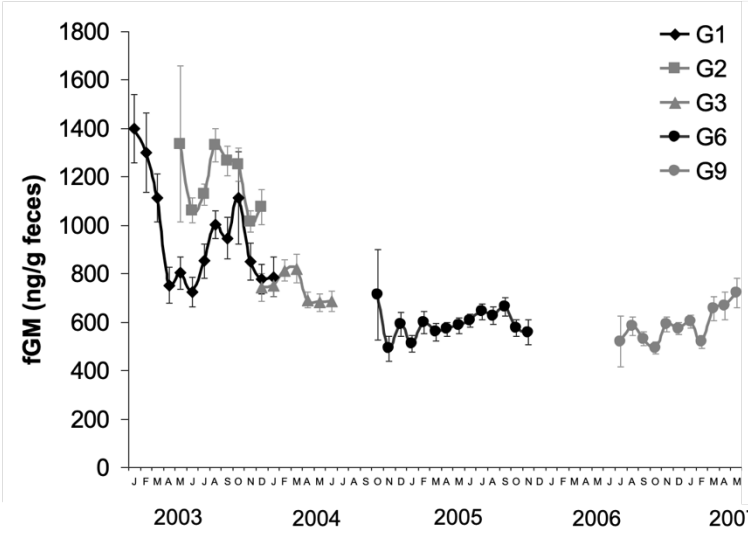

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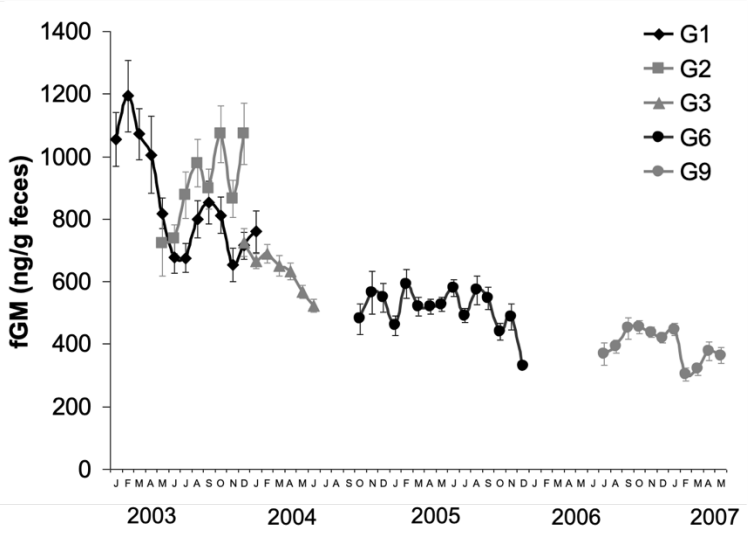
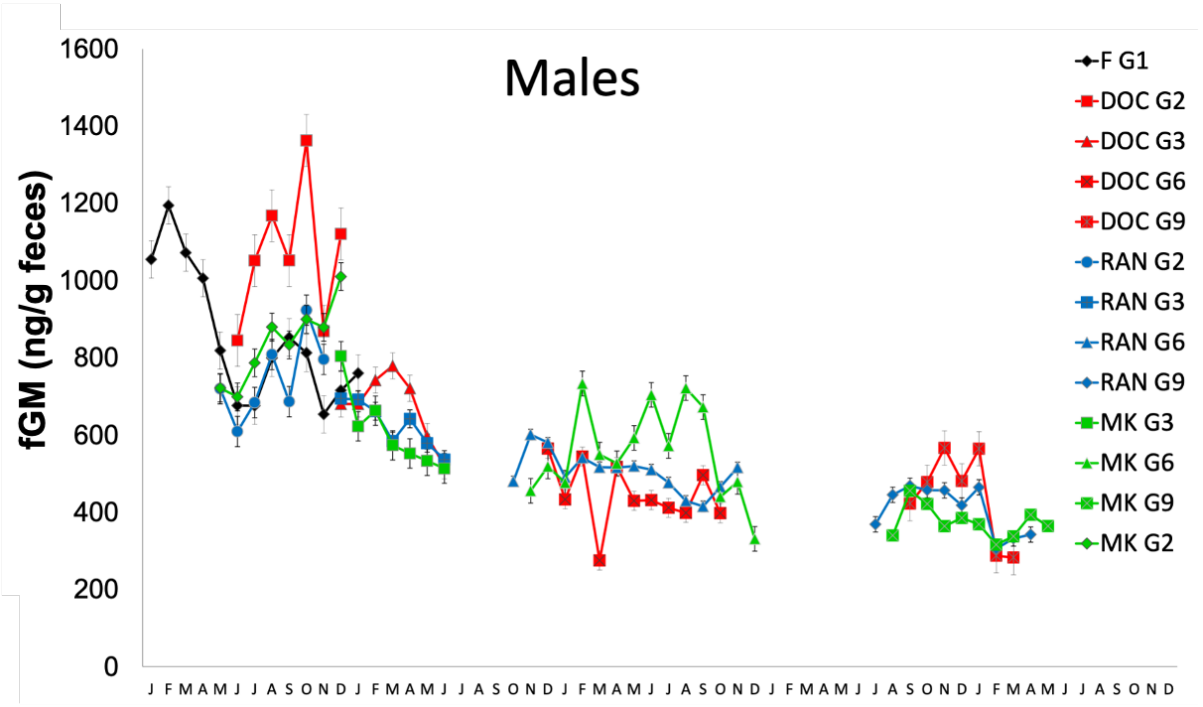


Figure 2.

A)


Males
fGM (ng/g feces)
0
200
400
600
800
1000
1200
1400
1600
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
F G1
DOC G2
DOC G3
DOC G6
DOC G9
RAN G2
RAN G3
RAN G6
RAN G9
MK G3
MK G6
MK G9
MK G2

B)

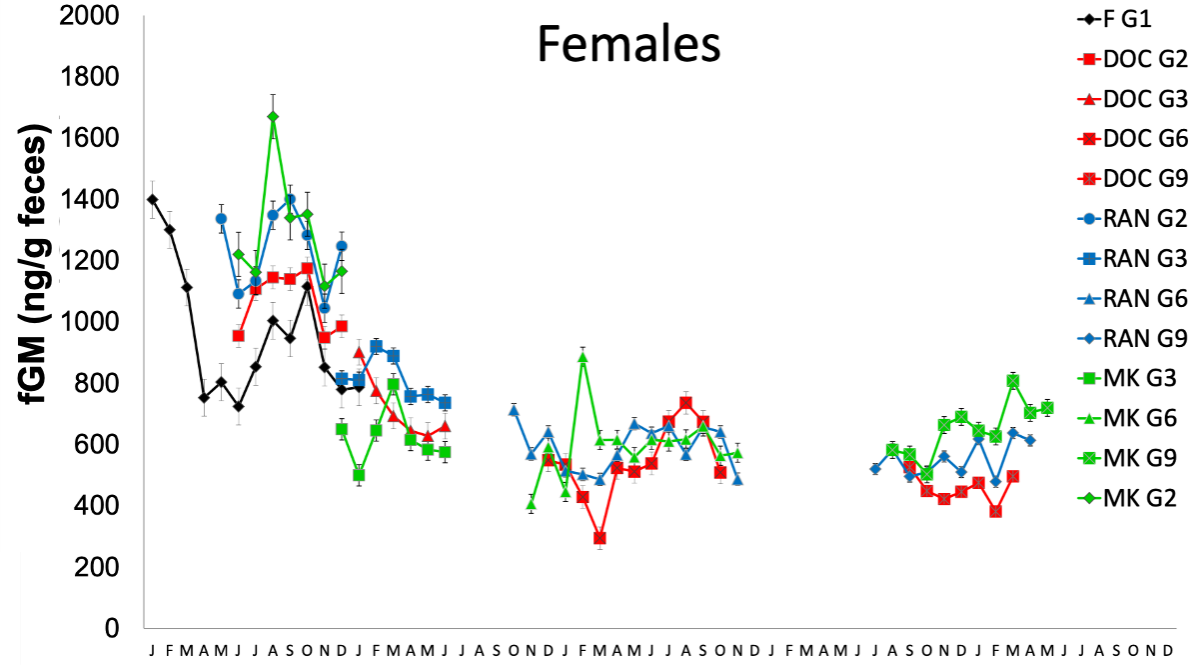
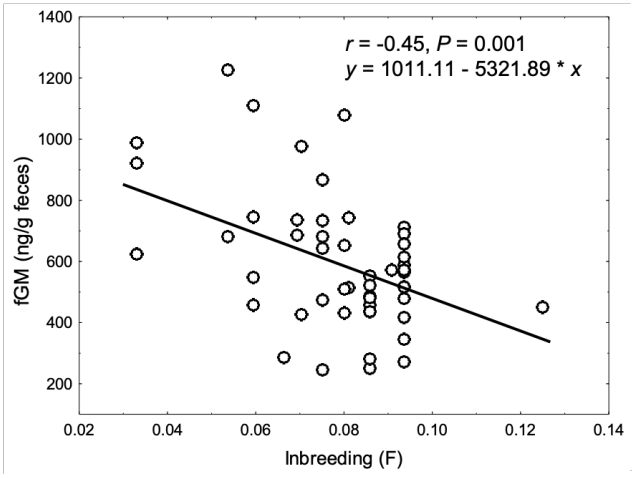
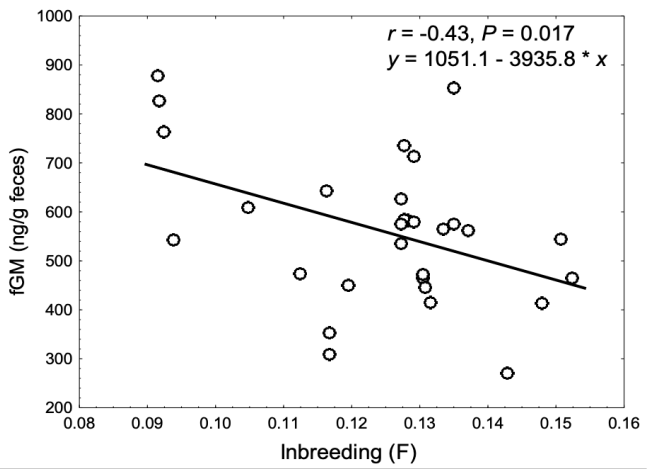

Females
fGM (ng/g feces)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
F G1
DOC G2
DOC G3
DOC G6
DOC G9
RAN G2
RAN G3
RAN G6
RAN G9
MK G3
MK G6
MK G9
MK G2

Figure 3.

A)


r = -0.45, P = 0.001
y = 1011.11 - 5321.89 * x
fGM (ng/g feces)
Inbreeding (F)
1400
1200
1000
800
600
400
200
0.02
0.04
0.06
0.08
0.10
0.12
0.14

B)


r = -0.43, P = 0.017
y = 1051.1 - 3935.8 * x
fGM (ng/g feces)
Inbreeding (F)
1000
900
800
700
600
500
400
300
200
0.08
0.09
0.10
0.11
0.12
0.13
0.14
0.15
0.16

Tables

Table 1. Number of mice used in the present study by generation, breeding protocol and sex. G stands for generation; G1 is the founder generation.

	G1	G2				G3				G6				G9				
		Doc	Ran	MK	**Total**	Doc	Ran	MK	**Total**	Doc	Ran	MK	**Total**	Doc	Ran	MK	**Total**	**Grand Total**
Females	**10**	16	12	8	**36**	6	12	6	**24**	18	21	26	**65**	6	12	12	**30**	**165**
Males	**10**	8	2	10	**20**	12	12	12	**36**	18	23	24	**65**	6	12	12	**30**	**161**
Total	**20**	**24**	**14**	**18**	**56**	**18**	**24**	**18**	**60**	**36**	**44**	**50**	**130**	**12**	**24**	**24**	**60**	**326**

Table 2. Summary table of the performance of the generalised linear models constructed in R using the *lmer* function to test the effect of habituation. The model with the lowest D.AICc is the most strongly supported. In the best model (top), the main effects of *date* (time in captivity)*, generation* and *sex* account for 17%, 12% and 5% of variation in baseline fGM levels (all $p < 0.001$), while the two interactions included, *date*generation (*P<0.001) and *sex*generation(*P=0.049) account for 7% and 1% variation, respectively.

Model	log(l)	K	AICc	D.AICc	wi	%DE
FGM ~ Date + Sex + Gen + Sex * Gen + Date * Gen + (1 | ID)	**-51672**	**6**	**103357**	**0.000**	**1.00**	**0.290**
FGM~ Date + Sex + Gen + Sex * Gen + (1 | ID)	-51707	5	103424	67.0	2.72e-15	0.220
FGM ~ Date + Sex + Gen + (1 | ID)	-51712	4	103432	75.5	3.90e-17	0.210
FGM ~ Date + Sex + (1 | ID)	-51715	3	103437	80.6	3.11e-18	0.210
FGM ~ Date + Gen + (1 | ID)	-51728	3	103462	104.8	1.68e-23	0.180
FGM ~ Date + (1 | ID)	-51731	2	103467	109.9	1.31e-24	0.170
FGM ~ Sex + Gen + Sex * Gen + (1 | ID)	-51740	4	103488	130.8	3.80e-29	0.160
FGM ~ Sex + Gen + (1 | ID)	-51745	3	103496	139.4	5.14e-31	0.150
FGM ~ Gen + (1 | ID)	-51762	2	103528	170.8	7.96e-38	0.120
FGM ~ Sex + (1 | ID)	-51807	2	103619	262.3	1.08e-57	0.028
Null	-51822	1	103646	289.1	1.60e-63	0.000

The top ranked model (in bold) was used as a base model to test for the effects of habituation, sex and generation. For each model, we present the log-likelihood, the number of parameters (*K*), the Akaike's Information Criterion corrected for finite sample sizes (*AICc*), the difference in Akaike's Information Criterion (*D.AICc*), Akaike weight (w*i*) and the deviance explained (*%DE*).

Electronic Supplementary Material

Inbreeding and adaptation to captivity depress the response to stress

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Contents:

S1. Physiological Assay Validation

An adrenocorticotropic hormone (ACTH) challenge was conducted to ensure that our assay detected biologically relevant changes in adrenal activity. ACTH gel (Corticotrophin, Wedgewood Pharmacy, Swedesboro, NJ) was administered as a single intra-peritoneal injection using the following calculation: 20 IU/kg which resulted in an average injection amount for each mouse of 0.44 IU (mouse average weight=22.2g). As control we injected the same amount of saline solution on the same mice at a different time. First, we injected ACTH in 3 mice (2 males, 1 female) and saline in another 3 mice (1 male, 2 females). Two weeks later we reversed the experiment. The two types of injections allowed us to ensure that the ACTH dose administered was sufficient to elicit an adrenal response, rather than solely a response to the injection itself. For both trials, daily fecal samples were collected for 7 days prior to the challenge and 7 days following the challenge. In addition, every individual sample excreted during the 24 hours following the injection was collected. This sampling schedule helped us to determine the lag time between changes in circulating hormone levels and changes in fecal hormone metabolite concentrations.

Figure S1. Example of ACTH challenge responses in 3 different individuals (2 females and 1 male) of *Peromyscus leucopus*.

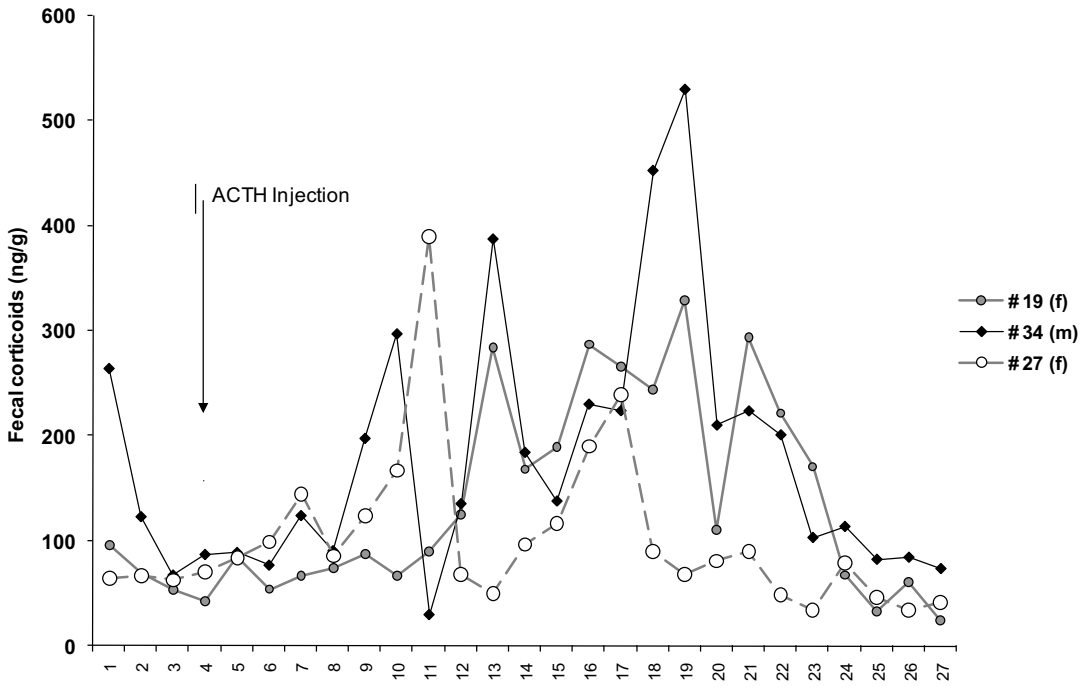

Figure S2. Means and standard errors of in-trap and post-trap fecal glucocorticoid metabolite concentrations in *Peromyscus leucopus* (N=44).

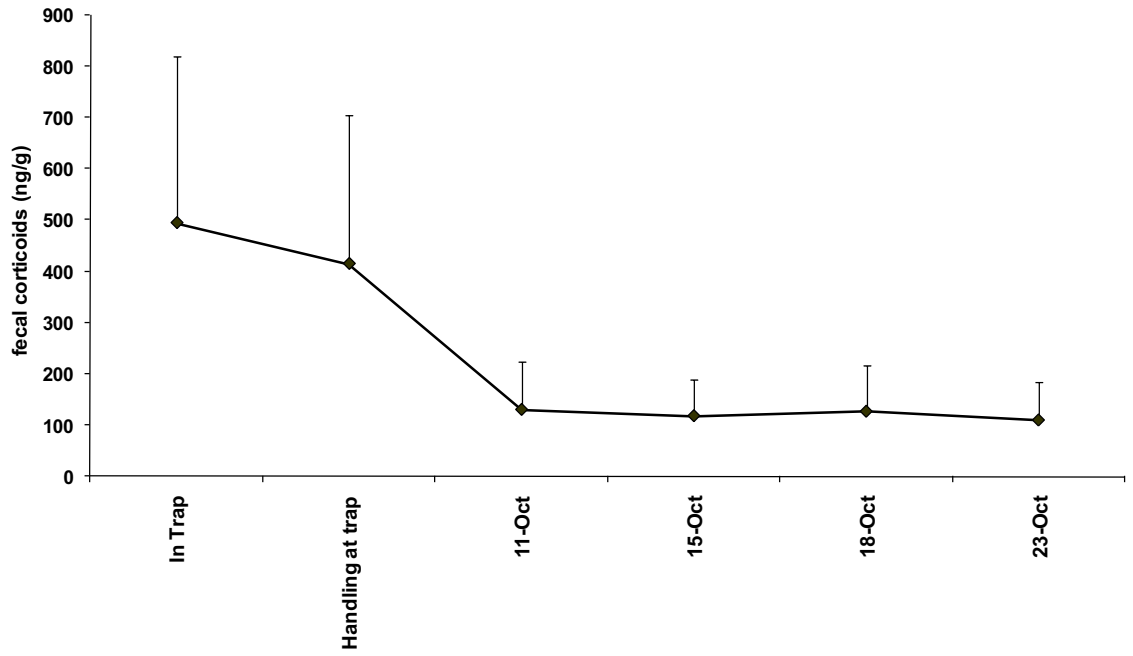

Figure S3. FGM level variation by generation and sex ($F_{4, 315}$=2.66, P=0.03). Females: open grey circles, males: black circles. Vertical bars denote 0.95 confidence intervals.

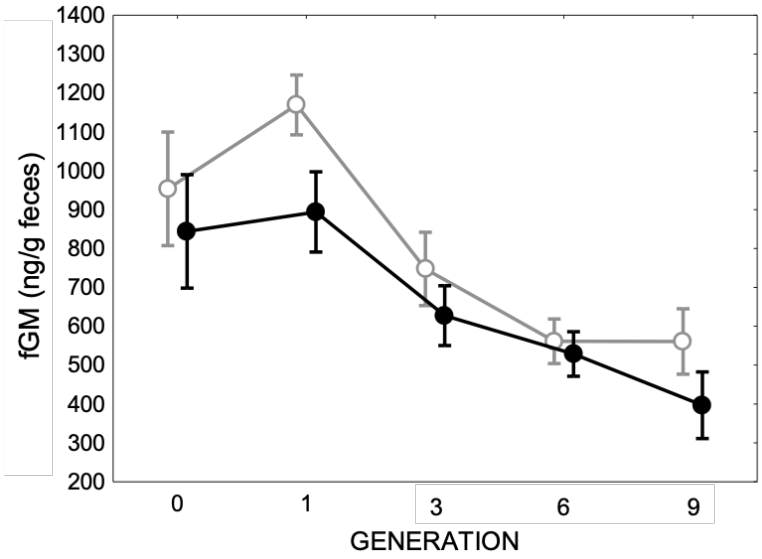

Figure S4. Seasonal variation in fGM levels by sex (founder generation was excluded). Bars denote 95% confidence intervals.

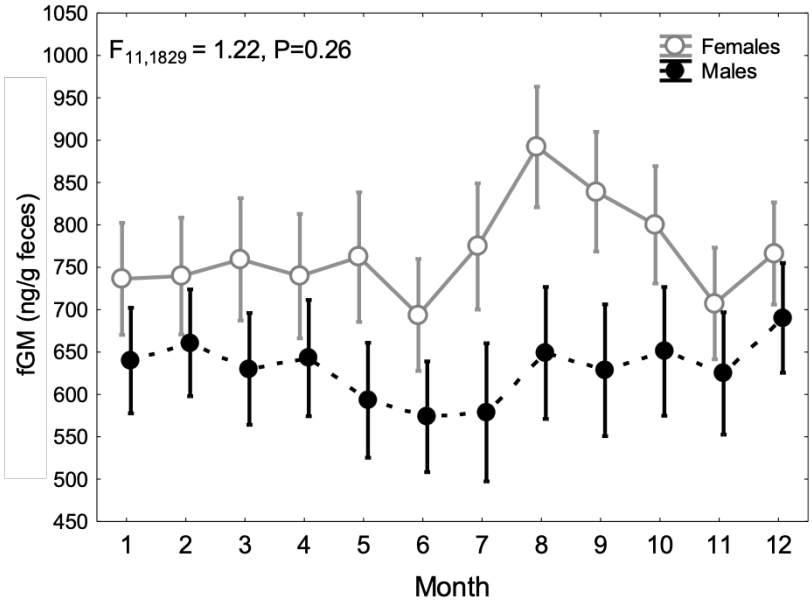

Figure S5. Correlation between juvenile and adult fGM levels in mice from generation number 6 (n=57). This relationship was also significant for both sexes; females (r = 0.44, p = 0.023; y = 497.66 + 0.21*x) and males (r = 0.52, p = 0.004; y = 387.81 + 0.29*x) when considered independently.