Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Environmental enrichment affects adrenocortical stress responses in the endangered black-footed ferret

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ARTICLE INFO

ABSTRACT

Potential stressors of wildlife living in captivity, such as artificial living conditions and frequent human contact, may lead to a higher occurrence of disease and reduced reproductive function. One successful method used by wildlife managers to improve general well-being is the provision of environmental enrichment, which is the practice of providing animals under managed care with environmental stimuli. The black-footed ferret (*Mustela nigripes*) is a highly-endangered carnivore species that was rescued from extinction by removal of the last remaining individuals from the wild to begin an *ex situ* breeding program. Our goal was to examine the effect of environmental enrichment on adrenocortical activity in ferrets by monitoring fecal glucocorticoid metabolites (FGM). Results demonstrated that enrichment lowered FGM in juvenile male ferrets, while increasing it in adult females; enrichment had no effect on FGM in juvenile females and adult males. These results correspond with our findings that juvenile males interacted more with the enrichment items than did adult females. However, we did not detect an impact of FGM on the incidence of disease or on the ability of ferrets to become reproductive during the following breeding season. We conclude that an environmental enrichment program could benefit captive juvenile male ferrets by reducing adrenocortical activity.

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1. Introduction

Stress responses in animals can occur when they experience internal and external demands that exceed their ability to respond to those demands [18,33]. A perceived stressor has an impact on the physiology and general health of an animal [53,59] by affecting the activities of the hypothalamic–pituitary–adrenal (HPA) axis, which controls the release of adrenocorticotropic hormone (ACTH), which, in turn, stimulates the secretion of glucocorticoids [2,59]. Chronic stress can lead to enhanced concentrations of ACTH and glucocorticoids [59], which can result in reduced reproductive function [1] and immune system suppression [26].

Stressors may include both physical challenges to homeostasis (the tendency of systems to maintain a steady state) and the threat of challenges, for example, aggressive movement towards an individual [33]. Captive animals face several potential unnatural stressors, including limited space, reduced ability to make choices, and artificial living conditions [28]. The stress of captivity can have a strong effect on the function of the HPA axis, elevating glucocorticoids in captive animals (e.g., as seen in Gambel's white-crowned sparrows, *Zonotrichia leucophrys gambelii* [39], and Grevy's zebras, *Equus grevyi* [22]). These stressors can have adverse effects on cognitive abilities, which are vital for survival in captive animals ultimately reintroduced into the wild [46]. Identifying potentially harmful stressors in captivity and developing strategies to overcome their negative effects is necessary to enhance the health and welfare of captive animals [59]. One mitigating strategy increasingly used by managers to reduce stress in captive animals is environmental enrichment, an approach that seeks to maximize psychological and physiological well-being by providing the appropriate environmental stimuli [42]. Behavioral observations [3,45] and measurement of glucocorticoid hormonal values [5,6] have suggested that enriched environments can reduce adrenocortical activity in captive animals. Enrichment can also reduce the expression of stereotypic behaviors that are indicative of poor well-being [42,45] and can enhance memory function and learning ability [41,48]. Environmental enrichment can be especially beneficial to endangered species programs, including those programs

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that produce individuals that are reintroduced to the wild, by promoting the development of healthy, reproductively successful, and behaviorally competent animals [45].

The black-footed ferret (Mustela nigripes; hereafter, "ferret") is one of the world's most endangered mammals [51] and is currently being reintroduced into the wild through an ex situ breeding program. The current ferret captive breeding program was initiated in 1986 and has since met with notable success [47]. Captive breeding has been an essential tool in the recovery of ferrets; without it, ferrets would likely be extinct. Nonetheless, high densities of animals in restricted living spaces, unnatural substrates and sounds, and frequent contact with humans are examples of potential sources of stress for ferrets in captive environments. The presence of humans in particular has been shown to increase glucocorticoid levels in other mustelids, including the European pine marten (Martes martes) [4], and may also impact adrenocortical activity in ferrets. Thus, ferrets may benefit from a well-designed environmental enrichment program. Vargas and Anderson [50] determined that ferrets exposed to greater environmental complexity, including enriched cages with hidden food to encourage food-searching behavior, were more likely to be successful at killing prey than ferrets raised in deprived cages. Success at killing may have important consequences for carnivores that are released into the wild and may determine whether an animal survives after reintroduction. Biggins et al. [11] established that ferrets raised in natural, outdoor pens with burrow systems and consistent exposure to live prairie dogs had the highest 30-day minimum survival rate (39%) after reintroduction, four times higher than the survival rate of ferrets raised in standard cages with limited exposure to prairie dogs. The animals raised in outdoor pens also appeared to disperse shorter distances, which may have decreased their risk of predation and resulted in higher survival.

The objective of this study was to evaluate whether a simple enrichment program would be effective in reducing adrenocortical activity and improving the physical and reproductive health of captive ferrets. Ferrets are solitary carnivores; hence, we manipulated their physical, but not social, environment. Because previous studies have established age and sex effects in stress responses in other species [24,25,27,43], we also examined different age and sex classes to determine how adrenocortical activity varies among these groups. Enrichment has had greater effects on males [20,36] and juveniles [12,45] in other species; thus, we predicted that younger, male animals would respond to enrichment by lowering adrenocortical activity.

We measured adrenocortical activity in ferrets by assaying fecal glucocorticoid metabolites (FGM), a technique increasingly used to measure adrenocortical activity which is less invasive and provides several advantages over the blood-based glucocorticoid assays that have been used historically [32]. FGM analyses: (1) reflect an average concentration of circulating hormones over a certain time period before collection, due to the slower excretion rate of metabolized hormones into the feces, while blood-based analyses measure glucocorticoids at the time of collection, so that the impact of capture, handling and restraint is immediately reflected in the blood sample [23,32]; and (2) do not require capture or handling so that many samples can be collected without disturbing the animals or placing them in danger [32,52].

2. Materials and methods

2.1. Study site

We conducted this study at the United States Fish and Wildlife Service National Black-footed Ferret Conservation Center in northern Colorado, which houses nearly two-thirds of all captive ferrets.

At the time of the study, the breeding facility consisted of four rooms, each containing 36 individual ferret cages, housing a total of 144 ferrets. From this population, 72 ferrets were chosen for this study. Each animal's enclosure consisted of an elevated wooden cage, measuring $1.2 \times 1.2 \times 0.6$ m, with two attached nest boxes, one upper and one lower. The upper box was attached directly to the cage while the lower box was placed on the floor and attached with a piece of ribbed, black tubing that simulated a tunnel between the cage and the box. Bedding material (ALPHA-dri, Shepherd Specialty Papers, Kalamazoo, MI) was placed in both nest boxes, and additional shelter was provided by suspending a 46-cm black plastic tube from the ceiling of each cage. Animals were fed once daily (Toronto Zoo Small Carnivore Diet, Milliken Meat Products Ltd., Scarborough, Ontario, Canada), and water was provided ad libitum.

2.2. Study design

The experiments took place from November 2007 to February 2008, the time period between the end of the kit weaning season and the beginning of the breeding season, when ferrets were singly housed in individual cages. Eighteen ferrets were chosen from each of the four rooms so that the numbers of animals in each age and sex class were evenly distributed in each room. We divided the study animals into two groups: one control group, represented by ferrets in rooms 1 and 2, and one treatment group, represented by ferrets in rooms 3 and 4. Of the 36 animals in each group, an equal number (nine) were each adult males, adult females, juvenile males, and juvenile females. Adults were >1 year of age, and juveniles were <1 year; juvenile ferrets are able to reproduce at the end of their first year. The animals in each of the two groups were separated in different rooms to minimize any effects of the control ferrets observing or hearing the enrichment items as they were being manipulated by the treatment ferrets.

We divided the study into three periods: a pre-enrichment phase of 3 weeks, an enrichment phase of 8 weeks, and a post-enrichment phase of 2 weeks. The control group did not receive any enrichment items during the entire study period. During both the pre-enrichment and post-enrichment phases, the treatment group did not receive any enrichment items. The pre-enrichment phase provided reference values for data collected when enrichment was provided, and the post-enrichment phase allowed us to evaluate changes in adrenocortical activity after enrichment was removed. During the enrichment phase, the ferrets in the treatment group received one of three enrichment items: a black plastic tube cap, a Nylabone® (Nylabone Products, Neptune, NJ), or a plastic noise ball (larger ball containing a smaller ball with a bell inside of it). We chose these three items because the ferret breeding program had either used them in the past or was considering them in the future as possible enrichment items. Further, the items represented different functional types of enrichment: the Nylabone® was a chew toy, and the tube cap and noise ball were noise makers. Nylabones® have been effective enrichment items for kenneled dogs [54], and ferrets have previously demonstrated an interest in noise makers. We rotated each of these three items every 2 days throughout the study (during regularly scheduled cage cleanings) so that a ferret was given each item again 5 days after it was removed. Each animal in the treatment group received the same item at each presentation, and we presented each item in the same order throughout the study: the tube cap was followed by the Nylabone® and then the noise ball, repeated continuously. When the cap and noise ball were provided, they were placed on the floor of the cage in the same location each time. When the Nylabone® was provided, it was attached, alternatively, to either the ceiling or a wall of the cage by carabiners and cable ties.

Each time an item was provided we scored enrichment use and ferret behavior using a coding system with a graduated scale. Scores from 0 to 4 were recorded based on increasing intensity of enrichment use: 0 = no use of enrichment, 1 = visual observation of ferret investigating (but not using) enrichment, 2 = visual observation of minor ferret use of enrichment (e.g., touching or biting the item), 3 = no visual observation of ferret interacting with enrichment but evidence of use of items (e.g., tube cap or noise ball moved, chew marks on Nylabone®), and 4 = visual observation of considerable ferret use of enrichment (e.g., playing with or manipulating the item). Evidence of use for code 3 varied considerably and represented intermediate use between codes 2 and 4; some ferrets were heard vigorously interacting with an item but were not observed, which would place them closer to a code 4, while other instances were based only on whether the item moved, which would place them closer to a code 2. Regular observations took place for approximately 5 min per ferret after an item was placed in the cage. Scores of enrichment use were assigned based on interactions observed during each of these periods and at the end of each 2-day period when the ferret cages were next cleaned (approximately 28 times over the enrichment period). Codes were averaged for an individual ferret over the enrichment phase, and each mean was considered to be an index of an animal's behavioral use of enrichment items, a measure that varied continuously from 0 to 4. Because observations were made when ferret cages were cleaned, and cages of control ferrets were cleaned as often as those of treatment ferrets, visitation rates were equal between the two groups.

In addition to the enrichment use observations, we also noted when a ferret contracted a disease and the type of illness that occurred. The reproductive status of each ferret was checked beginning in mid-January 2008 and periodically afterwards until the animal reached reproductive readiness. The reproductive status of females was checked by conducting vaginal lavages to detect the percentage of superficial cells, a high percentage indicating estrus [56]. The reproductive status of males was assessed by measuring testes firmness and sperm production.

2.3. Fecal sample processing

Fecal samples were collected throughout the study from each ferret at the time the cages were cleaned (every 2 days). Previous validations of fecal glucocorticoid assays for black-footed ferrets demonstrated a time delay in fecal hormone excretion of 20–44 h [58,59], which indicates that FGM values reflect adrenocortical activity from the previous 1–2 days. We stored all scat samples at −20 °C until processing.

Fecal samples were dried on a lyophilizer (ModulyoD, Thermo Fisher Scientific, Inc., Waltham, MA) and crushed, and aliquots were weighed between 0.01 and 0.0229 g, which was one-tenth of the amount used in previous reported methods [59] because of the smaller amount of feces collected. Next, 0.5 ml of 90% ethanol:distilled water was added to each aliquot. Samples were agitated on a mixer (Glas-Col, Terre Haute, IN) on setting 60 for 30 min and centrifuged (1500 rpm; 20 min); the supernatant was then poured into clean glass tubes. The fecal pellets were resuspended in 0.5 ml of 90% ethanol:distilled water, vortexed for 30 s, and centrifuged for 15 min. The supernatant was added to the first extract and was evaporated with air and heat (60 °C). The extracted samples were reconstituted in 0.5 ml of methanol, vortexed briefly, sonicated (Thermo Fisher Scientific, Inc., Waltham, MA) for 20 min, and diluted in dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, NaCl) before analysis. We used fecal samples from all 72 ferrets (n = 2965 samples, 41.2 samples per individual ±0.5 SE). We were unable to extract FGM for some samples (n = 335) because the amount of feces in each sample was insufficient (⩽0.01 g) [32].

2.4. Enzyme-immunoassays of fecal samples

We compared a previously used cortisol enzyme-immunoassay (EIA) [59] to a newly developed corticosterone EIA to determine the most prevalent FGM. We chose three individuals for the EIA comparison and analyzed FGM using previously described methods [34]. The corticosterone horseradish peroxidase (HRP) ligands and polyclonal antiserum (CJM006) were provided by C. Munro (University of California, Davis, CA). Antiserum and HRP to corticosterone were used at dilutions of 1:6000 and 1:20,000, respectively. Antiserum cross-reactivities for corticosterone were: corticosterone, 100%; desoxycorticosterone, 14.25%; tetrahydrocorticosterone, 0.9%; 11-deoxycortisol, 0.03%; prednisone, less than 0.01%; prednisolone, 0.07%; cortisol, 0.23%; cortisone, less than 0.01%; progesterone, 2.65%; testosterone, 0.64%; and estradiol 17β, less than 0.01%. The EIA for ferrets was validated by demonstrating: (1) parallelism between binding inhibition curves of fecal extract dilutions (1:5–1:2560) and the corticosterone standard (R^2 = 0.991); and (2) significant recovery (>90%) of exogenous corticosterone (1.95–1000 pg/well) added to fecal extracts (1:1250; $\hat{y} = 0.88x + 7.80$; R^2 = 0.992). Assay sensitivity was 1.95 pg/well and intra- and inter-assay coefficients of variation were <10%. Cortisol polyclonal antiserum and HRP (R4866; provided by C. Munro, Davis, CA) were used at a 1:8500 and 1:20,000 dilution, respectively. Cross-reactivity to the cortisol antiserum has been reported previously [59]. The cortisol EIA was validated by demonstrating: (1) parallelism between binding inhibition curves of fecal extract dilutions (1:5–1:1280) and cortisol standards (R^2 = 0.975); and (2) significant recovery (>90%) of exogenous cortisol added to fecal extracts (1:500; $\hat{y} = 0.86x + 26.9$; R^2 = 0.9829). Assay sensitivity was 3.9 pg/well and intra- and inter-assay coefficients of variation were <10%. Results demonstrated a greater amount of fecal corticosterone metabolites than cortisol metabolites with a dilution rate of 1:125 and 1:50, respectively. For the three individuals, the corticosterone and cortisol results were highly correlated with r-values of 0.785 ($P < 0.001$), 0.829 ($P < 0.001$) and 0.812 ($P < 0.001$), and both results revealed similar hormonal profiles. Because of the high prevalence of fecal corticosterone metabolites, the corticosterone EIA was used to analyze the remaining samples.

2.5. Statistical analyses

We statistically analyzed the effects of enrichment using a repeated measures framework, grouping the FGM data for each 2-day period into 6 time intervals: interval Pre1 for the pre-enrichment phase (3 weeks), intervals E1, E2 and E3 for the enrichment phase (each interval 2.66 weeks), and intervals Post1 and Post2 for the post-enrichment phase (each interval 1 week). We divided the time periods into multiple intervals to detect fine-scale temporal effects after the ferrets received the enrichment items and again after enrichment was removed. We performed a repeated measures test using a generalized linear mixed model (PROC GLIMMIX) in the SAS statistical program [40], with repeated measures of individuals across time intervals. We analyzed these data with room number and individual ferret number as random variables and treatment group (control or enrichment), time interval, age, and sex as fixed variables. For any significant interactions or main effects, we analyzed specific pairwise comparisons with a t-test by examining comparison-wise error rates [44]. One juvenile female was removed from the study towards the end of interval E3 (because of fighting with an adult male in an adjacent cage), and one adult female died at the end of interval Post1. For the repeated measures analysis, we had 429 observations (72 animals × 6 intervals less one animal removed from 2 intervals less one animal removed from 1 interval).

We used nonparametric statistics for all tests involving enrichment use scores because these data were not normal, determined by quantile–quantile plots and histograms. To relate adrenocortical activity of treatment animals to the extent to which they used the enrichment items, we conducted a Spearman Rank correlation test on the FGM values and scores of enrichment use, averaged for each individual over the study period, in SAS (PROC CORR). To examine age and sex effects on enrichment use, we conducted a Kruskal–Wallis test in SAS on the mean scores of enrichment use, with age and sex as predictor variables, and we analyzed pairwise comparisons among the different age and sex classes using Wilcoxon rank-sum tests. In addition, to examine if use varied with enrichment type, we conducted a Kruskal–Wallis test on the scores of enrichment use with type of enrichment item as the predictor variable, and we analyzed pairwise comparisons among the different enrichment items with Wilcoxon rank-sum tests.

To evaluate whether adrenocortical activity predicted disease status, we assessed FGM values of ferrets with and without diseases. Ferrets were checked daily for outward signs of disease, including uneaten food, diarrhea, or changes in behavior such as listlessness. Ferrets showing signs of disease were further examined or tested; those animals diagnosed with a disease were then placed on a treatment protocol. We averaged FGM values for each ferret contracting a disease for the 1-month time period before diagnosis and evaluated their relationship to values for all other ferrets averaged over the course of the study by conducting a logistic regression analysis to determine if FGM predicted disease state. We also used logistic regression to determine whether FGM values predicted reproductive readiness in ferrets during the 2008 breeding season. We defined females as reproductively ready when they reached estrus and males as reproductively ready when their testicles were firm upon palpation. For this analysis, we excluded two females, including one adult that died during the study and one juvenile that died after the study ended. Finally, we used logistic regression to determine whether FGM values predicted reproductive success (defined by the production of offspring) during the breeding season. For this analysis, we excluded 15 ferrets that were reproductively ready but were not paired with another ferret and one male ferret that was paired with a female who died shortly afterwards. We set the significance level to 0.05 for all statistical tests, which were two-tailed. However, due to the highly endangered status of ferrets and our concern about committing a Type II error of a false negative [19,44], we reported trends that were not significant (with *P*-values between 0.05 and 0.1) in addition to statistically significant findings in the Results.

3. Results

3.1. Effects of environmental enrichment on FGM

3.1.1. Age and sex effects

The mean FGM ± SE for juvenile males, adult males, juvenile females, and adult females in the control group were 7.7 ± 0.5, 7.3 ± 0.4, 7.3 ± 0.4, and 6.8 ± 0.3 μg/g dry feces, respectively. A trend occurred for males and females to differ in responses over time by treatment group (Table 1, trt∗interval∗sex interaction). Generally, FGM values for males decreased after receiving enrichment, and then increased after enrichment was removed, while FGM values for females increased after receiving enrichment. Details of the effects of enrichment on each sex are described in the sections below. Ferrets of different age and sex combinations also differed in responses over time (Table 1, interval∗age∗sex interaction). Juvenile males displayed a pattern of decreasing FGM values throughout the enrichment phase, while values in adult females increased during this period. However, this interaction pooled values over both

Table 1
Tests of main effects and interactions for the model analyzing fecal glucocorticoid metabolite data for captive black-footed ferrets during the study period. Predictor variables for the model included treatment group (trt), time interval (interval), age of ferrets, either adult or juvenile (age), sex of ferrets, either male or female (sex), and interactions among each of the four variables.

Effect	Numerator D.F.	Denominator D.F.	F	P[a]
trt	1	64	0.31	0.5820
interval	5	317	1.40	0.2233
trt ∗ interval	5	317	0.51	0.7715
age	1	64	0.61	0.4390
trt ∗ age	1	64	1.06	0.3063
interval ∗ age	5	317	1.85	0.1033
trt ∗ interval ∗ age	5	317	0.38	0.8625
sex	1	64	0.04	0.8425
trt ∗ sex	1	64	1.29	0.2595
interval ∗ sex	5	317	0.94	0.4567
trt ∗ interval ∗ sex	5	317	2.23	**0.0512**
age ∗ sex	1	64	0.14	0.7090
trt ∗ age ∗ sex	1	64	0.17	0.6802
interval ∗ age ∗ sex	5	317	2.60	**0.0255**
trt ∗ interval ∗ age ∗ sex	5	317	0.41	0.8435

[a] Bold denotes significance at the 0.05 level or near significant trend (0.05–0.1).

treatment and control groups. Because we were interested in the treatment effect of enrichment in this study, we did not further analyze this interaction. No other interactions or main effects were significant (Table 1).

3.1.2. Males

For male ferrets, we found no statistically significant differences in FGM between treatment and control groups, but our results indicated several trends. FGM values in the treatment group were lower than values in the control group during interval E2 (t_{317} = −1.76, P = 0.079), suggesting a delayed reduction in male FGM values in response to enrichment. For treatment males, but not control males, FGM values in interval E2 were lower than in interval Pre1 (t_{317} = −1.81, P = 0.071), interval E1 (t_{317} = −1.83, P = 0.069) and interval Post1 (t_{317} = −1.73, P = 0.085), suggesting that FGM values of male ferrets decreased several weeks after receiving enrichment and then increased just after enrichment was removed.

Juvenile and adult male ferrets differed in their adrenocortical responses. During interval E2, FGM values for juvenile males trended lower in the treatment than the control group (t_{317} = −1.81, P = 0.071; Fig. 1a). Values for juvenile males in the treatment group, but not the control group, were significantly lower during interval E2 than interval Pre1 (t_{317} = −2.08, P = 0.038; Fig. 1a). Thus, the decrease in male FGM values that occurred several weeks after receiving enrichment was experienced primarily by juvenile males (Fig. 1a). A trend occurred for juvenile males to have lower FGM values in interval Post1 than in interval Pre1 for the treatment group (t_{317} = −1.73, P = 0.085), but also lower FGM values in interval Post1 than in intervals E2 (t_{317} = −1.91, P = 0.057) and E3 (t_{317} = −1.69, P = 0.093) for the control group, suggesting that the lower values in interval Post1 for juvenile males were not likely due to a treatment effect (Fig. 1a). Values for juvenile males in the control group, but not the treatment group, subsequently increased significantly in interval Post2 (t_{317} = 2.83, P = 0.005; Fig. 1a).

In contrast to juvenile males, FGM values for adult males did not differ between treatment and control across all intervals (Fig. 1b). However, adult males in the treatment group, but not the control group, experienced a significant increase in values between intervals E3 and Post1, just after enrichment was removed (t_{317} = 2.18, P = 0.030; Fig. 1b); these Post1 values for treatment
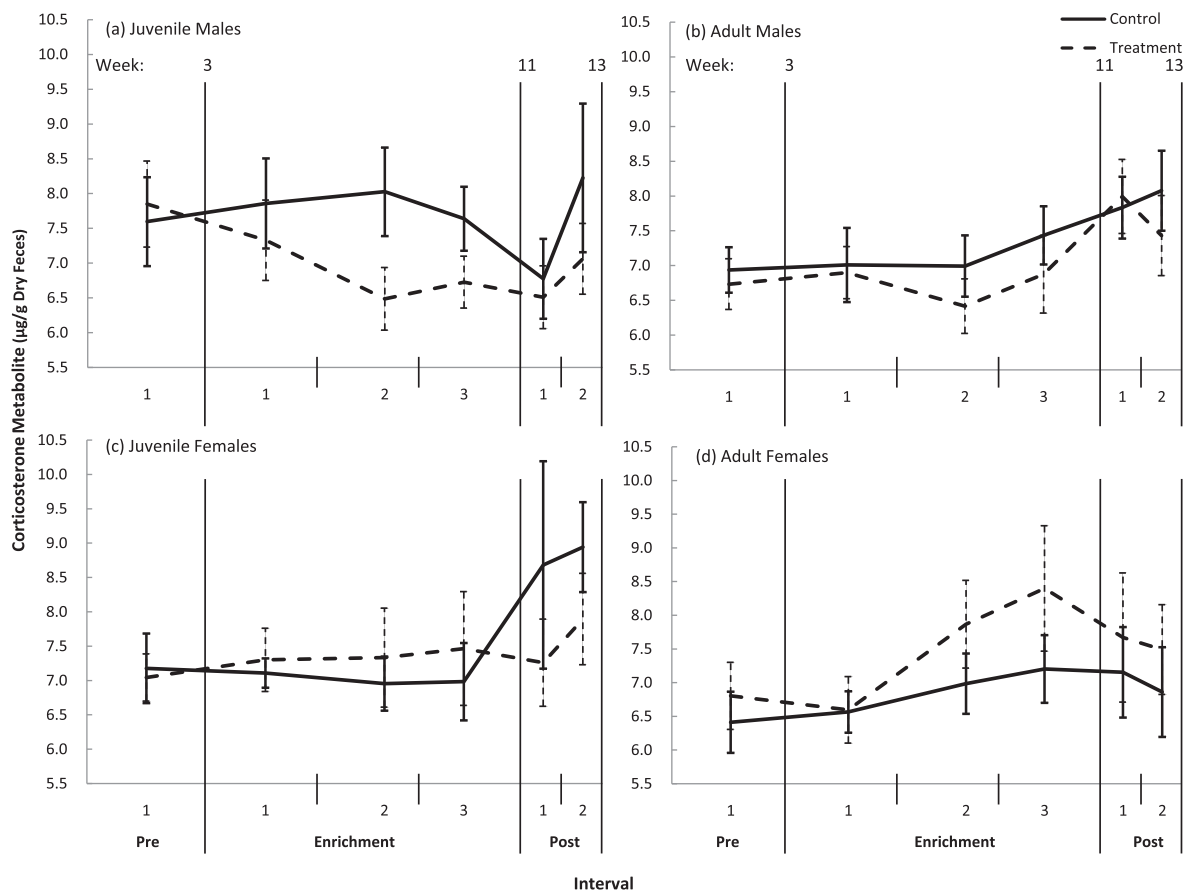
Fig. 1. Average fecal glucocorticoid metabolite values for (a) juvenile male, (b) adult male, (c) juvenile female, and (d) adult female black-footed ferrets in the control and treatment groups during the study period. Bars represent standard error around the mean, with sample size equal to number of individuals. Data points represent averages of each interval, and tick marks on the x-axis divide intervals in the enrichment and post-enrichment periods. See Section 3.1 for description of significant results and trends.

adult males also trended higher than Post1 values for treatment juvenile males (mean ± SE – adults: 7.99 ± 0.53 µg/g, juveniles: 6.51 ± 0.45 µg/g, t_{317} = 1.75, P = 0.081; Fig. 1a and b), indicating that the trend for an increase in FGM values for males immediately after enrichment was removed was experienced by adults, rather than juveniles.

3.1.3. Females

For female ferrets, FGM values for the treatment group were not significantly different than values for the control group across all intervals. However, a trend occurred for treatment females to have higher values than treatment males during intervals E2 (females: 7.60 ± 0.48 µg/g, males: 6.45 ± 0.29 µg/g, t_{317} = 1.92, P = 0.056) and E3 (females: 7.93 ± 0.61 µg/g, males: 6.80 ± 0.33 µg/g, t_{317} = 1.89, P = 0.060). This pattern was not evident for control females, which suggests that enrichment resulted in the trend for higher FGM values in females than males. Values for treatment, but not control, females, trended lower in interval E1 than in interval E2 (t_{317} = −1.80, P = 0.073) and were also significantly lower than interval E3 (t_{317} = −2.12, P = 0.034), indicating that female ferrets experienced an increase in FGM values a few weeks after receiving enrichment, which remained high throughout the enrichment phase.

As with males, juvenile and adult female ferrets differed in their adrenocortical responses. During both intervals E2 and E3, FGM values for adult females in the treatment group, but not the control group, trended higher than values for adult males in the treatment group (interval E2 – females: 7.87 ± 0.65 µg/g, males: 6.42 ± 0.39

µg/g, t_{317} = 1.71, P = 0.088; interval E3 – females: 8.40 ± 0.93 µg/g, males: 6.88 ± 0.56 µg/g, t_{317} = 1.80, P = 0.073; Fig. 1b and d) and were significantly higher than their values for interval E1 (interval E2: t_{317} = 2.48, P = 0.014; interval E3: t_{317} = 2.75, P = 0.006; Fig. 1d). Values for juvenile females in the control group, but not the treatment group, increased during interval Post1 and were significantly higher than interval E3 (t_{317} = 3.16, P = 0.002; Fig. 1c). However, in contrast to adult females, juvenile females did not experience increases in FGM values during the enrichment phase. Thus, FGM values that increased in treatment females a few weeks after receiving enrichment, which remained high throughout the enrichment phase, and which were higher than treatment males, were experienced particularly by adult females.

3.2. Enrichment use

We did not detect a relationship between the scores of enrichment use and FGM values (ρ = −0.16, P = 0.350). Age and sex predicted enrichment use (H_3 = 19.50, P < 0.001); juveniles used enrichment significantly more than adults (T = 461, N_1 = 18, N_2 = 18, P < 0.001), and a trend occurred for higher enrichment use in males than females (T = 277, N_1 = 18, N_2 = 18, P = 0.075; Fig. 2a). Further, juvenile males used enrichment significantly more than adult males (T = 52, N_1 = 9, N_2 = 9, P = 0.002) and adult females (T = 48, N_1 = 9, N_2 = 9, P < 0.001); juvenile male use of enrichment also trended higher than that of juvenile females (T = 65, N_1 = 9, N_2 = 9, P = 0.066; Fig. 2a). In addition, adult females used enrichment significantly less than juvenile females (T = 119,
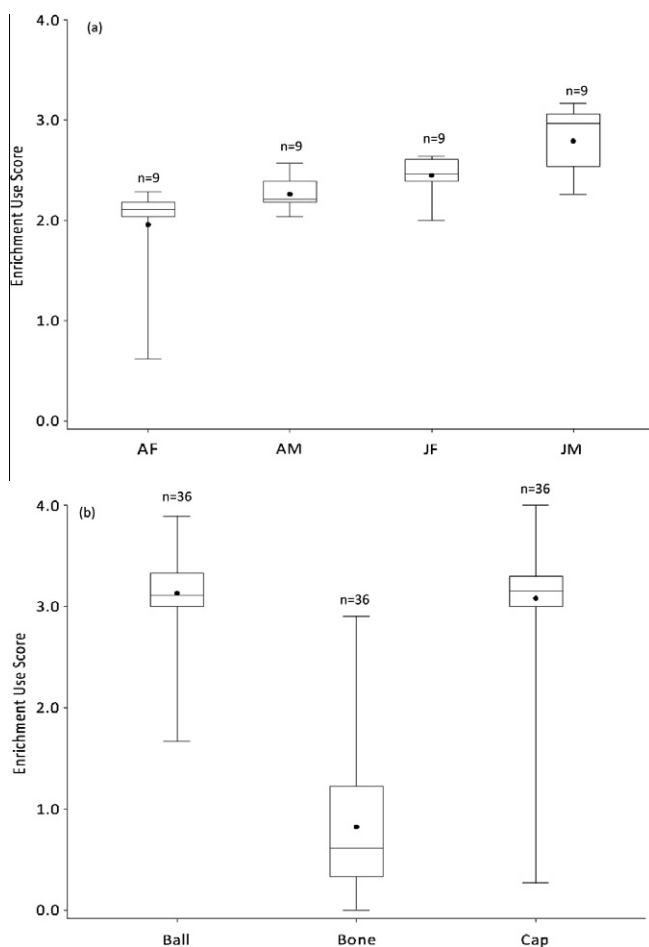
Fig. 2. Box plot of (a) enrichment use scores for adult female (AF), adult male (AM), juvenile female (JF), and juvenile male (JM) black-footed ferrets in the treatment group during the enrichment period and (b) enrichment use scores by type of enrichment item averaged for all black-footed ferrets in the treatment group during the enrichment period. The horizontal line represents the median, the dot denotes the mean, the box contains the range between the lower and upper quartiles and the whiskers represent the minimum and maximum observations.

$N_1 = 9$, $N_2 = 9$, $P = 0.002$) and also trended lower than adult males ($T = 107$, $N_1 = 9$, $N_2 = 9$, $P = 0.064$; Fig. 2a). This pattern of higher enrichment use by juvenile males and lower enrichment use by adult females was consistent with our findings of age and sex differences in adrenocortical responses to enrichment. In addition, type of enrichment item significantly predicted use ($H_2 = 66.62$, $P < 0.001$); the noise ball and tube cap were both used more than the Nylabone® (ball: $T = 1951$, $N_1 = 36$, $N_2 = 36$, $P < 0.001$; cap: $T = 1926$, $N_1 = 36$, $N_2 = 36$, $P < 0.001$; Fig. 2b). This preference was consistent for every individual in the study.

3.3. Disease

Five ferrets (7%) contracted a disease during the study period: two adult females, one adult male, one juvenile female, and one juvenile male. FGM values did not predict disease status ($\chi^2 = 0.40$, $P = 0.527$), but our sample size for diseased ferrets was low. Of these five ferrets, two were in the control group and three were in the treatment group.

3.4. Reproduction

Fourteen ferrets (19%) did not reach reproductive readiness during the 2008 breeding season: one adult female, one adult male,

nine juvenile females, and three juvenile males. FGM values did not predict reproductive readiness ($\chi^2 = 0.003$, $P = 0.954$). Of the 56 ferrets that reached reproductive readiness, 28 (50%) were in the control group and 28 (50%) were in the treatment group. In addition, of the 40 animals that were included in this analysis, 20 ferrets (50%) that were reproductively ready were not reproductively successful: nine adult females, six adult males, three juvenile females, and two juvenile males. FGM values did not predict reproductive success ($\chi^2 = 0.34$, $P = 0.558$). Of the 20 successful ferrets, 10 (50%) were in the control group and 10 (50%) were in the treatment group.

4. Discussion

Our results suggest that adrenocortical activity in male black-footed ferrets can be decreased through the provision of environmental enrichment, but enrichment provided to female ferrets may produce the reverse effect by increasing such activity, and that these results are mediated by an age effect. In particular, juvenile males responded positively to environmental enrichment by experiencing lower FGM values after enrichment was provided; adult males did not experience such a strong response to the provisioning of enrichment, but displayed an increase in FGM after enrichment was removed. Contrary to the juvenile male response, enrichment resulted in an increase in FGM for adult females, but did not affect FGM values of juvenile females. Importantly, these results correspond with our findings that both males and juveniles interacted more with the enrichment items than did females and adults, respectively, and, thus, received more benefit from these interactions through reduced FGM values.

Other studies have established sex differences in responses to environmental enrichment and novelty. Research conducted on laboratory rats has determined that male rats behaviorally respond more than females to physical enrichment, measured by decreased activity and increased habituation to novel environments [20], and to a novel stimulus (tunnel) placed in their environment [35]. Likewise, Platt and Novak [36] reported higher instances of habituation to videotapes used as enrichment among male rhesus monkeys than among females. Male ferrets may exhibit a stronger positive response to enrichment than females due to different sources of stress, including more vigorous territorial defenses by males [9,10,31,37]. Specifically, scent-marking is used to communicate with conspecifics and delineate territories and may have more impact on male ferrets, especially during the breeding season [10,30,37]. Although this study was conducted before the start of the breeding season, which typically occurs March to May, male ferrets can show development of the testes as early as November [14,55], and FGM values for control male ferrets in our study increased during the post-enrichment phase when the testes of several males were becoming firm. Because ferrets in captivity are in close proximity to one another in adjacent cages, enrichment may provide males with a way of coping with their close surroundings with other ferrets and, thus, decrease their stress. Although we are uncertain why FGM values in female ferrets increased with enrichment, one possibility might be that male ferret interaction with enrichment items and the accompanying sounds of their play may have caused increased sensory stimulation, and perhaps stress, in nearby females. Future research could address this hypothesis by measuring FGM values of females that do not receive enrichment when they are located in the same room as other ferrets that do receive enrichment. Another explanation for the increase in FGM values in females might be that female ferrets are accustomed to their daily routines and may become stressed with any modification to their environment, including the introduction of novel stimuli.

Previous studies have also shown differing responses to enrichment by age group. Swaisgood et al. [45] determined that adult, but not subadult, giant pandas preferred food-based enrichment devices to non-food-based items. Similarly, Bloomsmith et al. [12] discovered that use of rigid plastic balls by younger captive chimpanzees was higher than use of such balls by older chimpanzees. These results reflect the affinity of younger animals for play and the interest of older animals in feeding. Juvenile ferrets in the wild begin to disperse from their natal burrows soon after reaching adulthood in autumn [49], and males usually disperse longer distances than females [21]. Thus, compared to adults, juvenile ferrets in captivity (especially juvenile males) may experience restlessness and may need more stimulation from their environment on which to focus their energies. Therefore, enrichment may be an efficient technique of lowering stress in juvenile ferrets. Of the three enrichment items, ferrets preferred the tube cap and noise ball over the Nylabone®, likely because these two items could be moved and easily manipulated, and ferrets attempted to cache them down the tunnels of their cages into their nest boxes. Hence, the interest of juveniles in play might have contributed to the higher use recorded for these two items.

We did not detect an effect of FGM on either the onset of a disease in captive ferrets or the ability of ferrets to become reproductive. Disease was not common in our study; two ferrets contracted coccidiosis, one developed a urinary tract infection, one had a facial abscess, and one contracted amyloidosis, a disease in which proteins abnormally accumulate in organs and tissues, which was fatal in this case. Additionally, the number of nonreproductive animals was relatively low. Most (86%) of the 14 animals that did not reach reproductive readiness were juveniles which are less likely to become reproductive than adults. We also determined no effect of FGM on reproductive success. However, many factors influence the ability to produce young in captivity. For example, many males in the captive breeding program have failed to sire offspring, likely because of a combination of behavioral and physiologic factors (e.g., improper breeding posture), but not resulting from overall sperm quality [57]. Our results characterize only one breeding season; accordingly, future studies including multiple breeding seasons may reveal potential stress effects on reproduction. Moreover, additional research is needed to verify that chronic stress increases adrenocortical activity, and that such increases can negatively affect fitness in animals, especially those in captivity [13,15–17].

The FGM values in our captive ferrets should be compared to free-ranging ferrets in order to better understand the effects of captivity on stress. Other studies have shown that animals in captivity had increased stress over their free-living conspecifics [17,29]. Anecdotally, we collected scat from five wild ferrets living in Conata Basin, South Dakota, and found that their average FGM values (6.2 ± 0.6 µg/g dry feces) were lower than the average values for our 72 captive ferrets (7.2 ± 0.1 µg/g dry feces). However, the free-ranging samples consisted of only one scat from each of five individuals and were collected during a different season than those of the captive ferrets, which may influence adrenocortical activity [38]. Hence, collection of additional data from free-ranging ferrets is crucial to determine whether captive animals have increased stress, which would reinforce the importance of implementing appropriate techniques to mitigate stress in captivity.

In conclusion, an environmental enrichment program could benefit juvenile male ferrets by decreasing stress while in captivity. However, adult female ferrets did not respond to enrichment in the same manner; thus, enrichment may not be suitable as a tool for lowering stress in these animals, and future research efforts should be directed toward evaluating alternative methods that might reduce stress in female ferrets. Even though we could not detect effects of FGM on disease or reproduction, if facilities housing ferrets have resources that can be applied towards enrichment, then those items should be provided to juvenile male ferrets because they benefitted from enrichment. The enrichment items most likely to be used by ferrets are those that can be manipulated and moved. Enriched environments in captivity might also influence survival of ferrets that are later reintroduced. All ferrets that are reintroduced are given a much more dramatic form of environmental enrichment by being preconditioned in natural, outdoor pens prior to release. Biggins et al. [8] determined that for ferret kits born in cages and later placed in outdoor pens, the earlier they were placed in pens, where exposure to stressful conditions was reduced, the higher their minimum short-term survival after release. Hence, reducing stress at an early age, including during the time spent in cages, might affect post-release survival of juvenile ferrets. Further, because a ferret dam's behavior can influence the behavior of her kit, stressed females may adversely affect the early development of behavior in their young [7]. Thus, decreasing stress in adult females might influence the development of juveniles that are later reintroduced. Generally, reducing stress in captive ferrets can be essential in striving to meet certain husbandry goals, such as conservation of biodiversity [33]. Environmental enrichment can be one method used to achieve these goals and ensure the continued survival of this endangered species.

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