Using hair cortisol analysis to understand the biological factors that affect black-footed ferret (*Mustela nigripes*) stress physiology

R.M. Santymire¹,², *, N. Ali¹,², P.E. Marinari³ and T.M. Livieri⁴

¹Conservation and Science Department, Lincoln Park Zoo, 2001 N. Clark St, Chicago, IL 60614, USA
²Committee on Evolutionary Biology, University of Chicago, 1025 E. 57th St, Chicago, IL 60637, USA
³Center for Species Survival, Smithsonian Conservation Biology Institute, 1500 Remount Rd, Front Royal, VA 22630, USA
⁴Prairie Wildlife Research, PO Box 308, Wellington, CO 80549, USA

*Corresponding author: Georgia State University, 100 Piedmont Ave SE, 4th Floor, Atlanta, GA 30303, USA. Tel: +1 404 413 5397.
Email: rsantymire@gsu.edu

The black-footed ferret (*Mustela nigripes*) was driven to near extinction due to habitat loss and an introduced disease, sylvatic plague (*Yersinia pestis*). After 35 years of breeding in ex situ facilities, the black-footed ferret has been experiencing infertility with seminal traits declining in males and only about a third of breeding-aged females are whelping. Our goal was to use hair cortisol analysis to determine if the ex situ population was experiencing chronic stress that was affecting reproduction by comparing captive ferrets to wild individuals. Our specific objectives were to (i) compare hair cortisol concentrations (HCCs) between age classes (juveniles versus adults), (ii) compare the HCCs of in situ and across different ex situ facilities and (iii) determine the relationship between HCCs and reproductive success. Overall, wild juveniles had higher HCC than wild adults. Our generalized linear mixed model determined that the parameters that best predict HCC for adults were the interactions among sex, in situ versus ex situ facilities and season. During both seasons, wild females had higher HCCs compared to the ex situ females. During the breeding season, male HCCs across breeding facilities varied and males at the breeding facility with the largest ferret habitats had HCCs similar to wild males. At one breeding facility, HCC was higher in males that sired compared to those that did not sire. In conclusion, ex situ ferrets do not have higher HCC than wild individuals when controlling for season and ex situ habitat size, and ex situ males with higher HCC tended to sire. This suggests that HCC may be metabolically driven and/or that low HCC may be an indication of hypothalamus–pituitary–adrenal axis dysregulation and warrants further investigation both for laboratory validation and biological relevance.

Key words: Age, enzyme immunoassay, ex situ, glucocorticoids, season, sex, wild

Editor: Steven Cooke

Received 3 September 2020; Revised 1 February 2021; Editorial Decision 18 April 2021; Accepted 18 April 2021

Cite as: Santymire RM, Ali N, Marinari PE, Livieri TM (2021) Using hair cortisol analysis to understand the biological factors that affect black-footed ferret (*Mustela nigripes*) stress physiology. Conserv Physiol 9(1): coab033; doi:10.1093/conphys/coab033.

Introduction

Anthropogenic activities have put pressure on wildlife habitat and populations resulting in nearly one million species threatened with extinction (Brondizio et al., 2019). There are many strategies employed to offset these pressures; however, often times to prevent extinction an ex situ population needs to be established. This was the case for the black-footed...
ferret (*Mustela nigripes*), North America’s only endemic ferret species. This species was driven to near extinction due to habitat loss, disease and a decline in its main prey species, the prairie dog (*Cynomys* spp.). In an effort to prevent extinction, the last 24 black-footed ferrets were removed from the wild near Meeteete, WY, from 1985 to 1987. Of those, only 15 produced offspring that survived with seven considered to be genetic founders of the population (Graves et al., 2019). After nearly 35 years of captive breeding, which is overseen by the US Fish and Wildlife Service (USFWS), more than 9600 ferrets have been produced (Marinari, 2019). And since 1991, captive-born black-footed ferrets have been pre-conditioned and reintroduced into the wild throughout North America’s Great Plains (Jachowski and Lockhart, 2009). However, managing wildlife *ex situ* can be challenging because of the difficulty to completely simulate their natural habitat including both environmental and social conditions. Therefore, zoos and other breeding facilities design habitats that meet as many of the of the animals’ needs as possible using species natural history while striving to maximize reproductive success.

Facilities accredited by the Association of Zoos and Aquariums strive to maintain the genetic health of the more than 500 species in their care for the next 100 years without acquiring new individuals from the wild. However, this can be challenging especially when only 20% of the breeding recommendations are successful across all Species Survival Plans® (Faust et al., 2019). For the black-footed ferret, there are currently no novel genes to bring into the population naturally, but over time the black-footed ferret *ex situ* has maintained nearly 86% of the population’s gene diversity (Graves et al., 2019). However, we have observed signs of inbreeding depression, which is the result of increased mating between closely related individuals (Wright, 1977; Charlesworth and Charlesworth, 1987; Thornhill, 1993; Frankham, 2005). Species experiencing inbreeding depression are at a high risk for fertility issues, such as sperm degradation (Fitzpatrick and Evans, 2009; Brekke et al., 2010), reduced pregnancy rates (van Noordwijk and Scharloo, 1981; González-Recio et al., 2007) and negative impacts on sperm quality, including motility, concentration and morphology (Fitzpatrick and Evans, 2009). Carnivorans are known to be susceptible to inbreeding depression with case studies in African lions in the Ngorongoro Crater in Tanzania (Wildt et al., 1987; Munson et al., 1996) and the Florida panther (Wildt et al., 1994).

For the black-footed ferret, Santymire et al. (2019) examined a 20-year interval of captive breeding to investigate the effects of increasing inbreeding coefficient (F) on reproductive traits and found that as F increased, the percentage of normal spermatozoa in the ejaculate declined. Specifically, we have observed a decrease in the percentage of normal spermatozoa in ejaculates, which started at 50% in 1990 and declined to 23% by 2000 and remained consistent through 2007 (Santymire et al., 2019) and through 2019 (Per Comm. R.M. Santymire). For female reproductive success, pregnancy rates declined from 60% in the 1990s to 38% in 2019 (Marinari, 2019). However, wild black-footed ferrets, who are descendants of the *ex situ* population, have a higher percentage of normal sperm (45–55% normal sperm; Santymire et al., 2006). Further, an estimated 75% or more of wild females observed are associated with a litter in the summer and fall (Pers. comm: Travis M. Livieri). Therefore, *ex situ* black-footed ferrets could be experiencing environmentally-dependent inbreeding depression (EDID), where the magnitude of inbreeding depression is enhanced by environmental conditions, possibly mediated/influenced by the neuroendocrine stress response (Cheptou and Donohue, 2011).

Stress caused by the *ex situ* environment may be due to a number of factors, such as lack of exercise, range allowance, light conditions, proximity of conspecifics, sounds, odours, temperature and tactile experiences (Morgan and Tromborg, 2007). Research has shown that chronic stress induced by the *ex situ* environment can put individuals at risk of reduced health including shorter lifespans, abnormal estrous cycling and high infant mortality in elephants (Clubb et al., 2008; Mason and Veasey, 2010). High prevalence of disease, premature death and reproductive issues were also observed in *ex situ* cheetahs Acinonyx jubatus (Terio et al., 2004). Additionally, evidence has shown that *ex situ* cheetahs have elevated glucocorticoids (GCs), which are steroid hormones released through the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Reeder and Kramer, 2005), indicating chronic stress as a major contributor to their difficulty living *ex situ* (Terio et al., 2004). The abnormal production of GCs can result in the down regulation of all non-essential processes, including reproduction. Specifically, elevated GC concentrations can directly inhibit release of gonadotropin-releasing hormone, which can then reduce the synthesis and release of follicle-stimulating hormone and luteinizing hormone, thus, reducing the gonadal hormone production including testosterone in males and progesterone and estrogen in females (Whirledge and Cidlowski, 2017). However, the main function of GCs is to mobilize energy through increased gluconeogenesis, and they are affected by several biological factors including age, sex, nutritional status, circadian rhythm, circannual rhythm and thermoregulation (reviewed in Reeder and Kramer, 2005).

GC production analysis has traditionally been measured via cortisol or corticosterone in blood, faeces, urine and/or saliva, which reflect acute responses to a perceived stressor. Recent endocrinological research has used hair samples for GC analysis (Stalder and Kirschbaum, 2012). The advantages of using hair over traditional biomaterials include that samples can be stored at room temperature because the steroids deposited in the hair will not degrade under variable environmental conditions and a longer term perspective of GC production (weeks to months; Stalder and Kirschbaum, 2012). The mechanism by which hormones are deposited into hair is not fully understood, but it is likely that GCs diffuse across the plasma membranes from blood capillaries.
into the growing hair cell (Stalder and Kirschbaum, 2012) and remain in the shaft during the rest phase (Harkey, 1993; Henderson, 1993; Thieme et al., 2003). However, steroids may be produced locally within the hair follicle (Ito et al., 2005; Keckes et al., 2012); therefore, hormones measured in hair may be a combination of long-term steroids stored during growth and recent steroid production from the hair follicle or surrounding glands (reviewed in Kalliokoski et al., 2019; Koren et al., 2019). Additionally, repeated Adrenocorticotropic Hormone (ACTH) challenges have resulted in increases of hair cortisol concentrations (HCCs), thus demonstrating that hair does reflect integrated levels of circulating GCs (Endo et al., 2018).

Overall, hair steroid analysis can be effective at comparing GC production across populations if certain precautions are taken, including collecting hair from the same location across individuals at the same time of year since hair growth rate can vary across individuals, or within an individual across seasons and along body regions (Carlitz et al., 2015; Fourie et al., 2016). Here, our goal was to use hair cortisol analysis to determine if the ex situ population of black-footed ferrets was experiencing chronic stress that was leading to abnormal concentrations of GCs that were affecting reproduction and to compare results to those of a wild population. Previous research has demonstrated that the ex situ population of black-footed ferrets (n = 72) produced higher faecal glucocorticoid metabolites (FGMs) than wild ferrets (n = 5; Poessel et al., 2011). However, the wild samples were collected in a different season compared to the ex situ samples. Additionally, these populations eat differently, both in respect to food items, horsemeat diet for ex situ and prairie dogs for wild, frequency (daily for ex situ and unknown for the wild) and quantity, which can affect defecation rates and then faecal hormone metabolite analysis (Goymann, 2012; Palme, 2019). Our specific objectives were to (i) compare HCCs between age classes (juveniles versus adults), (ii) compare the adult black-footed ferret HCCs of in situ and ex situ populations as well as the HCCs across different breeding facilities to determine if ex situ black-footed ferrets were experiencing higher levels of stress than they would naturally and (iii) determine the relationship between HCCs and reproductive success. We predicted that juveniles would have higher HCC than adults as observed in other species (Fryce et al., 2002; Fourie and Bernstein, 2011) and that ex situ black-footed ferrets would have higher HCC than in situ individuals.

Materials and methods

Study animals

As obligate predators of the prairie dog, wild black-footed ferret populations are established only within the confines of prairie dog colonies (Biggs et al., 2006) and utilize prairie dog burrows. Black-footed ferrets are a polygynous species with a strict breeding season in the spring (February through May) as the photoperiod begins to lengthen. With the onset of spring, spermatogenesis is initiated in males, as is an increase in testosterone production, causing the testes to enlarge (Hillman and Carpenter, 1984; Carvalho et al., 1991; Williams et al., 1991; Brown, 1997). Male testes firmness is an indication of breeding readiness (Hillman et al., 1984; Carvalho et al., 1991; Williams et al., 1991). Males annually defend larger territories around smaller female territories (Livieri and Anderson, 2012; Eads et al., 2014). However, in the ex situ breeding program, males are paired with females based on a pedigree analysis and not on mate choice selection (Graves et al., 2019). After a 42–43 day gestation period, parturition of altricial kits occurs during May–June. Maternal care is intensive through late summer. In the wild, kits reach adult size in the early fall and disperse from the natal area (Biggs and Eads, 2017).

For the ex situ populations of black-footed ferrets used in this study (n = 126 males and 33 females; 700 g to 1000 g; < 1 to 4 years old), we sampled when individuals were anesthetized for either management sampling or physical examinations. These black-footed ferrets were housed individually at the National Black-footed Ferret Conservation Center (FCC; Carr, CO; n = 121, males = 99, females = 22), Smithsonian Conservation Biology Institute (SCBI; Front Royal, VA; n = 24, males = 13, females = 11) or Louisville Zoological Gardens (LZG; Louisville, KY, n = 14, males = 14, females = 0). At FCC and LZG, ferrets were housed in indoor cages (1.0 m × 1.3 m × 1.0 m) with upper and lower nestboxes filled with Alpha-dri® substrate (Shepherd Speciality Paper, Watertown, TN, USA). At SCBI, ferrets were housed in indoor enclosures (3.6 m × 6.0 m × 4.0 m) with a mulch substrate and contained nestboxes filled with Alphadri® substrate. At all facilities, lighting was both natural (provided by skylights) and artificial (via fluorescent or LED illumination; set to the natural photoperiod). Black-footed ferrets were fed 75 to 100 g of a commercially available horsemeat diet (TOR; product was handled according to manufacturer’s recommendations) or rodent carcass daily and were provided with ad libitum water.

Wild black-footed ferrets (n = 186; males = 107, females = 79) were all wild born and were trapped from the fall of 2014 through the spring of 2017 during monitoring surveys at the Conata Basin/Badlands National Park reintroduction site. The Conata Basin is a portion of the Buffalo Gap National Grasslands (43°46 N and 102°14 W), administered by the United States Forest Service, in southwestern South Dakota, USA, and is directly adjacent to Badlands National Park. These two federal land units encompass 58 222 ha of mixed grass prairie with more than 5340 ha of black-tailed prairie dog colonies (Cynomys ludovicianus). Black-footed ferret reintroduction began in 1994 and ended in 1999. Sixty or more litters were documented annually from 2000 to 2008 and the site is considered self-sustaining (Jachowski and Lockhart, 2009) but requires intensive disease management, vector control in prairie dog burrows and direct vaccination of black-footed ferrets, to reduce the incidence of sylvatic...
plague (Yersinia pestis). Trapping and immobilization followed protocols of the Black-Footed Ferret Recovery Implementation Team (Kreeger, 1998; Black-footed Ferret Recovery Team, 2016). Briefly, animals were cage-trapped at night and returned to the same location following examination and recovery from anesthesia, usually within 1 hr of capture. All trapping was authorized by the Black-Footed Ferret Recovery Implementation Team under permit #TE064682-1 and was conducted by the US Forest Service, National Park Service and Prairie Wildlife Research as part of routine population monitoring for Conata Basin. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Lincoln Park Zoo Research Committee (Chicago, IL) and USFWS (Carr, CO).

Sample collection and processing

Hair was collected during medical examinations while black-footed ferrets were anesthetized in an anesthesia induction chamber via inhalation of isoflurane (Kreeger, 1998). All hair samples were shaved from the ventral side of the neck, under the chin about 1 inch by 1 inch. This area was chosen because the individual could not reach it for licking/grooming, which could add salivary hormones onto the hair, thereby confounding our cortisol measurements. This was also the area that was shaved for blood collection via the jugular vein. All sample collection took place either during the breeding season (March and April) or during routine medical examinations (December and January). Shaved hair was placed in pre-labelled, individual opaque paper envelopes and stored at room temperature until analysis.

Each hair sample was washed with 5.0 ml of 90% MeOH (methanol:distilled water) and placed on a mixer (Glas-Col, Terre Haute, IN) for 1 min at setting 50 to ensure consistent mixing across all samples. The methanol was decanted and an additional 5.0 ml of MeOH was added to the hair. This process was repeated three times. Hair samples were then placed in individual plastic trays until completely dry (~3 days; Schell et al., 2017).

Hormone extraction

Dried hair was pulverized to a fine powder (Omni Bead Ruptor 24, Kennesaw, GA, USA; on settings: 6.8 m/s, four 50 s intervals with 15 s break). Pulverized hair weighing 0.02 ± 0.005 g was then placed into pre-labelled plastic tubes and combined with 2 ml of 90% MeOH, vortexed briefly and agitated on the Glas-col mixer for 4 hr at speed 50. Tubes were then centrifuged (Sorvall R-4, Thermo-Scientific, Waltham, MA) for 15 min at 500 × g at 10°C, and the supernatant was decanted into clean, pre-labelled plastic tubes. The supernatant was then evaporated in a hot-water bath (DC30; Fisher Scientific, Waltham, MA, USA; 60°C) with air blowers in each individual tube. For assay analysis, dried extract was reconstituted with 250 μl of assay buffer (0.2 M NaH2PO4, 0.2 M Na2HPO4, NaCl, pH 7.0) to produce a four times concentrated sample. Glass beads were added to each tube and vortexed briefly. Samples were then sonicated (FS220, Fisher Scientific) for 20 min before analysis (Schell et al., 2017).

Cortisol enzyme immunoassay

We measured HCC by modifying a previously described cortisol enzyme immunoassay (EIA; Loeding et al., 2011). Briefly, 96-well Nunc plates (Thermo-Scientific) were coated with a goat anti-rabbit antibody (1:1000, Arbor Assays, Ann Arbor, MI, USA) and incubated at room temperature for 1 day. Contents were poured out and a blocking buffer (Arbor Assays) was added and incubated at room temperature for 1 day. The contents were poured off and then the plates were dried and stored at 5°C until used. On the day of the EIA analysis, polyclonal cortisol antiserum (R4866) and cortisol-3-CMO-peroxidase (provided by C. Munro (University of California, Davis, California) were used at dilutions of 1:375000 and 1:200000, respectively, and then used in the EIA protocol described by Munro and Stabenfeldt (1984). The cross-reactivities for cortisol antiserum were the following: cortisol, 100%; prednisolone, 9.9%; prednisone, 6.3%; cortisone, 5%; corticosterone, 0.7%; deoxycorticosterone, 0.3%; 21-deoxycorticosterone, 0.5%; 11-deoxycortisol, 0.2%; progesterone, 0.2%; 17α-hydroxyprogesterone, 0.2%; pregnenolone, 17α-hydroxyprogrenolone, androstenedione, testosterone, androsterone, dehydroepiandrosterone, dehydroisoandrosterone-3-sulfate, aldosterone, estradiol-17β, estrone, estriol, spironolactone and cholesterol, 0.1% (Young et al., 2004; Loeding et al., 2011).

To biochemically validate the cortisol EIA (Möstl and Palme, 2005), we evaluated the parallel relationship, using the Spearman rank-order test, between binding inhibition curves of hair extract dilutions (neat, twice concentration and quadrupled concentration) and the cortisol standard (r = 0.999), and the significant recovery (88%) of exogenous cortisol (1.95–1000 pg/well) added to hair extracts (y = 0.955x − 0.913; R² = 0.999) using linear regression analysis. Assay sensitivity was 1.95 pg/well and the intra- and inter-assay coefficients of variation were <15%.

Statistical analysis

Our overarching goal was to build a generalized linear mixed model (GLMM) that explains how various factors (season, sex and ex situ versus in situ) influence black-footed ferret HCCs. We first evaluate the normality of our dataset through visual inspection of histogram, quantile plots and the Cullen and Grey graph using the fitdistrplus package (Delignette-Muller and Dutang, 2015), which uses Pearson's distribution and Grey graph using the fitdistrplus package (Delignette–Muller and Dutang, 2015). We then evaluated the normality of our dataset through visual inspection of histogram, quantile plots and the Cullen and Grey graph using the fitdistrplus package (Delignette–Muller and Dutang, 2015), which uses Pearson's distribution and Grey graph using the fitdistrplus package (Delignette–Muller and Dutang, 2015). We then evaluated the normality of our dataset through visual inspection of histogram, quantile plots and the Cullen and Grey graph using the fitdistrplus package (Delignette–Muller and Dutang, 2015), which uses Pearson's distribution and Grey graph using the fitdistrplus package (Delignette–Muller and Dutang, 2015).
The pairwise tests allowed us to further investigate the relationships established by our GLMM.

We used our log-transformed data in a GLMM to determine what factors may influence HCC in the black-footed ferret. Sex, *ex situ* versus *in situ*, season and their interactions were evaluated. Sample year, individual identity and locations (Conata Basin, SD for wild, SCBI, FCC and LZG) were used as random effects. This model was fit in RStudio (Version 1.1.412; RStudio Team, 2020) using the packages ‘lmerTest’ and ‘MuMIn’ (Kuznetsova et al., 2017; Barton, 2018). All figures show untransformed HCC results. All analyses were conducted in RStudio (Version 1.1.412) using the following packages: ggplot2, dplyr and onewaytests (Wickham, 2016; Dag et al., 2018; Wickham et al., 2019).

To determine the relationship between HCC and reproductive success, we used reproductive records compiled from each *ex situ* facility and matched them for years in which we had HCC data (FCC, SCBI and LZG where available). Groups were compared using Welch’s *T*-test in RStudio, as above.

**Results**

**Age**

No kits were available in our dataset for the breeding season, so all inferences regarding comparisons of age classes are for the non-breeding season. Overall, wild juveniles had higher (\(F_1, 139.16 = 84.56, P < 0.0001\)) HCC than wild adults during the non-breeding season (Fig. 1). Juvenile females had higher HCC than adult females (\(F_1, 67.28 = 25.35, P < 0.0001\)) and adult males (\(F_1, 70.70 = 63.56, P < 0.0001\); Fig. 1). Juvenile males had higher HCC than adult males (\(F_1, 66.16 = 71.79, P < 0.0001\)) and adult females (\(F_1, 73.62 = 27.07, P < 0.0001\)). Juvenile male and female HCC were similar (\(F_1, 74.44 = 0.50, P > 0.05\)); however, adult females had higher (\(F_1, 61.27 = 14.15, P < 0.005\)) HCC than adult males during the non-breeding season. Because age affected HCCs, we then compared adult HCC between *in situ* and *ex situ* populations.

**Explanatory model**

The parameters that best predicted HCC for adults were the interactions among sex, *in situ* versus *ex situ* and season; all predictors were found to be significant in the model except *in situ* versus *ex situ* (Table 1). The marginal R² (fixed effects only) for the model is 0.23 while the conditional R² (fixed and random effects combined) for the model is 0.82. This implies that the random effects (individual ID, sample year, location) explain a large proportion of the variance. Sample year explains 36.1% of the variation not explained by fixed effects, individual identity accounts for 23.6% and location accounts for 16.8% (Table 1).

**Effect of *ex situ* versus *in situ*, sex and season on HCC**

While our GLMM did not find *ex situ* versus *in situ* alone to be significant in determining HCC, the interaction of *ex situ* versus *in situ* and season was significant. Specifically, during the non-breeding season, wild adult females had the highest HCC compared to wild adult males (\(F_1, 61.27 = 14.15, P < 0.005\)), FCC females (\(F_1, 11.85 = 12.25, P < 0.05\)) and SCBI females (\(F_1, 18.52 = 49.92, P < 0.0001\); Fig. 2). Wild male HCC was similar to FCC females (\(F_1, 13.51 = 1.64, P > 0.10\)) but was higher than SCBI females (\(F_1, 23.37 = 12.94, P < 0.005\); Fig. 2). Adult female HCCs from both captive locations were similar (\(F_1, 15.19 = 1.42, P > 0.10\)).

During the breeding season, wild males and SCBI males had similar (\(F_1, 22.90 = 0.28, P > 0.10\)) HCC but had higher HCC than wild females (wild: \(F_1, 17.80 = 7.25, P < 0.05\); SCBI: \(F_1, 19.23 = 11.30, P < 0.001\)), LZG males (wild: \(F_1, 21.58 = 32.91, P < 0.0005\); SCBI: \(F_1, 23.30 = 40.99, P < 0.0005\)), FCC females (wild: \(F_1, 17.38 = 20.60, P < 0.005\); SCBI: \(F_1, 18.69 = 27.38, P < 0.0005\)) and FCC males (wild: \(F_1, 14.37 = 7.00, P < 0.05\); SCBI: \(F_1, 15.85 = 11.53, P < 0.001\); Fig. 3). LZG males had lower HCC than FCC males (\(F_1, 19.86 = 26.32, P < 0.0005\)) but similar to FCC females (\(F_1, 19.65 = 3.32, P > 0.05\)). Adult wild females had higher (\(F_1, 14.91 = 5.84, P < 0.05, P = 0.029\)) HCC than FCC females during the breeding season but were similar (\(F_1, 14.41 = 0.25, P > 0.05, P = 0.63\)) to FCC males (Fig. 3).

HCC for FCC females was similar (\(F_1, 15.69 = 0.94, P > 0.1\)) across the seasons. HCC also was similar (\(F_1, 12.57 = 0.00, P > 0.1\)) across the seasons for wild females. In contrast, HCC for wild males was higher (\(F_1, 14.92 = 23.49, P < 0.005\)) during the breeding season compared to the non-breeding season.
Table 1: Model parameters from the fitted general linear mixed effects model for black-footed ferret hair cortisol concentrations (HCCs)

| Fixed effects                        | Value | Standard error | DF  | t-value | P-value |
|--------------------------------------|-------|----------------|-----|---------|---------|
| (Intercept)                          | 1.24  | 0.22           | 14.79 | 5.67    | <0.0001 |
| In situ versus ex situ               | 0.39  | 0.21           | 7.54 | 1.82    | 0.11    |
| Sex (M versus F)                     | 0.26  | 0.11           | 209.82 | 2.40 | <0.05    |
| Season (breeding versus non-breeding)| -0.62 | 0.15           | 225.04 | -4.19 | <0.0001 |
| Sex * season                         | -0.72 | 0.14           | 217.40 | -4.97 | <0.0001 |
| In situ versus ex situ * season      | 0.62  | 0.16           | 232.40 | 3.79    | <0.0005 |

Random effects

| Standard deviation | % Variance explained |
|--------------------|----------------------|
| Animal ID          | 0.26                 | 23.6%                   |
| Location           | 0.22                 | 16.8%                   |
| Sample year        | 0.33                 | 36.1%                   |
| Residual           | 0.26                 | 23.4%                   |

Ex situ populations compared to in situ population, comparing males (M) and females (F) across seasons (breeding versus non-breeding).

Figure 2: Boxplot of median cortisol concentrations (HCC; ng/g hair) of adult male and female black-footed ferrets by location including the wild individuals (n = 31 females, n = 34 males) from Conata Basin and from ex situ facilities including FCC (n = 10 females) and the SCBI (n = 11 females) during the non-breeding season. The median value is the center line in each box. The upper whisker extends from Q3 to the largest value and does not extend further than 1.5 * IQR; the lower whisker extends from Q1 to the smallest value and does not extend beyond 1.5 * IQR. Any data points that fall beyond the upper and lower whiskers are depicted as outliers, which were included in our analyses. Letters indicate differences (P < 0.05) across location and sex.

FCC, SCBI and LZG male data were only available during the breeding season so could not be compared across season.

Reproductive outcomes

When determining the relationship between HCCs and reproductive success, we found that HCC from sires at FCC (n = 51; 6.43 ± 0.36 ng/g) had higher (F1, 105.02 = 4.66, P = 0.033) HCC than males who did not sire (n = 94; 5.38 ± 0.29 ng/g). This was not the case for SCBI or LZG males. SCBI sires (n = 11; 4.59 ± 0.98 ng/g) had similar (F1, 5.92 = 1.82, P > 0.10) HCC to males who did not sire (n = 5; 7.66 ± 1.94 ng/g). Likewise, HCC for males who sired
et al. Conversely, in Canadian lynx (Lynx canadensis; Terwissen et al., 2013) and brown hares (Lepus europaeus; Esposito et al., 2017), there was no significant effect of age on hair cortisol levels. The influence of life stages on glucocorticoid production is dependent on timing and species (Heimbürge et al., 2019). We were thus able to establish that age has a significant impact on HCCs in the black-footed ferret.

We then built a model to explain HCC variation in the adult black-footed ferrets and determined that sex, season and interactions between sex and season and ex situ versus in situ and season were significant predictors of HCC. For sex, HCCs in wild males and females reflected opposite trends depending on season. During the non-breeding season, females had significantly higher HCC than males whereas in the breeding season, males had significantly higher HCC than females. This difference may be driven by the different roles that each sex plays in reproduction. For instance, during the breeding season the males may be establishing territories since they are a polygynous species (Wingfield and Sapolsky, 2003; Livieri and Anderson, 2012). Santymire et al. (2020) measured FGM in male black-footed ferrets before, during and after the breeding season and observed a significant increase in FGMs during the breeding season (Santymire et al., 2020). In general, increases in cortisol may reflect allostatic adjustments to different energy requirements (Sapolsky et al., 2000; Romero et al., 2009), which would be especially true during the breeding season. For example, in Japanese macaques (Macaca fuscata), mating frequency of subordinate males was positively correlated with cortisol levels (Barrett et al., 2002). Wild females had higher HCC than the wild males during the non-breeding season potentially due to the increased energy demands from supporting their offspring before they reach adult size and disperse away from their mothers.

When comparing the HCC of in situ and ex situ females, during the non-breeding season, wild females had higher HCCs than females from two breeding facilities (FCC and SCBI), which were similar. Higher HCC may be due to the increased energetic demands of living in the wild. Studies have shown that moderate to higher intensity exercise causes increases in circulating cortisol levels (Hill et al., 2008; Gerber et al., 2012). Wild females live on prairies where they have access to open land and large burrows. Ex situ individuals live in pens supplemented with a nest box. Wild females also have to kill prey to feed their offspring, whereas ex situ females do not have to provide food to their offspring after weaning. Even during the breeding season, wild females had higher HCC than FCC females. This may be because they are experiencing different stressors, such as multiple encounters with males versus one male selected by staff to pair with the female and nutritional stress with wild females still having to hunt and kill prey. We were not able to collect hair from SCBI females in the spring because most facilities do not anesthetize females during the breeding season and samples that we used were from FCC females who were anesthetized for surgical artificial insemination. HCC for both FCC females and wild females was consistent between both seasons. Another study has observed an increase in stress hormone production (via FGMs) in FCC females as they entered the breeding season, but this study was able to collect multiple samples over consecutive weeks (Poessell et al., 2011).

Discussion

The black-footed ferret recovery program has been extremely successful, producing nearly 10 000 individuals from seven genetic founders in ~35 years (Marinari, 2019). However, with out any novel genes to introduce into the population at least, naturally, F has continued to increase over the years from an estimated 0 to 0.1305 (Graves et al., 2019). As population relatedness has increased, we have observed increased infertility (declining seminal quality and whelping success) in the black-footed ferret (Santymire et al., 2019). The etiology of this infertility is unknown but may be related to environmental conditions since individuals living in the wild have improved semen quality and larger testes than captive males (Santymire et al., 2004; Santymire et al., 2014). Therefore, our goal was to compare GC production across in situ and ex situ black-footed ferrets, hypothesizing that chronic stress may be impacting ex situ reproduction. We used HCC analysis to compare different breeding facilities to a wild population of black-footed ferrets. We attempted to eliminate the issues of using hair to analyse the stress physiology by comparing HCC between populations and facilities within the same month within the season and shaving hair from the same location (ventral side of neck). This strategy was to reduce variation in the HCC due to time of year and location on the body (Carlitz et al., 2015; Fourie et al., 2016).

Because age can affect reproductive success and can influence GC production, we first chose to compare HCCs between juvenile and adult black-footed ferrets. Age has been shown to affect HCC in several species from non-human primates to cattle (reviewed in Heimbürge et al., 2019). We did observe higher HCCs in juveniles (~90 to 120 days of age) compared to adults that were 1 to 4 years of age. Because adolescence is a time of rapid growth, GCs may be elevated to manage the greater metabolic demands. Additionally, age class is identified by a shift in neuroendocrine functioning (Romeo, 2010, 2013). For example, studies have shown that juveniles have higher GCs than adults in the rat Rattus rattus (Romeo et al., 2004), Egyptian mongoose (Herpestes ichneumon; Azevedo et al., 2019) and mountain goats (Oreamnos americanus; Dulude-de Brion et al., 2019). Conversely, in Canadian lynx (Lynx canadensis; Terwissen et al., 2013) and brown hares (Lepus europaeus; Esposito et al., 2017), there was no significant effect of age on hair cortisol levels. The influence of life stages on glucocorticoid production is dependent on timing and species (Heimbürge et al., 2019). We were thus able to establish that age has a significant impact on HCCs in the black-footed ferret.
For males, HCC during the breeding season was similar between wild and SCBI individuals but lower in FCC males and was the lowest in LZG black-footed ferrets. This could be driven by environmental conditions. Males were housed in smaller breeding cages at FCC and LZG, but black-footed ferrets at SCBI are maintained in larger pens with mulch for substrate allowing for greater intensity of activity. Previous research has determined that black-footed ferrets raised at SCBI have longer limbs, which the authors suggested was because the larger pens allow them more space to engage in physical activity (Wisely et al., 2005). While FCC males have more varied HCC, the majority of individuals are concentrated at lower levels than SCBI and wild males. LZG males had the lowest HCC even though their pens are identical in size to FCC pens. Here, lower HCC could be related to the management practices. For example, the building where black-footed ferrets are located at LZG has more human presence and continuous noise. FCC and SCBI are specialized breeding facilities maintained separately from other animals and away from excessive noise. LZG individuals may have become habituated to human disturbances, which might explain the significantly lower levels of HCC. Interestingly, wild male HCC increased during the breeding season compared to non-breeding season (cross-season data not available for other males). This could be related to establishing and defending territories around females. Increasing GC production during the breeding season has been observed previously in FCC males via FGM analysis (Santymire et al., 2020).

Overall, we did observe a relationship between HCC and reproductive success with sires at FCC having higher HCC. Our original hypothesis was that ex situ black-footed ferrets would have higher HCCs because it is presumed that animals in zoos have more psychological stressors such as visitor presence, the inability to disperse from social pressures, limited usable space and lack of natural diets (Morgan and Tromborg, 2007). However, zoos make great efforts to simulate the natural environment that stimulate natural behaviours for species they house. Interestingly, we found the inverse relationship with sires having higher HCC than males that did not sire that breeding season. Perhaps, low HCC is an indicator of an underlying issue of chronic stress-rated pathology where HPA axis is downregulated due to allostatic overload, which is the cumulative damage to bodily functions and health due to repeated attempts at maintaining homeostasis (reviewed in McEwen and Wingfield, 2010; Juster et al., 2010).

In addition to the evaluating biological relevance of the HCC for the black-footed ferrets, we should validate the biochemical analysis further. Here, we conducted the standard biochemical laboratory validations to ensure the sample’s antigens were binding to the cortisol antibody similar to the standards (the parallelism) and we confirmed that there was no interference with the sample’s hormones binding to the antibody (the percent recovery). However, future research efforts should include performing high-pressure liquid chromatography paired with simultaneous mass spectrometry (LC–MS/MS) to identify the conjugated and unconjugated immunoreactive steroids that are found in the black-footed ferret hair extracts. This will ensure that the EIA system is producing exact quantification of the steroids and that they are biologically relevant. Recent efforts have analysed the steroidal components for hair extracts from six different mammalian species including two carnivores and four omnivores using the same cortisol polyclonal antibody and HRP (Jewgenow et al., 2020). Interestingly, these authors determined that this cortisol EIA was over-estimating the amount of up to three times higher compared to the LC–MS/MS results; however, another EIA (cortisol-21-HS) produced 2.3 to 12 times higher HCC than this cortisol EIA. Because each analysis is species specific, it will be important to conduct the LC–MS/MS analyses on black-footed ferret HCC to validate the interpretations and to precisely identify the steroids that are being measured.

In conclusion, we demonstrated that juveniles have higher HCC than adult black-footed ferrets. In adults, HCCs were influenced by all factors in the model including sex, in situ versus ex situ and season. We determined that ex situ ferrets do not have higher HCC than wild individuals and high HCC were not related to poor reproductive success. In fact, it was almost the opposite relationship than what we predicted, with FCC males, who sired, with significantly higher HCC compared to the males who did not sire. This suggests that higher HCC may be important for reproduction and that low HCC may signal issues with allostatic overload or dysfunction of the HPA axis regulation. Further analysis is needed to determine the relationship between low HCC, HPA function and reproductive success in the black-footed ferret. Unfortunately, we were only able to compare the effect of location on female HCC across the seasons because we did not have any samples from ex situ males in the non-breeding season. In the wild, male HCC did increase in the breeding season. Santymire et al. (2020) observed an increase in FGMs during the breeding season in FCC males, so perhaps we would have observed a similar result in the HCC.

Ex situ black-footed ferret HCC did not necessarily reflect wild trends and seemed to be influenced by breeding facility. During breeding season, SCBI males exhibited similar HCCs to wild males, while FCC and LZG males had lower HCC. The low HCCs exhibited by LZG males cannot be explained by cage size alone, since FCC males live in similar sized cages, but may be related to management practices. Because the ex situ environments cannot completely mimic the wild environment, there may be unintended consequences (Fischer and Romero, 2019). For example, in the wild, black-footed ferrets are solitary except for a dam with her kits. Males and females typically encounter one another only during breeding season. In the ex situ environment, males and females live in separate cages but are housed adjacent to each other in the same room. Though not well studied, housing solitary, seasonal breeders together ex situ can lead to behaviour issues and sexual
incompatibility, such as was the case for captive cheetahs for many years (Wielebnowski et al., 2002; Schulte-Hostede and Mastromonaco, 2015; McDermott, 2019). It is a goal of zoo breeding programs to have individuals exhibit similar behaviours and physiological responses as their wild counterparts. This can impact the overall fitness of the species, which is especially critical in the case of endangered species. In the case of the *ex situ* black-footed ferret, fertility has been found to be reduced compared to wild counterparts (Santymire et al., 2004; Santymire et al., 2014). Although HCC was not found to be correlated with reproductive success, the fact that both HCC and reproductive measurements are significantly different between wild and some breeding facilities indicate that the variable environments may be increasing negative effects on fitness in the black-footed ferret, as posited by EDID theory (Cheptou and Donohue, 2011). Specifically, certain environmental conditions may increase the magnitude of inbreeding depression experienced by black-footed ferrets. While investigations are still underway to determine what specific genetic mutations the black-footed ferrets have compared with non-inbred related species, the significantly different reproductive and stress phenotypes exhibited by ferrets living under different conditions lends evidence to EDID as a culprit.

Alternatively, differential HCCs based on environmental differences can be a result of metabolic rate rather than perceived stress. As previously discussed, GCs are essential for energy mobilization, and access to larger cages and more exercise, paired with changes in seasonal breeding demands, may explain some of the differences we observed between locations (MacDougall-Shackleton et al., 2019). Additionally, GCs have pleiotropic effects on a large suite of genes (Xavier et al., 2016; MacDougall-Shackleton et al., 2019). It is possible that the varying levels of HCC indicate different metabolic demands and that these varying levels cause differential gene expression downstream. As aforementioned, that differential gene expression may impact fitness metrics such as reproduction.

With just an estimated 700 black-footed ferrets total living *in situ* and *ex situ*, it is important to continue to investigate the etiology of infertility. While stress does not seem to be directly impacting fitness in *ex situ* individuals, it could be contributing indirectly to causes of infertility and may be influenced by other factors, such as nutrition. For example, antioxidant deficiency can lead to oxidative stress, which has been shown to damage spermatozoa membranes and DNA (De Iulisi and Aitken, 2009; Wright et al., 2014). Abnormal morphology can prevent spermatozoa from reaching the ovum (Agarwal and Saleh, 2002; Pukazhenthi et al., 2006; Wright et al., 2014) and high levels of sperm DNA damage can prevent pregnancy or lead to offspring abnormalities (Ahmadi and Ng, 1999; Wright et al., 2014; Menezo et al., 2016). While oxidative stress is not measured by cortisol, diet may influence cortisol levels, and cortisol levels may thereby play a role in influencing expression in genes related to fertility (Whirledge and Cidlowski, 2017; von Krogh et al., 2019) that then compound with the symptoms of oxidative stress, impacting reproductive success. Studies are currently underway to understand how different diets in the black-footed ferret may promote or prevent antioxidant uptake and how this influences gene expression, oxidative stress and spermatozoa health.

The black-footed ferret program will continue to use science-based management to recover the species. Other tools of propagation are also being investigated including intra-species somatic cell nuclear transfer (Wisely, 2016). In 2021, a black-footed ferret was cloned using a cell line from 1988, marking the first ever cloning of a North American endangered species (Imbler, 2021). Additionally, we could incrementally increase gene diversity by using frozen–thawed semen from wild and/or *ex situ* founders or at least individuals that were in the population decades ago for artificial insemination as previously demonstrated (Howard et al., 2016). As more and more species need to be managed in *ex situ* breeding programs to avoid extinction, we can use information generated from the black-footed ferret recovery program to help inform our understanding of how to manage wild animals *ex situ*. The short generation time of black-footed ferret populations works to our advantage because we can obtain rapid results from management changes. Also, as one of the oldest and most successful *ex situ* programs, along with the California condor (*Gymnogyps californianus*) and Wyoming toad (*Anaxyrus baxteri*), we have multitudes of generations that have informed our research and that can provide information on phenotypic and genetic change in the *ex situ* breeding program that would otherwise only be available decades from now in other programs with species that might have longer generation times or a social hierarchy that prolongs breeding.

**Acknowledgements**

We thank Katherine Fowler and Ivy Yen for laboratory assistance. We also thank Robyn Bortner and Dr Della Garelle at FCC for assistance with sample collection. Finally, we thank the animal care staff at the FCC, LZG and SCBI for assistance with the project.

**Funding**

Funding was provided by The Davee Foundation, Association of Zoos and Aquariums and Prairie Wildlife Research.

**References**

Agarwal A, Saleh RA (2002) Role of oxidants in male infertility: rationale, significance, and treatment. *Urol Clin North Am* 29: 817–827.

Ahmadi A, Ng S-C (1999) Fertilizing ability of DNA-damaged spermatozoa. *J Exp Zool* 284: 696–704.
Azevedo A, Bailey L, Bandeira V, Dehnhard M, Fonseca C, de Sousa L, Jewgenow K (2019) Age, sex and storage time influence hair cortisol levels in a wild mammal population. *PloS One* 14: e0221124.

Barrett GM, Shimizu K, Bardi M, Asaba S, Mori A (2002) Endocrine correlates of rank, reproduction, and female-directed aggression in male Japanese macaques (*Macaca fuscata*). *Horm Behav* 42: 85–96.

Bartoń K (2018) MuMIn: Multi-Model Inference. R package version 1.42.1.

Brekke P, Bennett PM, Wang J, Pettorelli N, Ewen JG (2010) Sensitive males: inbreeding depression in an endangered bird. *Proc. Royal Soc. B.* 277: 3677–384.

Brondizio ES, Settele J, Díaz S, Ngo HT (2019) Global assessment Report on Biodiversity and Ecosystem Services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. IPBES, IPBES Secr.

Brown JL (1997) Fecal steroid profiles in black-footed ferrets exposed to natural photoperiod. *J Wildl Manag* 61: 1428–1436.

Carlitz EHD, Kirschbaum C, Miller R, Rukundo J, van Schaik CP (2015) Effects of body region and time on hair cortisol concentrations in chimpanzees (*Pan troglodytes*). *Gen Comp Endocrinol* 223: 9–15.

Carvalho CF, Howard J, Collins L, Wemmer C, Bush M, Wildt DE (1991) Captive breeding of black-footed ferrets (*Mustela nigripes*) and comparative reproductive efficiency in 1-year-old versus 2-year-old animals. *J Zoo Wildl Med* 22: 96–106.

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. *Annu Rev Ecol Syst* 18: 237–268.

Chetou P-O, Donohue K (2011) Environment-dependent inbreeding depression: its ecological and evolutionary significance. *New Phytol* 189: 395–407.

Clubb R, Rowcliffe M, Lee P, Mar KU, Moss C, Mason GJ (2008) Comprised survivorship in zoo elephants. *Science* 322: 1649.

Dag O, Dolgun A, Konar NM (2018) One-way tests: an R package for one-way tests in independent groups designs. *R J* 10: 175–199.

De Julis GN, Atken RJ (2009) On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* 16: 3–13.

Delignette-Muller M, Dutang C (2015) Fitdistrplus: an R package for fitting distributions. *J Stat Softw* 64: 1–34.

Dulude-de Broin F, Côté SD, Whiteside DP, Mastromonaco GF (2019) Faecal metabolites and hair cortisol as biological markers of HPA-axis activity in the rocky mountain goat. *Gen Comp Endocrinol* 280: 147–157.

Eads DA, Biggins DE, Livieri TM, Millsbaugh JJ (2014) Space use, resource selection and territoriality of black-footed ferrets: implications for reserve design. *Wildlife Biol* 20: 27–36.

Endo N, Yamane H, Rahayu LP, Tanaka T (2018) Effect of repeated adrenocorticotropic hormone administration on reproductive function and hair cortisol concentration during the estrous cycle in goats. *Gen Comp Endocrinol* 259: 207–212.

Esposito L, Auletta L, Ciani F, Pelagalli A, Pasolini MP, Lamagna B, Piscopo N, Amici A (2017) Hair cortisol levels in captive brown hare (*Lepus europaeus*): potential effect of sex, age, and breeding technology. *Eur J Wildl Res* 63: 62.

Faust LJ, Long ST, Perisín K, Simons J (2019) Uncovering challenges to sustainability of AZA Animal Programs by evaluating the outcomes of breeding and transfer recommendations with PMCTrack. *Zoo Biol* 38: 24–35.

Fischer CP, Romero LM (2019) Chronic captivity stress in wild animals is highly species-specific. *Conserv Physiol* 7. doi: 10.1093/conphys/coz093.

Fitpatrick JL, Evans JP (2009) Reduced heterozygosity impairs sperm quality in endangered mammals. *Biol Lett* 5: 320–323.

Fourie NH, Bernstein RM (2011) Hair cortisol levels track phylogenetic and age related differences in hypothalamic–pituitary–adrenal (HPA) axis activity in non-human primates. *Gen Comp Endocrinol* 174: 150–155.

Fourie NH, Brown JL, Jolly CJ, Phillips-Conroy JE, Rogers J, Bernstein RM (2016) Sources of variation in hair cortisol in wild and captive non-human primates. *Fortschr Zool* 119: 119–125.

Frankham R (2005) Genetics and extinction. *Biol Conserv* 126: 131–140.

Gerber M, Brand S, Lindwall M, Elliot C, Kalak N, Herrmann C, Jonsdottir IH (2012) Concerns regarding hair cortisol as a biomarker of chronic stress in exercise and sport science. *J Sports Sci Med* 11: 571–581.

González-Recio O, López de Maturana E, Gutiérrez JP (2007) Inbreeding depression and hair cortisol concentrations during the estrous cycle in goats. *J Dairy Sci* 90: 5744–5752.

Goymann W (2012) On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Methods Ecol Evol* 3: 757–765.

Graves G, Santymire R, Marinari P, Lynch C (2019) *Population Analysis and Breeding and Transfer Plan for the Black-Footed Ferret, Mustela nigripes*. Association of Zoos and Aquariums, Chicago, IL.

Harkey MR (1993) Anatomy and physiology of hair. *Forensic Sci Int* 63: 9–18.

Heimbürge S, Kanitz E, Ottew W (2019) The use of hair cortisol for the assessment of stress in animals. *Gen Comp Endocrinol* 270: 10–17.

Henderson GL (1993) Mechanisms of drug incorporation into hair. *Forensic Sci Int* 63: 19–29.
Hill EE, Zack E, Battaglini C, Viru M, Viru A, Hackney AC (2008) Exercise and circulating cortisol levels: the intensity threshold effect. J Endocrinol Invest 31: 587–591.

Hillman CN, Carpenter JW (1984) Breeding biology and behaviour of captive black-footed ferrets. Int Zoo Yearb 23: 186–191.

Howard JG, Lynch C, Santymire RM, Marinarri PE, Wildt DE (2016) Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret. Anim Conserv 19: 102–111.

Imbler S (2021) Meet Elizabeth Ann, the First Cloned Black-Footed Ferret. The New York Times.

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Paus R (2005) Hair follicle fibroblasts display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. FASEB J 19: 1332–1334.

Jachowski D, Lockhart J (2009) Reintroducing the black-footed ferret Mustela nigripes to the Great Plains of North America. Small Carniv Conserv 41: 58–64.

Jewgenow K, Azevedo A, Albrecht M, Kirschbaum C, Dehnhard M (2020) Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results. Conserv Physiol 8. doi: 10.1093/conphys/coaa009.

Juster RP, McEwen BS, Lupien SJ (2010) Allostatic load biomarkers of chronic stress and impact on health and cognition. Neurosci Biobehav Rev 35: 2–16.

Kalliokoski O, Jellestad FK, Murison R (2019) A systematic review of Koznetsova A, Brockhoff P, Christensen R (2017) lmerTest package: tests inlinearmixedeffectsmodels. J Stat Softw 82: 1–26.

Keckeis K, Lepschy M, Schöpper H, Moser L, Troxler J, Palme R (2005) Measurement of corticosterone metabolites in birds' droppings: an analytical approach. Ann NY Acad Sci 1046: 17–34.

Kuznetsova A, Brockhoff P, Christensen R (2017) lmerTest package: tests in linear mixed effects models. J Stat Softw 82: 1–26.

Livieri TM, Anderson EM (2012) Black-footed ferret home ranges in Conata Basin, South Dakota. West N Am Nat 72: 196–205.

Loeding E, Thomas J, Bernier D, Santymire R (2011) Using fecal hormonal and behavioral analyses to evaluate the introduction of two sable antelopes at Lincoln Park zoo. J Appl Anim Welf Sci 14: 220–246.

MacDougall-Shackleton SA, Bonier F, Romero LM, Moore IT (2019) Glucocorticoids and “stress” are not synonymous. Integr Org Biol 1: 1–7. doi: 10.1093/ioa/obz017.

Mariani P (2019) North American Regional Black-Footed Ferret Studbook. Association of Zoos and Aquariums, Front Royal.
Santymire RM, Livieri TM, Branvold-Faber H, Marinari PE (2014) The black-footed ferret: on the brink of recovery? In WV Holt, JL Brown, P Comizzoli, eds, Reproductive Sciences in Animal Conservation: Progress and Prospects. Springer New York, New York, NY, pp. 119–134.

Santymire RM, Marinari PE, Kreeger JS, Wildt DE, Howard J (2006) Sperm viability in the black-footed ferret (Mustela nigripes) is influenced by seminal and seminiferous. Cryobiology 41: 37–50.

Santymire RM, Marinari PE, Kreeger JS, Wildt DE, Howard J (2019) Inbreeding causes decreased seminal quality affecting pregnancy and litter size in the endangered black-footed ferret. Anim Conserv 22: 331–340.

Santymire RM, Lavin SR, Branvold-Faber H, Kreeger J, Che-Castaldo J, Rafacz M, Marinari P (2020) Influence of vitamin E and carcass feeding supplementation on fecal glucocorticoid and androgen metabolites in male black-footed ferrets (Mustela nigripes). PLoS One 15: e0241085.

Santymire RM, Howard JG, Wisely SM, Kreeger JS, Marinari PE, DE W (2004) Seminal characteristics of wild black-footed ferrets (Mustela nigripes). Defenders of Wildlife Carnivores-Expanding Partnerships in Carnivore Conservation 200.

Sapolsky RM, Romero LM, Muncyk AJ (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21: 55–89.

Schell CJ, Young JK, Lonsdorf EV, Mateo JM, Santymire RM (2017) Investigation of techniques to measure cortisol and testosterone concentrations in coyote hair. Zoo Biol 36: 220–225.

Schulte-Hostedde AJ, Mastromonaco GF (2015) Integrating evolution in the management of captive zoo populations. Evol Appl 8: 413–422.

Stalder T, Kirschbaum C (2012) Analysis of cortisol in hair – state of the art and future directions. Brain Behav Immun 26: 1019–1029.

Terio KA, Marker L, Munson L (2004) Evidence for chronic stress in captive but non-free-ranging cheetahs (Acinonyx jubatus) based on adrenal morphology and function. J Wild Dis 40: 259–266.

Terwissen CV, Mastromonaco GF, Murray DL (2013) Influence of adrenocorticotropic hormone challenge and external factors (age, sex, and body region) on hair cortisol concentration in Canada lynx (Lynx canadensis). Gen Comp Endocrinol 194: 162–167.

Thieme D, Anielski P, Grosse J, Sachs H, Mueller RK (2003) Identification of anabolic steroids in serum, urine, sweat and hair: comparison of metabolic patterns. Anal Chim Acta 483: 299–306.

Thornhill NW (1993) The Natural History of Inbreeding and Outbreeding: Theoretical and Empirical Perspectives. University of Chicago Press, Chicago.

van Noordwijk AJ, Scharloo W (1981) Inbreeding in an island population of the great tit. Evolution 35: 674–688.

von Krogh K, Bjarnadal GT, Nourizadeh-Lilabadi R, Ropstad E, Haug TM, Weltzien F-A (2019) Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary cultures dependent on stage of sexual maturation. Comp Biochem Physiol A Mol Integr Physiol 236: 110517.

Whirledge S, Cidlowski JA (2017) Glucocorticoids and reproduction: traffic control on the road to reproduction. Trends Endocrinol Metab 28: 399–415.

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer, New York.

Wickham H, François R, Henry L, Müller K (2019) dplyr: A Grammar of Data Manipulation. R package version 0.8.3.

Wielebnowski NC, Ziegler K, Wildt DE, Lukas J, Brown JL (2002) Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (Acinonyx jubatus). Anim Conserv 5: 291–301.

Wildt DE, Bush M, Goodrowe KL, Packer C, Pusey AE, Brown JL, O’Brien SJ (1987) Reproductive and genetic consequences of founding isolated lion populations. Nature 329: 328–331.

Wildt DE, Howard J, Barone MA, Roelke ME, Brown JL, Anderson AE (1994) Reproductive characteristics of male Florida panthers: comparative studies from Florida, Texas, Colorado, Latin America, and North American zoos. J Mammal 75: 150–162.

Williams ES, Thorne ET, Kwiatkowski DR, Anderson SL, Lutz K (1991) Reproductive biology and management of captive black-footed ferrets (Mustela nigripes). Zoo Biol 10: 383–398.

Wingfield JC, Sapolsky RM (2003) Reproduction and resistance to stress: when and how. J Neuroendocrinol 15: 711–724.

Wisely SM (2016) Genome resource banks pay conservation dividends. Anim Conserv 19: 114–115.

Wisely SM, Santymire RM, Livieri TM, Marinari PE, Kreeger JS, Wildt DE, Howard J (2005) Environment influences morphology and development for in situ and ex situ populations of the black-footed ferret (Mustela nigripes). Anim Conserv 8: 321–328.

Wright C, Milne S, Leeson H (2014) Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reprod Biomed Online 28: 684–703.

Wright S (1977) Evolution and the Genetics of Populations, Volume 3: Experimental Results and Evolutionary Deductions. University of Chicago Press, Chicago.

Xavier AM, Anunciato AKO, Rosenstock TR, Glezer I (2016) Gene expression control by glucocorticoid receptors during innate immune responses. Front Endocrinol 7: 31.

Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, Brown JL (2004) Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. Gen Comp Endocrinol 137: 148–165.