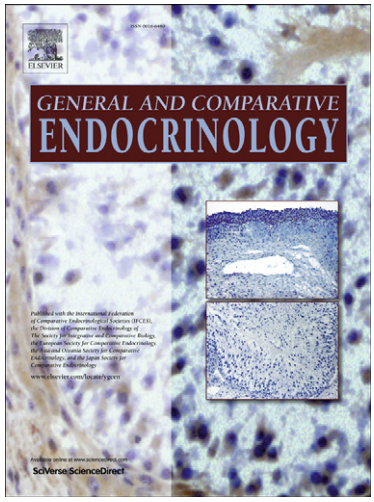


Analyses of fecal and hair glucocorticoids to evaluate short- and long-term stress and recovery of Asiatic black bears (*Ursus thibetanus*) removed from bile farms in China

K.D. Malcolm, W.J. McShea, T.R. Van Deelen, H.J. Bacon, F. Liu, S. Putman, X. Zhu, J.L. Brown

PII: S0016-6480(13)00054-3
DOI: http://dx.doi.org/10.1016/j.ygcen.2013.01.014
Reference: YGCEN 11390

To appear in: *General and Comparative Endocrinology*

Received Date: 20 September 2012
Revised Date: 8 January 2013
Accepted Date: 19 January 2013

Please cite this article as: Malcolm, K.D., McShea, W.J., Van Deelen, T.R., Bacon, H.J., Liu, F., Putman, S., Zhu, X., Brown, J.L., Analyses of fecal and hair glucocorticoids to evaluate short- and long-term stress and recovery of Asiatic black bears (*Ursus thibetanus*) removed from bile farms in China, *General and Comparative Endocrinology* (2013), doi: http://dx.doi.org/10.1016/j.ygcen.2013.01.014

Analyses of fecal and hair glucocorticoids to evaluate short- and long-term stress and recovery of Asiatic black bears (*Ursus thibetanus*) removed from bile farms in China

K.D. Malcolm*[a,b], W.J. McShea[b], T.R. Van Deelen[a], H.J. Bacon[c], F. Liu[d], S. Putman[b], X. Zhu[e], and J.L. Brown[b]

[a] *Department of Forest and Wildlife Ecology, University of Wisconsin, Madison, WI, USA*

[b] *Smithsonian Conservation Biology Institute, National Zoological Park, Smithsonian Institution, Front Royal, VA, USA*

[c] *Sichuan-Lonqiao Black Bear Rescue Centre, Animals Asia Foundation, Longqiao, Xin Du District, Chengdu, Sichuan Province, P.R. China*

[d] *Institute of Forestry Ecology, Environment, and Protection, Chinese Academy of Forestry, Haidian, Beijing, P. R. China*

[e] *School of Life Sciences, Peking University, Beijing, P.R. China*

*Corresponding Author, Address: University of Wisconsin – Madison, Department of Forest and Wildlife Ecology, 1630 Linden Drive, Madison WI 53706, e-mail: karl.d.malcolm@gmail.com

Abstract: Demand for traditional Chinese medicines has given rise to the practice of maintaining Asiatic black bears (Ursus thibetanus) in captivity to harvest bile. We evaluated hypothalamic-pituitaryadrenal (HPA) activity in Asiatic black bears on a bile farm in China by measuring cortisol in hair. We also monitored hair and fecal glucocorticoid metabolites as bears acclimated to improved husbandry at the Animals Asia Foundation China Bear Rescue Center (CBRC) after removal from other bile farms. Fecal samples were collected twice weekly for ~1 year, and hair was obtained from bears upon arrival at the CBRC and again >163 days later. Paired hair samples showed declines in cortisol concentrations of 12-88% in 38 of 45 (84%, $P < 0.001$) bears after arrival and acclimation at the rehabilitation facility. Concentrations of cortisol in hair from bears on the bile farm were similar to initial concentrations upon arrival at the CBRC but were higher than those collected after bears had been at the CBRC for >163 days. Fecal glucocorticoid concentrations varied across months and were highest in April and declined through December, possibly reflecting seasonal patterns, responses to the arrival and socialization of new bears at the CBRC, and/or annual metabolic change. Data from segmental analysis of hair supports the first of these explanations. Our findings indicate that bears produced elevated concentrations of glucocorticoids on bile farms, and that activity of the HPA axis declined following relocation. Thus, hair cortisol analyses are particularly well suited to long-term, retrospective assessments of glucocorticoids in ursids. By contrast, fecal measures were not clearly associated with rehabilitation, but rather reflected more subtle endocrine changes, possibly related to seasonality.

Keywords: Fecal glucocorticoids; Hair cortisol; Asiatic black bear; *Ursus thibetanus*; Bile farming; Stress

1. Introduction

As of 2010 there were approximately 97 facilities in China that maintained Asiatic black bears (*Ursus thibetanus*) and other bears (*Ursus* spp.) for production of bile to be used in traditional medicines [14]. Because bile farming is a sensitive issue, access to bears on farms is strictly controlled, and data on the physiologic effects of bile collection practices are limited. However, it is often assumed that many bears on bile farms experience significant stress resulting from poor management and bile extraction that impacts their health and welfare [14]. Documented evidence of compromised welfare in farmed bears includes a wide array of physical and mental ailments (e.g., growth retardation, ulcers, stereotypic behavior, self-mutilation, etc., [29]) consistent with chronic stress [39]. Most bears removed from bile farms seem to adjust well to conditions at rehabilitation facilities after relocation [2], although changes in adrenal hormone levels have not previously been quantified in these populations. This study capitalized on a unique opportunity to evaluate short- and long-term changes in activity of the hypothalamic-pituitary-adrenal (HPA) axis as an index of stress status and recovery in bears transitioning from prolonged captivity on bile farms to the Animals Asia Foundation China Bear Rescue Centre (CBRC).

Fluctuations in glucocorticoid concentrations are often associated with normal metabolic function, including the mobilization of stored energy [8], and serve to trigger physiological and behavioral changes that promote survival by allowing individuals to respond to stressors [45]. However, long-term production of elevated glucocorticoids in response to chronic stress is counterproductive and associated with diverse health problems [42, 46]. Among the potential consequences of prolonged stimulation of the HPA axis is the failure to elicit a normal response to continued or subsequent stressful stimuli [33, 48]. In contrast to the general association

between stress and increased HPA activity, these cases are characterized by low (i.e., at or below baseline) circulating levels of corticoids, which reflect the physiologic costs of protracted HPA stimulation.

Feces have long been used as a non-invasive tool for monitoring HPA activity in wildlife [6, 15, 34, 35, 37], and analyses of excreted glucocorticoid metabolites have helped identify environmental, management, and social variables that are potentially stressful [8, 27, 32, 40]. However, the logistical limitations of accessing farmed bears for stress studies have, to date, prevented similar investigations from being conducted in farm settings.

Contrasting feces, follicular cells of hair incorporate some fraction of circulating cortisol via diffusion as hairs grow [9]. Recent work in guinea pigs provides evidence of local corticoid synthesis in the hair follicles themselves [23]. Though much work remains to fully elucidate the pathways leading to the production and incorporation of glucocorticoids in hair, increases in circulating ACTH could theoretically lead to elevated hair cortisol via either or both mechanisms described above. In both cases a growth rate-dependent delay between high circulating ACTH and elevated hair cortisol should also be expected. Consequently hair is a medium uniquely appropriate for assessing HPA activity retrospectively [12]. Compared to other biological samples (e.g., blood, saliva, urine) that can vary within individuals over short time periods, hair cortisol concentrations reflect a pre-sampling timespan of weeks to months, depending on rates of hair growth and turnover [12, 26]. Achieving similar long-term assessments with other sample media, including feces, requires repeatedly sampling individuals [22], which is not always tenable in wildlife studies. Hair cortisol analysis, as a potential indicator of longer-term stress, may therefore be a more practical approach for studying wildlife in situations where access to animals is limited. Methods to quantify cortisol in hair have been developed for

several species (e.g., [1, 12, 25]), and in humans prolonged exposure to a noxious stimulus (chronic pain) was related to elevations in hair cortisol concentrations [44]. Macbeth et al. [31] measured cortisol in hair samples from grizzly bears (*Ursus arctos*), comparing a number of hair characteristics (e.g., color, age of bear, sex of bear, hair from different body regions, various hair types, etc.) to cortisol concentrations. More recently the technique has been applied to studies of polar bears (*Ursus maritimus*) in southern Hudson Bay and Greenland [4, 30]. However, no studies have tracked long-term patterns of hair cortisol in any bear species in relation to stressors stemming from their management.

In our study, we aimed to identify a suitable enzyme immunoassay for monitoring adrenocortical activity of captive Asiatic black bears using samples of hair and feces. Once validated, we applied the assay to address the following objectives: (1) determine if bears on bile farms exhibited higher hair cortisol concentrations than those after relocation and rehabilitation or exhibited signs of adrenal exhaustion, (2) determine if individual bears tracked for months or years responded to being removed from bile farms as reflected by a long term decrease in hair cortisol concentrations, (3) compare concentrations of cortisol in segments of hair samples over time, (4) evaluate HPA activity in bears during acclimation in a rehabilitation facility via longitudinal fecal glucocorticoid analysis, and (5) track concentrations of fecal glucocorticoid metabolites across seasons.

An understanding of how HPA activity is affected by bile farming practices could help guide captive bear management to improve welfare of captive bears. Methods for assessing long-term glucocorticoid production also could be incorporated into field-based studies that involve hair sampling, for analysis of archived samples, or when real-time sampling is not possible.

2. Materials and methods

2.1. Animals

At the start of the study in March 2008, the Animals Asia Foundation China Bear Rescue Centre (CBRC), located outside Chengdu in Sichuan Province, People's Republic of China, cared for 218 Asiatic black bears that were removed from bile farms starting in October 2000. An additional 52 Asiatic black bears were brought from bile farms to the CBRC during the study period, which continued through December 2010. Of the 270 bears that had resided at CBRC, 116 were included in our study based on survivorship, availability of historic samples, arrival to CBRC during the study period, and accessibility for sampling. All bears included in the study were obtained as adults and no efforts were made to attain more precise estimates of age. Housing conditions at CBRC met or exceeded international standards for holding bears in captivity by providing extensive enrichment (e.g., simulated natural foraging on a balanced and diverse diet of fruits and vegetables dispersed throughout the enclosures), opportunities for nesting and hibernation, and veterinary care [3]. Diet constituents were generally consistent and included a combination of dry dog food, tomatoes, cabbage, pumpkin and sweet potato, with a varied selection of seasonal fresh and dried fruit and vegetables in smaller quantities for enrichment and training purposes. Quantity fed per bear changed seasonally from as little as 150g dog food and 300g of produce once a day for dormant bears during winter up to 2kg of dog food and 6-8kg of produce per day for active bears exhibiting late summer hyperphagia. During the day, bears were housed communally in enclosures that ranged from 1,368 to 3,881 m^2, while nights were spent in 144 m^3 "den houses" that held up to 4 individuals. Males were castrated shortly after arrival at CBRC; females remained reproductively intact.

Another 20 adult bears (females: $n = 9$; males $n = 11$) were housed at a bile-producing facility in Dujiangyan, China where they were drained of bile twice every day. Bile was reportedly drained using the "free-drip" method in which a permanent hole or fistula was made in each bear's abdomen and gall bladder allowing bile to drip out freely through a collection tube. Bile draining generally occurred while bears were distracted by food. However, in some cases bears were restrained in holding cages (pressed to the floor) to facilitate bile collection. No permanent catheters or other extraction devises were observed in these 20 individuals. Bears on the bile farm were adults and reproductively intact, but exact ages were not known. They were reportedly bred in captivity, however many of the bears obtained by the CBRC from assorted bile farms showed scars consistent with capture from the wild via snaring. Forty-nine of the 247 (19.8%) bears at CBRC for which data were available had ligature scars or missing limbs. No such injuries were observed on any of the bears sampled at the bile farm. Bears on the farm were housed in communal concrete holding pens or small (approximately 4.5 m^3) cages where feeding and bile collection occurred with relatively little enrichment. Their diet consisted chiefly of a semi-liquid, rice-based slurry. Veterinary care was available on the bile farm; however**,** 4 of the 20 bears were noted as being in poor physical or mental condition at the time of sampling based on visual inspection (e.g., emaciated, alopecic, exhibited stereotypic behavior).

2.2. Fecal and hair sampling

Forty-six bears (females: $n = 29$; males $n = 17$) were monitored using serial fecal glucocorticoid analyses and comprised four groups based on their dates of arrival at the CBRC: 17 arrived on 31 March 2008 (females: $n = 11$; males $n = 6$); 12 arrived on 6 February 2009 (females: $n = 7$; males $n = 5$); 6 arrived on 19 April 2010 (females: $n = 4$; males $n = 2$). Eleven

that had been in residence at the CBRC for a year or more served as controls (females: $n = 7$; males $n = 4$). We collected fecal samples from bears as soon as possible after their arrival (range 0 – 6 days) at the CBRC and twice weekly thereafter, for a period of at least 6 months and up to 1 year. Feces from control bears were collected twice a week from April 2008 through December 2010. Due to the long-term sampling protocols, we obtained samples reflecting the entire calendar year (all seasons) for both groups. Long-term sampling was necessary to evaluate the effects of season on adrenocortical activity.

To aid in the identification of fecal samples from individual bears in group enclosures, a subset were fed colored MicroGrits (Micro Tracers, Inc., San Francisco, CA, USA), which were added to a fruit-based liquid meal and acted as a fecal marker. Fecal samples were collected from enclosures on the day of defecation and were stored frozen (-20°C) in plastic bags until processing and analysis.

Hair samples were collected from the abdomens of 7 bears (females: $n = 5$; males $n = 2$); that were euthanized at the CBRC due to their poor physical condition. They were severely malnourished, suffering from untreatable injury, and / or diagnosed with an advanced, terminal disease. Euthanized bears were included in the study to provide a biological validation of the hair immunoassay, with the validation based on the comparison of bears suffering from extreme illness (and therefore likely experiencing chronic stress) and their counterparts in the other groups described here. Abdominal hair samples were also collected from 45 bears (females: $n = 31$; males $n = 14$, including eight of those monitored via fecal glucocorticoids) during general health evaluations within 14 days of arrival at the CBRC. During subsequent health checks, ranging from 163 days to 8 years later (average sample interval = 4.4 years, 40 of 45 resampled less than 6 years after initial sampling), follow-up hair samples were collected from the same

183 individuals. The follow-up (acclimation) samples collected from these 45 bears were used for
184 the primary biological validation of our assay based on comparisons to the 7 euthanized bears.
185 Bears were anesthetized with tiletamine-zolazepam 100 mg diluted with 5 mg of
186 medetomidine (1.25 ml/100 kg body weight), and hair was collected by trimming as close to the
187 skin as possible using electric clippers. Nine bears (females: $n = 6$; males $n = 3$, including three
188 sampled for feces) were used to calculate the growth rate of new hair by trimming a 25 cm^2 area
189 on the abdomen and measuring regrowth to the nearest millimeter at subsequent handling
190 opportunities ranging from 15 to 394 days later. Those nine bears and an additional 11 (females:
191 $n = 4$, males $n = 7$) were used to determine the average length of untrimmed hair based on a
192 single measurement per bear. Thirty nine bears (females: $n = 22$; males $n = 17$, including 8
193 sampled longitudinally for feces) were used for segmental hair cortisol analysis, which was done
194 by cutting samples into proximal and distal halves. All bears included in the segmental analysis
195 had been at the CBRC for at least 19 months prior to sampling. Individuals were sampled
196 opportunistically for hair length, growth rate and segmental analyses while anesthetised for other
197 veterinary purposes. Hair samples from bears on the bile farm were collected in December 2009
198 by trimming with scissors as close to the skin as possible through the bottom of holding cages
199 while bears were distracted during feeding. All hair samples were stored in dry envelopes at
200 room temperature in the dark [13].
201
202 *2.3. Hair preparation and steroid extraction*
203 The undercoat was manually removed from stored hair samples and the guard hairs were
204 washed to remove surface contaminants similar to the approach described by Davenport et al.
205 [12]. Hair was washed three times in polypropylene tubes with 10 ml of isopropanol shaken by

hand for 3 minutes per wash, then dried under directed air and pulverized using a Retsch ball mill (mixer mill MM 200; 10-ml stainless steel grinding jars; 2, 12-mm stainless steel grinding balls) for 8-15 minutes at 30 Hz. Milling was sufficient to reduce hair to a fine powder that was grossly uniform across samples. Powdered hair from each sample (28 - 300 mg) was weighed into 16x125mm glass tubes and 6 ml of 100% ethanol was added. Samples that produced less than 28 mg of hair powder were excluded from the analysis based on patterns of proportionally higher glucocorticoid concentrations extracted from very small samples (< 20 mg) of feces in other studies [21, 34]. In further effort to account for any effect of small samples on hair cortisol concentration we ran a regression analysis comparing hair cortisol to sample mass after our assays were completed. The results of the analysis indicated that hair cortisol was not related to the amount of sample tested $R^2 < 0.01$, $F(1, 127) = 1.18$, $p = 0.28$. Tubes containing the ethanol and hair powder were vortexed briefly and placed in a hot water bath near the boiling point for ethanol (~78°C) for 20 minutes. Every 5 minutes samples were removed from the water bath and vortexed. After 20 minutes samples were centrifuged (2000 X g, 5 minutes) and the supernatant poured off into a separate tube. We repeated these steps and dried the combined supernatants from the three rounds of extraction for each sample under directed air. Dried extracts were stored frozen at -20°C until analysis.

2.4. Extraction from feces

Frozen fecal samples were thawed, dried using a conventional oven at 100°C for 12 hours, pulverized into a fine powder and sifted through a wire mesh to remove large particles (>2 mm). Hormones were extracted from fecal powder using established methods for adrenocortical steroids [19]. Briefly, 0.18 to 0.22 g of well-mixed fecal powder were weighed into a 12 mm

glass tube to which 4 ml of 90% ethanol was added. The fecal powder and ethanol were then vortexed briefly and boiled 85-90°C for 15 minutes with additional ethanol added as necessary to compensate for evaporation and retain an approximately consistent volume. Samples were centrifuged (2000 X g, 15 minutes) and the supernatant poured off into a new tube. The pellet was re-suspended in an additional 4 ml of 90% ethanol, vortexed for 1 minute, boiled, and centrifuged again. Combined ethanol supernatants were dried down under directed air and stored frozen at – 20°C until analysis. As with the hair cortisol data, a regression analysis was run comparing fecal glucocorticoid metabolite concentrations to dried fecal sample mass to ensure that the data were not influenced by variation in sample size. The results of the analysis indicated that sample mass had no effect on the concentration of hormone metabolites detected in samples, $R^2 < 0.01$, $F(1, 2094) = 1.98$, $p = 0.16$.

2.5. Cortisol enzymeimmunoassay

A cortisol enzymeimmunoassay (EIA) was used to analyze hair cortisol and fecal glucocorticoid metabolites following Munro and Lasley [36]. The cross-reactivities for the polyclonal antiserum (R4866; C. Munro, University of California, Davis, CA) were: cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%), and less than 1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, and rostenedione, androsterone, and 11-desoxycortisol. The cortisol EIA was physiologically validated for feces from Asiatic black bears in the previous work by Young et al. [49], which included monitoring fecal glucocorticoid concentrations in response to an ACTH challenge. Extracts were initially run at a 1:8 concentration based on trials with pooled samples, which bound near 50% at that dilution. Samples that bound at < 20% or > 80% were re-assayed after adjusting dilutions

targeting the middle of the curve. All samples were run in duplicate, and were re-assayed if coefficients of variation for paired aliquots exceeded 10%. High and low concentration control samples (n = 1 high, 1 low) were included in every assay for fecal and hair extracts, as were two standard curves. The inter-assay variations for assays of fecal extracts were 9.72% and 4.67% (n = 116 assays) at 30% (high control) and 68% (low control) binding, respectively. Inter-assay variations for hair samples were 6.13% and 3.33% (n = 21 assays) at 30% and 68% binding. Assay plates contained random assortments of samples collected from bears in all treatment groups and various time points. Assays were, in part, validated for feces and hair based on the parallelism of serial dilutions of extracts from both media relative to the cortisol standard curve (feces: $R^2 = 0.96$, $F(1, 4) = 131.70$, $p < 0.01$, hair: $R^2 < 0.99$, $F(1, 5) = 727.40$, $p < 0.01$). Slopes for the linear portions of the curve for dilutions of standards, fecal, and hair samples were -13.67%, -14.33%, and -11.29%, respectively. Standards were produced using hydrocortisone ≥98% (HPLC) supplied by Sigma Aldrich (catalog # H4001-1G, lot # 061M1142V). To ensure that our assay was accurate at high and low doses across dilutions, we plotted concentrations (as determined by EIA analysis) of known standards against their actual values. This plot was highly linear ($R^2 = 0.97$), and the formula for the line of best fit included a slope close to 1 (0.94) indicating consistency across variable concentrations of hormones and metabolites.

Bears from which hair samples were collected following euthanasia yielded elevated concentrations of hair corticoids relative to their conspecifics, providing a biological validation of our EIA (see Results). Matrix interference tested at the concentration of 1:8 (the same dilution at which our samples were initially run) was minimal as indicated by >90% recovery of cortisol standard (0.156–20 ng/ml) added to samples before extraction and analysis. The biological validity of the cortisol EIA for feces was demonstrated further in short-term (~20 day)

preliminary trials (n = 4 individuals) where fecal glucocorticoid concentrations were increased about 4-fold within a day after a known stressor (e.g., relocation to a new enclosure, introduction of new bears, an aggressive interaction). Glucocorticoid concentrations in hair and feces are expressed as nanograms per gram of dry, powdered sample material (ng/g).

2.6. Data analysis

The length of abdominal hair between male and female bears was compared using a *t* test, and the relationship between hair growth and time (growth rate) was evaluated using a generalized Michaelis-Menten equation [28]. To evaluate the biological validity of our EIA we compared hair cortisol in euthanized bears to bears acclimated to the CBRC using a *t* test. Subsequently, hair cortisol among bears on bile farms, bears euthanized due to their poor physical condition, newly arrived bears at the CBRC, and bears acclimated to the CBRC was compared using one-way ANOVA, with post hoc analysis by Tukey's Honestly Significant Difference (HSD) test. Hair cortisol concentrations were log-transformed to meet assumptions of normality.

Fecal samples collected within 24 hours of transport to and arrival at the CBRC were compared to overall means for each of the 20 bears (females: $n = 14$; males $n = 6$) sampled immediately at arrival to determine if the HPA axes of farmed bears remained responsive after farming and during shipment or suffered from adrenal exhaustion. Bears not sampled within 24 hours of arrival to the CBRC were excluded from this analysis because fecal glucocorticoid concentrations returned to baseline two days after ACTH stimulation in a previous study of this species [49]. ANOVA and *t* tests were completed using R version 2.12.1 [38]. For longitudinal profiles of fecal glucocorticoids, the following was calculated for each bear: (1) the overall

mean of all samples for the collection period; and (2) a mean baseline that included only values less than the overall mean plus 1.5 standard deviations. Concentrations of fecal glucocorticoids were considered significantly elevated when they exceeded the mean baseline plus 3 standard deviations [18, 49].

Mixed effects linear regression (also completed using R version 2.12.1 [38]) was used to determine the effects of sex, arrival group, month, and acclimation time on concentrations of fecal glucocorticoids in newly acquired bears. The random effects structure for the linear mixed effects models was determined by fitting and comparing differing saturated models using restricted maximum likelihood (REML [50]). Candidate random effects structures included or omitted individual bear (*BearID*) and / or year (*Year*) as random effects, and tested an AR1 autocorrelation term because samples were collected serially from known individuals. The optimal random effects structure included *Year* and the AR1 term. We compared models with varying fixed effects structures using maximum likelihood (ML) and Akaike information criterion (AIC) values to determine what explanatory variables had the strongest relationships to concentrations of fecal glucocorticoids [7]. The dependent variable, fecal glucocorticoid concentration, was log-transformed to meet assumptions of normality.

Based on results from mixed effects linear modeling, concentrations of fecal glucocorticoids as a function of month were compared using a one-way ANOVA and the post hoc Tukey HSD test. Hair cortisol concentrations in samples that were divided into halves for segmental analysis were compared using a paired *t* test. In all tests, significance was determined at the $p < 0.05$ level. Data are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Hair length and growth rate

The length of abdominal hair ranged from 49 to 88 mm and did not differ between sexes (females: 67.5 ± 4.37 mm vs. males: 72.50 ± 4.17 mm; $t_{18} = 2.10$, $p = 0.42$). After periods of regrowth between 15 and 394 days, hair on previously trimmed patches grew 10 - 70 mm. The optimal Michaelis-Menten equation that described hair growth as a function of time took the form $L = (90.63t) / (77.49 \text{ mm} + t)$ where L is the length of hair at time t, 90.63 is the Michaelis constant, and 77.49 mm is the estimated maximum hair length. The coefficient of determination for the Michaelis-Menten equation was 0.85 (Fig. 1). The minimum length of mature abdominal hair that we observed (49 mm) reflected a growth period of 155 days based on this equation. Therefore it took an estimated minimum of approximately 155 days for a hair follicle to reach full length, assuming constant, steady growth.

3.2. Hair cortisol concentrations

Given the estimated rate of hair growth, bears were included in the arrival / acclimation groups if they had >155 days of residence at the CBRC between arrival and follow-up (acclimation) hair sampling. This effort to ensure that adequate hair growth occurred between paired samples resulted in 45 bears being selected, with a range of 163-3,112 days elapsed between samples.

Comparisons of hair cortisol between bears that were sufficiently ill to warrant euthanasia and those acclimated to the CBRC indicated that our EIA approach was appropriate for detecting biologically meaningful differences in HPA activity. Hair from euthanized bears contained more than three times higher concentrations of corticoids (35.42 $\pm$ 9.18 ng/g) on average than hair

344 from acclimated bears (10.60 ± 1.21 ng/g) at the CBRC (t_{49} = -5.43, $p < 0.001$), even after
345 excluding data from an outlier euthanized bear whose hair contained nearly an order of
346 magnitude higher concentration of corticoids than any other hair sample in the study (425.78
347 ng/g). Data from the remaining 6 euthanized bears of both sexes were pooled because means did
348 not differ (t_4 = 0.09, p = 0.93). Hair cortisol also did not differ between non-euthanized males
349 and females at arrival or after acclimation (t_{43} = 0.12, p = 0.90), therefore data from both sexes
350 were pooled in those groups as well. In both sexes and at both points of measure (i.e., at arrival
351 and after acclimation), hair cortisol concentrations were highly variable among these 45
352 individuals. For the 31 female bears in the analysis, hair cortisol concentrations at arrival ranged
353 from 5.11 - 37.84 ng/g (18.87 ± 1.62 ng/g) compared to the range of 6.50 - 58.24 ng/g (19.32 ±
354 4.08 ng/g) for the 14 male bears. Samples collected from acclimated females contained cortisol
355 concentrations of 2.66 - 28.19 ng/g (10.70 ± 1.37 ng/g), while hair cortisol in acclimated male
356 bears, which had been castrated after arrival, ranged from 4.32 - 41.66 ng/g (10.38 ± 2.50 ng/g).
357 Although less prominent than the variance within groups, mean hair cortisol concentrations were
358 lower in samples collected from bears that had been at the CBRC for ≥163 days compared to
359 matched samples from the same individuals collected within 14 days of arrival (arrival: 19.01 ±
360 1.66 ng/g vs. acclimated: 10.60 ± 1.21 ng/g; t_{44} = 2.01, $p < 0.01$). Thirty-eight of the 45 bears in
361 the analysis (27 female, 11 male, 84%) exhibited declines of 12-88% in hair cortisol
362 concentration between arrival and acclimation, while the other seven bears (4 female, 3 male,
363 16%) had 24-208% increases in hair cortisol between arrival and acclimation. Declines
364 averaging 58% were noted in the three bears from which acclimated samples were collected less
365 than 1 year after arrival, indicating that measurable reductions in circulating glucocorticoids
366 occurred shortly after arrival in those individuals.

Cortisol concentrations in hair collected from bears housed at a bile farm in Dujiangyan, China were also highly variable among individuals, ranging from 10.28 to 23.44 ng/g (16.74 ± 1.36 ng/g) in females, and from 6.78 to 63.71 ng/g (17.73 ± 5.11 ng/g) in males. The sexes did not differ (t_{18} = -0.17, p = 0.87); therefore, data were pooled for further analysis. Hair cortisol concentration differed among the four study groups: euthanized, bile farm, newly arrived at the CBRC, and acclimated to the CBRC ($F_{3, 113}$ = 16.79, p < 0.001, Fig. 2). Post-hoc comparisons using the Tukey HSD test indicated that the mean hair cortisol concentration in bears that had been at the CBRC for ≥163 days (10.60 ± 1.21 ng/g) were significantly lower than in those that had recently arrived from bile farms (19.01 ± 1.66 ng/g, p < 0.001), those that were sampled on the bile farm (17.29 ± 2.82 ng/g, p = 0.01), and those that were euthanized (35.42 ± 9.18 ng/g, p < 0.001). Hair cortisol in newly arrived bears at CBRC did not differ from bears housed on the bile farm (p = 0.92) nor from bears that were euthanized due to their poor physical condition (p = 0.10), and bears that were on the bile farm were also not different from those that were euthanized, although the comparison approached significance (p = 0.06).

3.3. Segmental analysis of hair cortisol content

Differences in cortisol content were detectable between matched halves of hair samples (paired samples t test, t_{38} = -3.10, p < 0.01), with proximal sections (7.40 ± 1.13 ng/g) containing less cortisol than distal sections on average (9.15 ± 1.43 ng/g). Proximal sections contained lower concentrations of cortisol that distal sections in 28 of the 39 (72%) bears sampled, with differences ranging from 3-58%. In the divided samples from the remaining 11 bears, proximal sections contained more cortisol than distal, with differences ranging from 7-56%. Matched, whole samples collected concurrently contained intermediate concentrations of hair cortisol

(7.80 ± 1.43 ng/g); hair cortisol in female (n = 22) and male (n = 17) bears did not differ significantly at the time they were sampled (*t* test, $t_{37} = 1.56$, $p = 0.13$). All 39 samples divided into proximal and distal halves were collected during September-December, when fecal corticoids in captive bears at the CBRC were in decline (Fig. 3).

3.4. Longitudinal fecal glucocorticoid analysis

Overall mean fecal glucocorticoid concentrations in control bears averaged 38.84 ± 1.64 ng/g (range, 0.17 to 819.53 ng/g) and in newly acquired bears were 38.57 ± 1.40 ng/g (range, 0.70 to 1194.14 ng/g). After removal of values above 1.5 SD, baseline fecal glucocorticoid concentrations in control bears averaged 33.75 ± 0.72 ng/g (range, 0.17 to 108.50 ng/g) and in newly acquired bears were 32.63 ± 0.52 ng/g (range, 0.70 to 112.22 ng/g). Neither overall mean nor baseline fecal glucocorticoids varied between control and newly acquired bears ($p = 0.90$, $p = 0.16$, respectively).

Glucocorticoid concentrations in fecal samples collected immediately upon arrival at the CBRC were higher than overall averages in 16 of the 20 bears analyzed, with elevations ranging from 10 – 338% above mean values. Figure 4 depicts longitudinal profiles fecal glucocorticoids from two female bears, one representative of newly-acquired bears relocated from various bile farms to the CBRC and one representative control. Seven of 35 newly acquired bears whose fecal glucocorticoids were monitored exhibited at least one significant elevation (i.e., values exceeded mean baseline + 3SD) during the first 3 weeks after arrival.

Elevations in glucocorticoid metabolites noted shortly after arrival were not maintained for extended periods of time. In only one instance were consecutive significant elevations recorded. In that case the consecutive samples were collected on day 1 and day 8 after arrival at

413 the CBRC and subsequent samples were near baseline for that bear. Control bears that were
414 long-term residents also exhibited occasional spikes in circulating glucocorticoids which, in
415 some cases, coincided with events that were noted in veterinary records (e.g., relocation to a new
416 enclosure, introduction of new bears, an aggressive interaction, etc.). Overall, significant
417 elevations in glucocorticoid metabolites were recorded in profiles of 29 of the 46 bears included
418 in the analysis. Thirteen were observed in nine (81%) of the 11 control bears, while 25 were
419 recorded among 20 (57%) of the 35 newly-arrived bears. These peak concentrations averaged
420 220.45 ± 43.64 ng/g (range, 50.35 to 682.25 ng/g) across all individuals.
421 Median monthly fecal glucocorticoid metabolite concentrations appeared to have an
422 annual pattern for the newly acquired and control groups of bears (Fig. 5). Concentrations of
423 fecal glucocorticoids tended to be higher during the months of February through April than
424 during the summer and fall months. This seasonal pattern was reflected in results from mixed
425 effects linear modeling that indicated fecal glucocorticoid concentrations were related most
426 strongly to *Month*. Models that did not include *Month* as a covariate lacked support, with delta
427 AIC values $\geq$33.15 (Table 1). Mean fecal glucocorticoid concentrations increased steadily
428 between December and April, then decreased from April through December (Fig. 3). An
429 ANOVA comparing glucocorticoids as a function of *Month* indicated that the mean
430 concentration of fecal glucocorticoids was lower in December than during all other months and
431 higher in April than all months except March, ($p < 0.05$, Fig. 3).
432

4. Discussion

Access to bears on bile farms is generally limited, so assessing their endocrine status *in situ* has historically not been feasible. Thus, many assertions regarding the health and welfare of farmed bears have been based on anecdotes (e.g., observation of poor physical condition of bears removed from farms) and are difficult to quantify. Our comparisons of hair cortisol between bears that were presumed to be physically distressed (i.e., sufficiently malnourished, injured, diseased, or otherwise ill to require euthanasia) and their relatively healthy counterparts at the CBRC indicate that the EIA we used for detecting alterations in adrenocortical activity via hair was biologically meaningful. Although the euthanized bear group was limited to only 7 individuals (including an outlier), the samples they yielded contained some of the highest concentrations and the highest overall mean of hair cortisol recorded in our study (even after removal of the outlier). Mean hair cortisol in euthanized bears did not differ from bears at arrival to CBRC, or to those on the bile farm, but all of these groups were elevated relative to the acclimated bears that were re-measured after $\geq$ 163 days of acclimation (Fig. 2). The lack of a difference between euthanized bears and the other two groups (Arrival and Bile Farm) suggests that HPA activity is similar among bears that are extremely ill and those on bile farms. It is important to point out, however, that the euthanized bear group consisted of a relatively small sample size with a high variance in hair cortisol. Thus, differences may have emerged if more samples had been available from euthanized bears. Nevertheless, the patterns we detected indicate significantly elevated HPA activity in bears housed on bile production farms. Similar concentrations of cortisol in hair collected from bears shortly after removal from farms and those sampled while still on farms suggest that bears sampled after arrival at the CBRC yielded accurate data representing their historic HPA activity.

Comparisons between glucocorticoid concentrations in fecal samples collected at arrival and overall mean concentrations showed that many bears arrived at the CBRC with higher HPA activity, which subsequently declined. Regardless of the underlying stimulus (transport, handling, relocation, an artifact of response to farm conditions, etc., [20, 41]), these elevations indicate that responsiveness of the HPA axis was generally maintained during farming and throughout the process of relocation. Sporadic increases in fecal glucocorticoid concentration in over half of the bears studied are further evidence that bears generally had not suffered from a state of adrenal exhaustion or shutdown. Therefore, this appears to be a case in which chronic stress resulted in a consistent, long-term elevation of circulating corticoids, rather than a decline, and points to the resilience of the HPA axis in Asiatic black bears faced with protracted HPA stimulation.

We documented a decrease in hair cortisol concentrations of 12-88% in matched samples from 38 of 45 bears that had $\geq$163 days of acclimation to improved husbandry and environmental conditions. Changes of this scale in a sample medium that reflects coarse, long-term adrenal activity suggest that the HPA axes of bears housed on the bile farm we studied were stimulated over an extended period of time prior to sample collection. Elevations in hair corticoids may have resulted from prolonged, chronic stimulation by a constant stressor (e.g., poor nutrition, confinement, sleep deprivation, etc.) or by sporadic, acute stimuli (e.g., pain associated with bile extraction). Regardless of the finer scale temporal patterns underlying the coarse hair corticoid measurements we obtained, these findings could have important management implications. Housing conditions, diets, or bile collection procedures coincident with regular stimulation of the HPA axis could be sources of concern for the overall welfare of bears on bile farms. Because prolonged stress on farms could impair reproductive fitness and hinder captive breeding efforts,

these considerations may also pertain to long-term sustainability of some captive bear populations [42].

Differences in hair cortisol among individuals from the same groups were even more dramatic than overall differences among Euthanized, Bile Farm, Arrival, and Acclimation groups. The high degree of individual variation made matched samples particularly valuable for tracking changes in hair cortisol before and after acclimation to the CBRC. Although the clear pattern of long-term decline was detected in most of the bears we studied, a substantial number (7 of 45) exhibited increases in hair cortisol between arrival and acclimation to CBRC. Differences among bears whose hair cortisol declined and those whose increased may have resulted from variation in their ability to adapt to their new surroundings, inconsistent social settings relative to other members of their cohort, underlying disease or injury not detected or accounted for in our analysis, or variation in the conditions they experienced on the farms where they had previously been housed. Based on the high degree of variation we found among individual bears we caution against comparisons of hair cortisol that rely on small sample sizes or fail otherwise to account for this potentially important factor.

Fecal glucocorticoid concentrations were higher during the spring months and declined steadily into winter for all newly arrived and control bears. Three non-mutually exclusive explanations could relate to this temporal pattern. Disturbances associated with arrival of newly acquired bears may have played a role in elevating stress hormone levels across all bears in the spring, including those that were already acclimated to the CBRC. The arrival of new bears (most of which were eventually incorporated into shared enclosures) occurred between February and April during the three years of our study, and declines in excretion of fecal glucocorticoid metabolites in subsequent months could reflect bears at the facility adjusting either to their new

environments, to the new residents, or both. Considering the difference in mean fecal glucocorticoid concentrations between December and January, when no new bears were added to enclosures, this explanation fails to account for all of the variation observed. The social repercussions of adding unfamiliar bears to shared enclosures compromised, to an extent, our ability to maintain true controls for this portion of the study. We encourage future long-term studies of HPA seasonality in this species that include controls experiencing no social change.

An alternative explanation is that the trend in fecal glucocorticoids represents a true seasonal pattern of stress hormone production based on the natural history of Asiatic black bears. For example, increases in fecal glucocorticoids could have been related to the impending onset of the breeding season, which occurs during June and July [16]. Elevated glucocorticoids have been documented in other mammals during periods of territoriality, direct competition among males, dominance interactions, courtship, and breeding [10, 11, 24, 40]. Because the males in our study were castrated, our measures may have underrepresented the seasonal changes in glucocorticoids that naturally occur in reproductively intact male bears. Although they did not hibernate, bears at the CBRC transitioned from periods of relative dormancy (e.g., decreased food intake, extended periods of inactivity, etc.) during the winter months to higher activity during the spring. These changes reflect the natural seasonality of the species, driven by day length and food availability. In the wild, at least some Asiatic black bears hibernate during winter months [43]. Changes in metabolic rate over the season could be a key factor underlying patterns in glucocorticoid concentrations observed in this study, and are consistent with findings that the closely related American black bear produces increased metabolic hormones (e.g., growth hormone) during late-denning [5]. These hormones could play an important role in recovery following prolonged fasting. In Asiatic black bears, that recovery would occur during

the spring months, when we detected steady increases and our highest concentrations (in April) of fecal glucocorticoid metabolites.

Annual changes in metabolism also relate to the third possible explanation for the fluctuations we detected in concentrations of fecal glucocorticoid metabolites. Seasonal metabolic cycles can alter hormone metabolite concentrations in feces, irrespective of bioactive hormone levels [17], thus the trends we observed could have been shaped by metabolic change rather than trends in HPA activity. However, we were fortunate to have concurrent data from hair samples which were not dependent upon metabolic fluctuations. Divided hair samples collected during the fall corroborated the finding that HPA activity was in decline during the months leading up to sampling, providing additional evidence of true seasonality of glucocorticoid production in Asiatic black bears. Based on our estimated hair growth rate, samples collected for segmental analysis during September-December were growing, at least in part, during months when circulating glucocorticoids appeared to be in decline, based on fecal data. The pattern of decreasing fecal glucocorticoid concentrations leading up to hair sampling corresponded to higher concentrations of cortisol in distal than proximal sections of the divided samples we analyzed. Aside from supporting the hypothesis that season influences HPA activity in this species, these data demonstrate the potential for detecting changes in stress hormones over a coarse timescale using single hair samples. By contrast, Yang et al. [47], Davenport et al. [12], and Macbeth et al. [31] did not find differences in cortisol concentrations across hair segments in humans, macaques, and grizzly bears, respectively. A likely explanation for the difference in the present study is the aforementioned pattern of the hair samples we analyzed growing during a period of major, long-term (several months) change in circulation glucocorticoid levels.

We used a rather crude technique for establishing an estimate of hair growth rate in Asiatic black bears, recognizing that hair growth patterns are likely influenced by season, body region, sex, climate, and a host of other factors we did not account for in this study, and acknowledging that fluctuations in hair growth rate can influence concentrations of hair corticoids [12, 26]. Differences between proximal and distal portions of divided samples could theoretically have been attributable, in whole or part, to changing growth rates during the summer and fall months. Faster hair growth should result in lower (relatively diffuse) concentrations of corticoids in hair shafts, which were detected in proximal sections of samples collected late in the year. An increased hair growth rate during the fall could be advantageous for bears preparing for winter and correspond to our findings. While fluctuating hair growth rates may have played some role in eliciting the hair corticoid patterns we observed, the combined fecal and hair datasets provide overall support for seasonality in HPA activity in this species.

These findings underscore the potential value of applying a mixed sampling approach to concurrently examine coarse, long-term endocrine patterns and those occurring at relatively fine temporal scales. The long-term retrospective information gleaned from hair samples made it possible to evaluate adrenal function in a way that could not have been achieved by collecting single fecal samples after bears were removed from farms. Furthermore, evaluating hair cortisol in bears immediately upon arrival at the CBRC made it possible to determine prior stress hormone levels, and to discern how overall adrenal activity changed during acclimation with only two samples collected per bear. Dividing hair samples into halves for segmental analysis supported, in concert with data from fecal samples, our conclusion that HPA activity changes seasonally in Asiatic black bears. Comparisons of divided samples also demonstrated that

cortisol concentrations can vary detectibly across hair segments, a potentially important step towards more fine scale, temporal analyses using hair, which could have applicability in a host of wild and domestic species. By contrast, fecal glucocorticoid analyses clearly identified trends in adrenal activity that occurred at finer resolution. These characteristics render hair a preferable sample medium to feces when only isolated samples are available. The application of a combined, complementary sampling approach made it possible for us to track the endocrine responses of Asiatic black bears to bile farming and, subsequently, to improved environmental conditions. Ultimately these findings quantify the marked physiologic response that bile farming practices elicit in Asiatic black bears.

Acknowledgments

We are grateful for the dedicated assistance of the animal care and veterinary staff at the Animals Asia Foundation China Bear Rescue Centre. Caroline Nelson was particularly integral to orchestrating sample collection and storage for the duration of the study. We thank the staff at the Endocrinology Laboratory of the Chengdu Research Base of Giant Panda Breeding for allowing us access to their facilities in support of this work, and to the managers of the bear farm in Dujiangyan, China for granting us permission to collect hair samples from their bears. We also thank Gong Jien for providing logistical support throughout the study. Randall Malcolm, Carol Rindahl, Du Beibei, Li Jiao, Qi Ruijuan, and Wu Lan each made meaningful and extensive contributions in the laboratory. We thank Josh Posner, Teri Allendorf, and the Integrative Graduate Education and Research Traineeship (IGERT) program at the University of Wisconsin-Madison for their support. This research was made possible by the financial contributions from the Morris Animal Foundation and the Animals Asia Foundation.

References

[1] P.A. Accorsi, E. Carloni, P. Valsecchi, R. Viggiani, M. Gamberoni, C. Tamanini, E. Seren, Cortisol determination in hair and faeces from domestic cats and dogs, General and Comparative Endocrinology 155 (2008) 398-402.

[2] Animals Asia Foundation, Rescue Begins (2011) <http://www.animalsasia.org/index.php?UID=FZEISBAJQGI>. Downloaded on 25 May 2011.

[3] Animals Asia Foundation, General husbandry and management practice for Asiatic black bears (Moon Bears): enrichment (2011) <http://www.animalsasia.org/index.php?UID=1R4TBXVQ1NA> Downloaded on 7 August 2011.

[4] T.Ø. Bechshøft, C. Sonne, R. Dietz, E.W. Born, M.A. Novak, E. Henchey, J.S. Meyer, Cortisol levels in hair of East Greenland polar bears, Science of the Total Environment 409 (2011) 831-834.

[5] S. Blumenthal, R. Morgan-Boyd, R. Nelson, D. L. Garshelis, M. E. Turyk, T. Unterman, Seasonal regulation of the growth hormone-insulin-like growth factor-I axis in the American black bear (*Ursus americanus*), American Journal of Physiology - Endocrinology and Metabolism 301 (2011) E628-636.

[6] F. Bonier, H. Quigley, S.N. Austad, A technique for non-invasively detecting stress response in cougars, Wildlife Society Bulletin 32 (2004) 711-717.

[7] K.P. Burnham, D. R. Anderson, Model selection and inference: A practical information-theoretic approach, second ed. Springer-Verlag, New York, 2002.

[8] D.S. Busch, L.S. Hayward, Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142 (2009) 2844-2853.

[9] E.J. Cone, R.E. Joseph Jr., The potential for bias in hair testing for drugs of abuse. In: P. Kintz (Ed.), Drug testing in hair. CRC Press, Boca Raton, Florida, 1996. pp. 69-93.

[10] S. Creel, Social dominance and stress hormones, Trends in Ecology and Evolution 16 (2001) 491-497.

[11] S. Creel, Dominance, aggression, and glucocorticoid levels in social carnivores, Journal of Mammalogy 86 (2005) 255-264.

[12] M.D. Davenport, S. Tiefenbacher, C.K. Lutz, M.A. Novak, J.S. Meyer, Analysis of endogenous cortisol concentrations in the hair of rhesus macaques, General and Comparative Endocrinology 147 (2006) 255-261.

[13] L.A. Felicetti, C.C. Schwartz, R.O. Rye, M.A. Haroldson, K.A. Gunther, D.L. Phillips, C.T. Robbins, Use of stable isotopes to determine the importance of whitebark pine nuts to Yellowstone grizzly bears, Canadian Journal of Zoology 81 (2003) 763-770.

[14] K. Foley, C.J. Stengel, C.R. Shepherd, Pills, Powders, Vials, and Flakes: the bear bile trade in Asia, TRAFFIC Southeast Asia (2011) Petaling Jaya, Selangor, Malaysia.

[15] P.R.J. Garcia, D.J.M. Barbanti, J.A. Negrão, Effects of environmental conditions, human activity, reproduction, antler cycle and grouping on fecal glucocorticoids of free-ranging Pampas deer stags (*Ozotoceros bezoarticus bezoarticus*), Theriogenology 63 (2005) 2113-2125.

[16] D.L. Garshelis, R. Steinmetz, *Ursus thibetanus*. In: IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. <www.iucnredlist.org>. Downloaded on 18 April 2011.

[17] W. Goymann, On the use of non-native hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual, Methods in Ecology and Evolution 3 (2012) 757-765.

[18] W. Goymann, E. Möstl, T. Van't Hof, M.L. East, H. Hofer, Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta,* General and Comparative Endocrinology 114 (1999) 340-348.

[19] L.H. Graham, J.L. Brown, Cortisol metabolism in the domestic cat and implications for the non-invasive moniroting of adreno cortical function in endangered felids, Zoo Biology 15 (1996) 71-82.

[20] T. Grandin, Assessment of stress during handling and transport, Journal of Animal Science 75 (1997) 249-257.

[21] L.S. Hayward, R.K. Booth, S.K. Wasser, Eliminating the artificial effect of sample mass on avian fecal hormone metabolite concentration, General and Comparative Endocrinology 169 (2010) 117-122.

[22] J.M. Keay, J. Singh, M.C. Gaunt, T. Kaur, Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review, Journal of Zoo and Wildlife Medicine 37 (2006) 234-244.

[23] K. Keckeis, M. Lepschy, H. Schöpper, L. Moser, J. Troxler, R. Palme, Hair cortisol: a parameter for chronic stress? Insights from a radiometabolism study in guinea pigs, Journal of Comparative Physiological Biology 182 (2012) 985-996.

[24] D.C. Kersey, D.E. Wildt, J.L. Brown, Y. Huang, R.J. Snyder, S.L. Monfort, Parallel and seasonal changes in gonadal and adrenal hormones in male giant pandas (*Ailuropoda melanoleuca*), Journal of Mammalogy 91 (2010) 1496-1507.

[25] L. Koren, O. Mokady, E. Geffen, Social status and cortisol levels in signing rock hyraxes, Hormones and Behavior 54 (2008) 212-216.

[26] L. Koren, O. Mokady, T. Karaskov, J. Klein, G. Koren, E. Geffen, A novel method using hair for determining hormonal levels in wildlife, Animal Behavior 63 (2002) 403-406.

[27] P. Legagnuex, G. Gauthier, O. Chastel, G. Picard, J. Bêty, Do glucocorticoids in droppings reflect baseline level in birds captured in the wild? A case study in snow geese, General and Comparative Endocrinology 172 (2011) 440-445.

[28] S. López, J. France, W.J. Gerrits, M.S. Dhanoa, D.J. Humphries, J. Dijkstra, A generalized Michaelis-Menten equation for the analysis of growth, Journal of Animal Science 78 (2000) 1816-1828.

[29] B. Maas, The veterinary, behavioural and welfare implications of bear farming in Asia, World Society for the Protection of Animals, London, United Kingdom, 2000.

[30] B.J. Macbeth, M.R.L. Cattet, M.E. Obbard, K. Middel, D.M. Janz, Evaluation of hair cortisol concentration as a biomarker of long-term stress in free-ranging polar bears, Wildlife Society Bulletin (2012) doi: 10.1002/wsb.219.

[31] B.J. Macbeth, M.R.L. Cattet, G.B. Stenhouse, M.L. Gibeau, D.M. Janz, Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): considerations with implications for other wildlife, Canadian Journal of Zoology 88 (2010) 935-949.

[32] J.C. Mccoy, S.S. Ditchkoff, Patterns of fecal hormones in a fenced population of white-tailed deer, Wildlife Society Bulletin (2012) doi: 10.1002/wsb.190.

[33] P. Meerlo, M. Koehl, K. Van Der Borght, F.W. Turek, Sleep restriction alters the hypothalamic-pituitary-adrenal response to stress, Journal of Neuroendocrinology 14 (2002) 397-402.

[34] J.J. Millspaugh, B.E. Washburn, Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation, General and Comparative Endocrinology 138 (2004) 189-199.

[35] J.J. Millspaugh, R.J. Woods, K.E. Hunt, K.J. Raedeke, G.C. Brundige, B.E. Washburn, S.K. Wasser, Fecal Glucocorticoid Assays and the Physiological Stress Response in Elk, Wildlife Society Bulletin 29 (2001) 899-907.

[36] C.J. Munro, B.L. Lasley, Non-radiometric assays: Technology and application in polypeptide and steroid hormone detection, A.R. Liss, New York, 1988.

[37] D.M. Powell, K. Carlstead, L.R. Tarou, J.L. Brown, S.L. Monfort, Effects of construction noise on behavior and cortisol levels in a pair of captive giant pandas (*Ailuropoda melanoleuca*), Zoo Biology 25 (2006) 391-408.

[38] R Development Core Team, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria (2010) ISBN 3-900051-07-0, URL http://www.R-project.org/.

[39] B.S. Rabin, Stress, Immune Function, and Health: The Connection, Wiley-Liss, Wilmington, Delaware, 1999.

[40] D.M. Reeder, K.M. Kramer, Stress in free-ranging mammals: Integrating physiology, ecology, and natural history, Journal of Mammalogy 86 (2005) 225-235.

[41] M.J. Sheriff, B. Dantzer, B. Delehanty, R. Palme, R. Boonstra, Measuring stress in wildlife: techniques for quantifying glucocorticoids, Oecologia 166 (2011) 869-887.

[42] A.J. Tilbrook, A.I. Turner, I.J. Clarke, Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences, Reviews of Reproduction 5 (2000) 105-113.

[43] J.A. Trent, Ecology, habitat use, and conservation of Asiatic black bears in the Min Mountains of Sichuan Province, China, Master of Science Thesis, Blacksburg, Virginia, Virginia Polytechnic Institute and State University, 2010.

[44] S.H.M. Van Uum, B. Sauvé, L.A. Fraser, P. Morley-Forster, T.L. Paul, G. Koren, Elevated content of cortisol in hair of patients with severe chronic pain: a novel biomarker for stress, Stress 11 (2008) 483-488.

[45] J.C. Wingfield, A.S. Kitaysky, Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones, Integrative and Comparative Biology 42 (2002) 600-609.

[46] J.C. Wingfield, D.L. Maney, C.W. Breuner, J.D. Jacobs, S. Lynn, M. Ramenofsky, R.D. Richardson, Ecological bases of hormone-behavior interactions: The "emergency" life history stage, Integrative and Comparative Biology 38 (1998) 191-206.

[47] H.Z. Yang, J. Lan, Y.J. Meng, X.J. Wan, D.W. Han, A preliminary study of steroid reproductive hormones in human hair, J. Steroid chem. Mol. Biol. 67 (1998) 447-450.

[48] R. Yehuda, E.L. Giller, S.M. Southwick, M.T. Lowy, J.W. Mason, Hypothalamic-pituitary-adrenal dysfunction in posttraumatic stress disorder, Biological Psychiatry 30 (1991) 1031-1048.

[49] K.M. Young, S.L. Walker, C. Lanthier, W.T. Waddell, S.L. Monfort, J.L. Brown, Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses, General and Comparative Endocrinology 137 (2004) 148-165.

[50] A.F. Zuur, E.N. Ieno, N. Walker, A.A. Saveliev, G.M. Smith, Mixed effects models and extensions in ecology with R. Volume 1st Edition. Springer, New York, 2009.

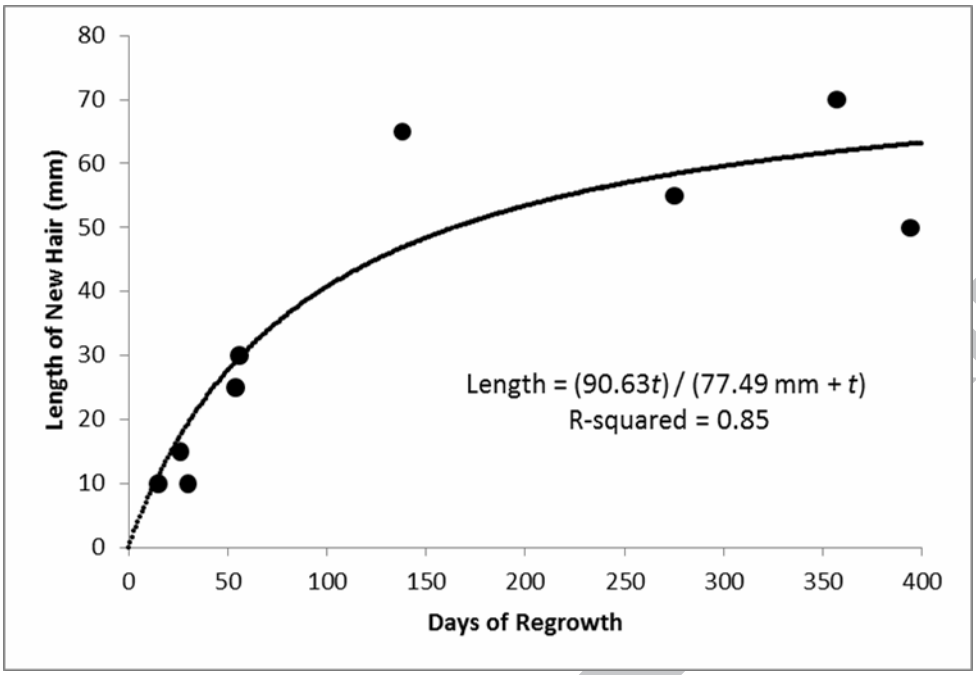
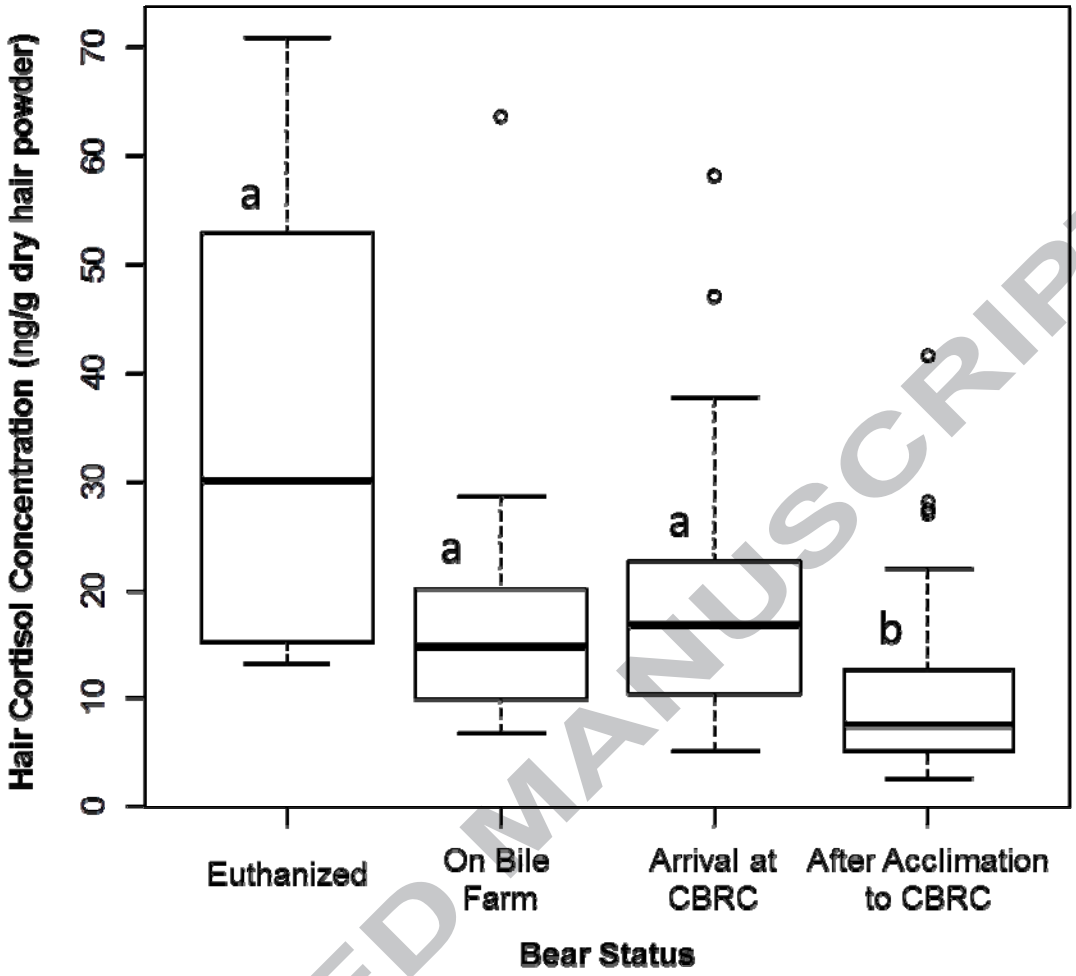


Fig. 1.



.

Fig. 2.

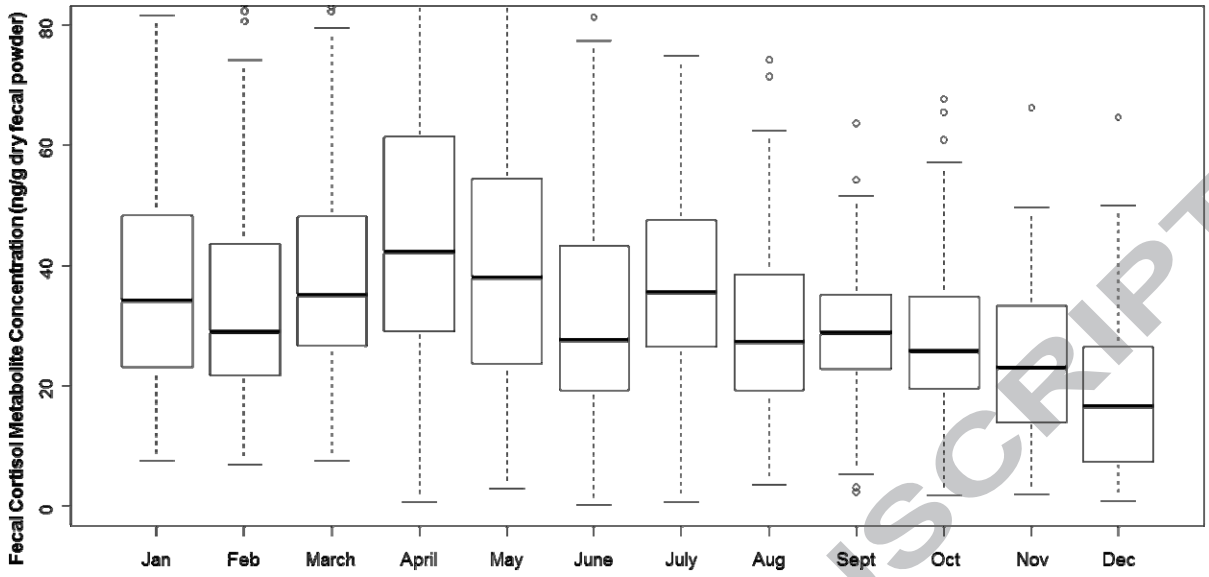


Fig. 3.

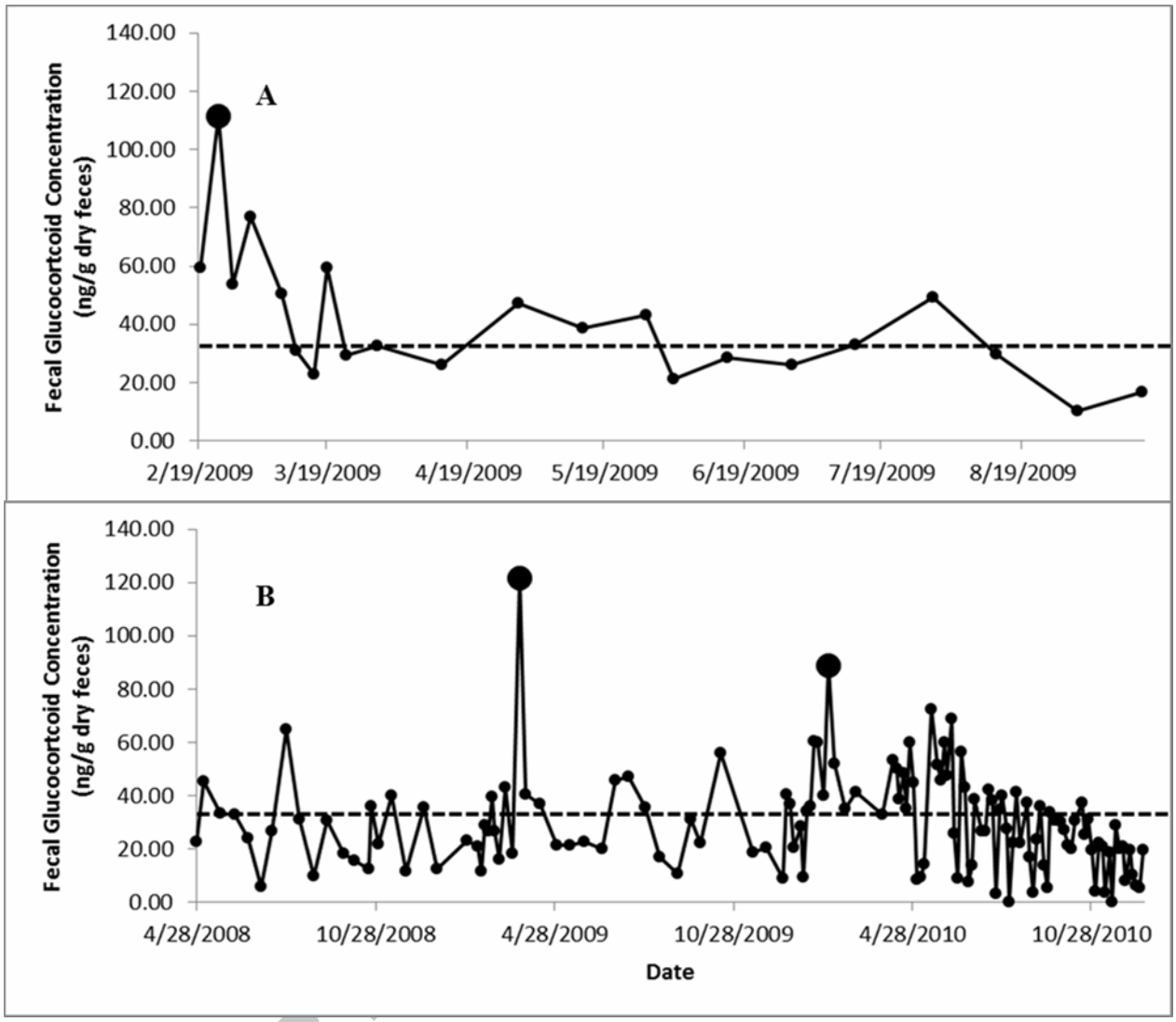


Fig. 4.

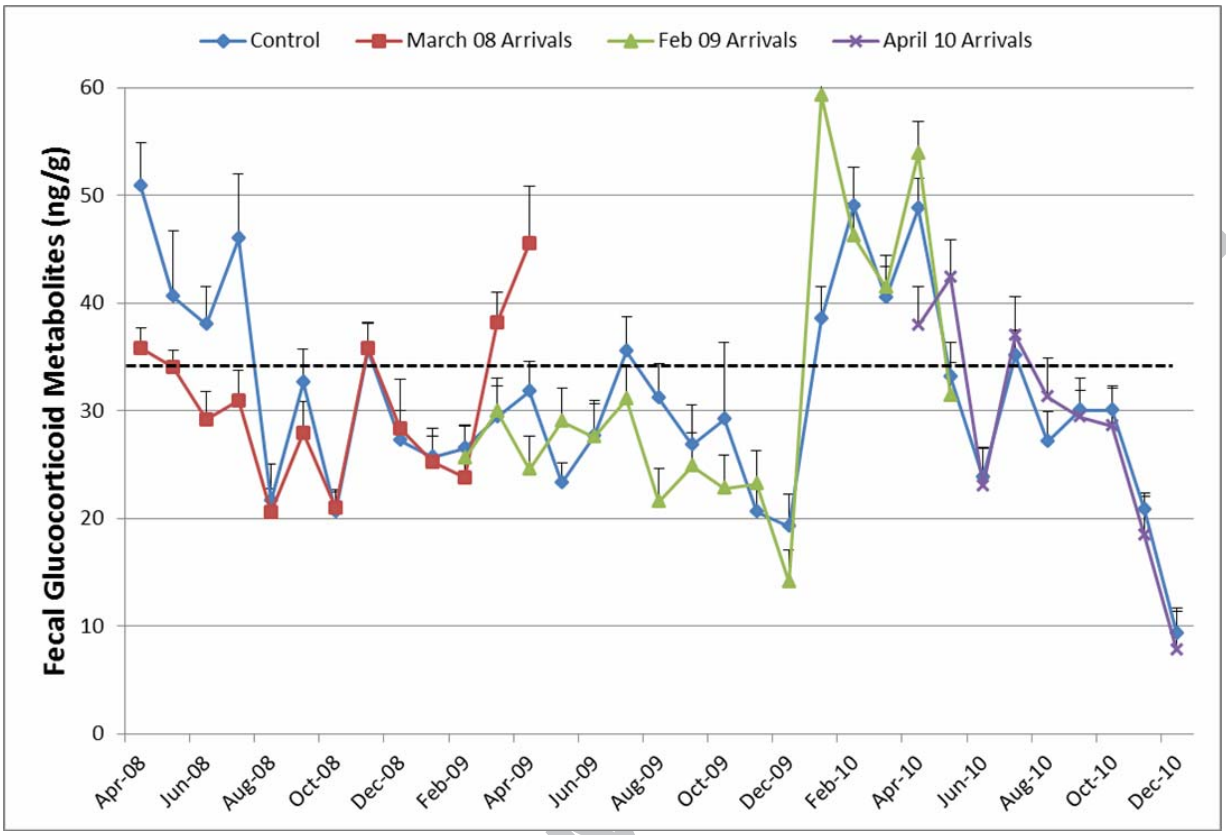


Fig. 5.

Figure Captions:

Fig. 1. Scatter plot and Michaelis-Menten growth curve illustrating the relationship between the length of regrown abdominal hair and days of regrowth among individual Asiatic black bears (*Ursus thibetanus, n* = 9) sampled at the Animals Asia Foundation China Bear Rescue Centre in Sichuan, China (2009-2010).

Fig. 2. Hair corticoid concentrations in samples collected from Asiatic black bears (*Ursus thibetanus*) that were sufficiently diseased, malnourished, or otherwise ill to warrant euthanasia (n = 6), those sampled on a bile farm (n = 20), those sampled immediately upon arrival at the Animals Asia Foundation China Bear Rescue Centre (CBRC, n = 45), and those resampled after ≥163 days of acclimation at CBRC (n = 45, same individuals as Arrival group). Different letters denote significance ($p < 0.05$).

Fig. 3. Seasonal pattern (medians) of fecal glucocorticoid metabolite concentrations in Asiatic black bears (*Ursus thibetanus*) sampled between 2008 and 2010 at the Animals Asia Foundation China Bear Rescue Centre in Sichuan, China. On average, fecal glucocorticoid metabolites were lower in December than all other months and higher in April than all months with the exception of March ($p < 0.05$).

Fig. 4. Longitudinal profiles of fecal glucocorticoid metabolites for a representative female Asiatic black bear (*Ursus thibetanus*) immediately after transport from a bile farm to the Animals Asia Foundation China Bear Rescue Centre in Sichuan, China (A) and a representative control

female (B). Enlarged points indicate that concentrations of fecal glucocorticoids exceeded the mean baseline plus three standard deviations for that individual and were thus deemed significant elevations. Dashed line indicates mean baseline concentrations of glucocorticoid metabolites.

Fig. 5. Monthly median concentrations of fecal glucocorticoid metabolites in Asiatic black bears (*Ursus thibetanus*) sampled between 2008 and 2010 at the Animals Asia Foundation China Bear Rescue Centre in Sichuan, China. Bears arrived in March 2008 (n = 17), February 2009 (n = 12), and April 2010 (n = 6), or were residents for at least one year prior to the start of the study and acted as controls (n = 11). Dashed line indicates mean baseline concentrations of glucocorticoid metabolites.

Model Name	Rank	Covariates	AIC	Par	ΔAIC	L	AIC Wt	Cumulative Wt
Sex & Month	1	β1 + β2(*Sex*) + β3(*Month*) + □| *Year*	2567.86	4	0.00	1.00	0.33	0.33
Month	2	β1 + β2(*Month*) + □| *Year*	2568.21	3	0.35	0.84	0.27	0.60
Acclimation Period & Sex & Month	3	β1 + β2(*Acclimation Period*) + β3(*Sex*) + β4(*Month*) + □| *Year*	2569.20	5	1.34	0.51	0.17	0.77
Acclimation Period & Month	4	β1 + β2(*Acclimation Period*) + β3(*Month*) + □| *Year*	2569.68	4	1.83	0.40	0.13	0.90
Group & Sex & Month	5	β1 + β2(*Arrival Group*) + β3(*Sex*) + β4(*Month*) + □| *Year*	2572.02	5	4.16	0.12	0.04	0.94
Group & Month	6	β1 + β2(*Arrival Group*) + β3(*Month*) + □| *Year*	2572.52	4	4.66	0.10	0.03	0.97
Saturated	7	β1 + β2(*Acclimation Period*) + β3(*Arrival Group*) + β4(*Month*) + β5(*Sex*) + □| *Year*	2573.99	6	6.14	0.05	0.02	0.99
Acclimation Period & Group & Month	8	β1 + β2(*Acclimation Period*) + β3(*Arrival Group*) + β4(*Month*) + □| *Year*	2574.49	5	6.63	0.04	0.01	1.00
Acclimation Period & Group & Sex	9	β1 + β2(Acclimation Period) + β3(Arrival Group) + β4(Sex) + □| Year	2601.00	5	33.15	0.00	0.00	1.00
Acclimation Period & Group	10	β1 + β2(Acclimation Period) + β3(Arrival Group) + □| Year	2602.63	4	34.77	0.00	0.00	1.00
Acclimation Period & Sex	11	β1 + β2(*Acclimation Period*) + β3(*Sex*) + □| *Year*	2652.08	4	84.22	0.00	0.00	1.00
Acclimation Period	12	β1 + β2(*Acclimation Period*) + □| *Year*	2652.29	3	84.43	0.00	0.00	1.00
Sex	13	β1 + β2(*Sex*) + □| *Year*	2662.59	3	94.73	0.00	0.00	1.00
Null	14	β1 + □| *Year*	2663.41	2	95.55	0.00	0.00	1.00
Group & Sex	15	β1 + β2(*Arrival Group*) + β3(*Sex*) + □| *Year*	2663.88	4	96.02	0.00	0.00	1.00
Group	16	β1 + β2(*Arrival Group*) + □| *Year*	2664.14	3	96.28	0.00	0.00	1.00

Table 1. Models of fecal glucocorticoid concentration in Asiatic black bears at the Animals Asia Foundation China Bear Rescue Centre in Sichuan, China (2008-2010) relative to month, arrival group, sex, and acclimation period.