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Research article

Faecal glucocorticoid metabolite concentrations during ACTH challenge tests in captive grizzly bears (*Ursus arctos horribilus*) and polar bears (*Ursus maritimus*)

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Abstract
The well-being of wild and captive animals is often assessed through the measurement of faecal glucocorticoid metabolite (FGM) concentrations. In order to determine the temporal relationships between faecal glucocorticoids and events that might elicit increases in glucocorticoid secretion, we conducted adrenocorticotropic hormone (ACTH) challenge tests on polar (*Ursus maritimus*) and grizzly bears (*Ursus arctos horribilis*) at the Louisville Zoo. FGMs were measured for three days before and after an injection of ACTH, using DA Corticosterone I-125 kits (ICN MP Biomedicals). FGM excretion increased 343–2258% over baseline concentrations. The latency to maximum excretion ranged from 4.5 h to 28.5 h. Five of the bears produced their maximum FGM excretion within 6.2 h of the ACTH injection. The first day's mean FGM concentration after ACTH injection for the six bears (mean ± standard error of the mean (SEM): 106.4±21.3 ng/g dry wt) was significantly higher than the mean baseline concentration (mean ± SEM: 22.3±5.3 ng/g dry wt). The results are consistent with the values obtained in other studies of captive mammals, including polar bears and grizzly bears. However, compared to previous publications, our results suggest that a shorter latency to peak response can occur in bears and that collection of faeces to evaluate peak responses should occur within hours rather than days of stimulation of the adrenal cortex with ACTH.

Introduction

The evaluation of well-being in captive animals commonly involves the assessment of glucocorticoids in body fluids and faeces (e.g. Mason and Veasey 2010). Increased concentration of cortisol and other glucocorticoids has become an accepted indicator of stress in a wide variety of vertebrate species (Keay et al. 2006). Training or anaesthesia are usually required to sample blood in species that pose a safety risk to humans. Collection of urine or saliva also requires training or specially designed cages. However, faecal samples can be collected within an animal's normal husbandry routine (Whitten et al. 1998).

We have measured faecal glucocorticoid metabolites (FGM) to monitor the well-being of three polar bears and three grizzly bears at the Louisville Zoo. In order to identify events that lead to increases in FGM concentration, it is necessary to estimate the time lag between the event and the hormone changes in the excreted faeces. This can be accomplished with an adrenocorticotropic hormone (ACTH) challenge test (Keay et al. 2006), which involves an injection of ACTH followed by periodic collection of faecal samples. The challenge test also establishes the physiological validity of the assay. Recently, Shepherdson et al. (2013) described the only ACTH challenge test on a captive polar bear. However, the assay used in this study is no longer available from the manufacturer. We have performed the ACTH challenge test on the polar and grizzly bears at the Louisville Zoo in order to provide data from a current assay protocol and to establish the excretion lag-time for these animals.

Methods

Subjects and materials

The Louisville Zoo maintained three polar bears (*Ursus maritimus*) and three grizzly bears (*Ursus arctos horribilis*) in a complex holding and exhibit space called Glacier Run (see Table 1 for demographic information about the animals). All

of the bears were fed commercial carnivore, bear and dog foods, combined with hard-boiled eggs, herring, grapes and a variety of enrichment foods. In addition, the grizzly bears were given apples and pears. The young female polar bear was also given formula and taurine supplement. The two sibling grizzly bears were housed together and their faecal remains were identified through the addition of coloured corn to their diet. The remaining four bears were kept physically isolated but were often able to see, hear and smell the other bears.

We conducted the study during November and December of 2012. On the morning of injection, each animal received 2.2 IU/kg body weight of ACTH (Corticotrophin LA 80 units/ml injection gel, Wedgwood Pharmacy, 405 Heron Dr., Suite 200, Swedesboro, NJ 37250 USA), using either a pole syringe or blow gun, between 0930 and 1015 (Hunt and Wasser 2003). Keepers collected baseline faecal samples before 1000 for three days prior to injection and on the morning of the injection. The mean FGM concentration of these four samples constituted the baseline concentration. The injected bear remained in a confined area without a pool. We examined the area for faecal deposits in the afternoon of the injection day (5.3–6.8 hr after ACTH) and in the mornings (before 1000) and afternoons (1410–1630) of the following three days. Each animal produced faecal samples in the afternoon following the injection and, at least, one sample on each of the following three days. All faecal samples were frozen at -20^{o} C until they were shipped frozen to the Endocrinology Laboratory at the Saint Louis Zoo.

Table 1. Individual bear characteristics and the FGM response to ACTH injection.

Identification (birth)	Age (years)	Sex	FGM concentration		Hours to peak response[1]
			Mean±SEM baseline (ng/g dry wt)	Peak excretion (ng/g dry wt)	
Grizzly bears					
IN (wild)	8	F	10.8±0.67	137.7	4.5
OT (wild)	3	M[2]	8.8±1.17	198.7	6.2
RI (wild)	3	F	12.9±3.44	118.4	6.2
Polar bears					
QA (wild)	2	F	29.6±8.29	216.8	28.5
SI (captive)	3	M	34.2±9.16	148.5	4.7
AR (captive)	28	F	37.6±9.50[3]	128.9	4.7

[1]These intervals reflect when we collected samples. Defaecation occurred earlier. [2]Castrated. [3]AR's first baseline day was excluded from the analysis because it was five standard deviations above her mean from the 118 samples previously assayed .

Faecal hormone extraction

Wet faecal material (0.4–0.7 g) was weighed then shaken overnight in 5 ml of a modified phosphate-saline buffer containing 50% methanol (Shideler et al. 1993). Liquid extracts were decanted and solids were removed through centrifugation at 4000*g.* Supernatants were then frozen at -80° C until assay. Faecal material was placed in a drying oven overnight at 100° C. The average amount of dried faecal material per sample was 0.18±0.004 g.

Glucocorticoid assay

FGM concentrations were determined using a commercially available corticosterone radioimmunoassay (DA Corticosterone I-125 kit, ICN MP Biomedicals, Solon, Ohio 44139, USA). Although cortisol is the primary circulating corticoid in the blood of most mammals including bears (e.g. Harlow et al. 1990), it is excreted as a mixture of corticoid metabolites. This assay has been shown to cross-react with glucocorticoid metabolites in a wide variety of species (Wasser et al. 2000).

The lower and upper detection limits of the assay were, respectively, 0.26 and 20 ng/ml. We followed the manufacturer's protocols with the exception that standard diluent was added to the faecal extracts and faecal extraction buffer (50% methanol/50% modified PBS; Shideler et al. 1994) was added to the standards. This ensured that the matrix of both the samples and standards was equivalent. Concentrations were determined as ng/ml, and then divided by the dry weight of extracted faeces to give the results as ng/g faeces. All samples were assayed in duplicate.

Assay validation

For both grizzly and polar bear samples, faecal extracts were tested for parallelism by diluting five samples that contained high levels of FGMs by 1/2, 1/4 and 1/8 with extraction buffer. Serial dilutions of grizzly and polar bear faecal extracts measured, respectively, an average of 90.82±3.28% and 90.38±4.30% of expected concentrations for corticosterone. Dilutions for both species were parallel to the standard curve (test of equal slopes, $P > 0.10$; Zar 1996). This indicates that no additional substances in the extract were cross-reacting with the antibody.

The accuracy of the assay was assessed by adding a known amount of corticosterone to five faecal extracts of each species containing low values of FGMs. At the three dosages, we recovered 100.83±1.63% of added corticosterone in the grizzly and 99.20±1.82% in the polar bear samples.

All statistical analyses except for the test of equal slopes used Statistix 9 (Analytical Software). Alpha was set at 0.05, and all tests were two-tailed except where noted. We evaluated the change from baseline through the four days following ACTH injection with a repeated measures analysis of variance (ANOVA). Subsequently, we compared each day of post-injection FGMs with the baseline concentration by using Dunnett's multiple comparison with a control.

Results

The maximum concentration of FGM following the ACTH injections ranged from 343 to 2258% of the respective bear's mean baseline concentration (see Table 1 for individual results). For five of the six bears, the maximum response occurred in less than 6.2 hr. We collected the youngest polar bear's maximum sample 28.5 hr after injection. However, her sample the morning after injection showed elevated excretion (470% above baseline), which was defaecated between 6.8 and 23.3 hr after the ACTH administration.

The baseline FGM concentrations appear to show a difference between the species. We have found no support for this in the samples assayed previously, which included over 100 samples from each bear. For this reason, our subsequent analysis combined the data from the polar and the grizzly bears. Although we did not have a large enough sample from either species for statistical evaluation, sex and age did not appear to be related to FGM baseline concentration or to the FGM response to the ACTH injection.

For the six bears, the mean FGM concentration on day one (106.4±21.3 SEM ng/g dry wt) after injection of ACTH was significantly higher than the mean baseline concentration (see Figure 1; baseline mean = 22.3±5.3 SEM ng/g dry wt; repeated measures ANOVA: $F = 4.20$; $df = 4,20$; $p = 0.01$; followed by

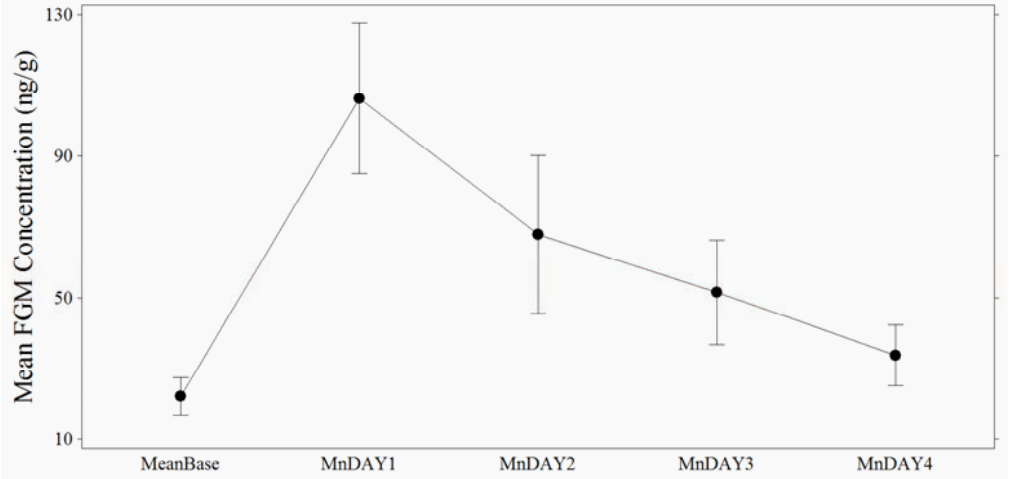

Figure 1. Combined FGM response of the six bears to the ACTH injection, with the means (±SEM) for the baseline samples (MeanBase) and each of the four days after the injection (MnDay1–4). Day 1 was the day of the injection and the mean for this day was significantly higher than the baseline.

Dunnett's T multiple comparison with a control; $p < 0.05$). The means for the other days were not significantly different from the baseline. Day 1 was not different from day 2, but was higher than days 3 and 4 (one-tailed test, $p < 0.05$).

Discussion

Each of the six bears showed the expected elevation in FGM concentration after the ACTH injection, demonstrating the physiological validity of the assay for these species. Five of the six bears had reached their peak excretion by six hours after injection. These excretion lags are shorter than those described in other studies of bears; however the assay used for analysis, the sampling frequency, and the methodology of the ACTH challenge experiment differs from other studies. Shepherdson et al. (2013) reported that an adult male polar bear produced his maximum response on day three after injection, but they did not sample shorter intervals. This bear was injected with ACTH during a physical examination, while in the present study, the challenge was performed without restraining or anaesthetising the animals. Although the double antibody radioimmunoassay in the study by Shepherdson et al. (2013) was conducted in the same laboratory as the assays for the present study, Shepherdson et al. employed a different antibody (Diagnostic Systems Laboratory, Webster, Texas, USA). Because the DSL assay is no longer available, the present study was necessary to establish the validity of the MP Biomedicals assay for polar bears. FGM concentrations from the DSL assay were substantially higher than from the MP Biomedicals assay. These differences may be the result of differences in the affinity of the antibodies for the FGMs.

Diet may also influence lag time from ACTH injection to peak excretion. Best (1985) found that all meat diets ranging from fish to various body parts of ringed seals (*Phoca hispida*) produced variation in polar bear gastrointestinal transit times (GTT), ranging from 12.3 to 18.6 h. In the present study, the polar bear diet contained a substantial proportion of plant material as part of the commercial feed, enrichment foods and fresh fruits. A vegetarian diet shortened GTT in grizzly and black bears (Pritchard and Robbins 1990).

Hunt and Wasser (2003) injected captive adult male and female grizzly bears with ACTH and found peak concentrations at 22 and 32 h, respectively. The magnitude of the peak responses was similar to the present study, but the lag time for the peak response was longer. As with our study, Hunt and Wasser employed the MP Biomedicals assay and fed their animals similar diets, but the proportion of plant and animal components may have differed. Plant and animal diets have been shown to have different GTTs in grizzly and black (*Ursus americanus*) bears (Pritchard and Robbins 1990). For both species, the GTT of a vegetarian diet (7 h) was nearly half that of a meat diet (13 h). They did not assess a mixed diet. The GTT of the vegetarian diet is close to the lag time for peak response in the present study. Our grizzly diet was approximately 40% plant material by volume.

A female Malayan sun bear (*Helarctos malayanus*) excreted peak FGM concentration 25 h after ACTH injection (Wasser et al. 2000), and a male giant panda (*Ailuropoda melanoleuca*) peaked at approximately 12 h (Kersey et al. 2010). Together, these studies and those described above suggest that there is considerable variation among several species of bears in the interval between ACTH injection and peak FGM concentration.

In a field study of Alaskan brown bears (*Ursus arctos horribilis*), von der Ohe et al. (2004) explored the effect of potential environmental stressors and diet on excretion of FGMs. Diet and season had significant effects on FGM concentrations, and the interaction of these variables revealed that some dietary items had a stronger effect in one season than in another. These variables may affect the baseline FGM concentrations in captive ACTH challenge tests and also may potentiate or add to the magnitude of the ACTH response.

With samples ranging from five to 18 individuals in each of five sex–age groups, von der Ohe et al. (2004) did not find any significant relationship between age–sex class and FGM concentrations. Our results are consistent with this. Neither the grizzly bears nor the polar bears exhibited a consistent age or sex relationship to FGM excretion or FGM response to ACTH.

A single animal may demonstrate that the assay is physiologically sensitive to the FGM excreted by the species, but is inadequate in establishing the time-lag for excretion. A study of seven jaguars (*Panthera onca*) illustrates this point by showing that five cats had their maximum excretion concentration on day 1, another on day 2, and a seventh never produced an identifiable peak (Conforti et al. 2012). This last animal was a sterilised female, which raised

the possibility that sterilisation my affect the adrenal response to ACTH. In our study, the castrated male grizzly had the strongest FGM response among the grizzlies. A study of six horses (*Equus ferus f. caballus*) in which all faeces were collected exhibited a range of peak FGM responses from 16.3 to 34.0 hr after injection (Möstl et al. 1999). The variability within species and within controlled conditions of a single study suggests that there are important unidentified individual differences contributing to the variation in excretion lag. Our results support the conclusion of Conforti et al. (2012) that "...long-term longitudinal monitoring of individuals is recommended if the impact of potential stressors is to be understood".

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