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Research article

Annual faecal glucocorticoid metabolite concentrations in pregnant and pseudopregnant polar bears (*Ursus maritimus*) in North American zoos

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Abstract

Polar bears (*Ursus maritimus*) face a tenuous existence in the wild due to climate change and in North American zoos due to a shrinking population and low reproductive success. A non-invasive diagnostic test that distinguishes pregnant from pseudopregnant (i.e. false pregnancy) bears is necessary to pinpoint when reproductive failure is occurring. However, faecal hormone metabolite patterns commonly used to determine pregnancy in other species are not diagnostic in this species. The goals of this study were to determine: 1) if faecal glucocorticoid metabolite (FGM) concentrations could be used to differentiate pregnant from pseudopregnant bears, 2) if baseline "normal" FGM concentrations excreted throughout the year, and 3) the seasonal impacts on FGM concentrations. Faecal samples collected from pregnant (n=3) and pseudopregnant (n=3) polar bears for approximately 12 consecutive months were analysed using an established cortisol EIA. Although mean FGM concentrations appeared slightly higher in pseudopregnant bears during the winter, spring and summer seasons, statistical analyses using a Friedman's test indicated there were no differences overall (P=0.10). There were no consistent changes in profile patterns exhibited by all individuals in each group. Additionally, there were no differences in FGM concentrations among seasons (P=0.896). Baseline FGM concentrations for pregnant and pseudopregnant bears ranged from 11.0–28.5 ng/g and from 36.8–42.2 ng/g, respectively. However, the individual profiles were dynamic, with spikes in FGM ranging from 161.4–416.8 ng/g. Study results indicate that FGM concentrations will not facilitate pregnancy diagnosis in polar bears.

Introduction

Polar bears (*Ursus maritimus*) are ice-dependent marine mammals. Their survival is tightly linked to their ability to utilise sea ice floating among vast expanses of water as a hunting substrate and a resting place. Polar bear populations are greatly influenced by climate change, as it causes early onset of the Arctic open-water season which pushes them inland, reducing hunting success and preventing the accumulation of sufficient fat reserves (Stirling and Parkinson 2006). In recent years, wild populations have declined due to nutritional stress, poor body condition and lower recruitment (Regehr et al. 2007). In fact, nutritional stress and the need to search for alternative food sources has become so great that intra-specific cannibalism and increased polar bear–human interactions have been documented (Amstrup et al. 2006). Associated with these challenges and exacerbating the negative impact on this species' survival is a decline in reproductive success in some wild populations (Rode et al. 2010; Molnár et al. 2011).

Ex-situ polar bear populations in US zoos also are declining. Although many of the challenges faced in the wild are alleviated in zoos, reproductive success and cub survival remain low and captive populations are not self-sustaining (Meyerson 2016). Therefore, reproductive research has been conducted on the ex-situ population over the past decade in an effort to better understand polar bear reproduction and to identify factors that may be interfering with its success. Most polar bear pairs are compatible, they demonstrate appropriate seasonal hormonal shifts and exhibit oestrus/mating activity in late winter and early spring (Stoops et al. 2012; Curry et al. 2012a); therefore, reproductive failure is likely due to lack of conception, embryo loss during diapause or implantation

failure. A non-invasive diagnostic test for pregnancy is necessary to more precisely elucidate the timing of reproductive failure, thereby exposing associated factors for further investigation. Several studies have been conducted to assess faecal steroid hormone metabolite monitoring of progestagens and androgens (Stoops et al. 2012) and urinary hormone metabolite monitoring of androgens, oestrogens and progestagens (Steinman et al. 2012; Knott et al. 2013) for diagnosing pregnancy in this species. However, to date, none of these avenues have led to a definitive test that reliably distinguishes pregnant from pseudopregnant (i.e. false pregnancy) bears. Most recent efforts targeting specific faecal proteins excreted in different quantities by pregnant versus pseudopregnant bears showed early promise (Curry et al. 2012b), but further research on one of the proteins revealed too much variation in faecal protein concentrations for accurate diagnosis (DeLorenzo et al. 2016).

Cortisol, a steroid hormone produced by the adrenal gland, has not been considered in previous polar bear reproductive studies; yet, in humans mean salivary cortisol starts to increase between the 25th and 28th week of gestation, and late in gestation reaches concentrations more than twice as high as in non-pregnant controls (Allolio et al. 1990). Faecal glucocorticoid metabolite (FGM) monitoring is a well-established method for detecting changes in adrenal activity using a variety of corticosterone and cortisol antibodies and EIA or RIA techniques (Mostl and Palme 2002; Millspaugh and Washburn 2004; Young et al. 2004), and these methodologies have previously been validated in polar bears (Shepherdson et al. 2013; White et al. 2015). However, FGM data must be interpreted cautiously since there are many potential confounding factors that can impact corticoid concentrations. For example, Owen and colleagues (2005a) documented both diurnal and seasonal variation in corticoid excretion in two giant pandas (*Ailuropoda melanoleuca*). Similarly, a larger study of serum cortisol concentrations in black bears (*Ursus americanus*) revealed seasonal differences with cortisol lowest in the summer and highest in the winter (Harlow et al. 1990).

The goal of this study was to retrospectively compare the FGM profiles of six female polar bears: three pregnant polar bears that produced cubs and three pseudopregnant polar bears. Specific objectives were to determine for all female polar bears: 1) consistent differences in FGM concentrations and/or profile patterns for pregnant versus pseudopregnant groups, 2) the baseline "normal" FGM concentrations that were excreted throughout the year, and 3) seasonal impacts on FGM concentrations. Two hypotheses were tested: 1) FGM profile patterns will differ between pregnant and pseudopregnant bears and 2) FGM will fluctuate seasonally in female polar bears.

Methods

Study animals

Six female polar bears were selected from five Association of Zoos and Aquariums (AZA) accredited zoos. All animals were of breeding age (7 to 20 years), two were wild-caught (PB1 and PB3), four were born in zoos, and all were considered to be in good health. Faecal samples were collected from female bears three times weekly for approximately 12 consecutive months. Three of the bears were pregnant and gave birth to cubs and the other three were considered pseudopregnant based on never being introduced to a male for mating but exhibiting faecal hormone metabolite profiles for progesterone and testosterone similar to those of polar bears that gave birth.

Hormone metabolite extraction and assay analysis

Faecal samples (n=805) for this study were previously collected approximately three times per week from each polar bear and lyophilised at the Center for Conservation and Research of Endangered Wildlife (CREW), Cincinnati Zoo and Botanical Garden, for reproductive studies. Approximately 0.5 g of faecal powder of each sample was sent in plastic bags, clearly labelled with the polar bear ID and date, to the Endocrinology Lab at the Center for the Science of Animal care and Welfare (CSAW), Brookfield Zoo, for cortisol analysis.

FGM were extracted using 90% methanol in distilled H2O. An aliquot weighing 0.2 g (± 0.02 g) of each faecal sample was transferred into 16 x 125 mm polypropylene tubes (Mettler balance, model #AB104-5). Then, samples were mixed with 2 mL of 90% methanol solution by first vortexing and then placing the tube on a rotator (Labline Maxi Rotator, model #4631) overnight for 14–18 hours. The following day, tubes were centrifuged for 15 min at 1500 rpm (Marathon 3000R centrifuge, model #120). Supernatant (1 mL) from each sample was diluted with 1 mL of assay buffer (0.1 M phosphate buffered saline containing 1% BSA, pH 7.0) in 12 x 75 mm polypropylene tubes. Tubes were capped tightly to avoid evaporation and stored frozen at –20°C until assay analyses.

Polar bear samples were analysed using a previously established and validated in-house cortisol EIA assay. Parallelism and recovery tests were used for the validation of the cortisol EIA for the study species. To establish parallelism, serial two-fold dilutions (1:8 to 1:512) of a sample pool were tested for comparison displacement curves. Recovery of exogenous hormone was measured by spiking a baseline diluted sample with the five highest standards, each containing a known amount of hormone. The percent recovery was calculated by dividing the measured concentration of hormone by the expected concentration of hormone multiplied by 100.

The cortisol antibody (R4866) and conjugate used for the in-house EIA were prepared and supplied by Coralie Munro (University of California-Davis, Davis, CA). Flat-bottom 96-well microtiter plates (Nunc maxisorp) were coated by adding 50 uL antibody (1:20,000) in coating buffer (50 mM sodium bicarbonate, pH 9.6) to each well and storing the covered plate at 4°C overnight. The cortisol plates were washed five times with wash solution (0.15 M NaCl containing 0.05% Tween 20) immediately prior to plate loading. Standards (0.078 ng/mL to 20 ng/mL), samples and controls diluted in assay buffer were added to each well in 50 uL aliquots according to plate set up, followed immediately by the addition of 50 uL per well of diluted horseradish peroxidase (HRP; 1:25,000). Plates were covered and incubated at room temperature for 2 hours. Following incubation, plates were washed five times to remove unbound antigen, blotted dry, and 100 uL of substrate solution (1.6 mM hydrogen peroxide, 125 uL 0.4 mM azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] in 0.05 M citrate buffer, pH 4.0) was added to each well. Plates were incubated at room temperature on a shaker (Titer plate shaker, model #4625) for 0.5 to 2 hours until optical density of maximum bound wells was approximately 1.0. Plates were read by a photospectrometer plate reader (Dynex MRX Revelation) at a wavelength of 405 nm.

The cross-reactivity of the R4866 cortisol antibody is as follows: 100% cortisol, 9.9% prednisolone, 6.3% prednisone, 6.2% compound S, 5.0% cortisone, 0.70% corticosterone and any other steroids were <0.50%. Assay sensitivity was 0.078 ng/mL and the intra-assay coefficient of variation was 6.34% at 67.42% binding. Inter-assay variation was 10.38% at 44.23% binding and 4.63% at 17.28% binding. Mean recovery of exogenous cortisol (1.25–20 ng/mL) was 110.84% (range 101.55–119.30%) in polar bear faecal extracts.

Data analysis

FGM concentrations for each individual were compiled into seasonal averages. Seasons were defined as winter: December, January and February; spring: March, April and May; summer:

Table 1. Average individual fecal glucocorticoid metabolite (FGM) concentrations ± SD across all seasons, across the entire data set, and baseline FGM concentrations for all study animals (PB1-PB3 were pregnant; PB4-PB6 were pseudo-pregnant). All FGM values are expressed in (ng/g feces).

Status	Polar bear	Age (years)	N	Winter FGM (± SD)	Spring FGM (± SD)	Summer FGM (± SD)	Fall FGM (± SD)	All FGM (± SD)	Baseline FGM
Pregnant	PB1	7	127	19.91 (± 9.90)	34.06 (± 11.60)	32.87 (± 13.02)	70.39 (± 40.91)	42.60 (± 30.68)	23.97
	PB2	10	136	33.17 (± 14.46)	47.83 (± 29.50)	37.99 (± 17.40)	34.34 (± 16.45)	39.00 (± 21.49)	28.46
	PB3	10	136	35.86 (± 36.00)	30.13 (± 22.05)	37.22 (± 25.68)	56.95 (± 40.59)	39.87 (± 33.61)	11.01
Pseudo-pregnant	PB4	20	114	107.73 (± 49.23)	86.02 (± 74.43)	54.60 (± 38.06)	72.64 (± 46.07)	78.53 (± 57.16)	42.24
	PB5	14	148	50.14 (± 25.13)	49.60 (± 44.71)	47.23 (± 27.76)	45.63 (± 21.49)	48.16 (± 30.91)	36.78
	PB6	14	144	45.55 (± 22.11)	52.33 (± 22.12)	80.86 (± 79.49)	59.99 (± 23.60)	59.83 (± 45.61)	37.43

June, July and August; and autumn: September, October and November. Averages of all FGM concentrations were obtained for each of the four seasons, and each individual's data sets were tested for normal distribution using the Shapiro Wilk test. Not all data were normally distributed, therefore non-parametric statistical tests were employed. A Friedman's test was run on FGM concentrations to test for significance across seasons. If significant differences were found, follow-up, pair-wise comparisons were performed (Cody and Smith 1997).

To determine individual baseline FGM concentrations, the data set for each female was tested against its own mean value and any values falling outside of ±1.5 SD were removed. This process was repeated until no values remained outside the standard deviation (Atsalis and Margulis 2006). Remaining data sets for each individual were analysed using the Shapiro Wilk test, and again, data were not normally distributed and therefore required non-parametric testing. Since there were only three bears per group, a Mann-Whitney U test was not possible. Instead, a phase design randomisation test was used to test for baseline differences between pregnant and pseudopregnant bears (Siegel and Castellan 1988).

To test the hypothesis that an increase in FGM concentrations associated with the progression of gestation could be used as an indicator of pregnancy versus pseudopregnancy, a Friedman's test was employed to analyse FGM concentrations in pregnant and pseudopregnant bears during three trimesters of pregnancy, each lasting 30 days. In polar bears, the interval from implantation to parturition is thought to be approximately 60 days (Stoops et al. 2012). Therefore, the first trimester was considered the control or pre-implantation period with the second and third trimesters encompassing the first and second halves of the implantation phase, respectively. For pregnant bears, day of parturition was considered Day 0; therefore, the third trimester encompassed the 30 days prior to Day 0 (Day 0 to Day -30); the second trimester Day −30 to Day −60; and the first trimester Day −60 to Day −90. For pseudopregnant bears, Day 0 was considered the day progestagen concentrations returned to baseline after the fall increase and the three trimesters were calculated as for pregnant bears using Day 0 as the reference point.

Statistical analyses were performed using SPSS, version 22 and Statview, version 5.0.1, with P≤0.05 considered significant for all tests.

Results

Individual FGM means were calculated for every season and these means varied between seasons and within individuals ranging from 19.9–107.7 ng/g faeces (Table 1). Both the highest and lowest mean values occurred during the winter season. Overall, there were no significant differences in FGM concentrations between seasons (P=0.896), therefore no follow-up testing was performed (Figure 1). Though not statistically different due to the high level of intra- and inter-individual variation, there was a tendency (P=0.10) for pseudopregnant bears to have higher FGM

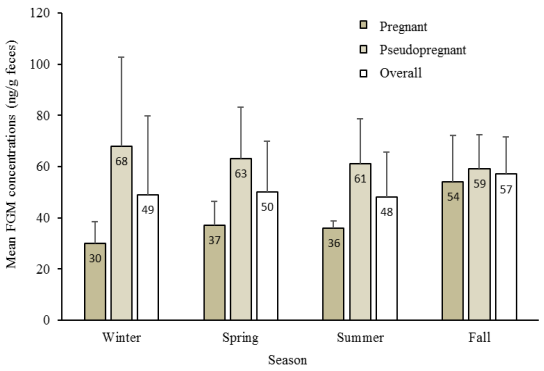

Figure 1. Mean (+SD) faecal glucocorticoid metabolite (FGM) concentrations for pregnant (n=3), pseudopregnant (n=3) and all bears combined (n=6) during each season of the year. There was a trend (P=0.10) towards higher corticoids in pseudopregnant bears but not a significant difference between bear groups or seasons overall (P>0.05).

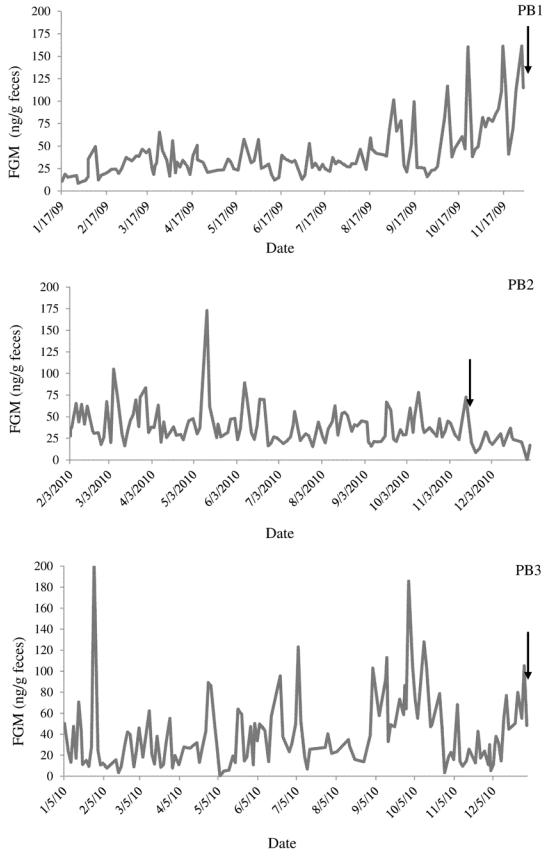

Figure 2. Faecal glucocorticoid metabolite (FGM) profiles for three pregnant polar bears. No consistent pattern or changes associated with pregnancy and/or parturition were identified. Arrows denote parturition.

concentrations than pregnant bears. However, the values for both groups were very similar in the autumn when implantation and true gestation occur in the pregnant bears (Figure 1 and Table 1). The oldest female (PB4) had the highest overall FGM yearly average as well as the highest FGM average during winter, spring and autumn (Table 1).

Mean FGM concentrations did not differ in female bears between the three trimesters of pregnancy or pseudopregnancy ($P>0.05$). Furthermore, there were no differences in FGM concentrations among trimesters when only pregnant bears were included in the analysis ($P>0.05$), likely due to significant inter-individual variation with regard to profile dynamics.

Baseline FGM concentrations ranged from 11.01–42.24 ng/g with an overall mean (± SD) of 29.98 ng/g ± 11.40 ng/g in female bears (Table 1). Pseudopregnant female baseline FGM mean (± SD) and range (38.82 ng/g ± 2.98; 36.78 ng/g–42.24 ng/g) appeared slightly higher than those for pregnant females (21.15 ng/g ± 9.06; 11.01 ng/g–28.46 ng/g), but the difference was not statistically significant ($P=0.24$). FGM peaks ranged from 161.4–416.8 ng/g across all bears or 618–1845% of baseline concentrations.

Because meaningful dynamic shifts in hormone profiles can get lost when data are compiled into mean values over time, data for the three pregnant bears are presented in individual longitudinal profiles (Figure 2). Although all bears exhibited dynamic profiles, no consistent pattern associated with pregnancy, implantation or pending parturition could be identified.

Discussion

This study is the first report of serial FGM concentrations in female polar bears throughout the year. The study is an extension of a larger body of work aimed at identifying a method for non-invasively diagnosing pregnancy in this species. Previous efforts to differentiate pregnancy from pseudopregnancy in polar bears have failed to yield a reliable, definitive diagnostic test, and have involved using oestrogen, testosterone and progesterone metabolites (Stoops et al. 2012), faecal proteins (Curry et al. 2012b; De Lorenzo et al. 2016), a sniffer detection dog (Curry et al. 2014), ceruloplasmin (Knott et al. 2013), relaxin (unpublished data), and PGFM (Dehnhard and Jewgenow 2013). Samples for this study were retrospectively selected from known pseudopregnant and pregnant bears to test the hypothesis that FGM concentrations or patterns of excretion would differ between these two groups. However, the resulting data do not support the hypothesis. Although distinct increases in serum and salivary cortisol occur in women during pregnancy, especially during the third trimester (Abou-samra et al. 1984; Meulenberg and Hofman 1990), and elevated FGM concentrations have been associated with late stage pregnancy in red deer (Pavitt et al. 2016), polar bear FGM concentrations were not higher in pregnant versus pseudopregnant bears. In fact, mean FGM concentrations for the two bear groups varied the least (54 and 59 ng/g) during the autumn season when the pregnant bears were experiencing implantation and the final stages of gestation; and the detailed comparison of FGM concentrations by trimester also revealed similar concentrations for pregnant and pseudopregnant bears during the third trimester (57 and 49 ng/g, respectively). Furthermore, there was no increase in FGM concentrations as pregnancy progressed. Although one bear (PB1) appeared to exhibit an increase in FGM towards the end of gestation and prior to parturition, another bear (PB2) exhibited no such increase, and PB3 exhibited higher FGM during her first trimester compared to the second and third trimesters. These variations in FGM concentrations in these three bears are more likely associated with their individual reactions to changes in their environment during these times rather than being a reflection of their physiological state of pregnancy.

Methods for employing cortisol and corticosterone EIA and RIA to measure FGM in many carnivorous species were previously successfully validated years ago (Young et al. 2004), and the ability to document physiological responses of polar bears to adrenal stimulation through FGM assessment has been validated more than once (Shepherdson et al. 2013; White et al. 2015). Although this study represents the first attempt to identify a relationship between FGM concentration and pregnancy in a bear species, the relationships between glucocorticoid metabolite concentrations in faeces or urine and environmental factors have been reported in several previously published studies in this taxon. For example, Shepherdson and colleagues (2013) found a positive relationship between frequency of stereotypic behaviours and FGM concentration in polar bears in zoological facilities. Additionally, urinary glucocorticoid concentrations were lower in giant pandas that had a choice of enclosures (Owen et al. 2005b), but concentrations did not increase consistently when pandas were subjected to high ambient noise (Owen et al. 2004). FGM concentrations in Asiatic black bears (*Ursus thibetanus*) reportedly decreased over time after the bears were moved from bile farms to rescue centres (Malcolm et al. 2013). Similarly, researchers studying wild bear populations employ FGM monitoring (Christina et al. 2004) and/or total hair cortisol content (Bechshøft et

al. 2011; 2013) as measures of stress under changing natural environmental conditions.

A challenge facing these correlative studies is in knowing what "normal" baseline values should be, given the number of factors that can impact FGM concentrations. For example, diurnal and seasonal shifts in glucocorticoids have been reported in the giant panda (Owen et al. 2005a) and shifts in FGM associated with dietary changes have been reported for brown bears (*Ursus arctos*) (Christina et al. 2004). Seasonal increases in cortisol during the winter have also been documented in black bears (Harlow et al. 1988). Therefore, it is essential when studying FGM concentrations in association with various environmental and physical factors for normal baseline data to be available for comparison and to minimise erroneous conclusions due to confounding factors. This study provides six examples of baseline data for female polar bears maintained in zoos.

This study has revealed substantial variation in FGM profiles between individuals, but no consistent seasonal effect was apparent, with mean values for each season ranging narrowly from just 49–57 ng/g. Although these results differ from those reported for the black bear and giant panda that exhibit elevated glucocorticoids in winter (Owen et al. 2005a; Harlow et al. 1990, it was suggested that the wild black bears experienced an increase in cortisol due to their fasting condition and obligate metabolism of fat for energy during hibernation (Harlow et al. 1988). In contrast, polar bears do not hibernate except when females give birth, and winter sampling for this study was conducted when females were without cubs. In giant pandas, it was suggested that the increase in glucocorticoids was associated with increased pacing behaviour, perhaps in anticipation of the impending breeding season (Owen et al. 2005a). Although polar bears under professionally managed care tend to breed from mid-February through early April (Curry et al. 2012a), behavioural monitoring was not included in this study, so no association between activity level and FGM concentration can be evaluated.

Although one might anticipate that the heat of summer at lower latitudes in the northern hemisphere could be a stressor to these Arctic bears, overall FGM concentrations were not elevated during the summer season. In fact, in only one of the six bears (PB6) was the individual FGM mean higher in the summer than during the other three seasons. It is possible that access to cool pools during the summer months alleviates heat stress in polar bears. Alternatively, they may be acclimatising to the warmer environments. In ruminants susceptible to heat stress, studies have shown that cortisol increases in response to acute exposure but then decreases during acclimatisation (Bernabucci et al. 2010). Since the polar bears would have been exposed to gradually warming temperatures as seasons change, it is possible that they hormonally acclimatise to shifting temperatures and therefore do not exhibit increases in cortisol that would be associated with acute exposure to high temperatures. However, acclimatisation to heat stress may have costs. Cows that acclimatise to heat stress yield less milk and exhibit reduced reproductive success compared to those not exposed to heat stress (Bernabucci et al. 2010).

Baseline FGM concentrations in this study (11.0–42.2 ng/g) were very similar to those reported for three polar bears in a different study (29.6–37.6 ng/g; White et al., 2015), despite the use of two different assays (cortisol EIA versus corticosterone RIA, respectively). However, it is more valuable to compare qualitative versus quantitative results when comparing endocrine data from two different labs and assays. Peak excretions recorded for individual bears in this study ranged from 618–1845% of baseline values and were similar to those reported by White et al. (2015) in response to the ACTH challenge (343–2258%). There was no significant difference in overall FGM concentrations between pregnant and pseudopregnant bears. However, there was a tendency (P=0.10) towards higher concentrations in pseudopregnant bears, and all three pseudopregnant bears exhibited a higher baseline concentration compared to pregnant bears, a nearly two-fold difference; though, due to low samples size and non-normal distribution of data, statistical testing was restrictive. Future studies using a larger sample size and greater statistical power could reveal baseline differences. Bear age was an inherent confounding factor in this data set since all three pseudopregnant bears were older than the pregnant bears. Similarly, the bear with the highest baseline value reported by White et al. (2015) was the oldest bear in the study. The age–cortisol relationship appears to differ between species, but wild polar bear and grizzly bear hair cortisol concentrations are not influenced by age (Bechshøft et al. 2011, 2013; Malcolm et al. 2010), and no age effect was noted in FGM concentrations of wild brown bears (Christina et al. 2004).

Although neither FGM concentrations nor patterns of excretion appeared useful for distinguishing pregnant from pseudopregnant polar bears in this study, the data have value for several reasons. First, results suggest that female polar bears maintained in zoos do not experience seasonal fluctuations in FGM concentrations. Second, individual profiles reveal a high level of variation throughout the year. Both these findings are important for researchers employing short-term FGM monitoring in behavioural and physiological studies. Finally, the study establishes baseline FGM concentrations for several female bears maintained in zoos, data that could be important to field researchers employing FGM monitoring to study the impact of pollutants and climate change on wild bears.

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