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FECAL CORTISOL METABOLITES: A NON-INVASIVE METHOD FOR MONITORING THE LONG-TERM HEALTH OF FREE RANGING BROWN BEARS

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By

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FECAL CORTISOL METABOLITES: A NON-INVASIVE METHOD FOR MONITORING THE LONG-TERM HEALTH OF FREE RANGING BROWN BEARS

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ABSTRACT

FECAL CORTISOL METABOLITES: A NON-INVASIVE METHOD FOR MONITORING THE LONG-TERM HEALTH OF FREE RANGING BROWN BEARS

By

Justin Antonio Pinero

Ecotourism is a rapidly growing industry worldwide and has been used as a tool that can promote conservation. While ecotourism can serve as a mechanism to help conserve natural areas, increases in visitors present challenges for managers tasked with balancing conservation goals while ensuring positive visitor experiences. As such, managers and ecologists are increasingly using fecal cortisol metabolites (FCMs) to index stress associated with ecotourism. In this study, I sought to (1) quantify the relationship between blood cortisol levels and FCM concentrations in brown bears (*Ursus arctos*), and (2) evaluate whether ecotourism elicits a measurable stress response in a free-ranging brown bears. For my first objective, I conducted an adrenocorticotropic hormone (ACTH) challenge on nine captive brown bears at the Washington State University Bear Research, Education, and Conservation Center to quantify the relationship between blood cortisol and FCM concentrations. For my second objective, I collected fecal samples from three designated bear viewing sites (Chinitna Bay, Shelter Creek, Silver Salmon Creek) across Lake Clark National Park and Preserve with variable ecotourism. I found that peak FCM concentrations occurred between 10h-27h following ACTH challenge. Additionally, I found no significant difference in average FCM among sites; however, bears at Chinitna Bay exhibited high variable in FCM concentrations, which may be a result of unpredictable human-interaction due to conflicting rules across land jurisdictions. This study highlights the importance of consistent bear viewing practices across bear viewing areas, providing bears with predictable human-bear interactions.

DEDICATIONS

I dedicate my master's thesis to my family. I would not be here without your support, encouragement, and trust.

I would also like to dedicate this to my partner Hannah, who has supported and sacrificed so much so that I can live my dream of doing meaning biological research. Your encouragement has helped me to push through all of the challenging moments I have faced in the process of completing my thesis. I am so very appreciative for all you have done for me.

I would also like to dedicate this thesis to my advisor, Dr. Diana Lafferty. Her constant drive, encouragement, and passion has always pushed me to do better. It is thanks to your guidance that I have grown and matured as a biologist and a person. Thank you for seeing the potential in me and trusting me to conduct this meaningful research.

To my pops, in loving memory.

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INTRODUCTION

Ecotourism is a rapidly growing industry worldwide and is often used as a tool to promote conservation (Balmford *et al*., 2009). Although ecotourism can generate substantial revenue to benefit protected area management, ecotourism can also create conflicts when managing wild populations (He *et al*., 2008; Patthey *et al*., 2008). Ecotourism can negatively impact native diversity and the natural behavior (e.g., resource acquisition, breeding) of wildlife (Czech *et al*., 2000, Hidinger 2001). As a result, natural resource managers have the difficult task of protecting wildlife resources while ensuring positive visitor experiences. As such, knowledge of how ecotourism impacts wildlife is essential for managers to make informed decisions that provide positive visitor experiences while upholding conservation goals for native species.

To understand the impacts of ecotourism on wildlife, fecal cortisol metabolites (FCMs) are increasingly used to index physiological stress. In response to a stressor, vertebrates activate the hypothalamic-pituitary-adrenal (HPA) axis, which stimulates the release of cortisol and other glucocorticoids into the blood stream (Mondol *et al*., 2020). HPA axis activation is an adaptive response, allowing individuals to respond to environmental perturbations; however, prolonged HPA axis activation is detrimental to individual health, and can result in immune suppression, muscle wasting, weight loss, and reduction or loss or reproduction (Blas *et al*., 2007; Charbonnel *et al*., 2008; Tilbrook, 2000). Cortisol circulating in the bloodstream is eventually metabolized by the liver and excreted in urine and feces as cortisol metabolites, resulting in a lag time between blood cortisol levels and fecal cortisol that is dependent on length of the gut, the rate of hepatic cortisol metabolism, and presence of food (Touma and Palme, 2005).

Expression of FCMs in wildlife is influenced by a variety of factors (Hadinger *et al.*, 2015). For example, sex directly impact FCM concentrations in coyotes (*Canis lantrans*), but is found to not impact FCM concentrations in polar bears (*Ursus miritimus*) (Leishman *et al*., 2022; Stevenson *et al*., 2018). Diet and season can indirectly affect FCM concentrations by affecting the transit of hormones through the gastrointestinal tract and the distribution of FCMs in feces (Lewis *et al*., 1997; von der Ohe *et al*., 2004; Ware *et al*., 2013). Increasingly, anthropogenic factors have been studied to determine whether a variety of human disturbances (e.g., number of humans, distance from humans to animals, road density) are correlated with FCM concentrations. For instance, human disturbance has been found to impact FCMs in elk (*Cervus elaphus*), Chamois (*Rupicapra rubpicapra*), and Europeon pine marten (*Martes martes*) (Barja *et al*., 2007; Millspaugh *et al*., 2001; Zwijacz *et al.*, 2013). In contrast, no correlation was found between human disturbance and FCMs in brown bears (*Ursus arctos*), Barbary Macaques (*Macaca sylvanus*), and red squirrels (*Sciurus vulgaris*) (Haigh *et al*., 2017; Maréchal *et al*., 2011; von der Ohe *et al*., 2004). As such, there is a lack of consistency regarding the impacts of tourism on the physiological stress in wildlife.

During this period of rapidly growing interest in ecotourism, it is critical for manager to have the information needed to make science-led decisions to ensure the health of wild populations while balancing needs to visitors. In my first chapter, I investigate the use of fecal cortisol metabolites as a means for indexing the physiological health of brown bears, as well as quantify the temporal relationship between HPA activation and the expression of cortisol metabolites in feces. In my second chapter, I applied the knowledge gained from my first chapter to assess the physiological impact of ecotourism on a wild population of brown bears within Lake Clark National Park.

1. CHAPTER 1: BLOOD CORTISOL AND FECAL CORTISOL METABOLITE CONCENTRATIONS FOLLOWING AN ACTH CHALLENGE IN UNANESTHETIZED BROWN BEARS (*URSUS ARCTOS*)

1. Introduction

Wildlife depend on a variety of internal and external cues to adaptatively respond to changing conditions. In vertebrates, environmental cues activate the hypothalamic-pituitary-adrenal (HPA) axis, which stimulates the release of cortisol and other glucocorticoids (GC) from the adrenal cortex to help individuals meet the demands imposed by environmental stressors (Mondol *et al*., 2020). For instance, GCs act to mobilize glucose, providing immediate energy in response to acute environmental pressures (e.g., 'fight or flight response'; Adamo 2014). While short-term HPA axis activation facilitates adaptive responses to environmental stress, chronic HPA axis activation can have detrimental health effects including immune suppression, muscle wasting, weight loss, and the reduction or loss of reproduction (Charbonnel *et al*., 2008).

In most mammals, cortisol is the predominant GC secreted in the blood in response to a stressor (Romero 2004). As such, elevated blood cortisol concentrations can provide a quantitative means for evaluating physiological stress in animals (von der Ohe and Servheen, 2002). Cortisol and cortisol metabolites are often measured using enzyme-linked immunoassays (ELISAs) in which specific antibodies bind with GCs and GC metabolites, allowing for quantification (Möstl *et al*., 2005). Cortisol can be extracted from a variety of animal matrices including blood, which requires more invasive procedures, whereas hair, feathers, saliva, and feces allow for non-invasive opportunities to obtain samples for the purpose of analyzing or indexing stress hormones (Palme 2012). Further, blood cortisol concentrations provide insight into the stress response at a single point in time, which can be highly variable based on time of

day, diet, or a recent stressful event (Davies *et al*., 2013). Additionally, use of blood measures of cortisol can be restrictive as animals must be captured first and occasionally chemically immobilized, potentially increasing an organism's stress response (Millspaugh and Washburn, 2004; Thompson *et al*., 2020).

While cortisol can be measured directly in blood, there is no free unbound cortisol in feces (Di Francesco *et al*., 2021). Circulating cortisol is metabolized by the liver and eventually excreted as cortisol metabolites in both urine and feces. Therefore, there is a time delay between peak blood cortisol concentrations following release from the adrenal gland and fecal metabolite concentrations depending on the rate of hepatic cortisol metabolism, length of the intestinal tract, secretion of GC metabolites into the intestinal tract, and presence of food (Touma and Palme, 2005). Importantly, rather than a single moment in time, fecal cortisol metabolites (FCMs) provide an integrated measure of fluctuating blood cortisol concentrations from the time FCMs are formed to when FCMs are excreted. Fecal samples can also be obtained noninvasively, thereby removing potential bias occurring as a result of animal capture and handling stress (Möstl and Palme, 2002). However, without validation that FCM concentrations reflect HPA axis activation and cortisol secretion into the bloodstream, the biological relevance of FCM expression may be spurious (Keay *et al*., 2006). As such, concurrent measures of blood cortisol and FCMs are needed to calibrate the relationship between blood cortisol concentration and subsequent FCM concentrations before drawing inference based on FCMs alone. Captive animals provide an ideal scenario to test this relationship. One method for validating the use of FCMs for non-invasive research purposes is to conduct an adrenocorticotropic hormone (ACTH) challenge. The injection of ACTH triggers the release of GCs in blood, which should then be mirrored in FCMs excreted in feces after a species-specific time lag.

In addition to calibrating the relationship between blood cortisol and FCMs, other factors such as sex, age, and time of day can affect their concentrations (Touma *et al*., 2003). For example, sex differences in FCMs have been found in Steller sea lions (*Eumetopias jubatus*) and coyotes (*Canis lantrans*) (Mashburn and Atkinson, 2004; Stevenson *et al*., 2018). However, other studies have found that sex had little or no effect on FCM concentrations, such as in brown bears (*Ursus arctos*) (von der Ohe *et al*., 2004). Thus, understanding how factors such as sex and age may influence FCM concentrations is critical for interpreting FCM concentrations as a tool for monitoring wildlife health.

In this study, I assessed blood cortisol concentrations and FCMs concentrations in nine captive brown bears. My primary objectives were to (1) determine the cortisol response in serum and FCM samples following an ACTH challenge and (2) quantify the lag time between HPA activation and the expression of FCMs in brown bears.

2. Methods

2.1 Subject and Materials

I conducted this study during June 2021 using nine captive brown bears (five females, four males) ranging in age from six to 20 years. Bears were housed at the Washington State University Bear Research, Education, and Conservation Center. For the duration of the experiment, bears were housed either individually or in pairs with indoor (3m x 3m x 2.5m) and outdoor (3m x 5m x 5m) access. The study bears had been trained previously to enter a holding crate and present a rear leg through the bars for blood collection. All bears were trained via positive reinforcement using dilute honey (in water), a method shown to not influence serum cortisol levels (Joyce-Zuniga *et al*., 2016). Bears were fed a commercial bear diet in the form of

kibble from Mazuri (Wild Carnivore Bear Plus), apples, and a small amount of meat (e.g. chicken, beef, or wild game).

Bears were challenged with 5µg/kg cortrosyn (Sandoz Pharmaceuticals and Amphastar Pharmaceuticals) injected intravenously (Cattet *et al*., 2021). 10mL of blood was collected from the metatarsal or lateral saphenous vein beginning at approximately 8:00am (0h) and then at 3h, 6h, 24h, 48h, and 72h following injection to measure changes in serum cortisol concentrations. Once collected, the blood was centrifuged and the serum stored at -80ºC until analyzed. Fecal samples were collected between 7:00am-8:00pm from 24h pre-ACTH challenge through 72h post-ACTH challenge and placed in a -20°C freezer until shipped overnight on dry ice to Northern Michigan University where samples were stored in a -80°C freezer until analyzed. Bears were under 24h video monitoring, individuals could be identified, and thus the time and source of each fecal deposition could be identified. Baseline serum cortisol levels were calculated as the average cortisol concentrations of plasma drawn at 0h for each bear. FCM baselines were calculated as the average FCM concentration of samples deposited prior to the ACTH challenge (time 0). Peak blood cortisol and peak FCM concentrations were identified as the sample with the largest concentration of cortisol or cortisol metabolites following ACTH challenge.

2.2. *Fecal Hormone Extraction*

Fecal samples were thawed at room temperature for 30 minutes prior to FCM extraction. I weighed 0.5±0.01g of wet feces and placed the feces in a 15mL centrifuge tube with 5mL of 80% methanol (Palme *et al.,* 2013). Samples were vortexed for one minute and then centrifuged at 2500g for 15 minutes. After being centrifuged, the supernatant was analyzed immediately via ELISA kit.

2.3. *Cortisol and Cortisol Metabolite Assay*

Serum cortisol concentrations and FCM concentrations were determined in duplicate using commercially available cortisol ELISA kits (Cortisol ELISA K003, ArborAssay, Ann Arbor, MI 48108, USA). The upper and lower detection limits of the assay were 45.4 and 27.6 pg/mL, respectively. Serum cortisol samples were brought to room temperature prior to being assayed, following the manufacturers protocol. For FCM samples, I modified the manufacturer's protocol by extending the time samples were on the plate shaker to an hour and a half to increase the time for FCMs to bind to the ELISA antibodies.

2.4. *Assay Validation*

Fecal extracts were tested for parallelism by diluting high FCM concentration samples (one for each sex) from 1:20 to 1:2.5 with assay buffer (Hein *et al.*, 2020). Dilutions were parallel to the standard curve (test of equal slopes, $p>0.30$), indicating no additional substances in the extract were cross-reacting with the antibody.

All statistical analyses were conducted in R (version 4.2.2, R Core Team 2022). Alpha was set at 0.05 and all tests were two-tailed. For both FCM and serum cortisol concentrations, I evaluated the change from baseline through four days following an ACTH challenge with a repeated measure analysis of variance (ANOVA). Additionally, I performed a two-way repeated measures ANOVA to determine the influence of sex (male, female) and day of feces collection/plasma collection, as well as age (young, old) and the day of feces collection/plasma collection before and after injection. I considered young individuals as bears six years old and younger, and old bears to be older than six years old. Next, I performed a post-hoc Tukey's test to determine which days of fecal collection were significantly different from one another.

3. Results

3.1. *Serum Cortisol Results*

Following injection of cortrosyn, serum cortisol concentrations peaked between 3h to 6h. (Figure 1.1). Serum cortisol concentrations increased from 4.5-10.4 times above baseline levels (Table 1.1). Serum cortisol concentrations at 3h and 6h post injection differed significantly from baseline cortisol ($p<0.001$ each); however, the 3h and 6h time period did not differ significantly from one another ($p=0.99$). Serum cortisol concentrations returned to baseline levels by 24h post-injection and did not differ from baseline at 72h following injection for the remainder of the study period (Tukey's HSD, $p>0.05$).

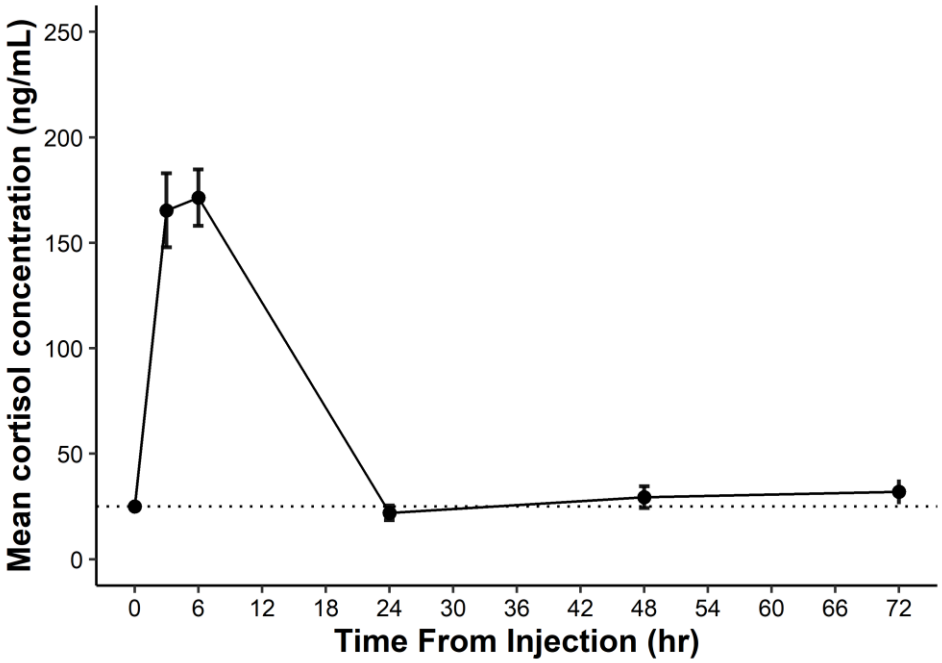


Figure 1.1. Time course of serum cortisol concentration (mean±SEM) for nine brown bears (*Ursus arctos*) following injection of 5 μg/kg (i.v.) of cortrosyn. The dotted line represents the population-level baseline concentration (24.96ng/mL).

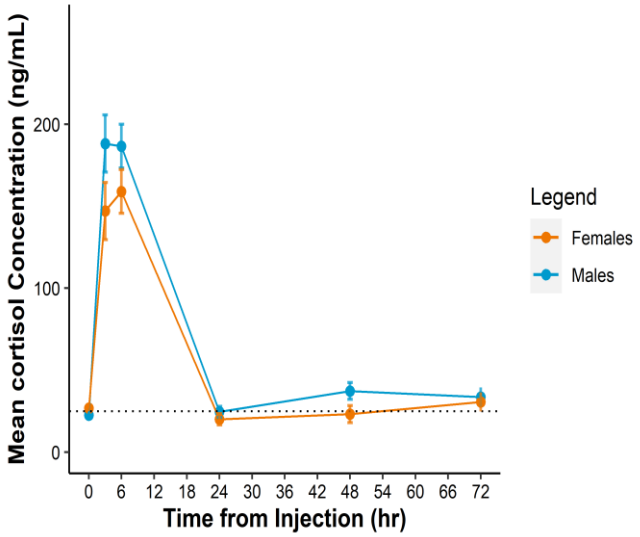
Table 1.1. Individual serum cortisol responses to intravenous injection of 5 μg/kg of cortosyn in nine unanesthetized brown bears (*Ursus arctos*).

			Plasma Concentration		
Identification	Age (years)	Sex	Time 0 (ng/ml)	Peak (ng/ml)	Hours to Peak Response
Adak	6	M	28.9	160.10	3.00
Dodge	6	M	20.0	174.50	6.00
Frank	20	M	16.1	246.10	3.00
John[1]	20	M	25.4	259.80	6.00
Kio	18	F	30.5	221.20	3.00
Luna	18	F	18.8	160.70	6.00
Peeka	18	F	23.6	187.20	6.00
Willow	6	F	35.4	112.10	6.00
Zuri	6	F	26.0	152.00	6.00

[1]In addition to bear kibble diet, John also received Hills prescriptive digestive care diet for dogs.

Serum cortisol concentrations did not differ significantly between males and females (Two-way ANOVA: factor time, $F=58.68$, $p<0.001$; factor sex, $F=3.37$, $p=0.07$; interaction, $F=0.80$, $p=0.55$; Figure 1.2). However, serum cortisol was significantly greater at 3h post-injection in old versus young bears (Two-way ANOVA: factor time, $F=89.17$, $p<0.01$, factor age, $F=16.82$, $p<0.01$, interaction, $F=3.23$, $p=0.01$). Serum cortisol concentrations did not differ between young and old bears at any other times (Tukey's HSD, $p>0.05$).

(a)



(b)

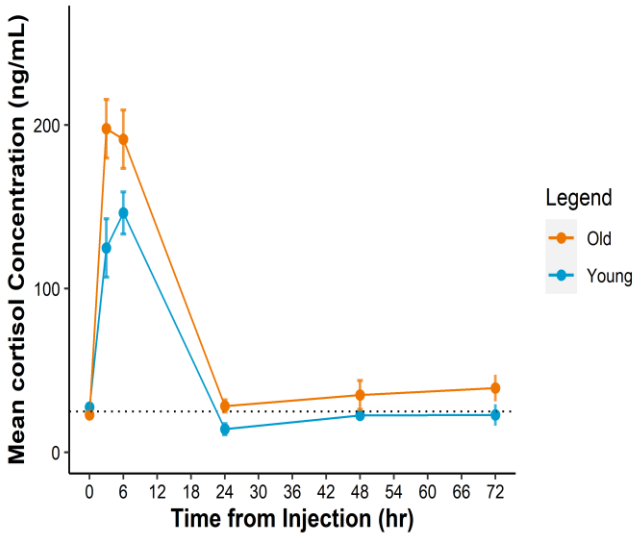


Figure 1.2. (a) Mean serum cortisol concentration (±SEM) for nine brown bears (*Ursus arctos*) by sex (4 males, 5 females) (b) Mean serum cortisol concentration (±SEM) of nine brown bears by age group (4 young, 5 old). Dotted line represents population-level baseline concentration (24.96ng/mL). Bears were injected intravenously with 5 µg/kg of cortrosyn.

Fecal Cortisol Metabolite Results

FCM concentration increased between 5-14 times from baseline (Table 1.2). Baseline FCM concentration for all bears averaged 21.9pg/g. On average, peak FCM occurred at 20.47h following ACTH injection. As expected, FCM patterns followed trends exhibited in serum. One

individual (i.e., Zuri) had an unexpected increase in FCM during the 24 hours prior to injection and on the final day of the study. Nevertheless, all animals were included in statistical analysis.

Table 1.2. Individual characteristics of nine brown bear (*Ursus arctos*) and the fecal cortisol metabolite (FCM) response to intravenous injection of 5 μg/kg of cortrosyn.

			FCM Concentration		
Identification	Age (years)	Sex	Time 0 (ng/g)[2]	Peak (ng/g)	Hours to Peak Response
Adak	6	M	3.54	172.41	13.42
Dodge	6	M	44.64	228.80	20.95
Frank	20	M	16.01	128.03	20.68
John	20	M	2.06	111.96	21.47
Kio	18	F	0.33	183.08	27.67
Luna	18	F	3.75	312.39	10.78
Peeka	18	F	1.53	146.11	27.08
Willow	6	F	9.85	115.13	22
Zuri	6	F	50.65	126.32	20.18[1]

[1]Zuri hours to peak response excluded the two peaks in FCM that occurred prior to injection. [2]Time 0 value are based on mean fecal cortisol concentration of individuals prior to injection.

FCM concentrations differed significantly from baseline during day one, and returned to baseline levels on day two and remained at baseline levels for day three and four ($p<0.01$, $p=0.46$, $p=0.99$, $p=0.91$, figure 1.3). However, daily mean FCM did not differ between males and females (Two-way ANOVA: factor day, $F=10.53$, $p<0.001$; factor sex, $F=0.36$, $p=0.85$; interaction, $F=0.23$, $p=0.92$; Fig. 1.4). Daily mean FCM concentrations also did not differ significantly between age groups (Two-way ANOVA: factor day, $F=10.93$, $p<0.001$; factor age, $F=0.14$, $p=0.71$); although, a significant time by age interaction was observed ($F=2.45$, $p=0.04$).

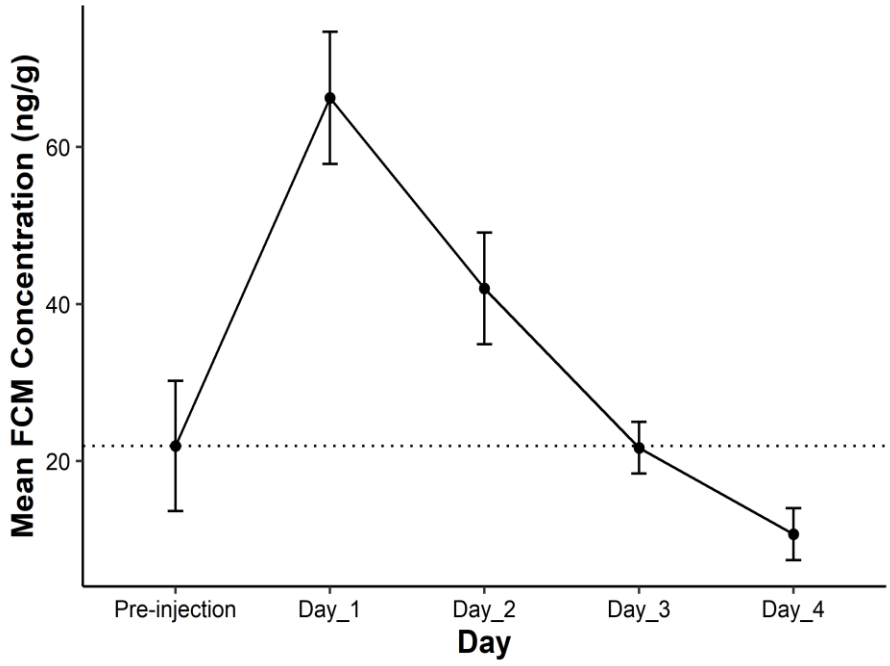


Figure 1.3. Daily mean fecal cortisol metabolite concentration (±SEM) for nine brown bears (*Ursus arctos*). Dotted line represents population-level baseline concentration (21.90 ng/g). Bears were injected intravenously with 5 µg/kg of cortrosyn.

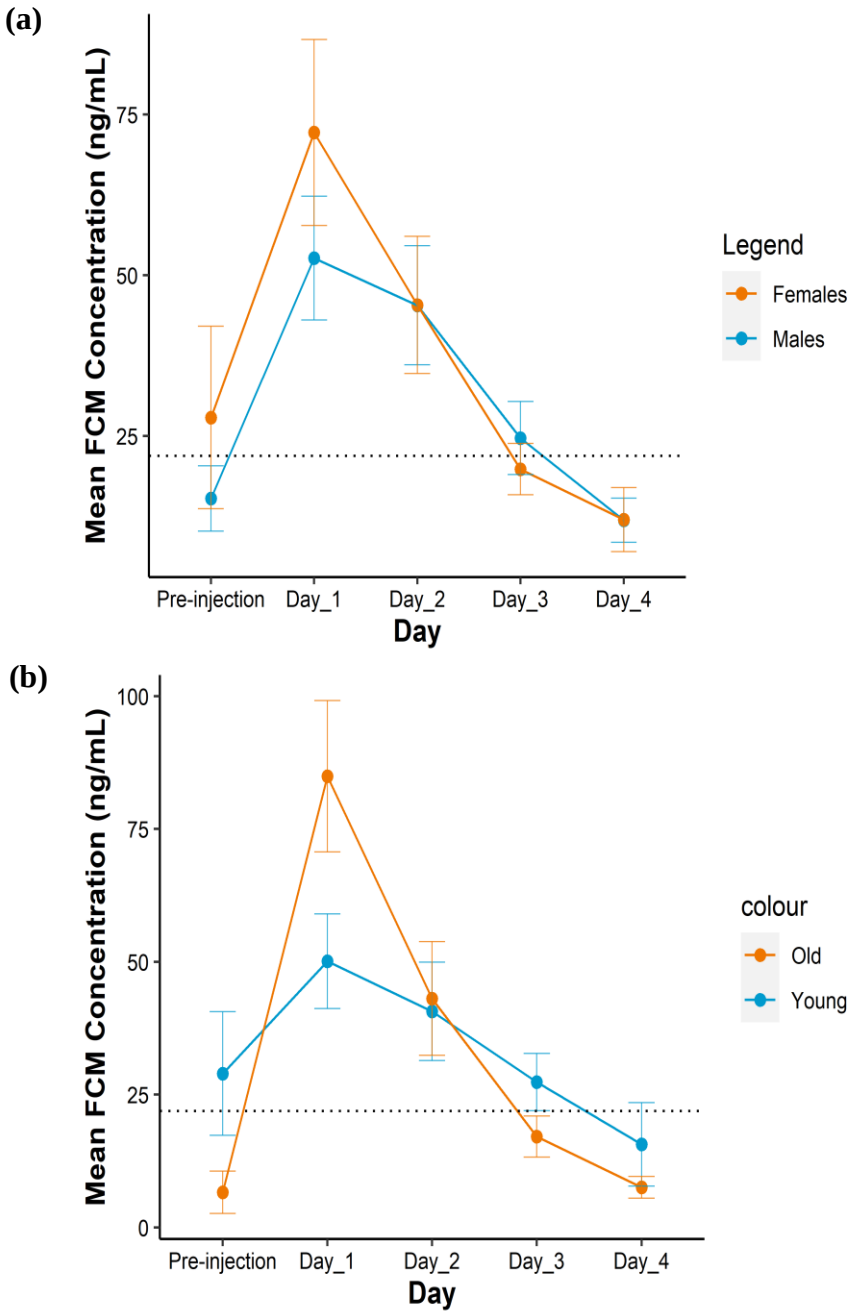


Figure 1.4. (a) Daily mean FCM concentration (±SEM) by sex (4 male, 5 female) for nine brown bears (*Ursus arctos*). (b) Daily mean FCM concentration (±SEM) for nine brown bears by age (4 young, 5 old). Dotted line represents population-level baseline concentration 21.90 ng/g. Bears were injected intravenously with 5 µg/kg of cortrosyn.

4. Discussion

I demonstrate that brown bear FCM concentration provide an alternative and ecologically meaningful index of circulating blood cortisol concentrations to draw inferences of physiological health of an organism. Peak FCM concentrations peaked on average 20.4 hours after administering cortrosyn. These peak times in the current study were considerably longer than the times described in White *et al.* (2015), who injected three brown bears with corticotrophin instead of cortrosyn (see Table 1.3 for details). Furthermore, White *et al.* (2015) chemically immobilized their animals and conducted their study in November and December when bears differ physiology from summer-active bears (Laske *et al.*, 2011, Ware *et al*., 2013). Hunt and Wasser (2003), conducted an ACTH challenge with a single male and female brown bear and observed peak FCM concentrations at 22h and 32h, respectively. A study conducted with using a single male giant panda (*Ailropoda melanoleuca*) found that peak FCM occurred around 12h following injection of cortrosyn (Kersey et al. 2010), while Wassar *et al*. (2000) found that peak FCM expression in a female Malayan sun bear (*Helarctos malayanus*) occurred roughly 25h following injection of ACTH via a slow releasing gel (ACTHAR). The magnitude of response in both studies were similar to those observed in the present study, suggesting the potential for high variation between individual endocrinology. The aforementioned studies may also suggest that individual bear species may vary enough that validation must be conducted separately.

Table 1.3. Summary of previous adrenocorticotrophic hormone (ACTH) challenge studies on fecal cortisol metabolites (FCM) in a variety of bear species.

Author	Species	Sample Size	Drug	Mean hours to peak FCM response
White *et al*., (2015)	Brown Bears (*Ursus arctos*) / Polar Bears (*Ursus maritimus*)	3 (brown bear) / 3 (polar bear)	Corticotrophin	5.63 (brown bear) / 12.63 (polar bear)
Hunt and Wassar (2003)	Brown Bears	2	ACTH	27
Kersey *et al.*, (2010)	Giant Panda (*Ailuropoda melanoleuca*)	5	Cortrosyn	12
Wassar *et al*., (2000)	Malayan Sun Bear (*Helarctos malayanus*)	1	ACTHAR Gel	25

Diet has also been shown to influence the lag time from injection to peak FCM expression. For example, Pritchard and Robbins (1990) found that mean gut retention time for vegetation in brown bears and black bears was 7h and while that for meat was 13h, suggesting a relationship between diet composition and digestive efficiency. Zhou *et al*. (2020) found that the macronutrient composition of foods eaten by giant pandas influenced FCM concentrations. Additionally, von der Ohe *et al*. (2004) found that diet and season interacted to affect FCM concentration in free ranging brown bears, but similar to my study, no sex or age effect was observed.

Daily and seasonal patterns may also influence cortisol concentrations in serum. Cortisol is indirectly influenced by light, leading to increases in serum cortisol concentrations during night and decreased levels during the day (Leproult *et al.,* 2001). Additionally, in brown bears, the daily means of serum cortisol have been found to vary significantly across seasons dependent

on the length of daylight (Ware *et al*., 2013). This may be of particular importance when conducting non-invasive studies on brown bears at high and low latitudes where length of day may differ dramatically by season.

In summary, my work adds to the knowledge of the biologically meaningful linkage between circulating serum cortisol and FCMs. Importantly, my results demonstrate that FCMs provide a potential index of stress in brown bears. The variability between lag time and magnitude of response by individuals within a controlled environment reinforces the importance of individual differences contributing to variation in the physiological response of animals after a disturbance event. My findings contribute empirical evidence to support the application of using FCMs to noninvasively monitor long-term stress of free ranging brown bear populations. Future studies should explore further the effect of seasonal variation in plasma and FCM concentrations, particular as bears experience hyperphagia and emerge from torpor.

5. Limitations and Considerations

Due to ethical considerations and the difficulty in defining stress, I was not able to experimentally compare the cortrosyn-induced elevations in serum cortisol and FCM to those of a defined stress. This is an important consideration and one that would also be relevant to field studies where human observations and timed fecal collections would be needed to draw firm conclusions. Another limitation of my work is the relatively infrequent collection times used to define the serum cortisol peak. Future studies should use more frequent blood sampling to define this with greater accuracy.

2. CHAPTER 2. EFFECTS OF ECOTOURISM ON FREE-RANGING BROWN BEARS (*URSUS ARCTOS*) FECAL CORTISOL METABOLITE CONCENTRATIONS.

1. Introduction

Ecotourism accounts for more than 9% of global revenue and supports roughly 277 million jobs worldwide (Newfarmer, Page, & Tarp 2018). Economic opportunities from ecotourism provide a mechanism to fund the conservation of natural resources, including the protection and management of public lands (Kiper 2013). However, high-levels of ecotourism can negatively affect native diversity and impact the natural behavior of free-ranging animals (Czech, Krausman, & Devers, 2000; Hidinger 2001; Reed & Merenlender, 2008). As such, challenges may arise as natural resource managers strive to balance the conservation of natural areas with ensuring positive visitor experiences. Thus, a better understanding of how ecotourism impacts free-ranging animals is critical for mangers to make science-informed decisions that promote both positive visitor experiences and conservation of natural areas.

To better understand the impacts of ecotourism on free-ranging wildlife, natural resource managers and ecologists are increasingly using cortisol and fecal cortisol metabolites (FCMs) to index the physiological stress of individuals and populations. When an animal perceives a stressor, the hypothalamic-pituitary-adrenal (HPA) axis is activated, stimulating the release of cortisol and other glucocorticoids (GCs) into the bloodstream (Jessop, Woodford, & Symonds, 2013). HPA axis activation is an adaptive response to mediate stress and aid in recovering homeostasis; however, chronic HPA axis activation can negatively impact an individual's health by suppressing immune function, cause muscle wasting, and in extreme cases can result in a reduction or loss of reproduction (Blas *et al*., 2007; Charbonnel *et al*., 2008; Tilbrook, 2000). While cortisol concentrations can be measured in blood, blood collection typically requires

capture and chemical immobilization, which may also elicit a stress response (Thompson *et al*., 2020). Cortisol circulating in the bloodstream is eventually metabolized by the liver and excreted as FCMs. As such, collection of fecal samples and the subsequent quantification of fecal cortisol metabolites provides a noninvasive approach for indexing a target taxa's physiological health.

Several factors can influence the expression of FCMs in free-ranging animals (Hadinger *et al*., 2015). For example, sex differences in FCM concentrations have been found in coyotes (*Canis lantrans*), but not in polar bears (*Ursus miritimus*) (Leishman *et al*., 2022; Stevenson *et al*., 2018). Diet may also affect the expression of cortisol metabolite concentrations. For instance, diet can affect the transit time of hormones and hormone metabolites through the gut, which in turn influences the reabsorption of GCs in the gut and subsequent concentration of hormone metabolites in feces (Lewis *et al*., 1997). In brown bears (*Ursus arctos*), diet has been correlated with cortisol in hair and cortisol metabolites in feces (Bryan *et al*., 2013, von der Ohe *et al*., 2004). FCM concentrations also can vary due to daily (i.e., circadian rhythm) and seasonal changes (e.g., active, hibernation) (Ware *et al*., 2013). In addition, numerous studies have explored the correlations between anthropogenic activities and physiological stress in diverse species as index by cortisol concentration in hair and blood, or the concentration of FCMs in feces (Shutt *et al*., 2014; Zwijacz *et al*., 2013). In elk (*Cervus elaphus*), for instance, Millspaugh *et al*. (2001) found that FCM concentrations were positively correlated with human disturbance (i.e., road density). Zwijacz *et al*. (2013) found that FCM concentrations in Chamois (*Rupicapra rupicpra tatrica*) were associated with both human presence and the number of human visitors in Tatra National Park in southern Poland. Similarly, in a nature park in Northwest Spain, wild populations of European pine marten (*Martes martes*) exhibited significantly higher FCM concentrations in areas with unrestricted human access compared to areas in which tourism is not

permitted and this index of stress was higher during the reproductive season (Barja et al. 2007). In contrast, von der Ohe *et al*. (2004) did not detect an association between the number of visitors and brown bear FCM concentrations at Katmai National Park and Preserve. In a study on Barbary Macaques (*Macaca sylvanus*), Maréchal *et al*. (2011) did not find an association between average number of tourists present and fecal glucocorticoid metabolites. Additionally, Haigh *et al*. (2017) did not find a correlation between FCMs and visitor numbers in red squirrel (*Sciurus vulgaris*). As such, there is no consistent pattern regarding the impacts of tourism on physiological stress in wildlife.

Brown bears are one of the most sought-after species in the world for ecotourism (Skibins, Hallo, Sharp, & Manning, 2012). Located in Alaska, Lake Clark National Park and Preserve (LACL) hosts one of the highest brown bear densities in the world, making it an ideal destination for bear-focused ecotourism. Due to the high brown bear density at LACL, visitation at designated bear viewing sites has increased five-fold in the past decade (Shepard, & Frith 2018). Designated bear viewing sites within LACL are located in resource rich areas where bears must contend with human presence when accessing critical food resources. While some predators may be elusive, brown bears are highly visible during foraging bouts and defecate numerous times a day. As such, the brown bear population inhabiting LACL is an exciting ecological model for monitoring the effects of human disturbance on FCM concentrations.

In this study, I use fecal samples to noninvasively index stress in a wild population of brown bears at Lake Clark National Park and Preserve in Alaska, USA. My objective was to evaluate whether ecotourism elicits a stress response in brown bears while also accounting for potential variation in measures of stress associated with diet and season. I hypothesized that if

ecotourism mediates physiological stress in brown bears, I would observe a positive correlation between the number of visitor present at bear viewing sites and brown bear FCM concentration.

2. Methods

2.1 *Study Area*

I conducted this study from June 4 through August 16, 2022 across three designated brown bear viewing sites along the Lake Clark National Park and Preserve coast: Chinitna Bay, Shelter Creek and Silver Salmon Creek (Figure 2.1). Chinitna Bay, Shelter Creek, and Silver Salmon Creek differ substantially in monthly and yearly human visitation, receiving ~4614, ~680, and ~6335 visitors annually, respectively for the 2022 ecotourism season (May through September). Chinitna Bay experienced the highest human visitation during June, whereas both Shelter Creek and Silver Salmon Creek experienced peak human visitation during July (Table 2.1). In addition to differences in human visitation, these sites differ in the type of bear viewing opportunities available. Chinitna Bay operates as a closed meadow, confining visitors to designated brown bear viewing areas situated at ground level along the edge of the meadow. However, if bears leave the meadow on National Park Service (NPS) land to access marine resources on the beach (e.g., razor clams, *Siliqua patula*), which is designated as state land, visitors can leave the viewing areas on NPS land and follow bears to the beach. On the beach, visitors can approach bears as closely as desired. Shelter Creek and Silver Salmon Creek operate as open meadows, where visitors can walk in the meadows alongside bears. Additionally, Chinitna Bay and Silver Salmon Creek have private ecotourism lodges on site, capable of hosting 20-30 visitors. The lodge at Chinitna Bay consists of a single inholding with a single elevated bear viewing platform, whereas the lodge at Silver Salmon Creek consists of multiple inholding spread across the edge of the meadow.

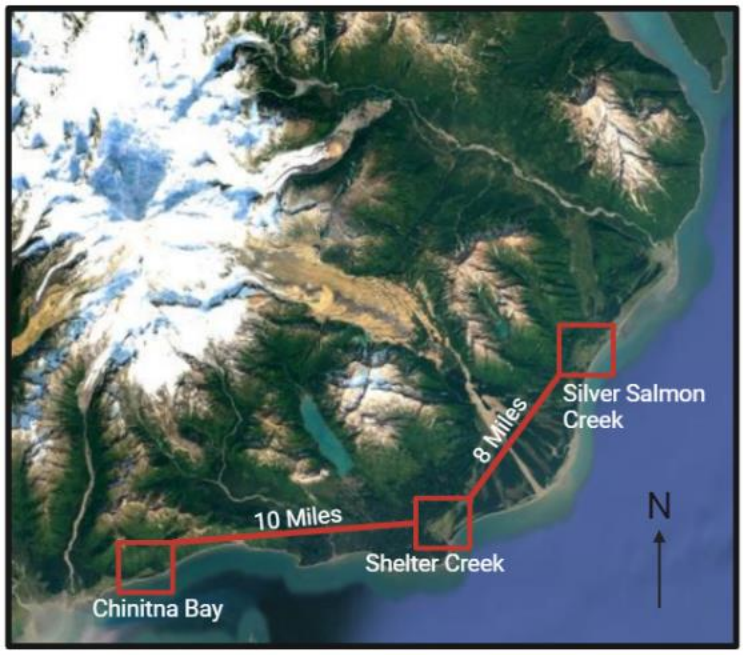


Figure 2.1. Location of three designated brown bear (*Ursus arctos*) viewing sites along the coast of Lake Clark National Park and Preserve, AK, USA. Map created using Google Earth.

Table 2.1. Summary of yearly human visitation data across three designated brown bear (*Ursus arctos*) viewing sites for May-September of 2022 at Lake Clark National Park and Preserve, AK, USA.

	Visitors					
Site	May	June	July	August	September	Total
Chinitna Bay	502	1509	1450	1060	93	4614
Shelter Creek	6	119	365	190	0	680
Silver Salmon Creek	41	1536	2166	1992	600	6335

2.2 *Fecal Sampling*

I randomly established thirteen 50m x 20m plots at each site for a total of 39 plots (Figure 2.2). On the first day of each site visit, I cleared all plots by scattering all feces by boot. I sampled each plot twice a day, once in the morning and once in the evening, to ensure that fecal samples were less than 12h old, thus minimizing bias associated with sample age. Each sample

was mixed thoroughly with a sterile wooden tongue depressor to homogenize the sample and I subsequently collected a 25-75g subsample. Samples were temporarily stored in a cooler backpack during sampling bouts until transferred to a -20°C freezer at camp twice a day. Additionally, I opportunistically collected samples when I observed brown bears defecating and collected samples along trails to and from established sampling plots when samples were determined to be fresh (i.e. absent from trail during previous sampling bout). During fecal sample collection, I recorded the site, plot number, GPS location, date, time, number of people visible from plot, and gross diet based on a visual inspection of feces in which I noted the dominant contents (i.e., vegetation, meat, mixed). I categorized diet as mixed when vegetation and meat appeared equally prominent. Additionally, I obtained the daily tally of human visitation collected via the National Park Service and subsequently categorized daily visitor data as low (0-50 people), medium (51-150 people), and high (150+ people). In addition to collecting fecal samples, I also recorded how often I observed brown bears being displaced due to human influence (e.g., humans approaching bears) and I used a range finder to determine distances between humans and bears. At the end of each site visit, fecal samples were transported to a -80°C freezer at the National Park Service headquarters in Anchorage, AK. At the end of my sampling period, all samples were shipped on dry ice overnight to Northern Michigan University where fecal samples were stored at -80°C until analyzed.

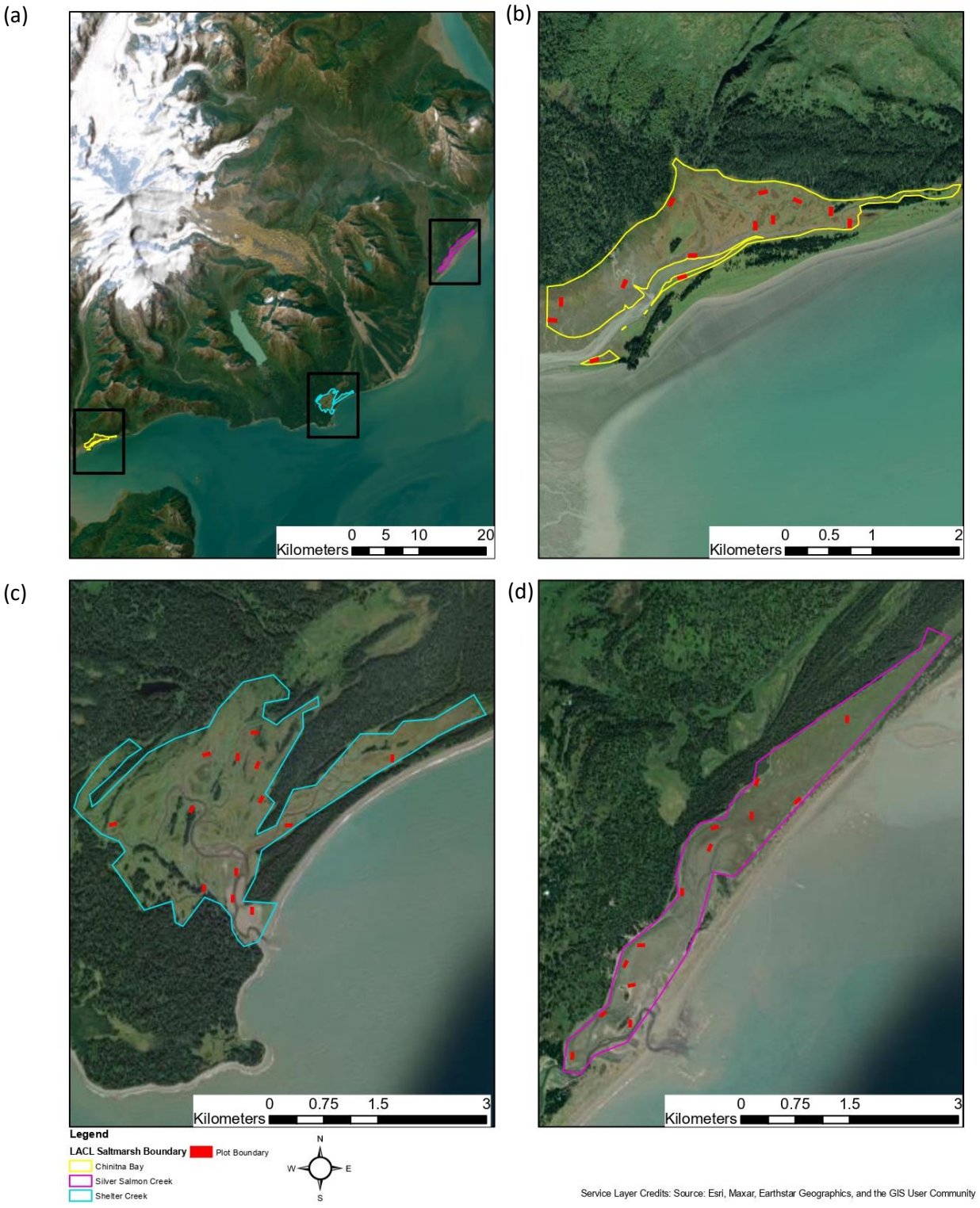


Figure 2.2. Location of brown bear (*Ursus arctos*) fecal sampling plots (N=13) within each designated brown bear viewing site at Lake Clark National Park and Preserve (LACL), AK, USA: (a) map of all three sites, (b) Chinitna Bay, (c) Shelter Creek, (d) Silver Salmon Creek. Map created in ArcMap 10.8.1.

2.3 *Fecal Hormone Extraction and Cortisol Metabolite Assay*

Fecal samples were thawed at room temperature for 30 minutes prior to FCM extraction. I weighed 0.5±0.01g of wet feces and placed it in a 15mL centrifuge tube with 5mL of 80% methanol (Palme *et al.,* 2013). I vortexed fecal samples for one minute and then centrifuged samples at 2500g for 15 minutes. Once centrifuged, the supernatant was analyzed immediately in duplicate via a commercially available cortisol enzyme linked immunoassay (ELISA; Cortisol ELISA K003, ArborAssay, Ann Arbor, MI 48108, USA). The upper and lower detection limits of the assay were 45.4 and 27.6 pg/mL respectively. I modified the manufacturer's protocol by extending the time samples were on the plate shaker to an hour and a half to increase the time for FCMs to bind to the ELISA antibodies.

2.4 *Assay Validation*

Fecal extracts were tested for parallelism by diluting high FCM concentration samples from 1:20 to 1:2.5 with assay buffer (Hein *et al.,* 2020). Dilutions were parallel to the standard curve (Test of equal slopes, $p>0.10$), suggesting that no additional substances in the extract were cross-reacting with the antibody.

2.5 *Statistical Methods*

All statistical analyses were conducted in R (version 4.2.2, R Core Team 2022). Alpha was set at 0.05 and all tests were two tailed. I began by testing whether mean FCM concentrations differed between samples collected inside and outside the randomly established sample plots. Because my data did not meet the assumptions for an analysis of variance test (ANOVA; Shapiro-Wilk Test: $W=0.60$, $p<0.05$; Levene's Test: $W=3.28$, $p=0.04$), I subsequently used a non-parametric version of a two-way analysis of variance test. To determine which factors

were associated with variation in FCM concentrations, I used a generalized linear mixed model (GLMM) with month, site, daily visitors (low, medium, high), and diet as fixed effects and plot nested within each site as a random effect.

3. Results

I collected 104 fecal samples from inside the designated sampling plots and 41 fecal samples were collected opportunistically outside of plots. There was no difference in mean FCM concentration between fecal samples collected inside versus outside the sampling plots at each site (Figure 2.3; non-parametric two-way ANOVA: F=0.63, p=0.53). As such, all samples (n=145) were used in the subsequent analyses.

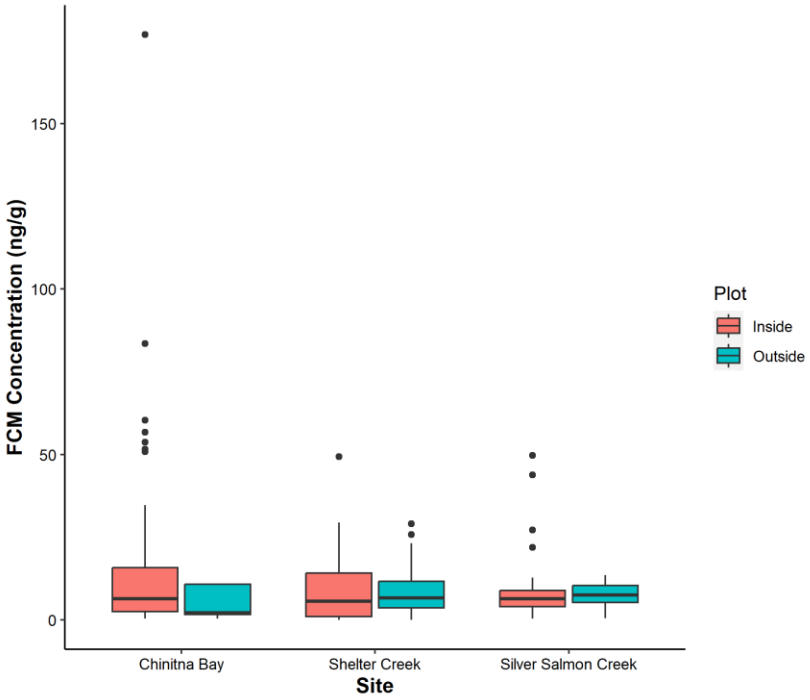


Figure 2.3. Concentration of fecal cortisol metabolites (FCMs) in brown bear (*Ursus arctos*) feces inside randomly assigned plots (n=104) and collected opportunistically outside assigned plots (n=41) across three designated brown bear viewing sites at Lake Clark National Park and Preserve, AK. USA. Samples were collected from June-August, 2022 at Lake Clark National Park and Preserve, AK, USA.

FCM concentrations ranged from <1 ng/g to 176ng/g, with an average FCM concentration of 12.25ng/g. Among sites, mean FCM concentration measured 16.6ng/g at Chinitna Bay, 10.1ng/g at Shelter Creek, and 9.31ng/g at Silver Salmon Creek, although these differences were not significant (Figure 2.4; GLMM: F=1.77, p=0.25). While mean FCMs did not differ among sites, the standard deviation of FCMs at Chinitna Bay (SD=29.4ng/g) was substantially greater compared to the standard deviations in FCMs from Shelter Creek (SD=11.0ng/g) and Silver Salmon Creek (SD=9.84ng/g) respectively (Table 2.2).

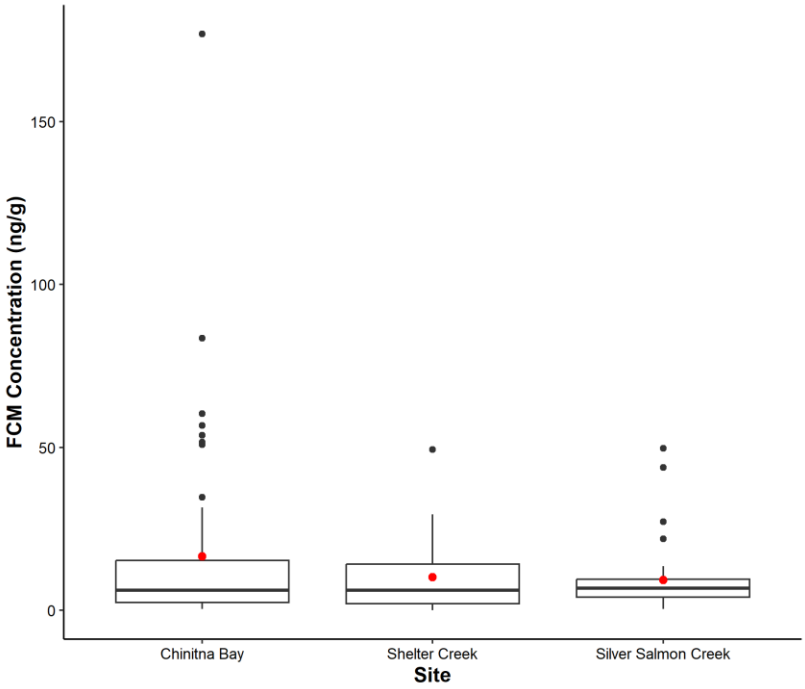


Figure 2.4. Concentrations of fecal cortisol metabolites (FCMs) in brown bear (*Ursus arctos*) feces (n=145) at Lake Clark National Park and Preserve, AK, USA. The red dot represents population-level mean per site: Chinitna Bay (16.6ng/g), Shelter Creek (10.1ng/g), Silver Salmon Creek (9.31ng/g)

Table 2.2. Summary of population-level brown bear (*Ursus arctos*) fecal cortisol metabolite concentrations derived from samples (n=145) collected across three designated brown bear viewing sites from June-August of 2022 at Lake Clark National Park and Preserve, AK, USA.

Site	Population Mean (ng/g)	Standard Deviation (ng/g)	Median (ng/g)

Chinitna Bay	16.6	29.4	6.17
Shelter Creek	10.1	11.0	6.19
Silver Salmon Creek	9.31	9.84	6.79

Month, site, daily visitors, and diet, did not explain a significant amount of variation in FCM concentrations in brown bear feces across the three viewing sites (Table 2.3). My model selection procedure identified four models with a $\Delta AIC<2$, which suggests that month, diet, and site separately may contribute to variation in FCM concentration, though not significantly. However, no models ranked about the null model.

Table 2.3. Generalized linear mixed models used to examine potential drivers of variation of fecal cortisol metabolite (FCM) concentrations (n=145) in brown bears (*Ursus arctos*) in relation to ecotourism at Lake Clark National Park, AK, USA from samples collected June-August 2022.

Model	Intercept	Std. Error	Fixed Effects	P-value	AIC	ΔAIC	R^2c
FCM~1+(1|Plot)	12.96	1.98	-	<0.01*	1278.5	0.00	0.08
FCM~Month+(1|Plot)	20.19	8.96	-	0.02	1279.3	0.77	0.08
			Month	0.19			
FCM~Site+(1|Plot)	16.67	2.81	-	<0.01*	1279.5	0.97	0.05
			Site	0.25			
FCM~Diet+(1|Plot)	13.11	1.99	-	<0.01*	1280.2	1.73	0.08
			Diet	0.60			
FCM~People+(1|Plot)	11.11	3.56		<0.01*	1282.2	3.66	0.07
			People	0.83			
FCM~Month+Diet+People+(1|Plot)	20.71	8.89	-	0.02*	1283.4	4.85	0.05
			Month	0.09			
			Diet	0.54			
			People	0.40			
FCM~Site+Month+People+(1|Plot)	22.46	11.44	-	0.05	1284.9	6.4	0.05
			Site	0.67			
			Month	0.32			
			People	0.72			
FCM~Site+Month+Diet+People+(1|Plot)	22.03	11.42	-	0.10	1285.0	6.4	0.09
			Site	0.63			
			Month	0.33			
			Diet	0.48			
			People	0.76			

Notes: Month was categorized as June, July, or August. Diet was assessed visually and consisted of three categories: vegetation, mixed, and meat. People represented daily counts of visitors that were subsequently categorized as low (0-50 people), medium (51-150 people) and high (150+ people). AIC: Akaike information criterion. R^2c represents the conditional R^2.

4. Discussion

I did not find support for my hypothesis that number of visitors present at bear viewing sites would have a positive correlation with brown bear FCM; however, I observed substantial among-individual variation in FCM concentrations at Chinitna Bay. Specifically, the FCM concentrations of the Chinitna Bay brown bear population had a standard deviation that was roughly three times higher than that of the other sites. A potential explanation for high among-individual variation of FCM concentrations at Chinitna Bay may be a result of different bear viewing practices (open vs closed meadows) and subsequent bear habituation to human activities. For example, in a study on orangutans (*Pongo pygmaeus*), unhabituated individuals exhibited a significant increase in FCM concentration after exposure to human visitors, whereas habituated orangutans did not exhibit a significant difference in FCM concentrations prior to and following human visitation (Muehlenbein *et al*., 2012). Brown bears at Chinitna Bay are exposed to variable human-bear interactions (e.g., visitor to bear proximity). Specifically, at Chinitna Bay when bears cross onto state and native lands that are adjacent to the National Park Service designated bear viewing areas, visitors approach bears within 10-50m, which are similar distances to those I recorded at Silver Salmon Creek, yet Silver Salmon Creek had substantially lower among-individual variation in FCM concentrations. At Chinitna Bay, however, I observed roughly 5% of bears being displaced by visitors. While some bears at Chinitna Bay may not be habituated to human presence, unhabituated individuals also may not be withdrawing from human encroachment, thus manifesting a higher stress response compared to conspecifics, resulting in greater among-individual variation in FCM concentrations at this site. Also, bears at Chinitna Bay may be accepting the tradeoff of greater human-bear interaction and subsequently higher stress for the perceived benefit of greater access to high quality resources. In contrast, at

Shelter Creek, which receives little ecotourism, my field team displaced approximately half of the bears encountered during sampling bouts, with some bears being displaced at distances as far as 200m away from my team. By withdrawing from humans at greater distances, bears at Shelter Creek may not be manifesting a measurable increase in HPA axis activation as indicated by higher concentrations of FCMs. At Silver Salmon Creek I regularly observed visitors within 10-50m from bears but I did not observe displacement of bears due to human encroachment. Perhaps bears at Silver Salmon Creek are habituated to people and as such, the bears that remain at this site and forage in close proximity to humans experience little stress from these human interactions. Simultaneously, bears that are intolerant of humans in close proximity may have already been displaced and are thus not represented in my sample population. Stress response may be exacerbated by habitat differences (i.e., distance from beach to forest cover) as bears on the beach have fewer cover and escape routes compared to when bears are in the meadow, which may result in increased stress for unhabituated individuals. Additionally, Silver Salmon Creek has multiple inholdings with a larger number of permanent structures (e.g., houses and barns) than Chinitna Bay, and thus bears at Silver Salmon Creek may be more accustomed to human presence.

Though diet, season, and the number of visitors did not affect FCM concentrations in brown bears across the three sites in my study; although, previous studies have shown that diet and season can affect FCM concentrations (Pokharel 2019; von der ohe *et al*., 2004). Sergiel *et al*., (2020) found that increased meat in brown bear diet was associated with higher FCM concentrations. Additionally, von der Ohe (2004) found that season and diet interacted to affect FCM concentration in brown bears at Brook River and Margot Creek in Katmai National Park. Notably, while brown bear populations at Brooks River experience high visitor numbers, Brooks

River has elevated platforms bear viewing platforms which may mitigate bear stress response. Although brown bear populations at Brooks River and Margot River congregate in large numbers to acquire salmon resources, both sites are inland sites that may also affect the variation in diet available to bears. The lack of support for diet being associated with FCM concentrations in my study may be due to limited variation in diet observed within and among sites. For example, fecal samples with meat (e.g., clam, fish) accounted for roughly 3.5% of my samples. Additionally, due to logistical issues (e.g., weather), I was unable to sample some of my sites multiple times throughout the season as originally intended. Repeated sampling of all sites throughout the season may provide more insight into how FCM concentrations fluctuate throughout the ecotourism season as bears transition from herbaceous vegetation and berries to salmon-dominated diets (Deacy *et al*., 2017). For example, Chinitna Bay experiences peak human visitation in June, which is when I sampled that site; however, I was unable to return to Chinitna Bay in August when visitation has decreased and diet may include more salmon.

In summary, variation in brown bear FCM concentrations was not explained by daily human visitation, diet, season, or site and the mean FCM concentrations among sites did not differ. However, at Shelter Creek and Silver Salmon Creek, sites with consistent bear viewing practices, brown bear FCM concentrations were less variable among individuals. At Chinitna Bay, where a mix of different bear viewing practices occur dependent on land jurisdiction (i.e., federal, state, native land), brown bears exhibited high variation in FCM concentration. These findings suggest that managers should consider the potential benefits of implementing consistent bear viewing practices across sites, thus providing bears greater predictability in human-bear interactions so that bears can better mitigate stress.

Funding

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SUMMARY AND CONCLUSION

I investigated the relationship between blood cortisol concentrations and fecal cortisol metabolites, as well as whether ecotourism elicited a measurable stress response in free-ranging brown bears. I demonstrated that FCMs provide a potential index of brown bear stress and found that lag time between peak blood cortisol and expression of fecal cortisol metabolites ranged between 10-27h following ACTH challenge. In addition, I found that in free-ranging brown bears at Lake Clark National Park and Preserve, ecotourism did not elicit a measurable stress response; however, I did find high variability in FCM concentration at one of my study sites that has less predictable human-bear interactions.

My research contributes meaningful empirical data in understanding the relationship between blood cortisol and FCM concentrations in brown bears, which is critical for monitoring the long-term stress of free-ranging wildlife. My findings also provide a framework for managers who are facing the challenge of balancing conservation goals with visitor experience. As such, my work will inform managers on the importance of implementing consistent management practices that allow for predictable human-wildlife interactions. Consistent management practices may allow wildlife to better mitigate human-mediated stress by allowing wildlife to dictate where visitors are, rather than visitors dictating where wildlife roam.

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APPENDIX A

IACUC FORM

Physiological response of brown bears to increasing visitation on the Lake Clark Coast: Development of physiological standards

ASAF# 6874 : Research : *Version 1*

Principal Investigator/Responsible Faculty Advisor

Refer to WSU IACUC Policy #22

WSU ID #	LAST NAME	FIRST NAME	E-MAIL	PHONE #
90115869	Robbins	Charles	ctrobbins@wsu.edu	509-335-1119

Area/College/Campus: COL AG HUMAN & NAT RES SCI (A)

Department: SCH OF THE ENVIRONMENT-CAHNRS (D)

Project Title

Physiological response of brown bears to increasing visitation on the Lake Clark Coast: Development of physiological standards

Three-Year Resubmission

Is this a three-year resubmission?

( ) Yes (✓) No

Conflict of Interest

Does the instructor or researcher or any other person responsible for the design, conduct, or reporting of this protocol, have a "significant economic interest" as defined in WSU's Conflict of Interest Policy? (For more information, please see WSU Policy and Procedures for more information)

( ) Yes (✓) No

Investigators Assurance

1. The information contained on this form provides an accurate description of my animal care and use protocol.

   I have read & agree with this statement: (✓) Yes ( ) No

2. All people using animals under my direction will be trained to use appropriate methods, and will have read and agree to comply with this protocol and with all regulations and policies concerning the use of animals, prior to commencing animal work associated with this protocol.

   I have read & agree with this statement: (✓) Yes ( ) No

3. Discomfort of animals will be limited to that which is unavoidable. Analgesic, anesthetic, and tranquilizing drugs will be used where indicated and appropriate to minimize pain and discomfort. Except as specifically described in this protocol, veterinary care will be provided to animals showing evidence of pain and discomfort.

   I have read & agree with this statement: (✓) Yes ( ) No

4. If animal uses or procedures described in this protocol should need to be revised or amended, I will notify the IACUC and gain IACUC approval for the modifications PRIOR to implementation following WSU IACUC Policy #24 . I understand that my failure to report significant changes may place the University and myself in violation of regulations and may result in suspension of my animal activities.

   I have read & agree with this statement: (✓) Yes ( ) No

5. I will notify the IACUC regarding ANY unexpected results that adversely impact the animals in this protocol. Any unanticipated pain or distress, morbidity or mortality will be reported to the Attending Veterinarian (or designee) and the IACUC. Guidelines for Reporting Adverse Events or Unanticipated Outcomes

   I have read & agree with this statement: (✓) Yes ( ) No

6. I understand that the approval of this ASAF in no way obligates the IACUC or the University to guarantee animal housing space, animals and/or equipment used to conduct the project.

   I have read & agree with this statement: (✓) Yes ( ) No

7. As required by regulations, I assure that the activities described herein do not unnecessarily duplicate previous procedures/projects.

   I have read & agree with this statement: (✓) Yes ( ) No

8. If the protocol is funded by a grant, I assure all procedures described in this protocol are covered by the grant.

   I have read & agree with this statement: (✓) Yes ( ) No

Is this project supported by extramural peer review funding? (✓) Yes ( ) No

ORSO # Pending

Congruency Review Notes

I have read the WSU IACUC Policy #27 and understand the need to maintain compliance in all animal care and use protocols (✓) Yes ( ) No

General Comments

The overarching goal of this project is to calibrate two enzyme immunoassays (EIA) for future use in the field that will allow us to non-invasively quantify the hormonal response of brown bears to psychological stress and nutritional stress, two factors that impact the health of brown bears.

Personnel

ALL individuals involved with animal contact MUST be included except students enrolled in courses associated with teaching protocols.
If personnel are not enrolled as a student or employed by WSU, they must be identified under Non-WSU Personnel.

1/8

Refer to WSU IACUC Policy #20

Will the study require the use of a Vet Pool ?

(✓) Yes ( ) No

Select your Vet Pool
- [x] OCV Vet Staff Pool
- [ ] VTH Vet Pool
- [ ] Vet Tech Pool
- [ ] USDA-ARS Vet Pool

Will the study require the use of approved personnel from a Personnel Protocol?

( ) Yes (✓) No

Personnel

Name	Role	Training Needed
Lafferty, Diana	Co-Investigator (Non-WSU)	

Email: <dlaffert@nmu.edu>

Institution: Northern Michigan University

Education Background (ex. Degrees, licenses, certifications)

BS, MS, PhD

PROCEDURES

Observation (research related) Injections Sample Collection Work Under Supervision

PROCEDURE SPECIFIC TRAINING

4 yrs field experience with brown bears in Alaska

Jansen, Heiko Co-Investigator

Email: heiko@wsu.edu

Education Background (ex. Degrees, licenses, certifications)

MS, PhD

PROCEDURES

Observation (research related) Injections Sample Collection Supervise/Train Personnel

PROCEDURE SPECIFIC TRAINING

30+ years experience with small and large animal experimentation (11 with bears) including, handling, surgery, blood and tissue sampling.

Evans Hutzenbiler, Brandon Other

Email: brandon.hutzenbiler@wsu.edu

Education Background (ex. Degrees, licenses, certifications)

BS

PROCEDURES

Observation (research related) Husbandry Injections Sample Collection

Work Under Supervision

PROCEDURE SPECIFIC TRAINING

Wildlife experience, current WSU Bear center manager. Brandon has been working closely with the bears for 5 years now. This includes drugging, feeding, general care and assistance with all procedures, veterinary health checks and research-related protocols. Brandon also performs daily enrichment duties during the active season. Brandon is performing behavioral training to enable blood sampling of our wild bears at the Center.

Robbins, Charles PI

Email: ctrobbins@wsu.edu

Education Background (ex. Degrees, licenses, certifications)

BS, MS, PhD

PROCEDURES

Observation (research related) Husbandry Injections Sample Collection

Supervise/Train Personnel

PROCEDURE SPECIFIC TRAINING

35 yrs working with captive and wild bears

Justification for Animal Use (in non-scientific terms)

Use non-scientific language to explain the goals and importance to human or animal health, the advancement of knowledge, or the good of society.

While many ecologists quantify stress hormone metabolite concentrations in the feces of diverse captive and free-ranging wildlife to infer relative stress levels within and/or across populations, an essential step in this process is to validate the assays used to measure hormone metabolite concentrations in feces relative to hormone concentrations in the blood because hormone concentrations in the blood are what impacts/represents an animal's physiological state. However, if we understand the quantitative link between fecal hormone metabolite concentrations and hormone concentrations in the blood, the concentration of fecal hormone metabolite concentrations can then serve as a powerful index for deducing the physiological state of the target animal. As such, completion of this project will serve to validate multiple EIA by quantifying the lag time between hormone secretions detected in the blood and excretion of the hormone metabolites in fecal samples. Further by conducting two hormone challenges (ACTH, TSH), I will provide data for other ecologists to use that are essential for teasing apart the interplay between psychological stress and nutritional stress. The ACTH challenge will allow me to validate assays to measure corticosterone responses by brown bears, which will give me the ability to measure their psychological response to disturbance stress, while the TSH challenge will allow me to validate assays for T3 and T4, which play important roles in regulating metabolism, blood pressure, body temperature and nutrition physiology; T3 and T4 are unresponsive to psychological stress. Validating assays for both corticosterone and T3/T4 measures will allow me to tease apart human-mediated disturbance stress versus nutritional stress, allowing me to better understand how these hormones collectively reflect biological function.

Why must live vertebrate animals be used rather than cell cultures, computer models, invertebrate animals, microbes, etc?

Project is specific to bears.

What characteristics of the animal species to be used justify their selection?

Brown bears are one of the most admired, enigmatic, and ecologically influential terrestrial mammals in North America. High-density brown bear populations such as those along the Lake Clark coast in Alaska draw huge crowds of humans each year for bear viewing. In fact, since 2007 human visitation at Lake Clark National Park and Preserve (LCNPP) bear viewing platforms has increased 5-fold. Managers at LCNPP are concerned that increased human presence is driving a stress response in brown bears at the designated bear view sites (e.g., increased vigilance, displacement) and managers would like to be able to measure and monitor changes in brown bear stress over time to develop management plans to minimize the negative impact of human visitation on this ecologically and economically important species. Additionally, brown bear feces can be safely and non-invasively collected. However, before I can non-invasively sample brown bears at LCNPP, I must first validate the hormonal assays needed to measure the physiological responses of brown bears to human presence. Further, because brown bear viewing occurs in areas with abundant food resources (e.g., salmon, saltmarsh meadows), I must be able to tease apart psychological stress from nutritional stress.

Please provide a statement to address the potential harm to the animals on this study (e.g Pain/Distress, Morbidity, Mortality) relative to the benefits to be gained by this study.

While negative effects of ACTH challenges have not been reported, animals injected with TSH, which can elicit a "hunger" response, could lead to increased aggression (Mondal et al. 2020). Thus, animals subject to the TSH challenge will be house individually for the duration of the experiment.

Species Table

If you're using client-owned animals, refer to WSU IACUC Policy #7 .
For a list of approved vendors, please refer to WSU IACUC Policy #14 or contact OCV.
For distress categories, refer to WSU Guidelines for pain and distress categories .
For guidelines on acquisition of animals, please refer to WSU IACUC Policy #5

Common Name	Genus	Species
Bears - Grizzly Bear	Ursus	arctos

Source	Distress Category	# Requested for 3 Years
WSU Bear Center	C	11

Duplication of Research

Does this work duplicate prior research?

( ) Yes (✓) No

Animal Justification

Please refer to the Guidelines for Rationale for Animal Numbers in Protocols

Has the possibility to reduce animal numbers been considered in this study?

(✓) Yes ( ) No

Justify the maximum total number of animals to be used in this protocol over the **three year period** . Please indicate if you have a preference for a particular gender and justify why that gender must be used. Provide statistical sample size justification for group size in this experiment. If no statistical method was used to determine sample size, please explain the criteria used to determine the number of animals required for this study.

Please provide power calculation details (variance measure, desired power, minimum detectable difference) used to determine group size, number of experimental groups in the protocol. Please refer to sample size determination document.
I propose to sample 11 brown bears at The Bear Center at Washington State University (4 adult males, 7 adult females). Specifically 2 males and 3 females will be subjected to the ACTH challenge, and 2 males and 4 females will be subjected to the TSH challenge. While a larger sample size would increase the statistical power, because this study is focused on assay validation additional animals are not necessary for assay calibration. Further, the proposed sample size is aligned with assay validation studies for other species (e.g., 6 Asian elephants, 2 Indian rhinos [Menargues and Mauri 2008] 6 western lowland gorillas [Shutt et al. 2012], 4 tigers [Mondal et al. 2020]). Given the small sample of animals used in this proposed study, analyses of data will be limited to an examination of trends in graphical presentations and calculating descriptive statistics.

Research Protocol

Please provide an abstract or a brief overall description of this research in layman terms:
While many ecologists quantify stress hormone metabolite concentrations in the feces of diverse captive and free-ranging wildlife to infer relative stress levels within and/or across populations, an essential step in this process is to validate the assays used to measure hormone metabolite concentrations in feces relative to hormone concentrations in the blood because hormone concentrations in the blood are what impacts/represents an animal's physiological state. However, if we understand the quantitative link between fecal hormone metabolite concentrations and hormone concentrations in the blood, the concentration of fecal hormone metabolite concentrations can then serve as a powerful index for deducing the physiological state of the target animal. As such, completion of this project will serve to validate multiple EIA by quantifying the lag time between hormone secretions detected in the blood and excretion of the hormone metabolites in fecal samples. Further by conducting two hormone challenges (ACTH, TSH), I will provide data for other ecologists to use that are essential for teasing apart the interplay between psychological stress and nutritional stress. The ACTH challenge will allow me to validate assays to measure corticosterone responses by brown bears, which will give me the

ability to measure their psychological response to disturbance stress, while the TSH challenge will allow me to validate assays for T3 and T4, which play important roles in regulating metabolism, blood pressure, body temperature and nutrition physiology; T3 and T4 are unresponsive to psychological stress. Validating assays for both corticosterone and T3/T4 measures will allow me to tease apart human-mediated disturbance stress versus nutritional stress, allowing me to better understand how these hormones collectively reflect biological function.

Thoroughly describe all animal use procedures/treatments in chronological order. This includes frequency and duration of each procedure/treatment to be performed on individual animals. :

Pre-hormone challenge observations, blood draws, and fecal collections: To establish baseline blood cortisol and T3/T4, and fecal cortisol and T3/T4 metabolite concentrations, two days prior to administering the ACTH and TSH hormone challenge, all 11 animals will be video recorded 24 hrs/day, a 10 ml blood sample will be collected from the dorsal metatarsal or lateral saphenous vein into a serum tube (10:00 – 11:00 am). Fecal samples will be collected individually within six hours of defecation, labeled (animal ID, date, time of defecation, time of collection) and frozen. All animal will be continuously video recorded/monitored.

Hormone challenges observations, blood draws, and fecal collections: On day three, immediately before ACTH or T3/T4 administration, blood will be collected from the dorsal metatarsal or lateral saphenous vein into a serum tube for each animal (10:00 – 11:00 am). Immediately following the blood draw, 3 females and 2 males will be injected with ACTH and the other 4 females and 2 males will be injected with TSH. Animals injected with TSH will be housed individually because TSH could lead to increased aggression (Mondal et al. 2020). Fecal samples will continue to be collected within 6 hours of defecation, labeled (animal ID, date, time of defecation, time of collection) and frozen. All animal will be continuously video recorded/monitored.

Post-hormone challenge observations, blood draws, and fecal collections: Post-hormone injection, blood will be collected from the dorsal metatarsal or lateral saphenous vein into a serum tube for each animal at 2 hrs., 6 hrs., 12 hrs., 24 hrs., 48 hrs., 72 hrs., 96 hrs., and at 120 hrs. Fecal samples will continue to be collected within 6 hrs. of defecation for 5 days post-hormone injection, labeled (animal ID, date, time of defecation, time of collection) and frozen. All animals will be continuously video recorded/monitored.

Please describe any expected adverse animal welfare condition (e.g Pain/Distress, Morbidity, Mortality) that may result in using animals on this protocol. Please review the Adverse Events Guidelines for more information. Examples of possible adverse events during research may include instrument or catheter failure, anesthetic complications, congenital abnormalities, etc.

While negative effects of ACTH challenges have not been reported, animals injected with TSH, which can elicit a "hunger" response, could lead to increased aggression (Mondal et al. 2020). Thus, animals subject to the TSH challenge will be housed individually for the duration of the experiment.

Method of Individual Animal Identification

Refer to WSU IACUC SOP #7

- [x] Cage/Pen/Tank/Stall Card
- [ ] Leg Band
- [x] Microchip (Refer to WSU IACUC SOP #7)

Microchip location

Shoulder

- [ ] Ear Notching
- [ ] Ear Tag
- [ ] Ear Punch
- [ ] Freeze Branding
- [ ] Halter/Collar
- [ ] Non-Invasive External Marking
- [ ] Tatooing
- [ ] Toe Clipping
- [x] Other

Please Describe:

Individual recognition by physical characteristics as described and illustrated in the photo album that will be available to OCV and IACUC personnel at all times and during semi-annual visits. Two bears (Cooke and Oakley) arrived at WSU with permanent tattoos applied by wildlife management agencies. All WSU bears including Cooke and Oakley have been microchipped as they were anesthetized for other procedures. Bears that might be difficult for OCV, IACUC, or Bear Program personnel to visually identify will have small patches of hair clipped in distinctive ways that will permit individual identification.

Behavioral and Neuroscience Studies

Does this protocol involve behavioral or neuroscience studies?

- ( ) Yes
- (x) No

Controlled Exercise Regimes

Does this protocol involve animals undergoing controlled exercise? (i.e. treadmills, forceplates, mazes, etc. are used) Note: The USDA considers forced exercise to be potentially painful and/or distressful.

- ( ) Yes
- (x) No

Use/Handling of Wild-caught/Non-domesticated Species

Will this protocol include contact with wild or non-domesticated animal species?

- (x) Yes
- ( ) No

If you will be using/handling these types of animals, please complete this section. If you are planning on working with certain species of wild rodents, you and your personnel must be knowledgeable of the zoonotic risks associated with Hantavirus. If more than one wild-caught/non-domesticated species is involved in this project/class, please address each part of this section **FOR EACH SPECIES**.

What zoonotic diseases and infectious agents could the species potentially carry? If you need assistance determining the potential zoonotic/infectious agent carried by a particular species, contact the Office of the Campus Veternarian at (509) 335-6246.

Bears may be carriers of a number of parasitic and bacterial agents (cryptosporidium, Leptospira, Echinococcus, visceral larval migrans from nematodes (Baylisascaris), Campylobacter and Salmonella) that could be transmissible to humans. In addition, the bear diet may include raw meats and carcass parts that may be contaminated with bacteria such as pathogenic E. coli. These are primarily fecal/urine contaminates, thus standard hand hygiene and food sanitation procedures will be followed.

What measures will be used to prevent transmission of potential infectious agents to personnel, other animals and the environment?

Coveralls or scrubs and rubber boots will be worn by anyone handling bears. Bear center laundry is conducted on campus. Disposable gloves are strongly encouraged but not always practical. All personnel even those not touching bears may come in contact with contaminated surfaces and are required to wash hands before leaving and before eating.

What potential physical hazards (i.e. bites, scratches, attacks, kicks, etc.) may be encountered in working/handling this species? Describe how personnel will be protected and risk(s) minimized.

Animals are trained by positive reinforcement to move from place to place in the facility. People are always behind protective fencing- only trained personnel are controlling gates and animal movement. Personnel do interact directly with young bears when hand-raising cubs but direct contact without protective fencing is stopped when the bear reaches the age of 2 years.

Trapping

Will this protocol include trapping of any animals?

○ Yes ☑ No

Special Diets

WSU IACUC Policy #35: Food, Fluid Restriction and Diet Manipulation

Will this protocol involve the use of non-standard or experimental or special diets?

○ Yes ☑ No

Food and/or Fluid Regulation

WSU IACUC Policy #35: Food, Fluid Restriction and Diet Manipulation (Excluding routine pre-surgical fasting up to 12 hours)

Will this protocol involve food and/or fluid restrictions or regulation?

○ Yes ☑ No

Restraint

Will this protocol include prolonged (>15 minutes) restraint of animals? This includes undersized caging, animals "tethered" with leash or catheter, animals held in stocks/restraint device etc.

○ Yes ☑ No

Imaging Procedures (use this section for radiographs, ultrasounds, CT, PET, MRI, endoscopy, fluoroscopy, etc.)

Will animals in this protocol undergo imaging procedures?

○ Yes ☑ No

Implants and Catheters

Will this protocol involve implanted catheters, cannula, or prosthetics?

○ Yes ☑ No

Blood Sampling

Refer to WSU Guidelines for Blood Collection

Will this protocol involve blood sampling in a live animal?

☑ Yes ○ No

Common Name	Vol/kg or % of blood volume	Route	Frequency
Bears - Grizzly Bear	10 mls	venous	Total of 10 samples in 8 days.

Please describe the method of collection:

10 mls of blood will be collected from the dorsal metatarsal or lateral saphenous vein into a serum tube for each animal at 0, 2, 6, 12, 24, 48, 72, 96, and at 120 hrs following ACTH or T3/T4 injections.

Over the period of two weeks, is the volume of blood to be collected greater than 10% of total blood volume?

○ Yes ☑ No

Genotyping

Will genotyping of the animals be performed? ○ Yes ☑ No

Other Tissue and/or Fluid Sampling from Live Animals

Please list all other samples below in the Tissue/Fluid sampling section

For multiple collection from the same individual live animal, please reference WSU IACUC Policy #10 . Note: This is only for non-blood sampling.

Will this protocol involve tissue and/or fluid sampling in live animals?

☑ Yes ○ No

Common Name	Tissue or Fluid Type	Amount/Volume	Method	Frequency
Bears - Grizzly Bear	Feces	Total defecation	Plastic bag	Multiple times/day

Will anesthesia be utilized?

○ Yes ☑ No

Method of Animal Disposition

Please refer to WSU IACUC Policy #5 and WSU IACUC Policy #28

- ☐ Released into the wild in accordance with the applicable permit and regulationss
- ☐ Euthanasia
- ☐ Slaughter at licensed/approved slaughter facility
- ☐ Sold at public auction
- ☐ Transfer to private individual
- ☐ Transfer to other institution
- ☑ Transfer to another approved ASAF
- ☐ Ownership retained by private owner (Please refer to WSU IACUC Policy 7)
- ☐ Other

Drugs and Chemicals

List all substances regardless the route of administration (topical, ocular, oral, aural, intraperitoneal, intravenous, intradermal, subcutaneous, intratracheal, intranasal, etc..). The substance should be listed no matter the route of administration (auricular, buccal, conjunctival, cutaneous, injected, oral, injection (intramuscular, intraperitoneal, intravenous, intradermal), applied to feed, auricular, buccal, instilled in nose or ear, etc.). All substances used in compounding/dilution of drugs and chemicals (e.g. saline, sterile water, etc.) should be included. Please refer to WSU IACUC Policy #29 for non-pharmaceutical grade drugs, WSU IACUC Policy #32 for the use of tribromoethanol (Avertin), and the WSU Guidelines for Drug and Chemical Administration.

Will animals in this protocol be administered or exposed to or treated with drugs and chemicals? Note: if you have indicated Anesthesia including local Anesthesia, it must be listed here. The use of expired drugs is not acceptable (see WSU IACUC Policy 11)

(✓) Yes ( ) No

Common Name	Drug/Compound Administered	Dose Range (mg/kg)	Route(s)	Frequency
Bears - Grizzly Bear	ACTH	4 mg/kg	im	No more than 4 times/mo

Purpose/Procedure:

Brown bears are one of the most admired, enigmatic, and ecologically influential terrestrial mammals in North America. High-density brown bear populations such as those along the Lake Clark coast in Alaska draw huge crowds of humans each year for bear viewing. In fact, since 2007 human visitation at Lake Clark National Park and Preserve (LCNPP) bear viewing platforms has increased 5-fold. Managers at LCNPP are concerned that increased human presence is driving a stress response in brown bears at the designated bear view sites (e.g., increased vigilance, displacement) and managers would like to be able to measure and monitor changes in brown bear stress over time to develop management plans to minimize the negative impact of human visitation on this ecologically and economically important species. Additionally, brown bear feces can be safely and non-invasively collected. However, before I can non-invasively sample brown bears at LCNPP, I must first validate the hormonal assays needed to measure the physiological responses of brown bears to human presence. Further, because brown bear viewing occurs in areas with abundant food resources (e.g., salmon, saltmarsh meadows), I must be able to tease apart psychological stress from nutritional stress.

Is the drug/compound pharmaceutical grade?

(✓) Yes ( ) No

Is the drug/compound particularly hazardous as defined by OSHA/EPA? Consult IBC for more details.

( ) Yes (✓) No

Common Name	Drug/Compound Administered	Dose Range (mg/kg)	Route(s)	Frequency
Bears - Grizzly Bear	TSH	1.5 mg	im	No more than 3 times/mo

Purpose/Procedure:

Brown bears are one of the most admired, enigmatic, and ecologically influential terrestrial mammals in North America. High-density brown bear populations such as those along the Lake Clark coast in Alaska draw huge crowds of humans each year for bear viewing. In fact, since 2007 human visitation at Lake Clark National Park and Preserve (LCNPP) bear viewing platforms has increased 5-fold. Managers at LCNPP are concerned that increased human presence is driving a stress response in brown bears at the designated bear view sites (e.g., increased vigilance, displacement) and managers would like to be able to measure and monitor changes in brown bear stress over time to develop management plans to minimize the negative impact of human visitation on this ecologically and economically important species. Additionally, brown bear feces can be safely and non-invasively collected. However, before I can non-invasively sample brown bears at LCNPP, I must first validate the hormonal assays needed to measure the physiological responses of brown bears to human presence. Further, because brown bear viewing occurs in areas with abundant food resources (e.g., salmon, saltmarsh meadows), I must be able to tease apart psychological stress from nutritional stress.

Is the drug/compound pharmaceutical grade?

(✓) Yes ( ) No

Is the drug/compound particularly hazardous as defined by OSHA/EPA? Consult IBC for more details.

( ) Yes (✓) No

Will this protocol involve administration of novel substances? Please refer to WSU IACUC Guidelines for using novel substances.

( ) Yes (✓) No

Please describe any expected adverse animal welfare condition (e.g Pain/Distress, Morbidity, Mortality) in using drugs and chemicals in this protocol. Please review the Adverse Events Guidelines for more information. Examples of possible adverse events may include congenital abnormalities, birthing complications, offspring rearing difficulties, etc.

While negative effects of ACTH challenges have not been reported, animals injected with TSH, which can elicit a "hunger" response, could lead to increased aggression (Mondal et al. 2020). Thus, animals subject to the TSH challenge will be housed individually for the duration of the experiment.

Please describe how pain, distress, adverse events, or animal welfare issues associated with this species/line will be monitored and addressed? Include clinical signs, frequency of monitoring (after hours/weekends/holidays), veterinary care intervention and treatments, and humane endpoints. (Note: All such animals should be included under "D" or "E" class).

All animals will be visually monitored continuously during the 12 hrs after each hormone challenge. We do not know of any adverse response that will be expected nor do we know of any intervention that will be needed.

Surgery

Please refer to WSU IACUC Policy 6 for all surgery requirements

Will this protocol involve animal surgery?

( ) Yes (✓) No

Biological Agents

Biological Agents are potentially biohazardous for humans, animals, or plants (including pathogens/infectious material and those with environmental or agricultural impacts); Recombinant or Synthetic Nucleic Acids (r/sNA); Genetically Modified Organisms (GMO); Biological Select Agents and Toxins (BSAT, as defined by the Federal Select Agent Program); human and primate blood, blood products, body fluids, cell lines, cells and tissues; and/or agents/materials that require federal permits. For more information about the oversight of potentially biohazardous agents by the Institutional

Biosafety Committee (IBC), please visit http://www.biosafety.wsu.edu/project.asp

Will animals in this protocol be subjected to treatment with biological agents that are considered potentially hazardous to humans, animals, or the environment as defined by WSU IBC?

○ Yes ✓ No

Genetically Altered Animals

The use of or creation of genetically altered animals fall under the NIH guidelines and will need WSU IACUC approval. This includes animals subject to recombinant or synthetic nucleic acid molecules, either heritable or inheritable.

Will animals in this protocol be subjected to treatment with r/sNA molecules that may or may not result in heritable changes (i.e. the use of vectors to modify gene expression siRNA, humanized mice) or/ and will you use a genetically altered animal or have one created for you?

○ Yes ✓ No

Radioactive Materials

Will this protocol involve the use of radionuclides or radiation on animals?

○ Yes ✓ No

Tumor Production (Not for Antibodies)

Refer to WSU IACUC Policy #8 and WSU IACUC Tumor Burden Guidelines

Will this protocol involve tumor production or transplantation?

○ Yes ✓ No

Antibody Production

Please reference WSU IACUC SOP 1 and WSU IACUC Policy #23

Will this protocol involve antibody production? (check all that apply)

☑ No
☐ Yes - At Non-WSU source
☐ Yes - At WSU

Transportation of Animals

Please reference WSU IACUC Policy #33

Will animals be moved outside of WSU building or off WSU property?

○ Yes ✓ No

Animal Housing

All animal housing locations MUST be approved by the IACUC prior to acquiring animals.
If your facility is not listed, please contact or.ora.iacuc@wsu.edu or call 509-335-7951.

Is this a field study? (A study conducted on free-living wild animals in their natural habitat.)

○ Yes ✓ No

Overnight Housing (holding animals > 12 hours)

Will animals be housed at WSU centrally-managed facilities? (for Pullman and Spokane Only)

✓ Yes ○ No

Centrally-Managed Facility Details:

- Pullman - Bear Center

Will animals be housed outside of a centrally-managed facility? Unapproved locations will need to be approved by IACUC prior to the protocol being approved.

○ Yes ✓ No

Are there any housing restrictions or special housing requirements (e.g. cage size exemptions, special bedding, change to cage cleaning intervals, special lighting)?

○ Yes ✓ No

Day Use/Study Area Locations (animals present <12 hours)

Will animals be taken to a laboratory/study/teaching area outside the animal housing facility OR involve the use of private animals at a private non-WSU location? (Locations such as euthanasia, behavioral testing, private farm, private vet clinics)

○ Yes ✓ No

Environmental Enrichment/Behavioral Management

Environmental Enrichment and behavior management are a part of the husbandry and care of teaching animals. Please refer to WSU IACUC Policy #30 for a description of the mandated enrichment program.

Are there any restrictions or changes on Environmental Enrichment?

○ Yes ✓ No

Will there be individual housing of a social species?

✓ Yes ○ No

Please explain and justify why this is necessary. Include other enrichment or behavioral management enhancements for the well-being of the animals.

Animals in the T

Veterinary Care

As per federal regulations and WSU policy, the WSU Office of the Campus Veterinarian **MUST be notified** of all abnormal animals - including emergencies, injuries, illness, and all adverse events that affect animal health and well-being. For more details, see WSU IACUC Policy #3

Provider of medical care to your animals: (emergencies, illness, preventive medicine)
- [x] Office of the Campus Veterinarian
- [ ] Veterinary Teaching Hospital
- [ ] Other

Attachments

1: SOP	
2: Client Consent Form	
3: Permit(s)	
4: Approved Protocols from Other Institutions	Diana Lafferty NMU ACTH_TSH Protocol 12_28_2020.docx
5: Syllabus/Training Material	
6: PI-maintained Housing (SAHL)	
7: Other	

;

WASHINGTON STATE UNIVERSITY

Office of
Research Assurances

4/12/2021

Dr. Charles Robbins
School of the Environment
Washington State University
Pullman, WA 99164-4236

Subject: Verification of Approval Letter

Dear Dr. Robbins,

Your protocol ASAF #6874, title "Physiological response of brown bears to increasing visitation on the Lake Clark Coast: Development of physiological standards", was approved by the IACUC on 1/29/2021 and is valid until 1/29/2024 with the submission of annual renewals. An approval by the IACUC means that the use of animals in your project has been approved.

Washington State University is an AAALAC accredited institution that operates its Animal Care and Use program under the Animal Welfare Assurance A3485-01 on file with the Office of Laboratory Animal Welfare (OLAW). The USDA certificate number is 91-R-0002.

If there are any questions regarding the approval status of this project, please do not hesitate to contact me.

Thank you,

Alan Ekstrand
Assistant Director- Animal Welfare Program
509-335-7951

PO Box 643143, Pullman, WA 99164-3143
509-335-7183 | Fax: 509-335-6410

APPENDIX B

IACUC EXEMPTION FORM

Exemption Request

Institutional Animal Care and Use Committee

NORTHERN MICHIGAN UNIVERSITY

Instructions: Use this form for projects involving vertebrate animals that are exempt from IACUC review. The animal uses must fall entirely within one of the categories listed in Part III. Include a brief description of the proposed animal use and explain why it should be exempt from IACUC review. Send this request electronically to IACUC@nmu.edu and IACUCChr@nmu.edu to ensure a prompt review. If the exemption is approved, the proposed animal use may commence once signatures from the principal investigator and the IACUC Chair have been obtained via RightSignature. Please contact the IACUC Chair (email: IACUCChr@nmu.edu) if you have any questions.

I. **Principal Investigator** (Must be a faculty member or Department Head): Diana Lafferty
Department: Biology

Phone number: 906-227-2227

Date: 2/10/22

II. **Project/Grant/Course Number and Title** (If you will be using external funds, please use the same title as the grant application; if work is for a course, please include the number of the course, title of the course, and a title for the work proposed): Tourism-induced psychological and nutritional stress modulate brown bear gut microbiomes

Funding Sources (External & Internal, if applicable): National Park Service

III. Exemption

The use of vertebrate animals involved in this project may only be exempt from IACUC review if it falls entirely in one or more of the categories below (check the box next to the appropriate category).

- ☐ Whole dead animals not regulated by the USDA [e.g. cold-blooded vertebrates, birds, rats (Rattus only), and mice (Mus only)].
- ☒ Non-intrusive field research (observation only, no significant manipulation of the animal or its environment).
- ☐ Faculty approved internship or field practicum in which animals are owned or under the legal responsibility of a non-NMU entity (e.g., institution, business).
- ☐ Demonstration, or similar short-term activity, conducted on NMU property involving animals that are not owned by or under the legal responsibility of NMU.

IV. Brief description of the project, and explanation of how the animal use qualifies for exemption from IACUC review: To investigate how variable human presence at bear viewing locations in Lake Clark National Park in Alaska impact the "gut brain axis" of brown bears, I will travel to Lake Clark beginning May 2022 until August of 2022 and collect bear feces at three established bear viewing locations. At each bear viewing location, I will sample bear feces from 10 randomly assigned 50x20

Revised August 2017

meter plots throughout the summer. I will clear all feces from each plot at the viewing location on day 1 and spend the following three days collecting feces from each plot. I will rotate among sites every 4-5 days throughout the summer. Upon completion of field work, samples will be transported back to NMU for laboratory analyses of fecal hormone levels and fecal microbiome diversity assessments. Because my research is noninvasive and will not disturb the anonymous fecal donors, my research merits exemption from IACUC review.

Signature: [redacted] 03/16/2022

Principle Investigator Date

V. Institutional Animal Care and Use Committee Approval

Based on the selected use categories, description and explanation of the proposed procedures, the IACUC Chair, on behalf of the IACUC, approves this request for exemption from IACUC review.

Signature: [redacted] 03/16/2022

Institutional Animal Care and Use Committee Chair Date

Revised August 2017

citrix | RightSignature

REFERENCE NUMBER
DA6C7984-2DFF-406B-A70F-277310F0C486

SIGNATURE CERTIFICATE

TRANSACTION DETAILS	DOCUMENT DETAILS
Reference Number DA6C7984-2DFF-406B-A70F-277310F0C486	**Document Name** Lafferty Iacuc Exempt on F na
Transaction Type S gnature Request	**Filename** afferty_ acuc_exmept on_f na .docx
Sent At 03/16/2022 10:52 EDT	**Pages** 2 pages
Executed At 03/16/2022 11:00 EDT	**Content Type** app cat on/vnd.openxm formats-off cedocument.wordprocess ngm .document
Identity Method ema	**File Size** 244 KB
Distribution Method ema	**Original Checksum** d8d377767cf368c2b9a46c5160ff90cbb3e7ee2a95c6c706871fb03a7c545d2e
Signed Checksum f68af76119789e8eda1885fd51b93e2b79f8fe8c613555f7c60f9d46bd3a5452	
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SIGNERS

SIGNER	E-SIGNATURE	EVENTS
Name John Brugg nk	**Status** s gned	**Viewed At** 03/16/2022 10:59 EDT
Email jbrugg n@nmu.edu	**Multi-factor Digital Fingerprint Checksum** d9f6b13b8b271c1d732a9153121d694a2da1fcff615cb61295e6affeb816db2d	**Identity Authenticated At** 03/16/2022 11:00 EDT
Signer Sequence 1	**IP Address** 35.24.3.167	**Signed At** 03/16/2022 11:00 EDT
Components 2	**Device** Chrome v a W ndows	
	Drawn Signature 	
	Signature Reference ID C964019E	
	Signature Biometric Count 229	
Name D ana Lafferty	**Status** s gned	**Viewed At** 03/16/2022 10:58 EDT
Email d affert@nmu.edu	**Multi-factor Digital Fingerprint Checksum** 6635a935c942fd607359070dcef3c6328706ffadcab00d123f619e3e181df513	**Identity Authenticated At** 03/16/2022 10:59 EDT
Signer Sequence 0	**IP Address** 35.24.94.71	**Signed At** 03/16/2022 10:59 EDT
Components 2	**Device** Chrome v a W ndows	
	Drawn Signature 	
	Signature Reference ID 0868D394	
	Signature Biometric Count 730	

AUDITS

TIMESTAMP	AUDIT
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03/16/2022 11:00 EDT	John Brugg nk (jbrugg n@nmu.edu) s gned the document on Chrome v a W ndows from 35.24.3.167.

APPENDIX C

R SCRIPT FOR CHAPTER ONE ANALYSIS

```
library(ggplot2)
library(dplyr)
library(magrittr)
library(tidyr)
library(tidyverse)
library(rstatix)
library(lme4)
library(lmerTest)
library(titanic)
library(MuMIn)
library(ART)
library(ARTool)
library(sjPlot)
library(ggforce)

#Set working directory

setwd("/Users/jpinero/Desktop/Thesis/Chapter 2")


#Chapter 1: Plasma Cortisol and Fecal Cortisol Metabolite Concentrations Following an ACTH Challenge in Unanesthetized Brown Bears (Ursus arctos).

Download CSV files

Bears<-read.csv("ThesisData.csv")
Blood<-read.csv("BloodAssay.csv")
BloodFigures<-read.csv("BloodFigures.csv")
DailyBear<-read.csv("MeanFCMDaily.csv")
Anova<-read.csv("FecalAnova.csv")
DailySA<-read.csv("DailyFCMSexAge.csv")

# Chapter 1 Figures

#Time course of serum cortisol concentrations
```

```
ggplot(BloodFigures)+
  geom_point(aes(Time,Mean),color="black",size=2,)+
  geom_errorbar(aes(x=Time,ymin=Mean-
SEM,ymax=Mean+SEM),width=1,color="black",alpha=0.9,size=0.7)+
  geom_line(aes(Time,Mean),color="black")+
  geom_hline(yintercept=24.96, linetype='dotted')+
  scale_x_continuous(name="Time From Injection (hr)",breaks=seq(0,72,6), limits=c(0, 72))+
  ylim(0,250)+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
        axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  ylab("Mean cortisol concentration (ng/mL)")+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
        axis.text.y = element_text(color="black"))

#Mean serum cortisol concentration by sex**

colors1<-c("Males" = "deepskyblue3","Females" = "darkorange2")

ggplot(BloodFigures)+
  geom_point(aes(Time,Male_Mean,color="Males"),size=2)+
  geom_point(aes(Time,Female_Mean,color="Females"),size=2)+
  geom_line(aes(Time,Male_Mean,color="Males"))+
  geom_line(aes(Time,Female_Mean,color="Females"))+
  geom_hline(yintercept=24.96, linetype='dotted')+
  geom_errorbar(aes(x=Time,ymin=Male_Mean-
SEM,ymax=Male_Mean+SEM,color="Males"),width=1,alpha=0.75,size=0.7)+
  geom_errorbar(aes(x=Time,ymin=Female_Mean-
SEM,ymax=Female_Mean+SEM,color="Females"),width=1,alpha=0.75,size=0.7)+
  labs(color1 = "Legend")+
  scale_color_manual(values = colors1)+
  ylim(0, 260)+
  scale_x_continuous(name="Time from Injection (hr)",breaks=seq(0,72,6),limits=c(0,72))+
  ylab("Mean cortisol Concentration (ng/mL)")+
  theme(plot.title = element_text(face="bold"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
        axis.title.y = element_text(size=12, face="bold", colour = "black"),
  )+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
```

```
      axis.text.y = element_text(color="black"))

#Mean serum cortisol concentration by age

colors2<-c("Young" = "deepskyblue3","Old" = "darkorange2")

ggplot(BloodFigures)+
  geom_point(aes(Time,Young_Mean,color="Young"),size=2)+
  geom_point(aes(Time,Old_Mean,color="Old"),size=2)+
  geom_hline(yintercept=24.96, linetype='dotted')+
  geom_line(aes(Time,Young_Mean,color="Young"))+
  geom_line(aes(Time,Old_Mean,color="Old"))+
  geom_errorbar(aes(x=Time,ymin=Young_Mean-
Young.SEM,ymax=Young_Mean+Young.SEM,color="Young"),width=1,alpha=0.75,size=0.7)+
  geom_errorbar(aes(x=Time,ymin=Old_Mean-
Old.SEM,ymax=Old_Mean+Old.SEM,color="Old"),width=1,alpha=0.75,size=0.7)+
  labs(color = "Legend")+
  scale_color_manual(values = colors2)+
  ylim(0, 260)+
  scale_x_continuous(name="Time from Injection (hr)",breaks=seq(0,72,6),limits=c(0,72))+
  ylab("Mean cortisol Concentration (ng/mL)")+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
      axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
      panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
      axis.text.y = element_text(color="black"))

#Daily mean FCM concentration

DailyBear1<-DailyBear
DailyBear1$Day<-factor(DailyBear1$Day,
                  levels = c("Pre-injection", "Day_1", "Day_2", "Day_3", "Day_4"))

ggplot(DailyBear1)+
  geom_point(aes(Day,Cort),color="black")+
  geom_errorbar(aes(x=Day,ymin=Cort-SEM, ymax=Cort+SEM),color="black", width=.1,
              position=position_dodge(0.05))+theme(panel.grid.major = element_blank(),
panel.grid.minor = element_blank(),
                                      axis.line = element_line(colour = "black"))+
  geom_line(aes(Day,Cort,group=1),color="black")+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
      axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  geom_hline(yintercept=21.9, linetype='dotted')+
```

```
  ylab("Mean FCM Concentration (ng/g)")+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
        axis.text.y = element_text(color="black"))

#Daily mean FCM concentraiton by sex

DailySA1<-DailySA
DailySA1$Day<-factor(DailySA1$Day,
                     levels = c("Pre-injection", "Day_1", "Day_2", "Day_3", "Day_4"))

colors<-c("Males" = "deepskyblue3","Females" = "darkorange2")
ggplot(DailySA1)+
  geom_point(aes(Day,Male.Cort,color="Males"),size=2)+
  geom_point(aes(Day,Female.Cort,color="Females"),size=2)+
  geom_line(aes(Day,Male.Cort,group=1,color="Males"))+
  geom_line(aes(Day,Female.Cort,group=1,color="Females"))+
  geom_errorbar(aes(x=Day,ymin=Male.Cort-
Male.SEM,ymax=Male.Cort+Male.SEM,color="Males"),width=0.25,alpha=0.75,size=0.25)+
  geom_errorbar(aes(x=Day,ymin=Female.Cort-
Female.SEM,ymax=Female.Cort+Female.SEM,color="Females"),width=0.25,alpha=0.75,size=0
.25)+
  labs(color = "Legend")+
  scale_color_manual(values = colors)+
  ylab("Mean FCM Concentration (ng/mL)")+
  geom_hline(yintercept=21.9, linetype='dotted')+
  theme(plot.title = element_text(face="bold"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
        axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
        axis.text.y = element_text(color="black"))

#Daily mean FCM concentration by age

colors2<-c("Young" = "deepskyblue3","Old" = "darkorange2")

ggplot(DailySA1)+
  geom_point(aes(Day,Cort.Young,color="Young"),size=2)+
  geom_point(aes(Day,Cort.Old,color="Old"),size=2)+
  geom_line(aes(Day,Cort.Young,group=1,color="Young"))+
  geom_line(aes(Day,Cort.Old,group=1,color="Old"))+
```

```
  geom_errorbar(aes(x=Day,ymin=Cort.Young-
Young.SEM,ymax=Cort.Young+Young.SEM,color="Young"),width=0.25,alpha=0.75,size=0.25
)+
  geom_errorbar(aes(x=Day,ymin=Cort.Old-
Old.SEM,ymax=Cort.Old+Old.SEM,color="Old"),width=0.25,alpha=0.75,size=0.25)+
  labs(colors2 = "Legend")+
  scale_color_manual(values = colors2)+
  geom_hline(yintercept=21.9, linetype='dotted')+
  ylab("Mean FCM Concentration (ng/mL)")+
  theme(plot.title = element_text(face="bold"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
        axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
        axis.text.y = element_text(color="black"))

#ANOVA Data


#ANOVA comparing FCM by day

Anova<-Bears
Anova$Day<-as.factor(Anova$Day)
FCMday<-aov(Cort~Day,data=Anova)
summary(FCMday)
TukeyHSD(FCMday)

#ANOVA comparing FCM for day, sex, and interaction between day and sex

Anova$Sex<-as.factor(Anova$Sex)
Anova$Age<-as.factor(Anova$Age)
FCMdaysex<-aov(Cort~Sex+Day+Sex:Day,data=Anova)
summary(FCMdaysex)
TukeyHSD(FCMdaysex)

#ANOVA comparing FCM by day, age, and interaction between day and age

FCMdayage<-aov(Cort~Age+Day+Age:Day,data = Anova)
summary(FCMdayage)
TukeyHSD(FCMdayage)

#ANOVA comparing blood cortisol by sex, time, and interaction between sex and time
```

```
Bloodanova<-Blood
Bloodanova$Time<-as.factor(Bloodanova$Time)
Bloodanova$Sex<-as.factor(Bloodanova$Sex)
Bloodanova$Age<-as.factor(Bloodanova$Age)

Bloodtimesex<-aov(Cort~Sex+Time+Sex:Time,data=Bloodanova)
summary(Bloodtimesex)

#ANOVA comparing blood cortisol by age,time, and interaction between age and time

Bloodtimeage<-aov(Cort~Age+Time+Age:Time,data=Bloodanova)
summary(Bloodtimeage)
TukeyHSD(Bloodtimeage)
```

APPENDIX D

R SRCIPT FOR CHAPTER TWO ANALYSIS

```
#Chapter 2: Effects of ecotourism on free ranging brown bear (Ursus arctos) fecal cortisol metabolite concentrations

library(ggplot2)
library(dplyr)
library(magrittr)
library(tidyr)
library(tidyverse)
library(lme4)
library(lmerTest)
library(titanic)
library(MuMIn)
library(ggforce)
library(sjPlot)
library(ART)
library(ARTool)
library(rstatix)

#Download CSV files

Bears<-read.csv("FCMData.csv")

#Chapter 2 Figures

#FCM concentration of samples collected inside vs outside of plots for each site

ggplot(Bears, aes(x = Site, y = Final.Cort..ng.g.)) +
  geom_boxplot(aes(fill=Plot))+
  ylab("FCM Concentration (ng/g)")+
  theme(plot.title = element_text(face="bold"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
        axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
        axis.text.y = element_text(color="black"))
```

```
#FCM concentration by site

ggplot(Bears, aes(x = Site, y = Final.Cort..ng.g.)) +
  geom_boxplot()+
  ylab("FCM Concentration (ng/g)")+
  theme(plot.title = element_text(face="bold"))+
  stat_summary(fun.y=mean, geom="point", shape=20, size=3, color="red", fill="red")+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(axis.title = element_text(size=12, face="bold", colour = "black"),
        axis.title.y = element_text(size=12, face="bold", colour = "black"))+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))+
  theme(axis.text.x = element_text(color="black"),
        axis.text.y = element_text(color="black"))

#ANOVA Data

#Non-parametric anova comparing FCM by site and samples collected inside of plots by samples collected outside of plots

Anova1<-Bears
Anova1$Site<-as.factor(Anova1$Site)
Anova1$Plot<-as.factor(Anova1$Plot)

m=art(Final.Cort..ng.g.~Site*Plot,data=Anova1)
anova(m)

#Generalized linear mixed model

GLMM<-Bears

GLMM$Site<-as.factor(GLMM$Site)
GLMM$Plot.Location<-as.factor(GLMM$Plot.Location)
GLMM$People<-as.factor(GLMM$People)
GLMM$Month<-as.factor(GLMM$Month)
GLMM$Diet<-as.factor(GLMM$Diet)

M1<-
lmer(Final.Cort..ng.g.~Site+Month+Diet+People+(1|Plot.Location),REML=FALSE,data=GLM
M)
M2<-
lmer(Final.Cort..ng.g.~Site+Month+People+(1|Plot.Location),REML=FALSE,data=GLMM)
M3<-
lmer(Final.Cort..ng.g.~Month+Diet+People+(1|Plot.Location),REML=FALSE,data=GLMM)
```

```
M4<-lmer(Final.Cort..ng.g.~Site+(1|Plot.Location),REML=FALSE,data=GLMM)
M5<-lmer(Final.Cort..ng.g.~Month+(1|Plot.Location),REML=FALSE,data=GLMM)
M6<-lmer(Final.Cort..ng.g.~Diet+(1|Plot.Location),REML=FALSE,data=GLMM)
M7<-lmer(Final.Cort..ng.g.~1+(1|Plot.Location),REML=FALSE,data=GLMM)
M8<-lmer(Final.Cort..ng.g.~People+(1|Plot.Location),REML=FALSE,data=GLMM)

AIC(M1,M2,M3,M4,M5,M6,M7,M8)

#Summary of all models

summary(M1)
anova(M1)
tab_model(M1)

summary(M2)
anova(M2)
tab_model(M2)

summary(M3)
anova(M3)
tab_model(M3)

summary(M4)
anova(M4)
tab_model(M4)

summary(M5)
anova(M5)
tab_model(M5)

summary(M6)
anova(M6)
tab_model(M6)

summary(M7)
anova(M7)
tab_model(M7)

summary(M8)
anova(M8)
tab_model(M8)

#Testing ANOVA assumptions

#Testing normality of data through Q-Q plots and Shapiro-Wilk test
```

```
BearsOutliers<-Bears %>%
  group_by(Site) %>%
  identify_outliers(Final.Cort..ng.g.)
View(BearsOutliers)

model<-lm(Final.Cort..ng.g. ~ Site, data = Bears)

qqnorm(Bears$Final.Cort..ng.g.)

shapiro_test(residuals(model))

#Testing for equal variance using Levene's Test

Bears$Site<-as.factor(Bears$Site)
levene_test(Bears,Final.Cort..ng.g.~Site)
```