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Noninvasive fecal monitoring of glucocorticoids in Alaskan brown bears (Ursus arctos horribilis)

Christina G. Von der Ohe
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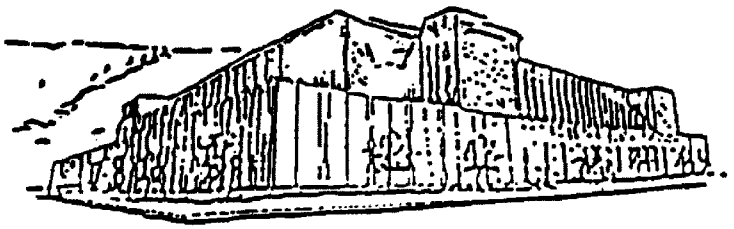

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NONINVASIVE FECAL MONITORING OF GLUCOCORTICOIDS IN ALASKAN BROWN BEARS *(Ursus arctos horribilis)*

By

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B.A. University of California, Berkeley, 1998

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for the degree of

Master of Science

THE UNIVERSITY OF MONTANA

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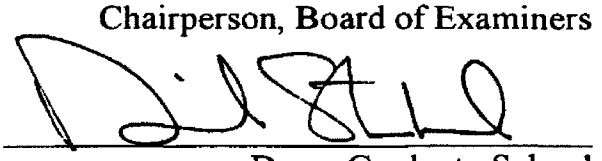

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Noninvasive fecal monitoring of glucocorticoids in Alaskan brown bears *(Ursus arctos horribilis)*

Director: Dr. Christopher W. Servheen

ABSTRACT

Brooks River in Katmai National Park is one of the most famous bear-viewing areas in the world. The close proximity of brown bears and humans at Brooks River poses a question vital to the management of these bears: do ongoing human activities at Brooks River induce stress in brown bears that rely on this fishing area for much of their nutritional intake? Fecal monitoring of glucocorticoids is a new and noninvasive technique for measuring stress in free-ranging animals and is being applied to an increasing number of species. This technique has never been performed on brown bears. Thus, the aim of this study was threefold: to validate the use of a radioimmunoassay (RIA) for quantifying glucocorticoid metabolite concentrations in feces from brown bears, to apply this technique to a study of impacts of human activities over a four-month period on physiological stress in brown bears along two rivers in Katmai National Park, AK, and to provide recommendations for management based on the results of stress analysis. An established RIA for corticosterone was tested for assay sensitivity, specificity, and sample matrix effects, and proved satisfactory. Fecal samples collected in the wild from brown bears of five month by location treatments were assayed for glucocorticoid metabolite concentrations and analyzed in the presence of covariates measuring current human and bear activities, as well as diet type of each sample. Fecal samples from identified bears were also collected and analyzed for differences among sex-age classes. We observed a significant interaction between the effects of diet types and treatments on fecal glucocorticoid concentrations. There was no evidence of a significant effect of human activities on fecal glucocorticoid concentrations, although confounding in this observational study limits inferences concerning effects of visitor use on bear stress. Sex-age class differences in fecal glucocorticoid concentrations were not observed. This study demonstrates that fecal glucocorticoid concentrations may be assessed in brown bears, but this technique may have limited application in species with complex or seasonal dietary habits.

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I. INTRODUCTION

The brown bear *(Ursus arctos horribilis)* has engaged human interest for thousands of years. Today, people congregate from many regions of the globe in areas that are renowned for their bear-viewing potential. Salmon-spawning rivers are particularly attractive to tourists who enjoy watching bears in their natural habitat, as bears reliably gather at these rivers in high concentrations when salmon are present. Brooks River, in Katmai National Park and Preserve, is one such popular bear-viewing river, and its discovery by the tourism industry has resulted in a large increase in human traffic in the last several years (National Park Service, 1996).

Katmai National Park and Preserve was originally designated as a National Monument in 1918 to protect the scientific and scenic value of the landscape created by an extremely violent volcanic eruption in 1912. It was subsequently enlarged and re-designated Katmai National Park and Preserve by the Alaska National Interest Lands Conservation Act (ANILCA) of 1980, which specifies that one of the salient purposes of the Park is to "protect habitats for, and populations of, fish and wildlife, including, but not limited to, high concentrations of brown/grizzly bears and their denning areas..."(ANILCA, 1980).

Katmai National Park and Preserve provides protected habitat for one of the largest surviving populations of brown bears. The stability of this population is closely tied to availability of salmon during the spawning season (Olson *et al.*, 1990). Brooks River is an important source of these salmon as it provides salmon for a longer duration than almost any other river in the park (Troyer, 1980). This river is a gathering area for bears during the salmon-spawning season, and is thus a popular area for bear-viewing.

The close proximity of brown bears and humans at Brooks River poses a question vital to the health and management of these bears: do ongoing human activities at Brooks River induce stress in the brown bears that rely on this fishing area for much of their nutritional intake? Olson *et al.* (1990) documented the behavior of non-habituated bears in Katmai (i.e. bears that have not

or can not become accustomed to the presence of humans). According to this study, non-habituated bears frequented river areas in inverse proportions to human numbers and exhibited a decrease in fishing efficiency caused in part by human disturbances. While these observations are suggestive of the effects of human activities on brown bear behavior, a quantitative measure of the degree of stress caused by human activities would provide a higher degree of objectivity and comparability, which is the intent of this study.

The profound physiological effects of stress are well understood. Adverse stimuli are known to activate the hypothalamic-pituitary-adrenocortical axis (HPA axis), resulting in the release of glucocorticoids from the adrenal gland, which is widely measured as an index of stress (Morton *et al.*, 1995). Plasma glucocorticoid levels can have opposing consequences upon health. On one hand, they can provide an animal with the resources necessary to cope with a stressor while maintaining vital homeostasis. On the other hand, chronically high levels may result in reduction of support for body functions such as growth (Klasing, 1985; Spencer and McEwen, 1990), reproduction (Doerr and Pirke, 1976; Bambino *et al.*, 1981; Barb *et al.*, 1982; Welsh *et al.*, 1982; Olsten and Ferin, 1987; Barbarino *et al.*, 1989), and immunity (Monjan, 1981; Munck *et al.*, 1984; Wiedenfeld *et al.*, 1990; Reichlin, 1993; Strauman *et al.*, 1993). Monitoring glucocorticoid levels can be a valuable tool for identifying stressors before the appearance of symptoms such as weight loss, infertility, or poor health.

Traditionally, glucocorticoid concentrations have been determined in blood plasma. The use of physiological stress measures on free-ranging animals has been limited due to the invasiveness and potential for bias of capturing and withdrawing blood from wild animals (Broom and Johnson, 1993), as this process of measuring stress is itself stressful. Moreover, corticosteroid secretion into blood varies diurnally and has pulsatile secretory patterns (Monfort *et al.*, 1993), causing sample plasma glucocorticoid concentrations to be highly variable. Fecal steroid measures now provide an appealing alternative to serum sampling. Samples for fecal

steroid analysis are relatively easy to collect and can be gathered with minimal disturbance of study subjects. This approach also provides a smoothed estimate of glucocorticoid concentrations over a longer time period than serum sampling, due to the pooling effect of adrenocorticosteroids in feces. Measurements of fecal glucocorticoid metabolite levels have been performed in a number of mammals (Miller *et al.*, 1991: bighorn sheep; Graham and Brown, 1996: several felids; Palme *et al.*, 1996: ponies and pigs; Jurke *et al.*, 1997: cheetahs; Palme and Möstl, 1997: domestic sheep; Monfort *et al.*, 1998: African wild dogs; Sousa and Ziegler, 1998: common marmosets; Whitten *et al.*, 1998: chimpanzees; Boinski *et al.*, 1999: brown capuchins; Goymann *et al.*, 1999: spotted hyenas; Strier *et al.*, 1999: muriquis; Wallner *et al.*, 1999: barbary macaques). Use of fecal glucocorticoid measures in brown bears has never been published. Validation of such a technique for brown bears would be a valuable contribution to the new field of fecal glucocorticoid metabolite research. This technique could prove useful for assessing the stressfulness of the close proximity of humans and bears at Brooks River and may also serve for future comparisons with other bear populations.

Objectives

In this study, glucocorticoid metabolite concentrations were studied from two populations of brown bears using salmon streams in high and low visitor-use areas of Katmai National Park and Preserve, AK. Objectives of this study were (1) to validate the use of a radioimmunoassay for quantifying cortisol metabolite concentrations in the feces of brown bears, (2) to apply this technique to a study of impacts of human activities on physiological stress in brown bears, and (3) to provide recommendations for management based on the results of the analysis.

Thesis Format

Chapter 2 is a detailed literature review of the glucocorticoid response to stress and identification of various factors effecting steroid excretion that have the potential to confound fecal glucocorticoid research. Chapter 3 details the methods and results of fecal glucocorticoid research performed on Alaskan brown bears and is formatted for submission to the journal of General and Comparative Endocrinology, save for an added description of the study area.

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II. REVIEW OF LITERATURE

The Physiology of Stress

Definition of Stress

Stress, as defined by Broom and Johnson (1993), is an environmental effect on an individual that overburdens its control systems, thus threatening to reduce fitness. The experience of stress is unique in each individual, relating to its cognitive interpretation of the situation and ensuing emotional arousal. Physiological responses to stress enable animals to escape such situations (Asterita, 1985; Moberg, 1985a). When confronting a potentially stressful situation, an animal's physiology is altered by initiation of the autonomic nervous system or the neuroendocrine system (Moberg, 1987). These systems have the capacity to alter metabolism, redirect blood supply to certain organs, modify digestion, and modulate numerous other biological systems. These physiological activities provide the animal with the resources necessary to cope with the stressor while maintaining vital homeostasis (Moberg, 1985a).

Physiological Response to Stress

An adverse stimulus is known to initiate a physiological cascade of responses, which mobilizes resources necessary to cope with the stressor (Moberg, 1985a). One such cascade that has been intensely investigated over the last half-century involves endocrine activation of the hypothalamic-pituitary-adrenal (HPA) axis (Asterita, 1985; Moberg, 1985a). When an animal perceives a stressor, its hypothalamus influences the anterior pituitary gland to secrete its respective trophic hormones, which in turn influence various target organs in the body to secrete their hormones (Asterita, 1985). Of particular interest, due to its utility in measurements of stress, is release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, which stimulates the adrenal cortex to increase synthesis and secretion of glucocorticoids, including

cortisol and corticosterone. The specific glucocorticoids synthesized and the pattern of their release varies between species (Broom and Johnson, 1993).

Glucocorticoid Metabolism and Excretion

Glucocorticoids are transported in the blood in free form or bound to albumin or corticosteroid-binding globulin (CBG). Only the free and albumin-bound fractions of glucocorticoids are available for metabolism and conjugation (Brooks, 1979). The liver is the chief site for glucocorticoid metabolism due to the presence of necessary enzymes, although significant metabolism also occurs in the kidneys, adrenals, placenta, connective tissues, fibroblasts, and muscles (Lipman *et al.*, 1962; Brownie, 1992). Steroid metabolism in the liver involves the conversion of biologically active glucocorticoids, which are those not bound to CBG (Brooks, 1979), to multiple metabolites. The steroids are reduced and conjugated with glucuronic acid, after which the inactivated hormone may either enter the blood to be excreted or reabsorbed by the kidney, or may be excreted in bile into the small intestine to be either excreted in feces or reabsorbed into the enterohepatic circulation (Taylor, 1971).

In the kidneys, reabsorption of glucocorticoids is passive, resulting in a linear increase of urinary free cortisol with plasma cortisol (Beisel *et al.*, 1964). Specifically, Scurry and Shear (1969) have estimated in dogs that 16-20% of the unbound cortisol that enters the kidney is reabsorbed. More polar metabolites are preferentially excreted in the urine in rats, rather than into the intestine (Marandici and Monder, 1985).

Most of the steroid metabolites that enter the small intestine via bile are deconjugated and some are reabsorbed (Palme *et al.*, 1996). Those metabolites that are not reabsorbed are excreted in feces. The influence of intestinal microflora on fecal steroid hormone metabolism has been demonstrated by Eriksson and Gustafsson (1970b) in rats. The role of the intestinal microflora is thought to be deconjugation of steroids, which enhances reabsorption, as evinced by studies in

which administration of antibiotics inhibited deconjugation of steroid conjugates, increased fecal steroid excretion, and decreased urinary steroid excretion (Adlercreutz *et al.*, 1984). Thus, the steroid metabolites found in the bowel are products of metabolism by the animal as well as the action of gastrointestinal microorganisms (Han *et al.*, 1983).

Physiological Effects of Glucocorticoids

Glucocorticoid hormones released during stressful events initiate numerous physiological reactions that enable an animal to cope with a situation. The effects of glucocorticoids are more widespread than other steroid hormones in terms of the number of tissues affected and the diversity of effects (Brooks, 1979). The primary influence of glucocorticoid activity is on carbohydrate, protein, and fat metabolism (Asterita, 1985; Broom and Johnson, 1993). Specifically, the most pronounced effects of glucocorticoids are on liver stimulation of gluconeogenesis, reduction of protein anabolism, increase of protein catabolism, and mobilization of fatty acids from adipose tissue. Apart from metabolic effects, glucocorticoids exert antianabolic and catabolic effects on lymphoid, bone, connective, and other tissues in the body. In large amounts, cortisol inhibits the inflammatory response of damaged or injured tissues by stabilizing lysosome breakdown, decreasing fibroblast activity, decreasing the permeability of capillaries, suppressing inflammatory mediators such as eicosanoids, bradykinin, seratonin, and histamine, and reducing lymphocyte migration by sequestering them in tissues (Cohen, 1972; Munck *et al.*, 1984). Glucocorticoids also help maintain vascular reactivity to catecholamines and are necessary for catecholamines to exert their full free fatty acid mobilization action (Ganong, 1987). The overall effects of glucocorticoid activities are to mobilize energy resources from tissues and increase glycogen storage in the liver in order to prepare energy necessary for a response and to minimize unnecessary energy expenditure in times of stress (Asterita, 1985; Moberg, 1985a).

General Adaptation Syndrome

Physiological effects of glucocorticoid activity on coping success become costly when an animal experiences chronic stress (Moberg, 1985b). Selye (1950) advanced the general adaptation syndrome (GAS) theory that described the series of nervous and endocrine gland activities during the chronic phase of stress activity. During the first, or alarm reaction phase, a stressor triggers the autonomic and neuroendocrine systems as described above. The second phase, namely that of resistance, is characterized by the body's attempt to maintain homeostasis in the presence of the stressor while maintaining high levels of glucocorticoids and intensified levels of body functioning. If stress continues, the individual experiences exhaustion of biological defense systems. Selye (1950) argued that various pathologies associated with prolonged stress develop during this final stage.

Pathologies Associated with Chronic Stress

Prolonged release of glucocorticoids can significantly endanger animal health. During the exhaustion phase of the GAS, the physiological expense of chronically elevated glucocorticoid levels can become evident (Asterita, 1985). The antianabolic and catabolic effects of glucocorticoids on several tissues in the body result in reduction of support for body functions such as growth, reproduction, and immunity (Moberg, 1985a).

Stress has been shown to lead to increased susceptibility to disease, and this increased susceptibility is due in part to alterations of immune function (Roth, 1985). Corticosteroid elevation is involved in the suppression of the immune system (Wiedenfeld *et al.*, 1990; Strauman *et al.*, 1993). Prolonged release of glucocorticoids has been shown to lead to increased susceptibility to infectious diseases due to inhibition of enzyme production, slowing of antigen processing, and by quantitatively reducing immune reactions and responses (Fauci, 1979; Monjan, 1981; Kiecolt-Glaser *et al.*, 1984a; Kiecolt-Glaser *et al.*, 1984b; Golub and

Gershwin,1985; Kelley, 1985). Specifically, suppression of various cellular immune parameters in blood lymphocytes appears to be importantly mediated by stress-induced elevation of glucocorticoids (Lysle *et al.*, 1990; Reichlin, 1993; Deguchi and Akuzawa, 1998). In addition, studies have demonstrated that glucocorticoids induce lymphocytopenia by promoting lymphocyte migration into tissues (Cohen, 1972). Other pathways activated during stress response also mediate immune suppression, such as reduction of natural killer cell activity of splenic lymphocytes due to secretion of corticotropin-releasing factor (CRF) from the hypothalamus (Irwin *et al.*, 1990) and suppression of mitogenesis in splenic lymphocytes by the sympathetic nervous system and peripheral catecholamines (Cunnick *et al.*, 1990). Finally, it has been suggested that glucocorticoids may also potentiate detrimental effects of neurotoxins (Sapolsky, 1985a).

The physiological response to stress may also have the potential to decrease reproductive capacity. Glucocorticoids have been shown to influence the secretion of gonadotropins and synthesis and secretion of gonadal steroids (Moberg, 1985b). Elevated CRF levels have been shown to contribute to decreased luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion and the disruption of reproductive function (Olsten and Ferin,1987; Barbarino *et al.*, 1989). In addition, ACTH or cortisol has been shown to block the preovulatory release of LH in swine (Barb *et al.*, 1982). Glucocorticoids can also act on the testes to decrease the concentrations of LH receptors and to directly suppress androgen biosynthesis (Bambino and Hsueh, 1981; Welsh *et al.*, 1982). Long term effects of glucocorticoids result in the suppression of secretion of testosterone, resulting in lower titers of this hormone (Doerr and Pirke, 1976; Moberg, 1985b). Through these mechanisms, the adrenal response to stress may have the potential to disrupt reproduction.

The physiological response to stress can impact growth, as well. Glucocorticoid action has been implicated in impaired growth due to skeletal muscle and lymphoid atrophy, as well as

depressed body weight due to both reduction of food intake and a decrease in food utilization efficiency (Klasing, 1985). A study of the effects of ethanol stress on rats by Spencer and McEwen (1990) demonstrated that in all experiments chronic ethanol stress blunted the increase in body weight, which is normally observed in rats of the strain and age used. These and numerous other pathologies have been shown to be associated with chronic stress.

Glucocorticoids as Indices of Stress

Quantitative measures of stress are particularly relevant in studies of animal welfare in light of potentially negative influences of glucocorticoids on health. Traditionally, glucocorticoid concentrations have been determined in blood plasma. The use of physiological stress measures on free-ranging animals has been limited due to the invasiveness and potential for bias of capturing and withdrawing blood from wild animals (Broom and Johnson, 1993). Moreover, corticosteroid secretion into blood varies diurnally and has pulsatile secretory patterns (Monfort *et al.*, 1993), causing sample plasma glucocorticoid concentrations to be highly variable. Fecal steroid measures now provide an appealing alternative to serum sampling. Samples for fecal steroid analysis are relatively easy to collect and can be gathered without disturbing study subjects. This approach also provides a smoothed estimate of glucocorticoid concentrations over a longer time period than serum sampling, due to the pooling effect of adrenocorticosteroids in feces. Measurements of fecal glucocorticoid metabolite levels have been performed for a number of mammals (Miller *et al.*, 1991: bighorn sheep; Graham and Brown, 1996: several felids; Palme *et al.*, 1996: ponies and pigs; Jurke *et al.*, 1997: cheetah; Palme and Möstl, 1997: domestic sheep; Monfort *et al.*, 1998: African wild dog; Sousa and Ziegler, 1998: common marmoset; Whitten *et al.*, 1998: chimpanzee; Boinski *et al.*, 1999: brown capuchins; Goymann *et al.*, 1999: spotted hyenas; Strier *et al.*, 1999: muriquis; Wallner *et al.*, 1999: barbary macaques). This new approach to stress analysis may prove to be extremely useful for identifying circumstances which cause

stress before the appearance of symptoms such as weight loss, infertility, or poor health. However, many confounding factors may confuse the results of stress analysis. Some of these factors will be discussed in more detail in the following sections.

Effects of Assay Techniques on Glucocorticoid Measures

To date there are countless methods for extracting steroid hormones from feces. This variability is primarily due to species-specific matrix effects of feces, requiring each researcher to discover the extraction technique that will extract the largest concentration of hormone. To a lesser extent, extraction methods vary due to laboratory preferences. For similar reasons, the specific radioimmunoassay employed to assess steroid hormone concentrations varies from study to study. Because the extraction methods determine how much steroid is recovered and radioimmunoassays vary in their specificity for hormones, data from different studies on the same species may only be compared if the techniques employed are identical. It behooves researchers to delineate their procedures in great detail so that data from future studies of the same species may be compared.

Species Specific Glucocorticoid Differences

The spectrum of glucocorticoids secreted into the blood varies between species, and it follows that the metabolites excreted via feces are dependent on species, as well (Brooks, 1979; Palme *et al.*, 1996). It has also been suggested that there are age and individual differences within species in glucocorticoid patterns, as well (Brownie, 1992). Potential reasons for such differences, as presented by Marandici and Monder (1984) include disparate liver abilities, differences of rate of movement from small intestine to large intestine, and variable rates of uptake for all organs, which may be due to receptor affinities or numbers. As differences in fecal glucocorticoid

metabolite concentrations between species are to be expected, inferences concerning physiological stress cannot be made between species from fecal data.

Effects of Gender on Glucocorticoid Concentrations

Gender differences in the pattern of glucocorticoid synthesis and secretion have been shown to exist (Taylor, 1971; Brooks, 1979). The sex hormones modify responses of the HPA axis to stress (Ganong, 1963; Bell *et al.*, 1991). Estrogens have been shown to enhance CRF gene transcription (Vamvakopoulos and Chrousos, 1993), stimulate ACTH secretion by enhancing anterior pituitary responsiveness to CRF-like activity and increasing the pituitary synthesis of ACTH (Kitay, 1963b; Coyne and Kitay, 1969), increase serum glucocorticoid concentrations (Peterson *et al.*, 1960; Kitay *et al.*, 1965; Lindholm and Schultz-Möller, 1973; Garris, 1986), impair glucocorticoid receptor-mediated feedback (Burgess and Handa, 1992), increase the levels of CBG (Sandberg and Slaunwhite, 1959; Brooks, 1979; Coe *et al.*, 1986; Rosner, 1990; Pepe and Albrecht, 1995) leading to a decrease in metabolic clearance rate of glucocorticoids (Peterson *et al.*, 1960, Kitay, 1963a, Pepe and Albrecht, 1995), and alter hepatic and intestinal metabolism of glucocorticoids (Lipman *et al.*, 1962; Eriksson and Gustafsson, 1970a; Colby and Kitay, 1972a; Colby and Kitay, 1972b; Brooks, 1979). Through these actions, estrogens influence plasma, urine, and fecal concentrations of glucocorticoids. Testosterone has been demonstrated to decrease pituitary ACTH secretion (Kitay, 1963b; Coyne and Kitay, 1971), increase adrenal responsiveness to ACTH in rats (Kitay, 1963b), increase glucocorticoid output (Colby and Kitay, 1972b), suppress CBG activity in the rat (Gala and Westphal, 1965a), increase biological half life of glucocorticoids (Kitay, 1963b), and alter hepatic function (Yates *et al.*, 1958; Kitay, 1963b). Progesterone impacts glucocorticoid concentrations independently of its effects on estrogens (Rodier and Kitay, 1974) by decreasing ACTH release via effecting either hypothalamic or pituitary sites (Mathews *et al.*, 1970; Rodier and Kitay, 1974, Vale *et al.*, 1978; Carr *et al.*, 1981),

reducing plasma concentrations of glucocorticoids (Rodier and Kitay, 1974; Hellman, *et al.*, 1976), antagonizing glucocorticoid negative feedback inhibition of rat anterior pituitary (Abou-Samra *et al.*, 1984a), and increasing CBG binding activity (Gala and Westphal, 1965a) due to competitive binding with glucocorticoids (Rosenthal *et al.*, 1969; Brooks, 1979). LH and FSH have also been shown to impact glucocorticoid levels by increasing their output (Vinson *et al.*, 1976). It is evident that differences in glucocorticoid secretion and excretion are influenced by the specific sex hormones present, and this must be considered when drawing comparisons between animals.

Effects of Reproductive Events on Glucocorticoid Concentrations

The studies mentioned above have demonstrated the impact of sex hormones on glucocorticoid levels. Due to the vast fluctuations of sex hormones during the various reproductive events, it is necessary to take such factors into account when analyzing glucocorticoid concentrations.

The ovarian cycle in all animals is generally regulated by the same interplay of pituitary and ovarian hormones. Despite some clear relationships between major sex hormones and the HPA axis, the interplay between these hormones and glucocorticoids during the female reproductive cycle is far from clear. Several studies suggest peak glucocorticoid activity in the late follicular and early luteal phase of the cycles, when levels of estrogens, LH, and FSH are high (Garris, 1986: guinea pigs; Saltzman *et al.*, 1998: marmosets), while others have found inverse results (Beck *et al.*, 1972: women) or no fluctuations at all (Carr *et al.*, 1979, Liu *et al.*, 1987: women). However, given the potential for glucocorticoid fluctuation in the ovarian cycle, such cyclicity must be taken into account in hormone analysis.

Mating behavior has been shown to be associated with fluctuations in sex hormones. In non-primate females, sexual activity occurs near the time of estrus, when circulating levels of

estrogens and LH are high. In males, such regular patterns do not exist. Rather, copulation has been correlated with increases in testosterone (Howland *et al.*, 1985; McDonald *et al.*, 1986; Borg *et al.*, 1992; Kenagy *et al.*, 1999; Strier *et al.*, 1999), as well with increases in glucocorticoid concentrations (Howland *et al.*, 1985: pygmy goats; Elias and Weil, 1989: camels; Borg *et al.*, 1991: bulls and boars; Borg *et al.*, 1992: rams; Levis *et al.*, 1995: boars; Strier *et al.*, 1999: muriqui monkeys), although a few studies noted no such changes (Bercovitch and Clarke, 1995: rhesus macaques; Kenagy *et al.*, 1999: degus). In cases where glucocorticoids increase during mating, psychosocial factors, rather than gonadal hormones are likely responsible for the increase. Physical aggression and psychosocial stressors cause increases in glucocorticoids in several species (Smith and French, 1997, Wallner *et al.*, 1999), whereas testosterone is not associated with a clear net effect on glucocorticoid concentrations (Coyne and Kitay, 1971). Copulation in females does not have a clear correlation with glucocorticoid levels. Several studies have documented no change in glucocorticoid levels associated with mating (Garcia-Villar *et al.*, 1985: ewes; Elias and Weil, 1989: camels; Kenagy *et al.*, 1999: degus), while others have shown an increase in such levels (Schiml and Rissman, 1999: musk shrews). Potential increases found during this period for females may be due to increases in concentrations of estrogens and LH, as well as any stress associated with the act.

Pregnancy is also characterized by a marked change in sex hormones, and the alterations in glucocorticoid concentrations approximate those expected with high concentrations of estrogens, more so than the effects of high progesterone. During pregnancy, CRF concentrations rise (Thomson and Smith, 1989; Lockwood *et al.*, 1996), ACTH levels increase (Carr *et al.*, 1981; Bell *et al.*, 1991; Lockwood *et al.*, 1996) due in part to an extra-pituitary source of ACTH not subject to feedback control (Rees *et al.*, 1975), total plasma glucocorticoids increase dramatically in humans (Cohen *et al.*, 1958; Rosenthal *et al.*, 1969; Burke and Roulet, 1970; Batra and Grundsell, 1978; Abou-Samra *et al.*, 1984b; Allolio *et al.*, 1990; Lockwood *et al.*, 1996) as well

as other species (Gala and Westphal, 1965b: rats; Rosenthal *et al.*, 1969: guinea pigs; Kriesten and Murawski, 1988: rabbits; Elias and Weil, 1989: camels; Ziegler *et al.*, 1995: cotton-top tamarins; Hodges, 1998: elephants), CBG binding capacity improves (Doe *et al.*, 1964; Gala and Westphal, 1967; Rosenthal *et al.*, 1969; Brooks, 1979; Abou-Samra *et al.*, 1984b; Selcer, *et al.*, 1991), retention of glucocorticoids in the intravascular compartment increases (Cohen *et al.*, 1958), breakdown of glucocorticoids by the liver is reduced (Martin and Mills, 1958), and metabolic clearance rate decreases throughout pregnancy (Brooks, 1979), with some studies demonstrating an increase near gestation (Sims and Krantz, 1958; Burke and Roulet, 1970; Oakey, 1975).

Glucocorticoids have been shown in a majority of species to rise near parturition, which is logical in light of their role in the initiation of lactation (Wilcox *et al.*, 1983). High glucocorticoid concentrations at the time of lactation onset have been found in many species including rats (Gala and Westphal, 1965b, Voogt *et al.*, 1969), cows (Schwalm and Tucker, 1978, Wilcox *et al.*, 1983), golden-mantled ground squirrels (Boswell *et al.*, 1994), and degus (Kenagy *et al.*, 1999). This increase occurs both in the total and unbound fractions of glucocorticoids (Gala and Westphal, 1967).

The potential influences of reproductive cycles and events on glucocorticoid concentrations can confound inferences of glucocorticoid research regarding stress. Researchers must take reproductive cycles into account when analyzing hormone levels and making comparisons between groups.

Effects of Dietary Intake on Glucocorticoid Concentrations

Dietary intake can impact urinary and fecal excretion of steroid hormone metabolites. The influence of vegetarian diets on steroid hormone levels in women has been documented (Goldin *et al.*, 1981; Goldin *et al.*, 1982). These researchers found that diet influenced the

excretion pattern of estrogens. Specifically, a positive correlation between fecal weight and fecal excretion of estrogens was found, with the vegetarian diet leading to both increased fecal weight and a two to three fold increase in fecal excretion of these hormones. However, there is potential for the increase in fecal bulk to mask increases in steroid hormone excretion (Wasser *et al.*, 1993). Goldin *et al.* (1982) postulated that the increased steroid hormone excretion was caused by the shielding of estrogens excreted in bile from deconjugation and reabsorption by the greater fecal bulk and nonabsorbed fiber in the intestine. Another hypothesis presented by these authors is that some characteristic of the vegetarian diet may decrease the ability of intestinal microflora to deconjugate bilary estrogen, which is necessary for reabsorption. These theories are strengthened by the finding of a significant inverse relationship between levels of estrogen in excreted feces and plasma found in the studies by Goldin *et al.* (1981) and Goldin *et al.* (1982) in subjects with a vegetarian diet. Thus, decreased intestinal reabsorption of steroid hormones seen with vegetarian diets may decrease steroid concentrations found in the enterohepatic circulation and lower plasma levels of circulating steroids. Similar studies have found that the ratio of protein to carbohydrate intake influences plasma concentrations of cortisol in man, with lower cortisol levels found during the high carbohydrate diet than the high protein diet, and this change was postulated to be due to influences of dietary factors on a number of aspects of steroid hormone metabolism (Anderson *et al.*, 1987). This study also demonstrated that parallel changes occur in CBG concentrations during the high carbohydrate diet, which may have been reflected in concentrations of steroid hormones. An impact of diet on urinary excretion of steroid hormones has also been investigated; Remer *et al.* (1998) have determined that the lactovegetarian diet decreases urinary cortisol metabolites. The influence of diet on excretion patterns requires researchers to be aware of any dietary confounding.

Fecal glucocorticoid metabolite concentrations are considered to represent a pooled fraction of plasma glucocorticoids, providing an estimate of adrenal status that smoothes the

effects of diurnal and pulsatory variations (Goymann *et al.*, 1999). However, variation in steroid hormone metabolite levels may be increased by dietary effects on excretion rates. Variations in gastrointestinal transit time may impact the degree of pooling of feces in the intestines. Palme *et al.* (1996) have suggested that the passage rate of digesta from the duodenum to rectum play an important role in the time course of excretion of steroids. Thus, diets that increase passage rate, such as those high in dietary fiber (Pritchard and Robbins, 1990), may decrease pooling time of steroids and increase metabolite concentration variability. The amount of food consumed may also impact pooling time due to the accelerated gastrointestinal transit time with increased quantity ingested (Palme *et al.*, 1996).

Ingested glucocorticoids from dietary sources, such as meat, may be absorbed by the body and impact plasma and fecal glucocorticoid metabolite concentrations. The excellent bioavailability of oral administration of cortisol has been well documented for rats and humans (Chanoine and Junien, 1984; Heazelwood *et al.*, 1984; Tauber *et al.*, 1986). The pattern of absorption of glucocorticoids from the diet depends on quantity of food intake, with food ingestion causing reduced and delayed peak plasma steroid levels (Barbhaiya and Welling, 1982). Thus, fecal samples from carnivores or omnivores should be analyzed with this potential influence on glucocorticoid levels in mind.

In the past, dietary differences have been adjusted for primarily by removing water from the sample, which accounts for differences in water content among the diets (Wasser *et al.*, 1993). However, large dietary differences may not be adjusted for merely by lyophilizing fecal samples, due to the ability of dietary intake to have impacts on the degree of reabsorption of metabolites, time of pooling, and exogenous augmentation of glucocorticoid levels. Wasser *et al.* (1993) suggested that a cholestanone index may be useful to improve serum to fecal correlation in longitudinal studies. Analysis of different diets independently may also reduce confounding. It is

necessary to know the intake of animals under investigation in order to control for potential dietary confounding.

Psychological Factors Influencing Glucocorticoid Concentrations

The activation of the HPA axis is contingent upon an animal's cognitive interpretation of an event as a threat. Psychological stimuli have been shown to be as effective as physical stimuli in activating the biological stress response in animals (Dantzer and Mormede, 1985). The pituitary-adrenal axis has been shown to be stimulated by stressors such as apprehension, frustration, conflict, disease states, pain, and several types of emotional conditions pertaining to loss of control (Dantzer and Mormede, 1985; Henry and Stephens-Larson, 1985). However, individual reactions to the above stressors may vary based on past experiences. Moberg (1987) maintains that factors such as age, genetics, prior experience, sex, and physiological conditions mold the nature of an animal's biological stress response. The effect of prior experience on reactions to stress can be demonstrated in habituated animals, which develop an internal representation of past events to deal with the environment. If the environment does not contain any new contingencies, the animal exhibits a progressive amelioration of physiological responses (Levine, 1985; Moberg, 1985a). In contrast, an animal exposed to stimuli that are unpredictable or noxious prepares for it by being constantly ready, which engenders a state of anxiety and, in extreme cases, may result in learned helplessness (Broom and Johnson, 1993). Clearly, the experience of stress is unique in each individual and relates to its cognitive interpretation of the situation and ensuing emotional arousal. Thus, a large degree of individual variation is to be expected in fecal studies of glucocorticoid metabolites.

Adaptation/Sensitization

The delicate interplay of physiological and psychological factors in elicitation of the physiologic stress response requires a closer examination of feedback mechanisms. Release of glucocorticoids into the bloodstream is performed through activation of the HPA axis. Down-regulation or negative feedback control exists at almost all levels of this system, with high circulating concentrations of glucocorticoids effecting ACTH and CRF synthesis and release, ACTH influencing CRF release, and possibly even an ultrashort loop feedback influence for CRF (Buckingham *et al.*, 1992). Adaptation, which is dependent on the number of exposures to a chronic stressor (Odio and Brodish, 1990), is accomplished by a reduction of the CRF-induced ACTH secretory response (Reisine and Hoffman, 1983). For example, Spencer and McEwen (1990) have shown that after repeated injections of ethanol into rats for 1-3 weeks, the amount of glucocorticoids released decreased. Adaptation has also been demonstrated using footshock in rats (Odio and Brodish, 1990).

Countering adaptation to repeated consistent stressors via glucocorticoid down-regulation, is hyperstimulation of the stress response. Facilitation may occur due to the enhancement of adrenocortical sensitivity to ACTH during chronic stress and depends on the duration of chronic stress exposure and the types of stressors (Odio and Brodish, 1990). This phenomenon is influenced by decreased sensitivity of glucocorticoid feedback, which may be accomplished by the interaction of glucocorticoid receptors with transcription factors induced by CRF and vasopressin (Aguilera, 1994). In the short term, restraint and injection stressors have been shown to induce facilitation of subsequently stimulated ACTH secretion in rats in at least a 12 h period, leading to a hyper-excitable pituitary-adrenal system (Dallman and Jones, 1973; Daniels-Severs *et al.*, 1973; Akana *et al.*, 1992). Another study of rats exposed to immobilization, light, and noise stressors demonstrated sensitization rather than adaptation over three weeks (Vogel and Jensh, 1988). One factor determining whether sensitization or habituation takes place

is individual experience, which is stressor-specific (Kant *et al.,* 1985). In addition, subtle changes in the characteristics of the stressors or in their regularity can greatly reduce adaptation of the glucocorticoid response (Marti and Armario, 1998). Novel stimuli presented during adaptation to a chronic stressor may actually result in hypersensitivity and faster response onset of the HPA axis (Sakellaris and Vernikos-Danellis, 1975; Aguilera, 1994), which is caused by enhanced adrenocortical sensitivity to ACTH, as discussed above. Severe stressors may cause sensitization rather than habituation due to the ability of ACTH secretion to dissociate from glucocorticoid secretion (Marti and Armario, 1998). Yet another cause of sensitization is the tendency of chronic exposure of rats to stressors to effect adaptation ability, as demonstrated by Sapolsky *et al.* (1984) and Reul *et al.* (1990). These researchers demonstrated that chronic exposure of rats to elevated levels of glucocorticoids damages the regulatory mechanism of the hippocampus and impairs the ability of the organism to reduce glucocorticoid levels after acute stress. In fact, long-term chronic stress can cause damage to the hippocampus to be long-lasting and possibly permanent, as was shown in a study of rats after a three-month treatment period (Sapolsky *et al.,* 1985b). Finally, the ability to adapt may be weaker in some individuals than in others. For example, one study showed that aged rats continued to show a significant corticosterone response long after young rats had developed complete tolerance to the stressor (Spencer and McEwen, 1997).

It is crucial, when conducting physiological stress studies, to consider the nature of the stressor or stressors presented. If the stressor is consistent and at regular intervals, physiological adaptation of the glucocorticoid response to the threat may occur, decreasing the concentrations of glucocorticoid metabolites found in feces. Analysis and interpretation of stress results require an understanding of the stressors presented to the animal.

Conclusion

Glucocorticoid concentrations have been widely used as an index of stress, and the new opportunity to study physiological stress noninvasively may prove to expand the range of such studies. However, the use of fecal samples to study stress requires careful consideration of all factors influencing secretion and excretion of steroids hormones, for these factors have the potential to confound inferences regarding stress. More studies will need to be performed to elucidate the extent of the impact of such factors on fecal glucocorticoid measures, as well as to identify other confounding factors not yet considered.

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III. NONINVASIVE FECAL MONITORING OF GLUCOCORTICOIDS IN ALASKAN BROWN BEARS *(Ursus arctos horribilis)*

ABSTRACT

The aim of this study was to validate a radioimmunoassay (RIA) for quantifying glucocorticoid metabolite concentrations in the feces of Alaskan brown bears and to use this technique to examine the impacts of human activities on physiological stress in brown bears at the Katmai National Park and Preserve, AK. We tested an established corticosterone RIA for assay sensitivity, specificity, and sample matrix effects of brown bear feces, and it proved satisfactory. We collected fecal samples from brown bears in the wild from five month by location treatments and assessed fecal glucocorticoid concentrations. Concentrations were analyzed in the presence of covariates describing current bear and human activities, as well as diet type of each sample. We also collected fecal samples from identified bears and analyzed them for differences among sex-age classes. We observed a significant interaction between the effects of diet types and treatments on fecal glucocorticoid concentrations. We did not observe a significant effect of human activities on fecal glucocorticoid concentrations, although the presence of confounding in this observational study limits inferences regarding human-induced stress. We did not observe sex-age class differences in fecal glucocorticoid concentrations. This study demonstrates that fecal glucocorticoid concentrations may be assessed in brown bears, but this technique may have limited application in species with complex or seasonal dietary habits.

INTRODUCTION

An adverse stimulus is known to initiate a physiological cascade of responses, which provides resources necessary to cope with a stressor (Moberg, 1985). One such response is the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, resulting in synthesis and

secretion of glucocorticoids by the adrenal cortex. Concentrations of these hormones have been used by many studies as a physiological index of stress in animals.

Quantitative measures of stress are particularly relevant in studies of animal welfare and conservation biology in light of potentially negative influences of glucocorticoids on health. Despite the importance of glucocorticoids for coping with stressors, chronically high levels may decrease fitness by disrupting normal physiological functions necessary for growth (Klasing, 1985; Spencer and McEwen, 1990), reproduction (Doerr and Pirke, 1976; Bambino *et al.*, 1981; Barb *et al.*, 1982; Welsh *et al.*, 1982; Olsten and Ferin, 1987; Barbarino *et al.*, 1989), and immunity (Monjan, 1981; Munck *et al.*, 1984; Wiedenfeld *et al.*, 1990; Reichlin, 1993; Strauman *et al.*, 1993). Because of the potentially detrimental effects of chronically high glucocorticoid levels, researchers have placed a high priority on developing and implementing reliable measures of this hormone. Such techniques could identify stressors before the appearance of symptoms such as weight loss, infertility, or poor health.

Human disturbance is an important potential stressor to some populations of Alaskan brown bears *(Ursus arctos horribilis)* that traditionally concentrate at what have become popular tourist destinations. Bears that rely on salmon spawning rivers for much of their summer and fall nutritional intake gather at these rivers, presenting excellent opportunities for bear-viewing. Health risks of high glucocorticoid levels, as well as other stewardship and safety considerations of a stressed animal population, prompted investigation into the nature of human impacts on physiological stress. Brooks River, at Katmai National Park and Preserve, is one bear-viewing area with a large increase in human traffic in the last few years (National Park Service, 1996). Management of these bears and their human viewers requires an understanding of whether ongoing human activities at Brooks River induce stress in the brown bears that rely on this fishing area for much of their nutritional intake. While behavioral studies performed on Brooks River have suggested an impact of human activities on brown bears (Olson *et al.*, 1990), quantitative

indexing of stress hormone levels may provide a degree of objectivity and comparability that managers will be able to consider in the ongoing challenge of balancing recreational and resource concerns.

Traditionally, quantitative measurements of physiological stress have involved assessing glucocorticoid concentrations in blood plasma. The use of physiological stress measures on free-ranging animals has been limited due to the invasiveness and potential for bias of capturing and withdrawing blood from wild animals (Broom and Johnson, 1993). Moreover, corticosteroid secretion into blood varies diurnally and has pulsatile secretory patterns (Monfort *et al.*, 1993), causing sample plasma glucocorticoid concentrations to be highly variable. Fecal steroid measures now provide an appealing alternative to serum sampling. Samples for fecal steroid analysis are relatively easy to collect and can be gathered without disturbing study subjects. This approach also provides a smoothed estimate of glucocorticoid concentrations over a longer time period than serum sampling, due to the pooling effect of adrenocorticosteroids in feces.

The application of fecal glucocorticoid metabolite assessments to studies of physiological stress in wildlife populations is relatively new, and as such, the technique involved has been described for a limited number of mammalian species (Miller *et al.*, 1991: bighorn sheep; Graham and Brown, 1996: several felids; Palme *et al.*, 1996: ponies and pigs; Jurke *et al.*, 1997: cheetahs; Palme and Möstl, 1997: domestic sheep; Monfort *et al.*, 1998: African wild dogs; Sousa and Ziegler, 1998: common marmosets; Whitten *et al.*, 1998: chimpanzees; Boinski *et al.*, 1999: brown capuchins; Goymann *et al.*, 1999: spotted hyenas; Strier *et al.*, 1999: muriquis; Wallner *et al.*, 1999: barbary macaques). Use of fecal glucocorticoid measures in brown bears has never been published. Its use may elucidate the influence of human activity on physiological stress of brown bears at Brooks River.

In this study, glucocorticoid metabolite levels were assessed from two populations of brown bears using salmon streams in high and low visitor-use areas of Katmai National Park and

Preserve, AK. Objectives of this study were (1) to validate the use of a radioimmunoassay (RIA) for quantifying cortisol metabolite concentrations in the feces of brown bears, (2) to apply this technique to a study of impacts of human activities on physiological stress in brown bears, and (3) to provide recommendations for management based on the results of the analysis.

STUDY AREA

Katmai National Park and Preserve is located on the Alaska Peninsula approximately 290 miles southwest of Anchorage (Fig. 1). The park has been shaped by glacial and volcanic activity, with topography ranging from glacial plains in the southwest to the rugged Aleutian Range in the east. Bordering these mountains is an extensive lake and river system, of which Naknek Lake is the largest.

The park encompasses several ecotypes including alpine and arctic tundra, boreal forests, and forested coastal areas. Vegetation communities of Katmai range from treeless tundra to spruce (*Picea spp.*), birch (*Betula spp.*), and poplar (*Populus spp.*) woodland and willow (*Salix spp.*) and alder (*Alnus spp.*) shrubland. The fauna includes assorted small mammals as well as moose (*Alces alces*), caribou (*Rangifer tarandus*), wolves (*Canis lupus*), and brown bears (*Ursus arctos*). Fish species include four species of Pacific anadramous salmon (*Oncorhynchus spp.*), rainbow trout (*Salmo gairneri*), char (*Salvelinus spp.*), arctic grayling (*Thymallus arctcus*), and arctic lamprey (*Lampetra japonica*). In sampling areas, sockeye salmon (*O. nerka*) are more abundant than all other species of salmon. The fish population provides an important source of nutrients to the park ecosystems.

The two rivers used in this study are a part of the Naknek drainage, which is the spawning ground and nursery for a significant portion of the salmon harvested in Alaska's Bristol Bay. Brooks River is a 2.5 kilometer (km) long drainage from Brooks Lake into Naknek Lake and serves as a major migratory route and spawning stream for sockeye salmon. This river is on

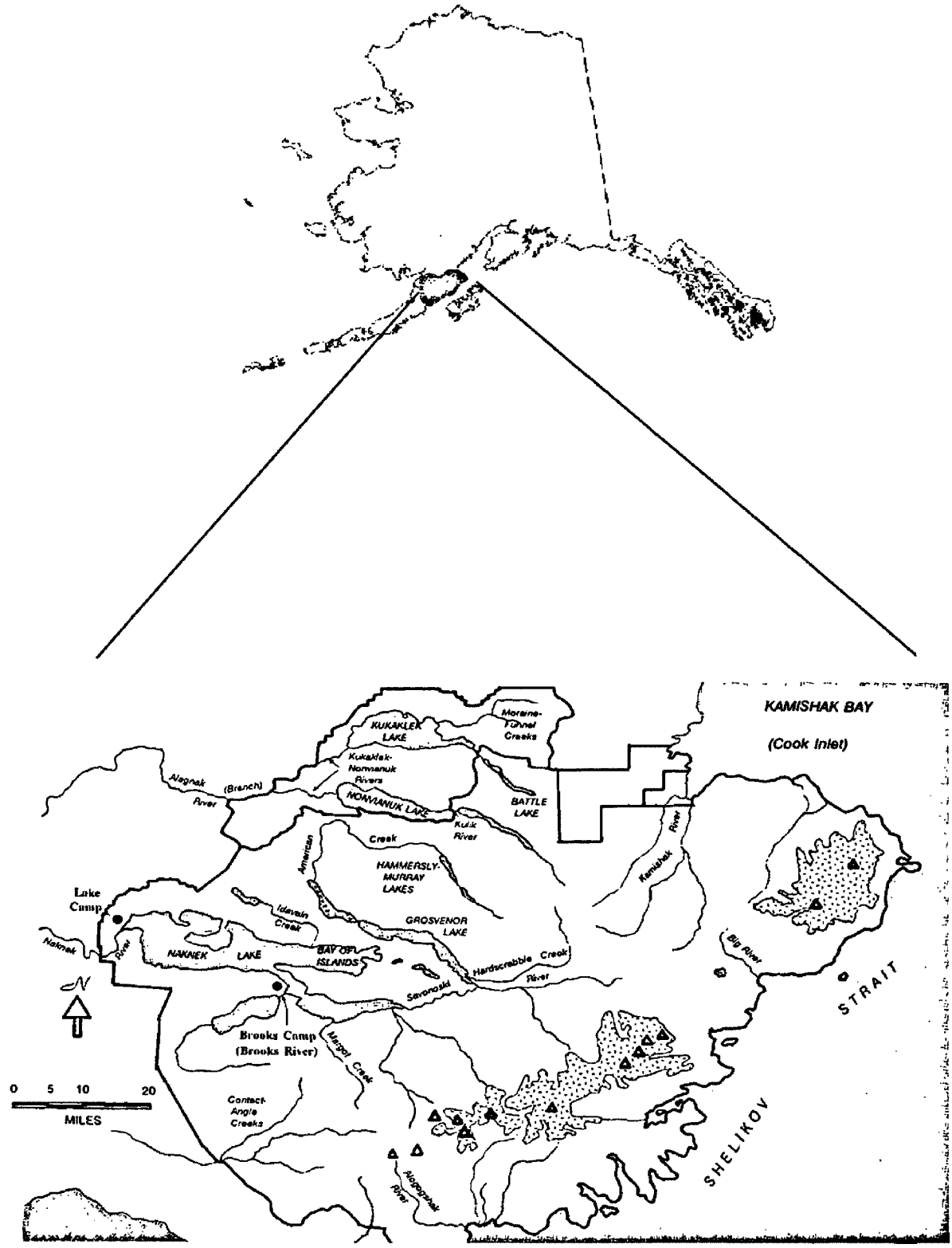

FIG. 1 Location of Brooks River and Margot Creek in the Katmai National Park and Preserve, Alaska.

average 155 feet (ft) wide and flows at 2.1 feet per second (ft/sec) (LaPerriere, 1996). Approximately midway up the river is a 2 meter (m) high waterfall, which migrating salmon attempt to ascend. Brooks River provides salmon to bears for a longer period (late June through late October) than any other river in the system, except the Savanoski River (Troyer, 1980).

Two major runs of sockeye salmon occur in Brooks River annually. The first run arrives in late June and heads to tributary streams that feed into Brooks Lake. These salmon are in excellent condition as needed to reach their relatively distant destination. The number of sockeye generally decline at the end of July, causing a lull in fish activity until the beginning of September, when the second run of sockeye appears. These fish spawn in Brooks River. As these fish are at the end of their journey and in the process of spawning and dying, they are generally in poor physical condition.

Bear activity closely parallels the rhythm of salmon activity in Brooks River. Few bears are seen near the river before the salmon arrive. However, when the first run of salmon appears in July, bears gather along the river. Many bears congregate at the Brooks River Falls, where the fish are vulnerable to predation as they attempt to ascend the waterfall. There are limited prime fishing spots, and these tend to be dominated by large adult male bears. Bears do fish along the entire length of the river during this time, although the healthy condition of fish compounded by river depth makes the capture of live fish difficult. As the first run of salmon leaves Brooks River and salmon become available for fishing in shallower rivers in August, bears leave the area.

Bear activity along Brooks River increases in late August, when the second run of salmon enters Brooks River and nearby salmon runs are depleted. Bears disperse along the length of the river to feed intensively on the dead and dying fish throughout September and October. Bear use of the river mouth is heavy during this period, as bears feed on the spawned-out salmon that drift downstream.

Fishing and bear-viewing opportunities attract many visitors to Brooks River. Brooks Camp, located at the mouth of Brooks River and along the Naknek shoreline, provides facilities for these visitors, as well as for concession and National Park Service (NPS) employees. The camp consists of a rustic lodge that can hold up to 60 guests, a visitor center, concessions and park housing, and support facilities. An NPS campground is located approximately one half kilometer north of the camp with 21 tent plots and an elevated food/garbage cache. Visitors access Brooks Camp via float planes or boats. Most float planes land several hundred meters offshore of Brooks Camp and taxi to shore.

The mouth of Brooks River receives the majority of human traffic throughout the season. Asides from float planes and boats that often moor in the mouth, a floating bridge about 200 meters upstream from the river mouth serves as a conduit of human activity: bus tours leaving for the Valley of Ten Thousand Smokes, the hike to the falls bear viewing platform, and the activities of park and lodge employees, all keep the bridge in almost constant use. Visitors also gather downstream of the bridge because hesitancy of fish to swim beneath the bridge creates an excellent fishing hole for both visitors and bears. Dead or injured fish that are floating downstream are also often caught against the bridge. Interactions between bears and humans occur here frequently throughout the season.

A short hiking trail located on the south side of Brooks River leads to Brooks River Falls. At the falls there is an elevated platform built for bear-viewing and photography. The trail to the falls platform is another area where bears and people come into close proximity, as the trail cuts through areas of high bear activity. Daily interactions between bears and humans along the trail are common, particularly in July, when bear activity is concentrated at the falls.

Below the falls and towards the mouth of Brooks River is a hairpin turn in the river that contains a deep hole ideal for anglers, as well as an island where bears feed on dead fish that are

caught while floating downstream. This is an area in which frequent conflicts between anglers and bears have been observed during periods of heavy use (Braaten and Gilbert, 1987).

The control area, Margot Creek, is located approximately 12 km southeast of Brooks River. Margot Creek is on average 128 ft wide and flows at 1.9 ft/sec (LaPerriere, 1996) into the Naknek Lake system, making it comparable in width and flow rate to Brooks River. In August, salmon spawn on the lower reaches of this river, because a steep falls 4.5 miles above the outlet serves as a barrier to salmon migration. Salmon are vulnerable to predation by bears along Margot Creek because of numerous natural weirs and shallow areas where the river widens. Concentrated bear activity along the river begins a short time after salmon enter the creek, with peak activity occurring during August and usually dropping in early September. Despite heavy bear activity, this creek receives almost no use by visitors due to its inaccessibility.

METHODS

Data Collection in the Field

This study was carried out at Katmai National Park and Preserve during the 1999 salmon spawning season in high visitor-use (Brooks River) and low visitor-use (Margot Creek) rivers that were deemed comparable in flow velocity, suspended solid content, and bear activity (Troyer, 1980; La Perriere, 1996). We randomly selected 10 50m x 15m plots along both rivers in which to sample bear feces. All plots along each river were visited on alternating days from June through September, 1999. All brown bear fecal samples were collected at each plot by mixing thoroughly with a gloved hand and collecting a random subsample. Factors potentially influencing fecal glucocorticoid metabolite levels were recorded: plot number in which the sample was found, date of collection, numbers of bears visible from the plot, total number of different bears seen each day, numbers of humans visible from the plot, total number of daily new visitors registered at Brooks Camp, numbers of vehicles (float planes, boats, automobiles) audible

or visible from the plot, and daily average times for bears to catch fish (minutes/fish catch). The dietary composition of each sample was noted during collection and grouped into "grasses," "berries," or "flesh," when the sample visibly consisted of only one of these three components, or "mixed" if the sample contained a mixture of these three primary diet types. To assess sex-age class differences in fecal glucocorticoid metabolite concentrations, we collected samples when bears of known sex-age groups (adult male, adult female, subadult, yearling, cub) were observed defecating. All fecal samples were stored in a freezer at -20° and remained frozen until analyzed. Dietary composition of samples were confirmed in the laboratory by observation while sifting feces through a wire mesh.

Fecal Steroid Extraction

Fecal samples were extracted and analyzed at the Center for Wildlife Conservation, WA. Fecal samples were lyophilized for 130-140 h at -20° to control for variable water contents of diets. Dried feces were then pulverized and a portion of the resulting powder (0.200±0.015 g) was weighed and extracted with 4ml 90% methanol. After vortexing for 30 min in a pulsing vortexer, the sample was centrifuged (2500g, 20 min, 4°) and the methanol supernatant collected and stored frozen (-20°) in labeled cryovials until assayed. The percent extraction efficiency of titrated ^{3}H cortisol of grizzly bear feces for this extraction method at the Center for Wildlife Conservation is 89.5%. The extract coefficient of variation was determined by running an assay in duplicate with 10 unique glucocorticoid extractions from fecal pools of each of the three representative diet types (grasses, berries, flesh) and subtracting the respective intra-assay coefficient variation.

Fecal Radioimmunoassay

Fecal samples were analyzed for cortisol metabolites using a double-antibody ^{125}I RIA kit for corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA 92626) with high affinities for grizzly bear fecal cortisol (Wasser *et al., in prep*). Samples were analyzed in duplicate. Methanol extracted samples were diluted 1:32 in steroid diluent (phosphosaline gelatin buffer with rabbit gamma globulins). 50µl of each methanol extracted sample were pipetted into 12 x 75 mm glass tubes, to which 100µl ^{125}I corticosterone and 100µl rabbit antiserum were added. After incubation for 2 h (22°-25°), 250µl precipitant solution (mixture of PEG and goat anti-rabbit gamma globulins contained in TRIS buffer) were added and the mixture vortexed. The tubes were centrifuged (2500g, 20 min, 4°) and decanted, and the resulting precipitate was counted for 2 min in a Crystal Multi-Detector Gamma System (United Technologies, Packard). Results were compared to standard curves and expressed in ng/g of dry fecal matter.

Radioimmunoassay Validation

An assay validation determines whether the values obtained with the radioimmunoassay are accurate and correct. This corticosterone assay was validated by testing assay specificity, sensitivity, and sample matrix effects. Specificity, whether the substance measured reacts in a similar manner to the analyte of interest, was assessed by testing equality of slopes for assays of serial dilutions (1:4–1:2048) of brown bear fecal samples from the three representative diet types (grasses, berries, flesh) against the standard. Sensitivity, the minimum amount of analyte which can accurately be distinguished, was assessed by extrapolating to dose from the lower 95% confidence limit of percent bound at zero dose for these same slopes. Sample matrix interferences with the antibody, reagents, or detection system were evaluated by assaying samples containing 50µl of each of the six standards and 50µl of pooled fecal samples and testing homogeneity of regression. Intra- and inter-assay coefficients of variation were assessed by running eight

duplicates of pools from each of the three representative diet types in one assay and eight duplicates of the pools through different assays, respectively.

Statistical Analysis

Statistical analyses were conducted using Excel 97 (Microsoft, Seattle, WA) and SPSS 10.0 (SPSS Inc., Chicago, IL). All hormone metabolite concentrations were log transformed to normalize the data and to decrease heterogeneity of variances. Effects on log fecal glucocorticoid concentrations of five month by location treatments and the covariates people visible/plot, new visitors/day, bears visible/plot, total different bears seen/day, vehicles audible or visible/plot, fishing time/day, and diet type were analyzed using a nested analysis of covariance (ANCOVA) with the random (plot) factor nested within the fixed (treatment) factor. Effects on log fecal glucocorticoid concentrations of sex-age class and the covariates new visitors/day, total different bears seen/day, fishing time/day, month by location, and diet type were also analyzed using ANCOVA. Variable selection was performed using a variety of criteria: F tests of model and parameter significance at α=0.05, lowest MSE, an R^2 value higher than that with a model with one fewer variable but only slightly lower than one with an additional variable, highest adjusted R^2, and the lowest difference between Mallows C_p (1973) and p. The need for higher order terms of selected variables was determined using residual plots. Multiple comparisons between means were made using the Tukey-Kramer procedure (Ott, 1993).

RESULTS

Assay Validation

Serial dilutions of these pooled samples yielded antibody displacement curves parallel to that of the standards at a statistical threshold of 0.05, demonstrating specificity. Cross-reactivity of the ICN-corticosterone antibody was reported by ICN Biomedicals for corticosterone (100%),

desoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.03%), progesterone (0.02%), androstenedione (0.01%), 5α-dihydrotestosterone (0.01%), and less than 0.01% for 12 other steroids tested. Assay sensitivity at α=0.025 was 15.27 ng/ml extract. Homogeneity of regression for additive effects of analyte to a given amount of each sample matrix revealed that sample matrix effects did not differ among the three unique diet types at α=0.01. The inter-assay coefficients of variation (% SD/Mean, 8 replicates in different RIA's) for pooled samples from each of the three representative diet types were 8.30%: grasses, 10.63%: flesh, and 10.51%: berries. Estimates of intra-assay coefficients of variation (% SD/Mean, 8 replicates from the same pools in one RIA) were 4.95%: grasses, 4.26%: flesh, and 4.86%: berries. The extract coefficients of variation (% SD/Mean, 10 replicates from the same pools in one RIA) were 0.17%: grasses, 1.95%: flesh, and 1.44%: berries.

Classification Treatments and Covariate Data

Four of the month by location treatments took place during different months along Brooks River (June, July, August, September), and only one treatment was observed at Margot Creek (August). Sample sizes during these treatments ranged from a high of 324 samples collected during July along Brooks River to a low of 29 samples collected during June along Brooks River (Table 1). The number of people seen per plot ranged from 0 to 43, while new visitors per day ranged from 0 to 264. Vehicular disturbance ranged from 0 to 7 vehicles visible or audible per plot. Bear activities varied, as well, with numbers of bears visible per plot ranging from 0 to 25, and total different bears seen each day ranging from 0 to 19. Fishing time varied between 0.33 and 45 minutes, although during certain periods, bears were never observed catching fish. Samples from all of the four diet types were collected, although flesh and berry samples were not available for collection during June along Brooks River, and samples of the grasses diet type were not available during September along Brooks River (Table 1).

Treatment Effects on Fecal Glucocorticoid Concentrations

A total of 749 samples within plots were collected. The nested ANCOVA did not detect any variability among the replicated plots ($F(38,694)=1.115$, $P=0.294$). Thus, the values from the plots were pooled post hoc for the analysis of the effects of treatments on fecal glucocorticoid concentration. A 0.058 increase in R^2 from a model with only treatment and a 0.005 decrease from a model with treatment, diet, and new visitors/day indicated that both treatment and diet type explained variation in log fecal glucocorticoid concentrations and that the variable new visitors/day did not explain much of the variation beyond that explained by treatment and diet type ($R^2=0.076$). The lowest difference between Mallows C_p (1973) and p, as well as F tests of model and parameter significance ($\alpha=0.05$), concurred with the above variable selection. The covariates new visitors/day, people seen/plot, total different bears seen/day, bears seen/plot, vehicles audible or visible/plot, fishing time/day did not explain a significant ($P>0.05$) amount of the variation of log glucocorticoid metabolites in feces, given that diet type and treatment were in the model. In contrast, highest values of adjusted R^2 and lowest MSE suggested that the variables treatment, diet type, bears/plot, and new visitors/day should be selected. However, given the high correlation between new visitors/day and treatment ($r=-0.847$, $P<0.0005$), and the small size of the increase in MSE (0.001) and decrease in adjusted R^2 (0.001) from this model to the previous one, the former set of variables was selected. Higher R^2, adjusted R^2, lower MSE, and F tests of significance also indicated that the variable treatment was more effective at explaining variability in log fecal glucocorticoid concentrations than new visitors/day both with and without diet type in the model. Inspection of residual plots of the selected variables treatment and diet type revealed that an interaction term between treatment and diet type was appropriate, and this was confirmed by F tests of significance of the interaction ($F(9,732)=7.262$, $P<0.0005$). R^2 for the final model including treatment, diet type, and an interaction between these two variables was 0.152.

Significance of main effects of treatments on log fecal cortisol concentrations could not be assessed due to the presence of this higher order interaction (Fig. 2). However, estimates of mean log fecal glucocorticoid concentrations were calculated at each treatment by diet type combination (Table 1), as well as the 95% confidence intervals for the sizes of the differences for all pairwise comparisons between these values at a constant diet type or treatment (Fig. 3). Mean log fecal glucocorticoids from samples consisting of berries were consistently, but not significantly ($P>0.05$) lower at each treatment sampled than any other diet, and the point estimate for this difference was high for the comparison of berry samples with mixed samples (0.676 log ng/g) and grass samples (1.021 log ng/g) during August at Brooks River, although the small sample sizes limited precise estimation of this difference. Mean log fecal glucocorticoid concentrations were slightly, but not significantly ($P>0.05$) higher at this diet type for Brooks River in September and Margot Creek in August than for Brooks River in both July and August. Bears that had eaten flesh had a small, but not statistically significant ($P>0.05$) increase in mean log fecal glucocorticoid levels over those that had eaten berries across all treatments tested. These levels were slightly, but statistically significantly lower in the flesh diet type for Brooks River in July than for both Brooks River during September ($P<0.01$, 95% CI: 0.041, 0.655) and Margot Creek in August ($P<.05$, 95% CI: 0.011, 0.450). Samples of the grass diet type had slightly higher mean fecal log cortisol concentrations than those that had eaten flesh across all treatments sampled and this difference was statistically significant at Brooks River in July ($P<0.01$, 95% CI: 0.130, 0.564) and both larger and significant along Brooks River in August ($P<0.01$, 95% CI: 0.140, 1.613). Mean log concentrations of fecal glucocorticoids in samples of the grass diet type showed a slight and not statistically significant ($P>0.05$) increase from Brooks River in June to Brooks River in both July and September and Margot Creek in August, and a significant and large increase to Brooks River in August ($P<0.01$, 95% CI: 0.220, 1.638). Samples of mixed feces had slightly higher mean log fecal glucocorticoid concentrations than those with grasses for

Brooks River in June and July and lower levels than grass samples for the remaining treatments. Although none of these comparisons were statistically significant ($P>0.05$), the point estimate for the effect size of the difference was large along Brooks River during June (0.715 log ng/g). Log concentrations of fecal glucocorticoids were significantly higher for the mixed diet type than the flesh type at Brooks River in July ($P<0.01$, 95% CI: 0.284, 0.738). Samples along Brooks River in September and Margot Creek in August of the mixed diet type had slightly lower mean log fecal cortisol concentrations than those in the remaining treatments, and this difference was statistically significant, although small in effect size, only for the difference in mean log fecal cortisol concentrations between Brooks River in July and Margot Creek in August ($P<0.05$, 95% CI: 0.026, 0.431).

Brooks River in July had lower log fecal cortisol concentrations than Margot Creek in August for flesh, berry, and grass samples, and this difference was statistically significant, although small in effect size, for samples from the flesh diet type ($P<0.05$, 95% CI: 0.011, 0.450). This trend reversed significantly for samples of the mixed diet type ($P<0.05$, 95% CI: 0.026, 0.431), although the point estimate for the magnitude of the effect size was small (0.228 log ng/g). Brooks River in July also had lower log fecal glucocorticoid levels than Brooks River in September for samples of the flesh and berry diet types, and this effect was significant at the flesh diet type ($P<0.01$, 95% CI: 0.041, 0.655). Similar to the relationship between samples from Brooks River in July and Margot Creek in August, mean log fecal glucocorticoids during July on Brooks River rose slightly, but not significantly over Brooks River during September for the mixed diet type ($P>0.05$, 95% CI: -0.039, 0.542). Likewise, Margot Creek in August was characterized by slightly, but not significantly ($P>0.05$) lower log fecal glucocorticoid concentrations than Brooks River in September at the flesh and berry diet types, and once again this comparison reversed for the mixed diet type, although this trend was neither statistically significant nor large ($P>0.05$, 95% CI: -0.418, 0.464). Fecal samples from Brooks River in June

and August revealed that the former had lower log fecal cortisol levels than the latter for grass samples ($P<0.01$, 95% CI: 0.220, 1.638), and this effect reversed slightly, but not significantly ($P>0.05$) for the mixed diet type ($P>0.05$, 95% CI: -0.697, 0.959).

Sex-Age Patterns in Fecal Glucocorticoid Metabolite Levels

A total of 57 samples from bears of known sex-age class were collected. The ANCOVA for the effects of the fixed factor (sex-age group) on log hormone level was analyzed with the covariates new visitors/day, different bears seen/day, fishing time/day, month by location of collection, and diet type. A 0.084 increase in R^2 from a model with only diet type and a 0.005 decrease from a model with diet type, month by location, and new visitors/day indicated that both month by location of collection and diet type explained variation in log fecal glucocorticoid concentrations and that the variable new visitors/day did not explain much of this variation beyond that explained by month by location and diet type ($R^2=0.187$). The lowest difference between Mallows C_p (1973) and p, highest adjusted R^2, and lowest MSE concurred with the above variable selection. However, F tests of parameter significance ($\alpha=0.05$) indicated that none of the variables tested explained a significant amount of the variation of log glucocorticoid metabolites in feces. Thus, model selection criteria used indicated that sex-age did not explain a significant amount of the variation of log fecal glucocorticoid concentrations both in a model with other covariates, as well as without any measured covariates ($F(4,52)=0.257$, $P=0.904$) (Fig. 4). Adult males (N=5) and spring cubs (N=12) had the highest mean log fecal cortisol concentrations, and subadults showed the lowest levels (N=11), although none of these differences were statistically significant at $\alpha=0.05$, nor were the magnitudes of the differences large.

TABLE 1 Mean fecal glucocorticoid concentrations log (ng/g) at each treatment by diet type combination ± SEM (N).

	Diet Type			
	Flesh	Mixed	Berries	Grasses
Brooks–June	NA[a]	2.209±0.317 (4)	NA[a]	1.494±0.082 (25)
Brooks-July	1.445±0.045 (64)	1.956±0.048 (112)	1.269±0.000 (1)	1.792±0.039 (147)
Brooks-August	1.546±0.097 (17)	2.078±0.064 (13)	1.402±0.048 (2)	2.423±0.157 (5)
Brooks–September	1.793±0.056 (34)	1.705±0.063 (32)	1.621±0.092 (3)	NA[a]
Margot-August	1.676±0.031 (136)	1.728±0.037 (94)	1.544±0.039 (52)	2.033±0.184 (8)

NA [a] - Samples at this treatment by diet type combination were not found.

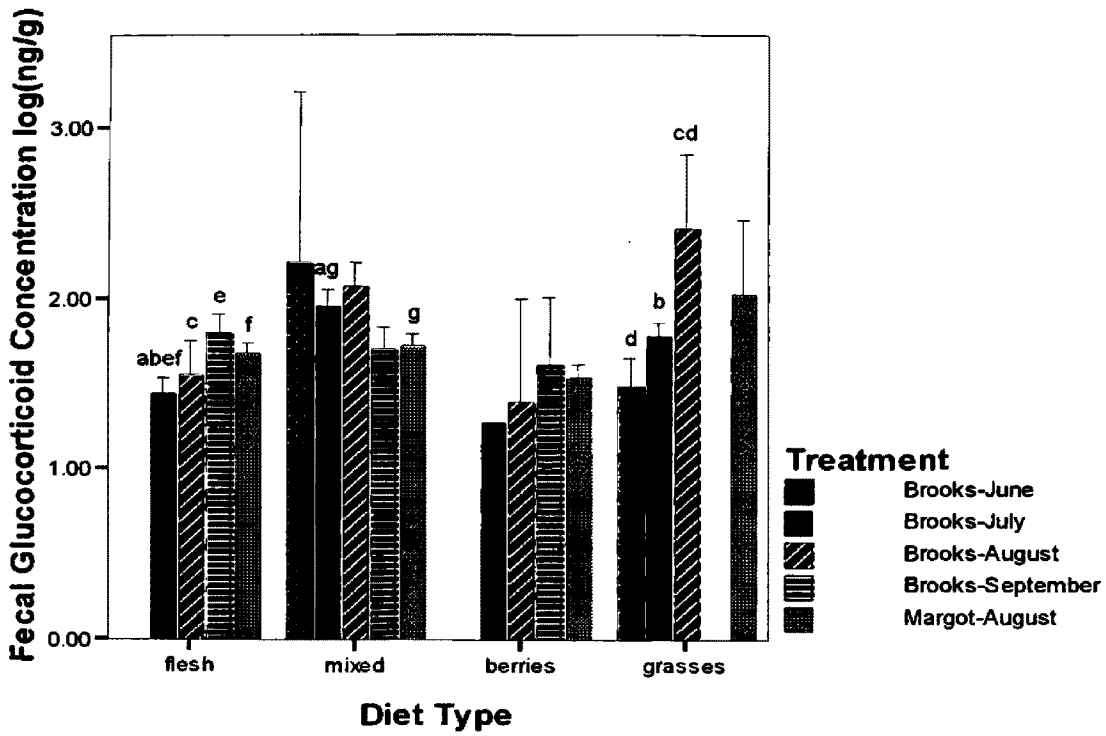

FIG. 2 Mean + upper 95% confidence limit of fecal glucocorticoid concentrations log (ng/g) for each treatment by diet type combination.

aa - comparison significant at α=0.01, 95% confidence interval for the difference: (0.284, 0.738)
bb - comparison significant at α=0.01, 95% confidence interval for the difference: (0.130, 0.564)
cc - comparison significant at α=0.01, 95% confidence interval for the difference: (0.140, 1.613)
dd - comparison significant at α=0.01, 95% confidence interval for the difference: (0.220, 1.638)
ee - comparison significant at α=0.01, 95% confidence interval for the difference: (0.041, 0.655)
ff - comparison significant at α=0.05, 95% confidence interval for the difference: (0.011, 0.450)
gg - comparison significant at α=0.05, 95% confidence interval for the difference: (0.026, 0.431)

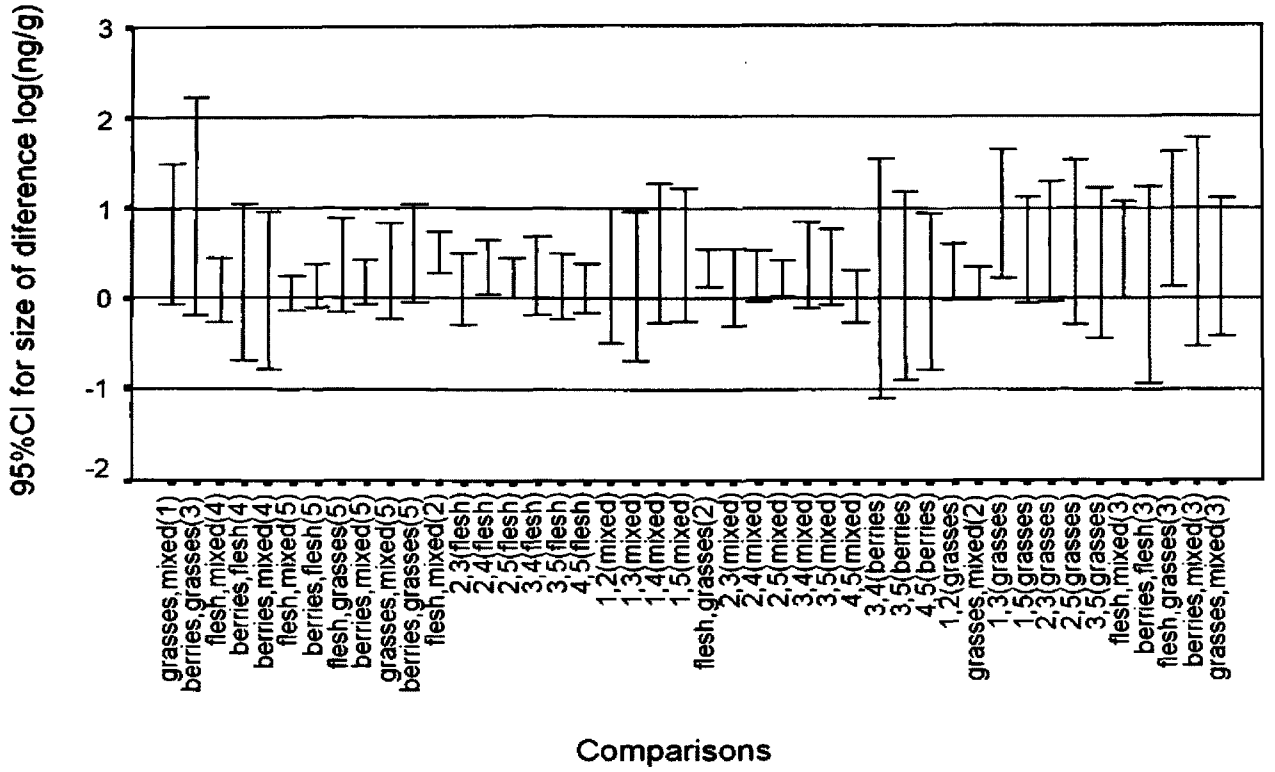

Fig. 3 Tukey-Kramer (Ott, 1993) 95% confidence intervals for the effect sizes of pairwise comparisons at each treatment and diet type.

Levels of the factor compared are separated by commas and the level of the other factor, at which they are compared, follows in parentheses.
1 – Brooks - June
2 – Brooks - July
3 – Brooks - August
4 – Brooks - September
5 – Margot - August

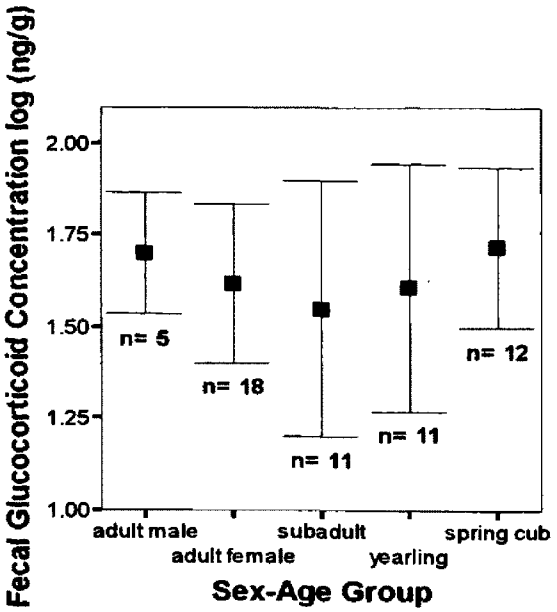

FIG. 4 Mean ± 95% confidence interval of fecal glucocorticoid concentrations log (ng/g) for each sex-age group.

DISCUSSION

We validated the use of a RIA for quantifying glucocorticoid metabolites in brown bear feces by evaluating assay sensitivity, specificity, and sample matrix effects of the three representative diet types. Although cortisol is secreted by the brown bear adrenal gland in greater quantities than corticosterone (Wasser *et al., in prep*), immunoreactivity was detected using a RIA for corticosterone, suggesting that the antibody was cross reacting with other, as yet unidentified, corticoid metabolites. To address physiological relevance of this technique, an adrenocorticotrophic hormone (ACTH) challenge for brown bears is being performed at the Center for Wildlife Conservation (Wasser, *pers.comm.*).

Human activities did not explain a significant portion of the variation in brown bear fecal glucocorticoid concentrations in Katmai National Park and Preserve. Although visitor use did not explain as much of the variation in fecal glucocorticoid concentrations as did treatments, the high correlation between visitor-use and treatments suggests that the treatments may have accounted for some of the effects of humans on these steroid levels. This confounding in our observational study prevented us from parsing out the effects of human activities on bear stress.

We observed that the month by location treatments had an effect on brown bear fecal glucocorticoid concentrations that differed across the diet types. No other studies of fecal glucocorticoids in free-ranging mammals (Creel *et al.*, 1997: African wild dogs; Strier *et al.*, 1999: muriquis; Wallner *et al.*, 1999: semifree-ranging barbary macaques) have addressed such interaction, likely because study subjects maintained a consistent diet. However, brown bears in the wild rely on a wide range of food sources, from plant matter to terrestrial and marine meat, and selection of specific foods changes seasonally (Pritchard and Robbins, 1990; Mattson, 1991; Clevenger, 1992; Hilderbrand *et al., in press*). In addition, intake volume in brown bears has been shown to increase in the fall relative to the spring (Nelson *et al.*, 1980; Hissa, 1997; Hilderbrand *et al., in press*). Dietary variability of brown bears demands a close look at the ways in which

diet-related patterns of fecal glucocorticoid excretion may confound inferences regarding physiological stress.

Dietary fiber has been shown in other studies to impact fecal excretion of steroid hormone metabolites. In vegetarian women, a positive correlation between fecal weight and fecal excretion of estrogens was found, with the vegetarian diet leading to both increased fecal weight and a two to three fold increase in fecal excretion of these hormones (Goldin *et al.*, 1981; Goldin *et al.*, 1982). These researchers postulated that increased steroid hormone excretion was caused by shielding of estrogens excreted in bile from deconjugation and reabsorption by the greater fecal bulk and nonabsorbed fiber in the intestine. Another hypothesis proposed by these researchers is that some characteristic of the vegetarian diet may decrease the ability of intestinal microflora to deconjugate bilary estrogen, which is necessary for reabsorption. Dietary fiber may have a similar influence on glucocorticoid reabsorption. Anderson *et al.* (1987) demonstrated that the ratio of protein to carbohydrate intake influences plasma concentrations of cortisol in humans, with lower plasma cortisol levels found during the high carbohydrate diet than the high protein diet. These studies suggest that high dietary fiber intake by bears, by ingestion of grasses, for example, may increase the concentration of glucocorticoid metabolites recovered in the feces. This characteristic of high fiber diets may explain the large effect size of the rise in fecal glucocorticoid concentrations for samples of the grass diet type over those of the flesh diet type along Brooks River during July and August. This rise may have been present in the remaining treatments, as well, although the small sample sizes and missing data for some of the diet types prevented precise estimation of differences. Alternatively, bears eating grass may have had higher stress-induced circulating glucocorticoid concentrations than those eating flesh during July and August along Brooks River, as those bears eating grass may have been unable to catch fish, and may have experienced frustration as result of their inability to fish successfully. The result that the berry diet, a relatively high fiber diet (Pritchard and Robbins, 1990), consistently showed the

lowest fecal glucocorticoid concentrations across all treatments is puzzling in light of the above information, and may reflect some aspect of this diet type to increase steroid reabsorption or reduce stress. The point estimate of the effect size of the difference between grass and berry diets is large, although the estimates were not precise due to small sample sizes collected at the berry diet type.

For bears consuming a mixture of flesh and vegetation, fecal glucocorticoid concentrations were significantly higher than those just eating flesh at Brooks River in July, although the magnitude of this effect was not large. This trend may have held across all treatments, other than Brooks River in September, however the effect and sample sizes were too small to detect this difference. This trend for flesh to have lower fecal glucocorticoid concentrations than that for the mixed diet type may be explained by differences in excretion patterns, physiological stress, or by sample matrix effects. The lack of independence of dietary effects for mixed diets has been studied primarily with regard to ruminant digestion (Robbins, 1983) and such associative effects may take place in bears that consume a mixed diet. Facilitation of fecal steroid excretion may occur due to the higher percent dietary fiber in these mixed fecal samples than flesh samples for reasons described above, thus slightly increasing fecal glucocorticoid concentrations in samples with a mixture of flesh and vegetable matter over those with merely flesh. Alternatively, those bears eating a mixed diet may have had higher circulating concentrations of glucocorticoids than those eating flesh due to unidentified stress associated with having to incorporate vegetation into the flesh diet. Sample matrix effects of such mixtures may have also influenced the results, as such influences for the mixed diet type were unfortunately not assessed and compared to the other three diet types in this study.

Significantly higher fecal glucocorticoid concentrations were found in samples of the grass diet type along Brooks River in August over those found in Brooks River in June, and the magnitude of this difference was large. Glucocorticoid metabolite increases observed may have

been due to a rise in circulating glucocorticoids caused by stress or seasonal changes, or due to excretory differences at these two treatments. Bears during August along Brooks River may have experienced greater stress during this time for reasons not described by any of the other measured covariates, such as inability to catch salmon during this time and changes in nutritional value of vegetation. However, seasonal effects on circulating glucocorticoids may also help to explain the results. Circulating concentrations of glucocorticoids have been shown to increase in two adult male black bears in the fall relative to summer (Palumbo *et al.*, 1980), and such increases have been associated with pre-hibernatory fattening in true hibernators (Armitage, 1991: yellow-bellied marmosets; Boswell *et al.*, 1994: golden-mantled ground squirrels; Shivatcheva *et al.*, 1998: European ground squirrel). Glucocorticoids enhance seasonal fattening via two modes of action. First, glucocorticoids stimulate lipogenic enzymes in the liver, thereby having an anabolic effect (Berdanier, 1989), particularly on brown adipose tissue accumulation by hibernators (Strack *et al.*, 1995). Second, glucocorticoids have been shown, in rodents, to be necessary for hyperphagia and excessive weight gain (King, 1988; Green *et al.*, 1992), and are thought to induce food intake increases via their ability to influence central regulation of appetite (Tataranni *et al.*, 1996). Thus, the increase in fecal glucocorticoid concentrations seen along Brooks River in August over that in June may have been due to the effects of pre-denning preparations on glucocorticoid levels. The increase in food intake documented in bears prior to denning (Nelson *et al.*, 1980; Hissa, 1997; Hilderbrand *et al.*, *in press*) itself has the potential to impact the concentration of fecal glucocorticoids that are excreted. Increasing consumption accelerates gastrointestinal transit time (Palme *et al.*, 1996), which may influence digestion (Brody and Pelton, 1998) and also reabsorption of steroid hormone, although this likely increases fecal bulk, as well. The rise of fecal glucocorticoid levels in Brooks River in August may be partly caused by such increases in intake volume, contributing to the magnitude of the effect.

In contrast to expectations based on high levels of human and bear activities along Brooks River in July, samples collected during September at Brooks River and August at Margot Creek both showed higher fecal glucocorticoid concentrations than those in Brooks River in July for the flesh diet type, although point estimates for these differences were not large. Possible reasons for these differences include increases in circulating cortisol levels due to stress-related or seasonal effects, or increases in fecal excretion of these steroids. Unmeasured psychological stressors may have been present during these treatments. Seasonal effects on circulating glucocorticoids may also have occurred, as collection in July occurred before the other two months. Such seasonal patterns may be due to the effect of increasing glucocorticoid concentrations on pre-denning fattening, as discussed above, or to the following dietary influence. Ingested glucocorticoids from dietary sources, such as meat, may influence glucocorticoid concentrations in bears due to potential absorption and excretion by the body. The excellent bioavailability of oral administration of cortisol has been well documented for rats and humans (Chanoine and Junien, 1984; Heazelwood *et al.*, 1984; Tauber *et al.*, 1986) and may also be true for bears feeding on cortisol-containing flesh. The predominant glucocorticoid in sockeye salmon is cortisol (Hane and Robertson, 1959) and a five-fold increase in cortisol concentrations has been demonstrated during their journey from the mouth of the river to their spawning grounds (Idler *et al.*, 1959), potentially increasing ingested and excreted cortisol of bears feeding on them. Rising consumption by bears in the fall (Nelson *et al.*, 1980; Hissa, 1997; Hilderbrand *et al.*, *in press*), when salmon are spawning, may compound potential dietary glucocorticoid absorption, as increased intake delays and reduces peak absorption of steroids (Barbhaiya and Welling, 1982) and also decreases gastrointestinal transit time, as discussed above. It is likely that bears feeding on spawning salmon, such as those during September along Brooks River and during August on Margot Creek, are ingesting and absorbing higher concentrations of glucocorticoids than those

feeding on fresh salmon, as in Brooks River in July, and that such increases are partially responsible for the rise in fecal glucocorticoid concentrations in these treatments.

Interestingly, this trend reverses for the mixed diet type, with higher fecal cortisol concentrations found along Brooks River in July than those collected along Margot Creek in August. Such an increase occurs despite potential seasonal effects discussed above. This result may be due to unmeasured stressors during this treatment that were masked by effects of the other diet types. One such stressor may have been the mating activities that took place early in the season, as mating has been associated with an increase in glucocorticoid concentrations for males (Howland *et al.*, 1985: pygmy goats; Elias and Weil, 1989: camels; Borg *et al.*, 1991: bulls and boars; Borg *et al.*, 1992: rams; Levis *et al.*, 1995: boars) and an unclear relationship for females (Garcia-Villar *et al.*, 1985: ewes; Elias and Weil, 1989: camels; Kenagy *et al.*, 1999: degus; Schiml and Rissman, 1999: musk shrew). Alternatively, it is likely that some shift in the composition of mixed samples over time took place that may have influenced fecal glucocorticoid concentrations, as we did not perform detailed analyses of the contents of each sample. For example, this mixture may have been composed predominantly of vegetation during June, July, and August along Brooks River, when salmon were either impossible or extremely difficult to catch. Composition may have shifted in favor of flesh during September along Brooks River and August along Margot Creek, when salmon were easy to catch because they were dead or dying due to spawning activities. Those feces comprised primarily of high fiber vegetation early in the season may have had higher concentrations of glucocorticoid metabolites than those with less dietary fiber later in the season, due to potential effects of dietary fiber on steroid excretion discussed above. The decreasing size of the difference between mixed and flesh mean log fecal glucocorticoid levels from June, July, and August along Brooks River to August along Margot Creek and September along Brooks River strengthens this theory.

Sex-age confounding of these results is not likely, based on information from our model selection. Results from this analysis suggest that sex-age class did not explain a significant portion of the variation of fecal glucocorticoid concentrations. However, gender differences in patterns of glucocorticoid synthesis and secretion have been shown to exist in humans and rats (Taylor, 1971; Brooks, 1979) due to sex hormone modifications of the HPA axis (Ganong, 1963; Kitay, 1963b; Colby and Kitay, 1972b; Rodier and Kitay, 1974; Bell *et al.*, 1991) and hepatic and intestinal metabolism of glucocorticoids (Kitay, 1963a; Eriksson and Gustafsson, 1970; Colby and Kitay, 1972a; Colby and Kitay, 1972b; Brooks, 1979). In addition, sex-age class differences may be anticipated due to potentially disparate social stressors placed on these different groups. Our failure to observe differences among these sex-age groups may be due to the small magnitude of these differences and our inability to measure these differences precisely due to sample size limitations.

We observed that the effect of month by location treatments on brown bear fecal glucocorticoid concentrations differed across the diet types. We have no evidence that human activities significantly effect bear fecal glucocorticoid levels, although the presence of confounding in this observational study limits inferences regarding human-induced stress. Further studies are required to elucidate the degree of stress imposed by human activities along Brooks River in order to make recommendation for management. Fecal monitoring of glucocorticoids is a relatively new technique and it is useful to identify potential pitfalls in this research so that valid inferences can be made and future research designs can employ techniques to parse out potential confounding factors. Numerous factors are capable of influencing fecal excretion of glucocorticoids, some of which are presented in this paper. Only one of these factors is stress. Future designs for brown bear stress research using fecal glucocorticoids as an index should take into consideration potential complex dietary influences resulting from their varied diet and

changing intake volume. Further studies are required to assess the nature of the relationships between dietary intake, temporal variations, and fecal glucocorticoid excretion.

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