Fecal Glucocorticoid Concentrations of Free-Ranging Stone's Sheep

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Abstract: Wild sheep do not readily expand their ranges or colonize new areas, making them especially susceptible to local anthropogenic and environmental disturbance. High levels of glucocorticoids can compromise the immune system and potentially increase susceptibility to diseases such as pneumonic pasteurellosis, the most serious infectious disease of wild bighorn sheep. Fecal glucocorticoids currently serve as the best measure for monitoring the physiological response of stressors with non-invasive samples. Our goal was to define baseline levels and seasonal variation in concentrations of glucocorticoids for Stone's sheep (*Ovis dalli stonei*). We compared fecal samples from sheep in two areas that differed in anthropogenic access and development, predicting that glucocorticoid concentrations would be higher with greater human disturbance. A secondary objective was to examine the relationship between cortisol and corticosterone, two glucocorticoids that commonly are used to describe stress in vertebrates. Concentrations of cortisol and corticosterone in feces from Stone's sheep were higher in summer than late winter, but did not differ between the two areas. We recommend measuring corticosterone concentrations for describing fecal glucocorticoid levels in Stone's sheep because of easy recovery and lower within-season variation than cortisol.

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Wild sheep are particularly susceptible to disturbance and exhibit physiological and behavioural responses to humans and aircraft in close proximity (MacArthur et al. 1982, Stockwell et al. 1991, Bleich et al. 1994, Papouchis et al. 2001, Frid 2003). These disturbances have been recognized as imposing energetic costs on sheep and may alter habitat use, increase susceptibility to predation, or increase nutritional stress (Stockwell et al. 1991, Bleich et al. 1994). Chronic environmental stress is believed to contribute to initiation of pneumonia epizootics in bighorn sheep (*Ovis canadensis*) (Kraabel and Miller 1997). Although epizootics have not been observed in wild thinhorn sheep (*Ovis dalli*) and disease has not been identified as a factor limiting thinhorn populations (Nichols and Bunnell 1999), Dall's sheep (*O. d. dalli*) developed pneumonia from *Pasteurella haemolytica* under experimental conditions (Foreyt et al. 1996). Also, lungworms (*Protostrongylus* spp.) which can damage

lung tissues and potentially set up secondary invasion by bacteria (Bunch et al. 1999) have been identified in Stone's sheep (*O. d. stonei*) (Luckhurst 1973, Seip 1983, Jenkins et al. 2005). The susceptibility to disease, philopatric nature, and inability to readily disperse or expand ranges (Geist 1971, Worley et al. 2004) make Stone's sheep particularly sensitive to disturbance. With increasing resource development of sheep habitat and access to sheep ranges, stressors imposed on Stone's sheep are likely to escalate with potentially serious consequences (Paquet and Demarchi 1999).

Stress elicits physiological and behavioural responses that can be invoked by physical or psychological stressors (Reeder and Kramer 2005). Response to stressors culminates in the release of adrenaline and glucocorticoids from the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal axis (HPA). Both systems play a role in the fitness of an individual by enabling the animal to deal with short-term (SNS) and long-term (HPA) challenges (Reeder and Kramer 2005). Prolonged production of glucocorticoids, however, can be detrimental to the health of an animal (Breazile 1987, Reeder and Kramer 2005). Chronic stress can impede reproduction, alter feeding behaviour and efficiency, cause hypertension and ulceration, and suppress the immune system (Breazile 1987).

Monitoring environmental and anthropogenic stress in animals is difficult because of the stress placed on the animal by the act of sampling (Moberg 1987). Traditionally, measures of stress have been obtained from glucocorticoids (i.e., corticosterone and cortisol) in blood serum or plasma (Harlow et al. 1987, Moberg 1987), but values often were inflated because of the rapid response to stress during handling (Moberg 1987). Plasma glucocorticoids can increase within 2-3 min of an animal being induced with a stressor (Sapolsky et al. 2000). In contrast, fecal excretion of glucocorticoids is determined largely by the time needed for glucocorticoids to travel through the digestive system (Millspaugh and Washburn 2004). Sheep and other large ruminants have relatively long digestive systems with slow passage rates (Millspaugh and Washburn 2004). Millspaugh et al. (2002) documented a temporal delay in glucocorticoid response in fecal samples of at least 10-12 hrs, following adrenocorticotropic hormone (ACTH) challenges on white-tailed deer (*Odocoileus virginianus*). Within 30 hrs of the induced stressor, fecal glucocorticoid measures returned to pretreatment levels. Bighorn sheep responded similarly under comparable ACTH treatments (Miller et al. 1991). The temporal lag between glucorticoid secretion in blood and excretion in feces limits the ability of fecal glucocorticoids to reflect circadian periodicity (observed in desert bighorn sheep (*O. c. nelsonii*), Turner 1984). This indicates that fecal measures better reflect average concentrations of circulating glucocorticoids and, therefore, are ideal for measuring long-term stress in wild animals (Millspaugh and Washburn 2004). In addition, collection of samples can be accomplished without disturbing or handling study subjects (Wasser et al. 2000, Millspaugh et al. 2002, Reeder and Kramer 2005).

Fecal glucorticoid assays have been used with numerous vertebrate taxa, as reviewed in Millspaugh and Washburn (2004). Miller et al. (1991) validated the assays in bighorn sheep and monitored responses of chronic stress in fecal and urine samples using cortisol concentrations. Even though sampling is non-invasive, sampling protocols and biological factors can influence measures of fecal glucocorticoids (Millspaugh and Washburn 2004).

Sampling issues include sample selection, age, condition, storage and transportation, weight, and assay type. Known biological issues influencing fecal glucocorticoid concentrations of free-living mammals are sex, age, diet, body condition, and reproductive status of sampled individuals (Millspaugh and Washburn 2004). Seasonal trends in glucocorticoid concentrations also are common in most mammals (Romero 2002). None of these biological factors has been quantified for wild sheep.

Our goal was to define baseline levels and seasonal variation in concentrations of fecal glucocorticoids in Stone's sheep. In comparing samples from two areas that differed in anthropogenic access and development, we predicted that glucocorticoid concentrations would be higher near greater human disturbance. A secondary objective was to examine the relationship between cortisol and corticosterone, the two glucocorticoids most often measured to describe stress in vertebrates (Moberg 1987).

Study Area

The study area was in the Besa and Prophet River watersheds of the Muskwa-Kechika Management Area (MKMA) in northern British Columbia (Fig. 1), between 57° 20' and 57° 40'N and 123° 10' and 123° 45'W (additional description in Walker [2005]). The 6.4 million-ha MKMA is distinguished by protected areas (i.e., provincial parks) and special management zones that accommodate industrial development as long as wildlife and other socio-environmental values are recognized. Although the Besa and Prophet River watersheds are largely unprotected, Stone's sheep are found throughout this mountainous region. Recreational activity is confined primarily to the southern portion of the study area where there is a permanent outfitter camp and a government designated all-terrain vehicle (ATV) trail. The trail is used from spring through fall and extends the length of the Neves valley in close proximity to several easily accessible mountains inhabited by Stone's sheep. The majority of ATV activity occurs during the summer and fall, with some snowmobile activity during winter. Although there is currently no significant industrial development, increased oil and gas exploration is probable in the southern portion of the study area. Several seismic lines are established in the Neves valley. The northern portion of the study area, encompassing Duffield Creek, is extremely remote and lacks any permanent anthropogenic development. The Neves and Duffield drainages are separated by the Besa River and data from GPS-collared Stone's sheep indicated no animal movements between these areas (Walker 2005).

Methods

Fecal samples were collected during early winter (December and January), late winter (March and April), and summer (July) of 2002 and 2003. Samples in early winter were taken from captured adult Stone's sheep ewes throughout the study area. Ewes were captured by helicopter and radio-collared, in accordance with the guidelines of the Canadian Council on Animal Care (2003), as part of a research project evaluating resource selection strategies of Stone's sheep (Walker 2005). Stone's sheep segregate sexually (Geist 1971, Luckhurst 1973, Seip 1983) with rams occupying distinct ranges or portions of a range away from ewes most of the year except during the breeding season (Geist 1971).

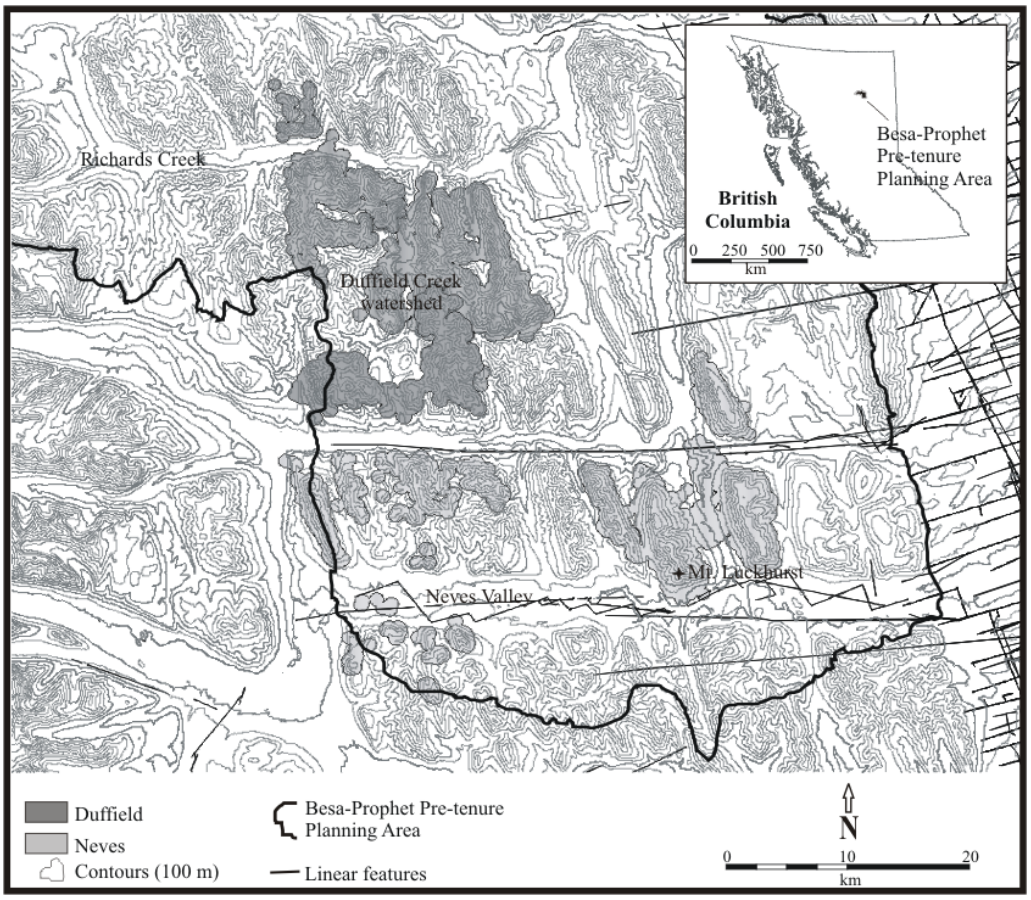

Figure 1. Study area within the Besa-Prophet Pre-tenure Planning Area in the Muskwa-Kechika Management Area of northern British Columbia.

Samples from late winter and summer were collected opportunistically from ranges frequented by maternal females. To minimize sampling the same individuals, we selected at least three different sites occupied by sheep within the Neves and Duffield ranges each year. We tried to alleviate confounding issues associated with age of the sample and sex of the animals by selecting only fresh samples from sites recently or still occupied by female sheep. During early winter samples were fresh because sheep often defecated in response to capture. During late winter we only collected pellets that were on top of the last snowfall and which were not frost-burnt or discolored by weathering. Summer samples were fresh if still moist. We did not collect samples from lambs (easily distinguished by small pellet size) and only went to ranges unoccupied by rams. With the exception of the fecal samples obtained directly from captured sheep (which would not have had time to indicate immediate stress), we minimized the influence of aerial disturbance by collecting samples more than 2 days after aircraft activity near collection sites. Aircraft activity was considered influential if an aircraft flew at or below the uppermost elevational ranges in the study area. Because of the remoteness of the study area and our central location within it, we were aware of all low-level aircraft activity around sheep ranges during periods of sampling.

All 85 fecal specimens were frozen within 2 hrs of collection until subsequent analyses for glucocorticoid content by

Prairie Diagnostic Services, Saskatoon, Saskatchewan. Fecal samples (10-12 pellets) were lyophilized in 20-ml vials and then ground. Approximately 0.25 g of each dry fecal sample were combined with 5 ml of 90% AnalaR grade methanol and inverted frequently for 24 hr. Following refrigeration overnight, samples were centrifuged for 20 min at 1500 g. One-ml aliquots of each methanol supernatant were then dried under air. Each aliquot was reconstituted with 100:1 absolute ethanol and 1 ml of steroid diluent from the corticosterone ^{125}I RIA assay kit (ImmuChem^{TM} Double Antibody, MP Biomedicals, Costa Mesa, California), capped, spun, and left overnight.

Corticosterone content of 50-µl aliquots was determined using the ICN corticosterone RIA antibody (MP Biomedicals, Costa Mesa, California), which is effective in detecting endogenous adrenal activity in a wide array of species (Wasser et al. 2000). Samples (50 µl) also were quantified for cortisol using the DPC Cortisol Coat-A-Count radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California). Results were calculated to give ng/g feces. Sample concentrations were multiplied by 2 for the 50-µl sample size, multiplied by 5 for the 1 ml of methanol originally dried, and divided by the weight of the original fecal sample to give final units of ng glucocorticoid/g feces.

We compared glucocorticoid values between Neves and Duffield populations using a two-way ANOVA of fixed effects with population nested within three seasons. Values were log-transformed after examining assumptions of normality and homogeneity of variance (Levene's test). Tukey's honestly significant difference (HSD) test was used as a post-hoc comparison of main effects within significant models (Zar 1999). The relationship between corticosterone and cortisol was described using Pearson's correlation coefficient (Zar 1999). Statistical significance was assumed at $\alpha \leq 0.05$ and all statistical procedures were conducted using Statistica 6.0 (Statsoft Inc., Tulsa, Oklahoma).

Results

Seasonal differences were observed for both corticosterone ($F_{2,79} = 24.28$, $P < 0.001$) and cortisol ($F_{2,79} = 3.62$, $P = 0.031$) (Fig. 2). Corticosterone levels across all sheep increased from early winter (33.5 ± 1.94 ng/g feces, mean ± SE) through late winter (41.0 ± 1.85 ng/g feces) to summer (56.0 ± 2.94 ng/g feces) and all seasonal comparisons were significant after post-hoc analysis. Average cortisol levels were similar from early winter to late winter and between early winter and summer, but levels in late winter were significantly lower than in summer. Average fecal glucocorticoids of Stone's sheep in the Neves and Duffield Creek drainages followed similar seasonal change and were not significantly different for either corticosterone ($F_{3,79} = 0.96$, $P = 0.418$) or cortisol ($F_{3,79} = 0.11$, $P = 0.954$). Cortisol levels were much more variable than corticosterone. Across seasons, cortisol ranged from 3.6 to 111.8 ng/g of feces in summer and early winter, respectively, with variation averaging 63% of the mean. The variability in cortisol was higher than the range (21.5 to 94.2 ng/g) and coefficient of variation (36%) for corticosterone. In spite of differences in variation and temporal patterns, corticosterone and cortisol measures were positively correlated ($r = 0.68$, $n = 85$, $P < 0.001$) (Fig. 3).

Discussion

Glucocorticoid concentrations are recognized as a physiological index for monitoring stress responses in mountain sheep (Harlow et al. 1987). Corticosterone and cortisol were detected readily in the feces of Stone's sheep. Typically one

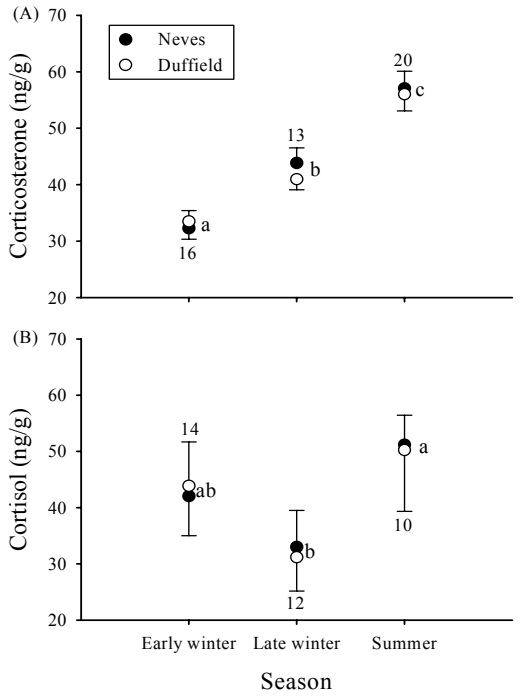

Figure 2. Corticosterone (A) and cortisol (B) concentrations (mean ± SE) in fecal samples from Stone's sheep in 2002 and 2003. Sample size adjacent to error bars in Neves Valley (A) and Duffield (B). Mean values sharing the same letters were not significantly different.

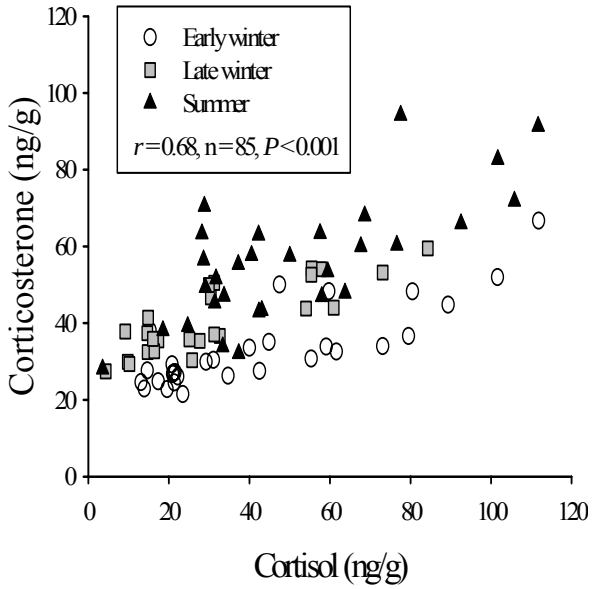

Figure 3. Seasonal relationship between corticosterone and cortisol concentrations in fecal samples from Stone's sheep in northern British Columbia during 2002 and 2003.

hormone tends to be more prevalent than the other in a given species, but both may persist in measurable quantities (Millspaugh and Washburn 2004). Their relationship to each other has been poorly described and trends between cortisol and corticosterone differ between captive and free-ranging desert bighorns (Turner 1984). Cortisol is generally the most prevalent glucocorticoid of large mammals (Millspaugh and Washburn 2004). In Stone's sheep, however, corticosterone provided a less variable measure of glucocorticoid concentrations than cortisol in every season. This may be due largely to the ability of the assay to cross-react or recover corticosterone more consistently than cortisol, as noted by Wasser et al. (2000). The variation exhibited in fecal corticosterone was still considerably greater than the 10% coefficient of variation described for fecal assays used on bighorn sheep under experimental conditions (Miller et al. 1991).

Contrary to our predictions, the glucocorticoid concentrations in the Neves and Duffield populations of sheep were similar even though anthropogenic access to the Neves Valley is greater. The glucocorticoid concentrations probably represent relatively undisturbed levels of stress or habituation by individuals in the Neves Valley or indicate that the disturbance did not elicit a response by sheep.

Fecal glucocorticoid concentrations in Stone's sheep fluctuated seasonally with higher levels in summer than late winter. Elk (*Cervus elaphus*) from Custer State Park in South Dakota also experienced highest fecal glucocorticoid concentrations during summer when air temperatures and anthropogenic disturbance were highest (Millspaugh et al. 2001). These factors, as well as seasonal metabolic rhythms and vulnerability of offspring to predation, may contribute to the elevated glucocorticoid

concentrations in Stone's sheep during summer. Female sheep with lambs generally forage less efficiently, spending less time foraging and more time vigilant than nonmaternal ewes (Risenhoover and Bailey 1985, Frid 1997). Compared to the 1.5 million annual visitors to Custer State Park (Millspaugh et al. 2001), the anthropogenic influence on sheep in our study area was minimal. In northern B.C., temperatures are highest during the summer months but snow is common during any month of the year (Meidinger and Pojar 1991). Thus thermal stress by high temperatures is unlikely.

Seasonal variability in glucocorticoids has been described primarily during the breeding season and to a lesser extent during parturition in mammals (Romero 2002, Millspaugh and Washburn 2004). These periods generally are associated with increases in adrenal activity of most vertebrates. But no seasons are associated consistently with elevated glucocorticoid concentrations across mammalian taxa (Romero 2002). In golden-mantled ground squirrels (*Spermophilus saturatus*), seasonal patterns in corticosterone and cortisol can be associated with changes in body mass and fat deposition (Boswell et al. 1994). Corticosterone levels in female ground squirrels were highest in June during lactation, coinciding with increased lean body mass. Cortisol appeared to mediate corticosterone levels because an increase in fat deposition occurred simultaneously with increased cortisol and decreased corticosterone concentrations. The change in mass gain from muscle to fat occurred well after peak lactation (Boswell et al. 1994). Although the feedback mechanisms among cortisol, corticosterone, and mass dynamics are not confirmed, the inferences may provide insight into why late winter levels of Stone's sheep did not follow similar seasonal patterns. Corticosterone levels in Stone's sheep may remain high in late winter in order to increase lean muscle mass to compensate for the loss of protein reserves during winter and gestation. Although we were unable to collect samples from female Stone's sheep during late summer and fall, we would expect a marked reduction in corticosterone concentrations as females weaned their lambs into the fall if patterns were similar to those in ground squirrels. Cortisol concentrations also should increase with the deposition of fat prior to the fall breeding season. More research is needed to clarify the biologically inherent variation and relationships between these two glucocorticoids.

Romero (2002) described three hypotheses for explaining seasonal patterns in glucocorticoid concentrations. The energy-mobilization hypothesis predicts that glucocorticoid concentrations will be elevated during energetically expensive seasons such as breeding, or mid- to late gestation (Robbins 1993). The behaviour hypothesis infers that glucocorticoids exert control over behaviour and that the stressor is irrelevant. The preparative hypothesis posits that glucocorticoids prepare the individual for seasonal life history changes and that changes in seasonal concentrations are evolutionary reflections preparing an individual for upcoming challenges. These hypotheses are not mutually exclusive and all likely contribute to the seasonal glucocorticoid rhythm of a species (Romero 2002). Selecting the hypothesis that best explains the seasonal trends in Stone's sheep is difficult considering fecal samples were not collected throughout the year. Increased movement rates by Stone's sheep during summer (Walker 2005) and the high energy costs of lactation (Gittleman and Thompson 1988) lend support to the energy-mobilization hypothesis. Stone's sheep ewes in the Besa-Prophet also experienced the greatest mortality during lambing and

early summer (Walker 2005). If female Stone's sheep perceive themselves or their young to be at increased risk of mortality, then the preparative hypothesis also may apply. Determining the range of acceptable concentrations and duration of chronic stress an individual can withstand without experiencing the deleterious effects (Millspaugh and Washburn 2004) is fundamental to understanding the effects of disturbance on fecal glucocorticoids. Glucocorticoids are important to an animal's well-being (Romero 2002, Reeder and Kramer 2005) and elevated levels do not automatically equate to reduced fitness. Without understanding normal variation and effects, inferences regarding the consequences of elevated glucocorticoids are inappropriate (Millspaugh and Washburn 2004). Continued research on baseline glucocorticoid measures throughout the life history of a species is required to enhance our understanding of the physiological status of disturbance-sensitive species in the wild. Our study documents the first baseline information on glucocorticoid levels and the range of naturally occurring variation during three seasons for Stone's sheep in an area where future disturbance associated with resource extraction and increased access is likely to occur.

Management Implications

Wild sheep do not readily expand their ranges or colonize new areas (Geist 1971, Worley et al. 2004), which makes them especially susceptible to local anthropogenic and environmental stressors. Increases of glucocorticoids under captive conditions can increase the susceptibility of bighorn sheep to pneumonic pasteurellosis (Kraabel and Miller 1997), the most serious infectious disease of wild bighorn sheep (Bunch et al. 1999). By describing baseline levels of glucocorticoids in Stone's sheep, we provide a reference to gauge the physiological cost of potential disturbance from environmental or anthropogenic sources. Anthropogenic disturbances can elevate glucocorticoid concentrations in other large mammals (Wasser et al. 2000, Millspaugh et al. 2001, Creel et al. 2002). We recommend measuring corticosterone concentrations rather than cortisol for describing fecal glucocorticoid levels in Stone's sheep because of lower within-season variation and easy recovery. Fecal glucocorticoids currently serve as the best measure for monitoring the physiological response of stressors with a non-invasive and easily attainable source of data (Wasser et al. 2000, Millspaugh and Washburn 2004). For fecal glucocorticoids to be most useful, however, more research is needed to identify the levels of glucocorticoids that are deleterious to individuals and that indicate a potential impact on population health.

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