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RELATIONSHIPS AMONG FECAL LUNGWORM LOADS, FECAL GLUCOCORTICOID METABOLITES, AND LAMB RECRUITMENT IN FREE-RANGING ROCKY MOUNTAIN BIGHORN SHEEP

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ABSTRACT: Most wild Rocky Mountain big-horn sheep (*Ovis canadensis canadensis*) in northern latitudes are infected with lungworms. Indirect effects of lungworms on bighorn sheep are unknown, but high pulmonary burdens might increase stress (i.e., elevated glucocorticoid levels), and chronic stress could in turn decrease fitness. We hypothesized that high lungworm burdens in Rocky Mountain bighorn ewes increase stress, thereby increasing lamb mortality. To test our hypothesis, one subherd of bighorn sheep in Custer State Park, South Dakota was provided a free-choice loose mineral mix containing the anthelmintic fenbendazole every six weeks from March 1999 to August 2000 to eliminate lungworms; another subherd served as the control. Daily, individually marked ewes were located telemetrically from the ground and uniquely marked animals were observed until they defecated. After the herd moved from the area, fecal samples were collected and stored at −23 C. A consistent number of samples per season per herd ($\bar{x}$=16.56±3.99 samples) were collected. Fecal larval lungworm levels (LPG) in the treatment subherd were lower than levels in the control subherd; however, there was no difference in fecal glucocorticoid metabolite (FGM) levels between the two subherds. Fecal glucocorticoid metabolite levels varied by season in both subherds, with levels in winter lower than during the other three seasons. Lamb:ewe ratios were not different between the control and treatment subherds at the end of summer 1999. In contrast, the treatment group had a lower lamb:ewe ratio at the end of summer 2000 despite having lower LPG. However, this result was attributed to lower ewe production, not lower lamb survival. The LPG levels were not correlated with FGM concentrations; instead, FGM levels might reflect normal seasonal patterns. Other factors, including contagious ecthyma, were more important for determining lamb mortality than LPG and FGM levels during our study. We suggest further experimental work over a longer duration to address these relationships.

Key words: Bighorn sheep, fecal corticosterone, glucocorticoid metabolites, lamb recruitment, lungworm, South Dakota, stress.

INTRODUCTION

Nearly all wild Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in northern latitudes are infected with lungworms (Fougere-Tower and Onderka, 1988). In Custer State Park (Custer), South Dakota, two subherds of Rocky Mountain bighorn sheep are naturally infected with lungworms. These bighorn sheep primarily shed *Muellerius capillaris* (Pybus and Shave, 1984) and secondarily *Protostrongylus* spp. (Goldstein, 2001). Lungworms are often found in lungs of bighorn sheep that have died of pneumonia; however, they are not always present, and no conclusive evidence indicates that lungworms lead to pneumonia (Samson et al., 1987; Miller et al., 2000). Effects of lungworms on bighorn sheep survival outside the lungworm–pneumonia complex have not been clearly established. A weakened ewe might be unable to suppress parasite reproduction, have insufficient resources to ensure optimal development of her fetus, or produce an inadequate quantity and quality of milk, leading to early

lamb mortality (Samson et al., 1987). Samson et al. (1987) suggested that lamb mortality is likely affected by a combination of lungworm infection and immunological depression from malnutrition and/or stress. Whether lungworm infection in bighorn sheep influences the stress response, has not been evaluated.

Over the short term, the stress response is adaptive in helping an animal maintain homeostasis through suspension of some activities (e.g., foraging) in favor of survival (Wingfield et al., 1995). However, stress might decrease overall fitness if homeostasis is not achieved and stress is chronic. Prolonged stress responses might result in a number of physiological changes that compromise immune function and resistance to disease, reproductive output, and survival (Dunlap and Schall, 1985; Wingfield and Farner, 1993; Sapolsky et al., 2000). Studies on domestic sheep lambs reported that individuals infected with parasites had higher cortisol levels, and in fence lizards (*Sceloporus occidentalis*), infected individuals had higher cortisol spikes in response to capture stress than uninfected individuals (Dunlap and Wingfield, 1995; Fleming, 1997). Thus, determining whether stress responses are related to lungworm loads might help us understand the relationship between lungworms and bighorn population dynamics. Stress responses are generally characterized by measuring glucocorticoids (i.e., cortisol) in blood and saliva (Bubenik and Brown, 1989; Millspaugh et al., 2002) or glucocorticoid metabolites in urine and feces (Wasser et al., 2000; Millspaugh et al., 2002).

Several anthelminthic drugs reduce lungworm loads in bighorn sheep, including cambendazole, ivermectin, and fenbendazole, and might consequently reduce stress (Schmidt et al., 1979). When administering these drugs on a free-choice basis, fenbendazole is preferred because it is available in several forms, has no apparent unpleasant taste, and is extremely safe for a wide variety of animals, including domestic sheep (Roberson, 1982). In contrast, adverse side effects have been observed for cambendazole (Roberson, 1982). Currently, no drug has long-lasting effects or is effective against all life stages of lungworm parasites. Ivermectin and fenbendazole act only on L4 and adults; therefore, L1 or L3 in a host will not be affected during treatment and will likely mature, reproduce, and perpetuate the cycle. Additionally, bighorn sheep will continue to reinfect themselves while grazing by incidentally ingesting intermediate host snails after drug treatment is complete. In order to achieve long-term reductions in lungworm levels, the drug must be administered repeatedly. Based on lungworm maturation rates, dosing should occur once every four to six weeks (Schmidt et al., 1979).

We hypothesized that high lungworm burdens, as measured by the number of lungworm larvae per gram of dried fecal material (LPG), in Rocky Mountain bighorn ewes elevates stress, thereby reducing lamb recruitment. We had two interrelated objectives, which were tested experimentally, to address our hypothesis: 1) determine the relationship between LPG and stress as measured by fecal glucocorticoid metabolites (FGM), and 2) document the relationship between stress in ewes and lamb recruitment.

MATERIALS AND METHODS

Custer State Park, South Dakota (43°45′N, 103°25′W) (Custer), encompasses 29,150 ha in the southern portion of the Black Hills region of southwestern South Dakota, USA. Custer features a wide variety of the Black Hills topography, ranging from mountains in the northwest to prairies in the southeast. Coniferous forests, dominated by ponderosa pine (*Pinus ponderosa*), comprise 12,355 ha of nonfire affected land within Custer.

French Creek Canyon bisects Custer from west to east. Dominant grasses along French Creek Canyon, where bighorn sheep reside, include western wheatgrass (*Pascopyrum smithii*) and blue grama (*Bouteloua gracilis*) (Morgan, 1987). Canyon rims in the east are characterized by open meadows, and canyon walls have large cliff faces interspersed with ponderosa

pine forests. French Creek ranges up to approximately 1m in depth, and riparian vegetation is dominated by poison ivy (*Toxicodendron rydbergii*), bur oak (*Quercus macrocarpa*), and quaking aspen (*Populus tremuloides*) (Morgan, 1987). Attributes of the west end are similar, although there are fewer meadows, and forests tend to be denser. Other large mammals in this study area include mountain lions (*Puma concolor*), bison (*Bison bison*), elk (*Cervus elaphus*), and mule deer (*Odocoileus hemionus*).

This study focused on two subherds, one in the east end and one in the west end of French Creek Canyon. These two subherds remain as separate groups, but have occasional contact where their ranges overlap. There were between 38–45 adult ewes and yearlings in the east group; 14 of those ewes were marked and used in this study. In the west group, there were about 45–55 adult ewes and yearlings; seven of the ewes were marked and used in this study. In both groups, lambing occurs from late May through mid-July, with a peak during the first two weeks of June. Custer's bighorn sheep population was estimated to be 180 individuals (E. Goldstein, unpubl. data). Low lamb survivorship and recruitment have been observed in Custer bighorn sheep for the past 20 years (Brundige, 1985; Merwin, 2000). A single predominant factor has not been discovered, although several contributing proximate causes including predation and contagious ecthyma have been identified.

All data collected were from free-ranging marked adult ewes that occupied the two ends of French Creek Canyon. The subherd residing in the east end of French Creek Canyon was selected as the treatment group for eliminating lungworms. Panacur® (Hoechst–Roussel Agri-Vet, Somerville, New Jersey, USA) granules containing 222 mg of the anthelmintic fenbendazole per gram of granule, added to a loose mineral mixture specifically designed for park bison (Lautt's Feed and Supply, Harvey, North Dakota, USA), was provided on a free-choice basis to the treatment group. We attempted to administer 3.0 g of Panacur® to each ewe. Treatments occurred every six weeks from March 1999 through August 2000. Treatment intervals were based on maturation time of immature larvae that survive an initial treatment. Each treatment lasted an average of nine days. The subherd residing in the west end of French Creek Canyon served as the control group and received neither Panacur® nor the loose mineral mix.

Herd composition data were obtained by recording total numbers of bighorn sheep, and age and sex of each individual in every group encountered. The majority of groups were found while radiotracking specific ewes to obtain fecal samples. Only counts where observers felt they had an equal probability of detecting lambs, yearlings, and ewes were used to calculate subherd compositions. We used weighted means to calculate numbers of lambs per 100 ewes (L:E ratio) in each subherd during each time period. Each observation in a given time period was weighted by the total number of lambs and ewes per group.

Fecal samples were collected from 21 of the individually marked ewes (14 treatment ewes, seven control ewes) that were identified with color-coded radio collars, uniquely numbered ear tags, or unique physical characteristics. On a daily basis, individually marked ewes were located telemetrically from the ground. When a marked individual was encountered, the animal was observed until she defecated. After the herd moved away from the area on their own accord, fecal samples were collected and placed into 2 oz. Nasco whirl-pak® bags (Modesto, California, USA). They were placed in a freezer within 12 hrs of collection and stored at −23 C. Multiple samples were collected from the same individual during the same time period to account for high variation in LPG between samples from the same individual. These fecal samples were used to measure both LPG and FGM levels.

Forrester and Lankester's (1997) methods for extracting lungworm larvae from feces was used to determine LPG. We modified their procedure by etching lines on the bottom of the petri dish that divided it into eight wedge-shaped sections. A piece of acetate with parallel lines drawn in ballpoint pen was taped to the bottom of the petri dish to increase counting accuracy. Forrester and Lankester's (1997) protocol was developed for bighorn sheep infected with *Protostrongylus* spp.; we examined certain parameters for applicability to *M. capillaris.* We were able to validate the technique after determining pellet-to-pellet variation and laboratory counting error for *M. capillaris,* and differences in wet weight to dry weight ratios among pellets of a given fecal sample (Goldstein, 2001).

Eight fecal pellets per sample were used to extract lungworm larvae, and five pellets per sample were air dried for a minimum of 24 hr to obtain a wet weight to dry weight ratio. Once larvae were extracted from the fecal sample the number of larvae in one eighth of the petri dish was counted, after verifying that larvae were randomly distributed when poured into the petri dish. The proportion of *M. capillaris* to *Protostrongylus* spp. was determined while counting all larvae under a dissecting microscope. Distinction between the two genera was made

based on length, width, and behavior of the larvae.

The remaining frozen fecal material was shipped to the University of Missouri for FGM analysis. There, approximately 25 g of fecal material was placed in a lyophilizer (Freeze-dry Specialties, Inc., Osseo, Minnesota, USA) for 24 hr. Once freeze-dried, each sample was ground, then sifted through a stainless steel mesh to remove large particles, and thoroughly mixed. We extracted FGM from bighorn sheep feces using a modification of Schwarzenberger et al. (1991). Dried feces (~0.2 g) were placed in a test tube with 2.0 ml of 90% methanol, vortexed at high speed in a multi-tube vortexer for 30 min, centrifuged at 500 × G for 20 min, and the supernatant stored at −84 C until assayed.

Corticosterone I^{125} radioimmunoassay (RIA) kits (ICN #07-120103, ICN Biomedicals, Costa Mesa, California, USA) were used to quantify bighorn sheep FGM concentrations. Fecal samples were analyzed in five assays. The ICN protocol for the corticosterone I^{125} RIA was followed, except all reagent volumes were halved (Wasser et al., 2000). A standard assay validation was conducted, including assessment of parallelism, recovery of exogenous analyte, intra- and interassay precision, and assay sensitivity (Jeffcoate, 1981; Grotjan and Keel, 1996; O'Fegan, 2000) to confirm the assay accurately and precisely measured FGM in bighorn sheep feces. We conducted parallelism and recovery of exogenous corticosterone validation assays on two pooled fecal extract samples (low and high; each pool consisted of feces from five individuals). Parallelism ensures the assay maintains linearity under dilution, and recovery of exogenous corticosterone verifies accurate measurement throughout the working range of the assay (Jeffcoate, 1981). Three bighorn sheep fecal samples were selected and analyzed in each assay; interassay variation was calculated from these three samples. Intra-assay variation was calculated by averaging the coefficient of variation (CVs) of replicate tubes from 20 randomly chosen samples.

A natural log transformation of ln(LPG+0.5) was used to normalize LPG data for both subherds, adding 0.5 as a means of incorporating "0" values into the analysis (Zar, 1996). Average values for ewe fecal samples were the sample unit. A generalized linear mixed model in SAS was used to determine if differences existed in LPG (using ln[LPG+0.5] transformed data) or FGM levels between subherds (control vs. treated) and across seasons (Crowder and Hand, 1990). Correlation analysis (Devore and Peck, 1986) was used to determine if a relationship existed between LPG and FGM. Differences in L:E ratios between subherds during the beginning of September 1999, the end of September 1999, and the beginning of September 2000 were tested using *z*-tests for weighted means. We used SAS (SAS Institute, 1985) to perform all statistical analyses and considered test results significant for $P<0.05$.

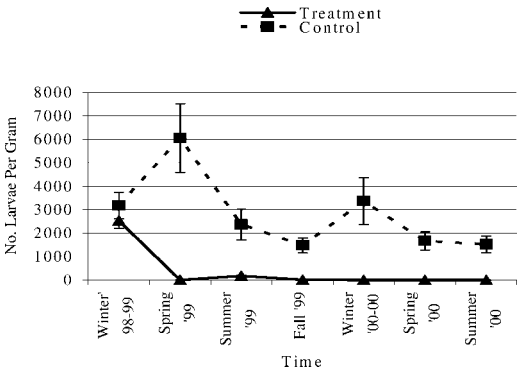

FIGURE 1. Number of lungworm larvae per gram in free-ranging Rocky Mountain bighorn sheep during winter of 1998 to summer of 2000 in Custer State Park, South Dakota. The treatment subherd was provided a free-choice loose mineral mix containing the anthelmintic fenbendazole starting in spring 1999. The control subherd received no treatment for lungworm.

RESULTS

Anthelmintic treatment reduced LPG in free-ranging Rocky Mountain bighorn sheep in Custer (Fig. 1). The LPG decreased (all $P<0.0001$) from pretreatment baseline levels following the application of anthelmintic treatment in all seasons; pretreatment levels did not differ between control and treatment subherds ($P=0.26$). In the treated subherd, LPG decreased from a mean of 2,541 to less than 200, whereas LPG ranged from 1,481 to 6,057 during corresponding seasons in the control subherd (Fig. 1). Differences were evident in LPG across all seasons in both years ($P<0.0001$).

The assay accurately and precisely measured FGM in bighorn sheep samples. Serial dilutions (1:2 up to 1:128) of bighorn sheep fecal extracts yielded displacement curves that were parallel (all $P>0.54$) to the corticosterone standard curve (Fig. 2). Mean recovery of added exogenous corti-

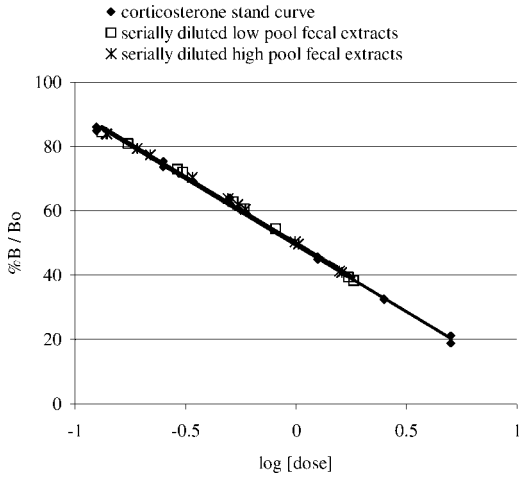

FIGURE 2. Parallelism of fecal glucocorticoid metabolite results for fecal extracts from Rocky Mountain bighorn sheep. Curves of percent binding of I^{125} tracer (%B / Bo) versus serially diluted (log-transformed doses of 1:2 to 1:128) low pool (n=2) and high pool (n=2) fecal extracts from wild bighorn sheep were parallel (test of equal slopes, all P>0.54) to corticosterone standard curves (log-transformed doses of 0.125 to 5.0 ng ml^{-1}). Corticosterone standard curve points are represented by diamonds, points from serially diluted low pool fecal extracts are represented by squares, and points from serially diluted high pool fecal extracts are represented by stars.

costerone (range=0.5–1.25 ng/ml) was 94.2% (SE=3.65, n=12). Acceptable recovery of exogenous corticosterone (within 90–110%) and demonstration of parallelism suggested no sample matrix effects (Jeffcoate, 1981; Grotjan and Keel, 1996; O'Fegan, 2000). Assay sensitivity was 1.25 ng/g. The manufacturer's reported cross-reactivity of the antisera was 100% with corticosterone and <1% for other steroids. Interassay variation for five assays was 5.3%, and average intra-assay variation was 1.6%.

The FGM levels were similar in both subherds of bighorn sheep in Custer during all pre- and posttreatment seasons (Fig. 3). Throughout the study, FGM levels in the treated subherd were not different ($F_{1,\ 219}$=0.83, P=0.36) from those in the control subherd, despite differences in LPG. The FGM concentrations in bighorn sheep were not related to LPG (r=−0.09, P=0.20). The FGM levels varied by season in both herds ($F_{6,\ 219}$=21.79, P<0.0001). Bighorn sheep FGM levels in winter periods were significantly lower (all

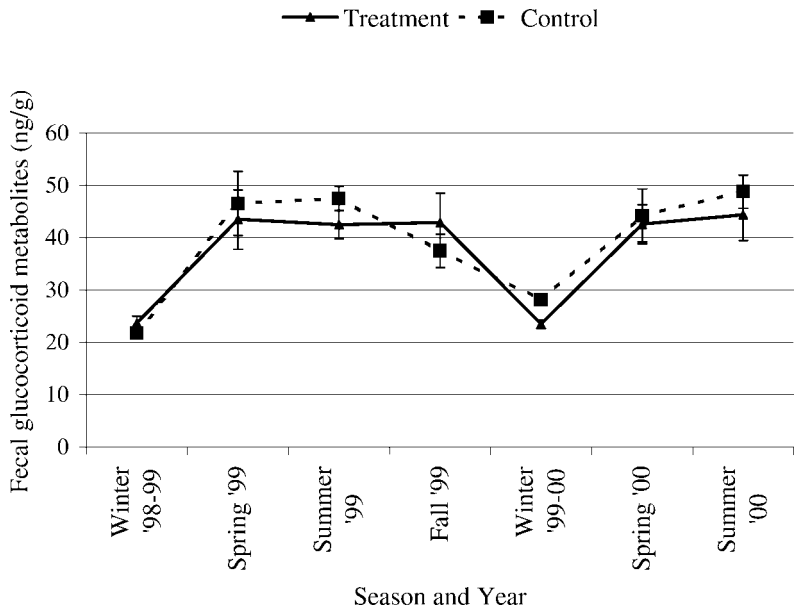

FIGURE 3. Fecal glucocorticoid levels (ng/g) in free-ranging Rocky Mountain bighorn sheep during winter 1998 to summer 2000 in Custer State Park, South Dakota. The treatment subherd was provided a free-choice loose mineral mix containing the anthelmintic fenbendazole starting in spring 1999. The control subherd received no treatment for lungworm.

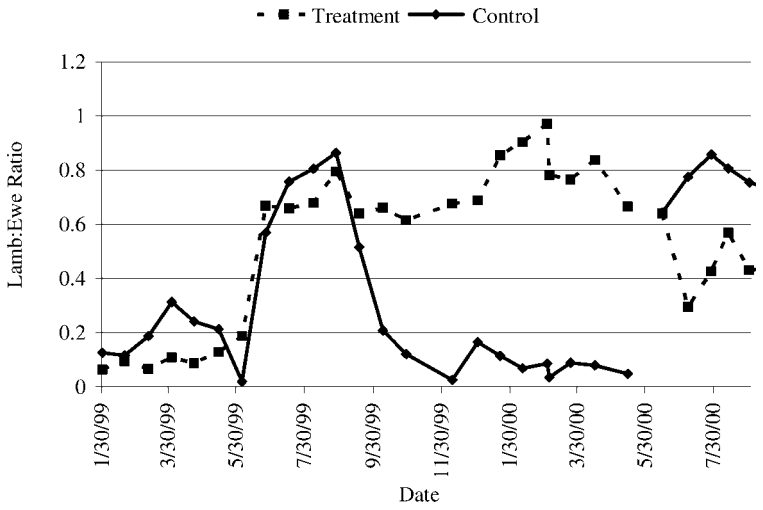

FIGURE 4. Lamb:ewe ratios in free-ranging Rocky Mountain bighorn sheep from February 1999 through August 2000 in Custer State Park, South Dakota. The treatment subherd was provided a free-choice loose mineral mix containing the anthelmintic fenbendazole starting in spring 1999. The control subherd received no treatment for lungworm.

$P<0.0001$) than in the other three seasons (Fig. 3).

The L:E ratios in Custer were not consistent between subherds or years (Fig. 4). Natality was high during 1999 when 20 of 21 marked reproductive treatment subherd ewes bore a lamb, and all nine marked control subherd ewes bore a lamb. Summer L:E ratios observed in the treatment subherd (63:100) were similar to ratios observed in the control subherd (86:100), with no significant difference through mid-September ($z=0.78$, $P=0.22$). On 13 September 1999, lambs with symptoms of contagious ecthyma were observed in the control subherd, and over the next three weeks L:E ratio dropped sharply. Consequently, we noted differences in L:E ratios between treatment (66:100) and control (20:100) subherds at the end of September ($z=-7.77$, $P<0.001$). L:E ratios in the control subherd continued to drop through October to about 8:100 (Fig. 4). Control subherd natality remained high in 2000, with 10 of 11 marked reproductive ewes bearing a lamb. However, birth rates were low in the treatment subherd with only four of 16 marked reproductive ewes seen with a lamb. An analysis of fecal progesterone levels suggested eight of 10 marked treatment ewes observed without a lamb were not pregnant, whereas results from the other two ewes were inconclusive (Goldstein et al., 2002). Consequently, L:E ratios in the treatment subherd were much lower than in the control subherd; by the beginning of September, treatment subherd L:E ratio (42:100) was lower than the control subherd L:E ratio (75:100) ($z=2.83$, $P=0.002$) (Fig. 4).

DISCUSSION

Drug treatments were highly successful in reducing LPG. Average posttreatment LPG was near 0 for the treatment subherd, and was always significantly lower than control subherd levels. This result was anticipated as laboratory experiments have demonstrated the safety and efficacy of this drug (Roberson, 1982). In addition, this drug has greatly reduced or eliminated LPG in numerous field applications (Schmidt et al., 1979; Jones and Worley, 1997). Deviations from 0 LPG resulted from difficulties in sufficiently dosing a wild population using a free-choice mineral mix. The non-normal distribution of the data indicates that most ewes con-

sumed adequate quantities of fenbendazole to eliminate essentially all lungworms, but a few individuals apparently did not.

Multiple studies, including this one, over several years have demonstrated that LPG in Custer bighorn sheep exhibit a seasonal cyclical trend with highest levels in winter, followed by lowest values in summer (Brundige, 1985; Merwin, 2000). Cyclical seasonal patterns of LPG occur in many bighorn sheep herds, but patterns vary geographically even in similar temperate zones (Uhazy et al., 1973; Arnett et al., 1993). Some studies have correlated precipitation and adult lungworm loads (Forrester and Littell, 1976), but others failed to demonstrate a relationship between precipitation and fecal larval load (Festa-Bianchet, 1991; Goldstein, 2001). Cyclical LPG trends have been linked with snail density patterns (Forrester and Senger, 1964; Wishart et al., 1980). However, a cyclical fluctuation in intermediate host populations might not result in a corresponding change in lungworm levels in bighorn sheep. Adult lungworms have a life span of up to seven years (Olsen, 1974), so a temporary reduction in snail numbers should have little influence on parasite loads in the primary host in the short term. Seasonal fluctuations in LPG are more likely a function of adult lungworm reproductive rate. During summer, bighorn sheep are in better body condition and should be better able to suppress lungworm reproduction, whereas in winter the opposite is true. Average monthly LPG in the control herd ranged from 500–3,575. Although these levels are much higher than what has been reported for *Muellerius* by Cabaret et al. (1980) for domestic sheep in Morocco and for *Protostrongylus* in other free-ranging bighorn populations (Forrester and Senger, 1964; Festa-Bianchet, 1989; Aune et al., 1998), it is unclear if these levels indicate a high degree of virulence.

In contrast to our prediction, there was no difference in FGM levels between the treatment and control subherds, despite the significant difference in LPG. Our results suggest that other general and physical stressors within Custer, or normal seasonal patterns, are more important determinants of FGM levels than LPG. The seasonal pattern of FGM levels we observed in bighorn sheep is consistent with FGM levels of elk in Custer (Millspaugh et al., 2001). Millspaugh et al. (2001) attributed these patterns to human activities, temperature, and/or normal seasonal metabolic changes in ungulates. However, other studies examining glucocorticoids in ungulates have not reported such variations. For example, Bubenik et al. (1975) reported constant blood cortisol secretion for seven captive white-tailed deer (*O. virginianus*) in Ontario. Caribou (*Rangifer tarandus*) and moose (*Alces alces*) also had no clear seasonal pattern of cortisol secretion (Franzman et al., 1975; Ringbert, 1979). Although other studies found seasonal variation in ungulate cortisol and FGM levels, the timing of changes is inconsistent with our study. For example, Reyes et al. (1997) reported two peaks in male pudu (*Pudu pudu*) cortisol secretion, one in winter and another in fall. Huber et al. (2003) noted highest FGM values in winter in red deer (*Cervus elaphus*), which they attributed to winter temperatures and snow. These discrepancies are likely due to different seasonal and site-specific stressors that are most important to an animal. Despite these contradictory findings, the pattern of FGM concentrations we observed could simply be a normal seasonal pattern (Millspaugh and Washburn, 2004).

Confounding effects of contagious ecthyma on lamb survival render it difficult to evaluate impacts of lungworms on lamb recruitment after the beginning of September 1999. We found no apparent relationship among FGM levels, contraction of contagious ecthyma, and increased lamb mortality. However, during summer 1999 lamb survival was very high in both subherds, implying that lungworms did not impact lamb survival. During the contagious ecthyma outbreak, treatment sub-

herd lambs apparently never contracted the virus. Fenbendazole should have had no direct influence on bighorn sheep susceptibility to contagious ecthyma. However, the severity of this disease may be related to condition and may have more severe effects on bighorn in poorer condition. Because no symptoms were observed in the treatment group, they likely did not have contact with the virus. Low natality in the treatment group during summer 2000, which could be attributed to the high weaning rates in 1999 (Festa-Bianchet et al., 1998), makes it difficult to evaluate LPG on lamb recruitment. However, we also observed very high weaning rates in 1999, and a low natality rate was not observed in 2000 in the control group. In addition, lamb survival in both subherds was high, again implying that lungworms did not have an impact on lamb survival. Although L'Heureux et al. (1996) reported contagious ecthyma does not result in bighorn sheep mortality, Merwin and Brundige (2000) observed severe lesions and scabbing associated with a high lamb mortality rate in 1997 and 1998 in Custer bighorn sheep.

Our experiment suggested no relationship among LPG, FGM levels, and L:E ratios. Fenbendazole treatment was effective in reducing LPG in adult ewes; however, no change in FGM was noted. Instead, a cyclical pattern of FMG in bighorn sheep was evident with highest values in summer and lowest values in winter. Our data suggested that lungworm infections do not cause chronic stress in bighorn sheep, and administering fenbendazole to bighorn sheep does not reduce lamb mortality. We suggest further experimental work over a longer duration to address these relationships.

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