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EFFECT OF SIMULATED STRESS ON SUSCEPTIBILITY OF BIGHORN SHEEP NEUTROPHILS TO *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN

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ABSTRACT: We examined the effects of simulated stress on susceptibility of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) neutrophils to *Pasteurella haemolytica* leukotoxin in a blocked, crossover experiment. Ten captive-raised bighorn sheep were sampled 10 hr after separate administrations of long-acting adrenocorticotrophic hormone (ACTH) gel and normal saline (control). We then compared in vitro leukotoxin-dependent neutrophil death rates after exposure to culture supernatants from four unique *P. haemolytica* isolates (one from domestic and three from bighorn sheep). Simulated stress effects were evidenced by elevated ($P = 0.002$) mean plasma cortisol concentrations, more neutrophils ($P = 0.037$), and fewer lymphocytes and eosinophils ($P \leq 0.043$) in ACTH-treated bighorn sheep. Maximum leukotoxin-dependent neutrophil death rates were ≥61% for three of four *P. haemolytica* isolates tested. For all three cytotoxic isolates, neutrophil death rates at 150 μg/50 μl supernatant were about 1.13 times higher ($P = 0.0001$) after bighorns received ACTH; for two of these, overall neutrophil death rates were higher ($P \leq 0.001$) in ACTH-treated bighorn sheep. Although variable leukotoxin production among *P. haemolytica* strains appeared principally responsible for differences in leukotoxin-dependent neutrophil death rates, susceptibility of bighorn sheep neutrophils to leukotoxin was increased by prior exposure to elevated plasma cortisol concentrations. It follows that if similar processes occur in neutrophils and alveolar macrophages in vivo, they could contribute to greater susceptibility of stressed bighorn sheep to pneumonic pasteurellosis.

Key words: *Pasteurella haemolytica*, pasteurellosis, adrenocorticotrophic hormone, stress, bighorn sheep, *Ovis canadensis canadensis*, cytotoxin, leukotoxin, ribotype, hematology.

INTRODUCTION

Respiratory disease remains the most serious health problem affecting survival and population dynamics of bighorn sheep (*Ovis canadensis*) populations in the western United States and Canada (Onderka and Wishart, 1984; Festa-Bianchet, 1988; Hobbs and Miller, 1992). *Pasteurella haemolytica* has been identified as a principal cause of pneumonia in wild bighorn sheep throughout their range (Hobbs and Miller, 1992). *Pasteurella haemolytica* can be isolated from most clinically healthy bighorn sheep (Wild and Miller, 1991) and appears to act as both an opportunistic and primary pathogen in bighorns (Miller et al., 1991b).

Pasteurella species are also common commensals of the nasopharynx in a variety of domestic animals, in which they act as opportunistic pathogens (Confer, 1988; Gilmour and Gilmour, 1989). *Pasteurella haemolytica* is particularly invasive due to a soluble leukotoxin released into the area surrounding colonization (Shewen and Wilkie, 1982). This toxin appears specific for ruminant leukocytes and may contribute to strain virulence (Shewen and Wilkie, 1982). The diseases caused by *P. haemolytica* in domestic ruminants are often precipitated by stress and have been well characterized (Frank and Smith, 1983; Gilmour and Gilmour, 1989). Pneumonic pasteurellosis is classically associated with severe physical stress such as shipment, inclement weather, or dietary changes (Frank and Smith, 1983; Gilmour and Gilmour, 1989). Stress or concurrent infection with respiratory viruses apparently enhances proliferation of *P. haemolytica* in the nasal cavity and predisposes the lung to colonization through reduction in bacterial clearance and immunosuppression (Frank et al., 1987). Subsequent inhalation of large numbers of *P. haemolytica* deep into the respiratory tract results in colonization of alveoli, proliferation of bacteria, and production of disease in the susceptible host (Wilkie and Shewen, 1988).

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Similar factors may contribute to pasteurellosis epizootics in bighorn sheep as well (Spraker et al., 1984; Miller et al., 1991b). Based on several accounts of pneumonia epizootics in wild bighorn sheep populations, environmental disturbances sometimes precede disease epizootics (Feuerstein et al., 1980; Spraker et al., 1984, Festa-Bianchet, 1988). Although corticosteroid-induced immunosuppression and accompanying increased susceptibility to infectious agents provide plausible explanations linking environmental stress to pneumonia epizootics in wild bighorn sheep, the mechanisms underlying these explanations remain poorly understood. Our objective was to test the hypothesis that elevated plasma cortisol concentrations stimulated by prior administration of adrenocorticotrophic hormone (ACTH) gel would not alter susceptibility of bighorn sheep neutrophils to *P. haemolytica* leukotoxin.

MATERIALS AND METHODS

We examined the effects of simulated stress on susceptibility of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) neutrophils to *Pasteurella haemolytica* leukotoxin in a blocked, crossover experiment. We used ten captive raised bighorn sheep (four adult females, four lamb females, and two lamb males) from a healthy captive herd housed at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (Fort Collins, Colorado, USA; 40°355′N, 105°10′). Bighorn sheep resided together in 3 to 7 ha pastures before acclimation and sampling periods. Grass/alfalfa hay mix and a pelleted high-energy supplement were provided throughout the study as prescribed under established feeding protocols for bighorn sheep in respective age and sex classes (Miller, 1990); fresh water and mineralized salt blocks were provided ad libitum, and natural forage dominated by buffalo grass (*Buchloe dactyloides*), blue gramma (*Chendrosum gracilis*), and cheatgrass brome (*Bromus tectorum*) was available to pastured bighorns.

We assigned bighorns to pairs by age and sex. On 19 November 1994, we randomly selected one adult female pair and two lamb pairs (one pair female, the other pair male) and placed each pair in a 100 m^2 isolation pen. On the fourth day of acclimation, at about 10 hr prior to sampling, one bighorn from each pair was randomly selected and injected subcutaneously (SC) with ACTH gel (Vedco®, Anthony Products, Arcadia, California, USA; 40 Units/ml; 0.5 units/kg); the other received the same volume of normal (0.9%) saline SC. Five days later, treatment assignments were switched and we repeated the experiment; in previous studies, Miller et al. (1991a) had established that effects of ACTH gel in bighorn sheep lasted <24 hr, and consequently we assumed that a 5 day interval was sufficient to preclude interference from prior ACTH treatment. We replicated this same acclimation, treatment, and sampling regime with the remaining two bighorn sheep pairs beginning 1 December 1994.

Ten hr after treatments were administered, we manually restrained each bighorn sheep and collected about 22 ml of blood via jugular venipuncture; for each individual, <2 min elapsed between initiation of handling and completion of sampling. We added about 20 ml to 5 ml of citrate phosphate dextrose solution (Sigma Chemical Company, St. Louis, Missouri, USA) and placed the remaining 2 ml in ethylendiaminetetracetic acid (EDTA) Vacutainer® tubes (Becton Dickinson and Company, Cockeysville, Maryland, USA). The blood was stored at 4 C for approximately 30 min until plasma was separated and neutrophils isolated. Oropharyngeal and nasal swabs were also collected 10 hr after treatment, placed in transport tubes containing modified Cary and Blair media (Port-a-cul®, BBL Microbiology Systems, Becton Dickinson and Company), and shipped overnight on ice to the University of Idaho's Caine Veterinary Teaching and Research Center (CVTRC) (Caldwell, Idaho, USA).

Blood in citrate phosphate dextrose was centrifuged at 1500 × G for 15 min. Plasma was removed and stored at −20 C until assayed for cortisol. Red cells in the centrifugate were hypotonically lysed by adding 25 ml of 0.001 M sodium phosphate monobasic for 50 sec followed by 12 ml of 0.001 M sodium phosphate and 2.7% sodium chloride to stop the reaction. Following centrifugation at 600 × *G* for 10 min, we repeated the lysis and centrifugation steps. The final pellets were resuspended in Hanks Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island, New York, USA) containing 1% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah, USA). We quantified cells using a hemocytometer (American Optical Corporation, Buffalo, New York) and determined viability by trypan blue exclusion (Boyse et al., 1964). Typical yields were >96% neutrophils, and >92% of these cells were viable. Cells were adjusted to a final concentration of 5×10^8 cells/ml in HBSS and 1% FBS.

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TABLE 1. Genotypic and phenotypic traits of *Pasteurella haemolytica* isolates used as sources of leukotoxin for in vitro assays.

Ribotype	Biotype	Serotype	Hemolysis	Biogroup[a]	Origin	Source
$B_{(O)}$	T	4	−	$4_{(C,D,S)}$	Tarryall Mountains, Colorado	bighorn
$E_{(O)}$	T	10	+	$4_{(C,D,S)}$	Alamosa Canyon, Colorado	bighorn
$AA_{(O)}$	A	6, 8, 9, 11, 12	+	$16_{(E)}$	Waterton Canyon, Colorado	bighorn
WSU-1	A	2	+	$1_{(G)}$	Washington State University	domestic sheep

[a] Biogroup variants classified based on Caine Veterinary Teaching and Research Center-modified criteria of Bisgaard and Mutters (1986).

Four distinct strains of *P. haemolytica* (Table 1) were used as sources of leukotoxin. Leukotoxins were isolated from crude culture supernatant beforehand using methods described by Shewen and Wilkie (1982). Briefly, individual *P. haemolytica* isolates were streaked onto 5% sheep blood agar plates (Becton Dickinson Microbiological Systems) and incubated for 18 hr at 37 C. Several morphologically similar colonies were used to inoculate 100 ml of brain-heart infusion broth (Difco Laboratories, Detroit, Michigan, USA) and incubated at 37 C. Logarithmic phase growth was determined by an optical density reading of 1 at 600 nm using a model U-1000 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Bacteria were centrifuged at 6,000 × *G* for 10 min, and culture supernatant was removed and filter sterilized in a 0.22 μm filter (Sigma Chemical Company). Culture supernatants were dialyzed to exhaustion against distilled water using dialysis tubing with pore size of 6,000 to 8,000 molecular weight (Spectrum Medical Industries, Inc., Los Angeles, California). Remaining supernatant was then lyophilized and stored at −70 C until used in the leukotoxin assay.

Neutrophils from ACTH-treated and control bighorn sheep were incubated with culture supernatants from each of the four *P. haemolytica* isolates using methods modified from Silflow et al. (1993), as follows. We resuspended lyophilized bacterial supernatant in HBBS and 1% FBS at concentrations of 0.5, 5, 25, 50, 100, and 150 μg/50 μl. We added 50 μl of each supernatant preparation containing leukotoxin to 96-well plates (Corning Glass Works, Corning, New York), and followed by adding 2.5×10^5 neutrophils in 50 μl of HBBS and 1% FBS to each well. After 1 hr incubation at 37 C, we added 100 μl of lactate dehydrogenase substrate to quantitate cytotoxicity of bacterial supernatants (Korzeniewski and Callewaert, 1983). Quantification of reduced LDH substrate was measured on a model 450 96-well plate reader (Biorad Labs, Hercules, California). All samples were compared to neutrophils treated with 0.05% Triton® detergent (Sigma Chemical Company) (maximal release) and to untreated cells (background release). Results were recorded as a percentage of LDH released from treated cells. Although we recognize that dialyzed culture supernatants used in this assay contained some amount of endotoxin, we assumed that the vast majority of observed cytotoxicity was caused by *P. haemolytica* leukotoxin (Confer et al., 1990).

Select hematological parameters were assessed on EDTA-treated blood at Colorado State University's Clinical Pathology Laboratory (Fort Collins, Colorado, USA). Total white blood cell counts ($\times 10^3$ cells/mm^3) were made using electrical impedance (ZBI, Coulter Electronics, Hialeah, California); differential counts were estimated manually, packed cell volumes (PCV) (%) were measured by microhematocrit centrifugation, and total protein concentrations (mg/dl) were estimated by refractometry (Jain, 1993). Plasma cortisol concentrations were measured by the Endocrine Laboratory, Department of Physiology, Colorado State University using an extracted double-antibody radioimmunoassay previously described and validated for use in bighorn sheep (Miller et al., 1991a); results were reported in ng/ml.

At CVTRC, oropharyngeal and nasal swabs were plated on both Columbia blood agar (CBA) (Difco Laboratories, Detroit, Michigan, USA) with 5% citrated ovine blood and selective plates containing Columbia blood agar with 5% citrated bovine blood and antibiotics selective for *Pasteurellaceae* (Ward et al., 1986). Suspected *P. haemolytica* colonies were selected after 48 hr incubation at 37 C in 5% CO_2 and further propagation was carried out on CBA. Plates were examined for hemolysis. Identities and biochemical profiles of colonies yielding Gram-negative rods or coccobacilli that fermented triple sugar iron, glucose, and sucrose were further examined to determine species and biogroup identifications (Kilian and Frederiksen, 1981; Bisgaard and Mutters, 1986). The biogrouping schema of Bisgaard and Mut-

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TABLE 2. Mean (standard error) plasma cortisol concentrations, packed cell volumes, protein concentrations, and differential white blood cell counts in ACTH-treated bighorn sheep as compared to untreated controls.

	Control		ACTH-treated	
	Lamb (n = 6)	Adult (n = 4)	Lamb (n = 6)	Adult (n = 4)
Cortisol (ng/ml)	9.5 (0.7)[b]	7.3 (2.7)	7.5 (2.6)	42.4 (9.0)[c]
Packed Cell Volume (%)	48.5 (1.0)	48.3 (1.4)	48.2 (1.5)	43.0 (9.0)
Protein (mg/dl)	6.8 (0.3)	7.4 (0.2)	6.7 (0.2)	6.7 (0.2)
Total White Blood Cells[a]	8.3 (0.8)	9.3 (1.9)	10.7 (1.4)	8.1 (2.6)
Neutrophils[a]	4.2 (0.6)	6.4 (2.0)	7.8 (1.1)[c]	8.8 (1.3)[c]
Lymphocytes[a]	3.6 (0.3)	2.3 (0.2)	2.4 (0.6)[c]	1.4 (0.3)[c]
Monocytes[a]	0.2 (0.1)	0.5 (0.2)	0.5 (0.1)	0.3 (0.1)
Basophils[a]	0.03 (0.02)	0.03 (0.03)	0.05 (0.03)	0 (0)
Eosinophils[a]	0.2 (0.1)	0.2 (0.1)	0.03 (0.03)[c]	0 (0)
Platelets[a]	359.0 (35.8)	265.3 (13.0)	356.3 (45.4)	317.8 (17.2)

[a] Values are $\times 10^3$ cells/mm^3 of blood.
[b] Values are mean (standard error).
[c] Values differ ($P \leq 0.05$) from control values.

ters (1986) was modified by the CVTRC to allow the use of negative test results to separate isolates into additional biogroups. Select isolates identified as *P. haemolytica* were further characterized by serotype using rapid plate agglutination (Frank and Wessman, 1978).

We compared neutrophil death rates, plasma cortisol concentrations, and white blood cell count data using least squares analysis of variance (ANOVA) for general linear models (SAS Institute, Inc., 1989). Differences in neutrophil susceptibility to *P. haemolytica* leukotoxin were analyzed with ANOVA for a randomized complete block design with a repeated measures structure. We used treatment (ACTH vs. control) and sequence of treatment (control followed by ACTH vs. ACTH followed by control) as main effects and combined as an interactive term; individual animal nested within a sequence was used as the error term in hypothesis tests related to sequence. Varying doses of leukotoxin (0.5 to 150 μg) were treated as a within subject effect using a multivariate approach to repeated measures (Morrison, 1976). We compared plasma cortisol concentrations and white blood cell count data post hoc by analysis of variance using treatment (ACTH vs. control), age, and sequence of treatment as main effects and combined as interactive terms. Fisher's exact probability tests (Mielke and Berry, 1992) were used in comparing *P. haemolytica* isolation rates between ACTH-treated and control bighorns. For all comparisons, we used $\alpha = 0.05$ in assessing statistical significance.

RESULTS

Simulated stress effects were evidenced by elevated ($P = 0.002$) mean plasma cortisol concentrations in ACTH-treated bighorns, although age (Table 2) and age × treatment interactions also affected cortisol concentrations 10 hr after ACTH treatment ($P \leq 0.003$). White blood cell counts from ACTH-treated sheep were typical stress leukograms, characterized by increased neutrophil numbers ($P = 0.037$) and decreased numbers of lymphocytes and eosinophils ($P \leq 0.043$) compared to differential counts of controls (Table 2); age ($P = 0.01$) and age x sequence interactions ($P = 0.046$) also affected lymphocyte numbers.

Culture supernatants from three of the four *P. haemolytica* isolates (E_{CO}, AA_{CO}, WSU-1) caused neutrophil death rates ≥61% at 150 μg supernatant irrespective of ACTH treatment (Fig. 1B, C, D). For all three cytotoxic isolates, neutrophil death rates at 150 μg/50 μl supernatant were about 1.13 times higher ($P = 0.0001$) after the bighorn sheep received ACTH (Fig. 1B, C, D); for two of these (E_{CO}, WSU-1), overall neutrophil death rates were higher ($P \leq 0.001$) in ACTH-treated bighorn sheep (Fig. 1B, D). Sequence x treatment interaction affected neutrophil death rates for isolate AA_{CO} ($P = 0.019$), but not for the three other isolates ($P \geq 0.193$). We detected no difference (P =

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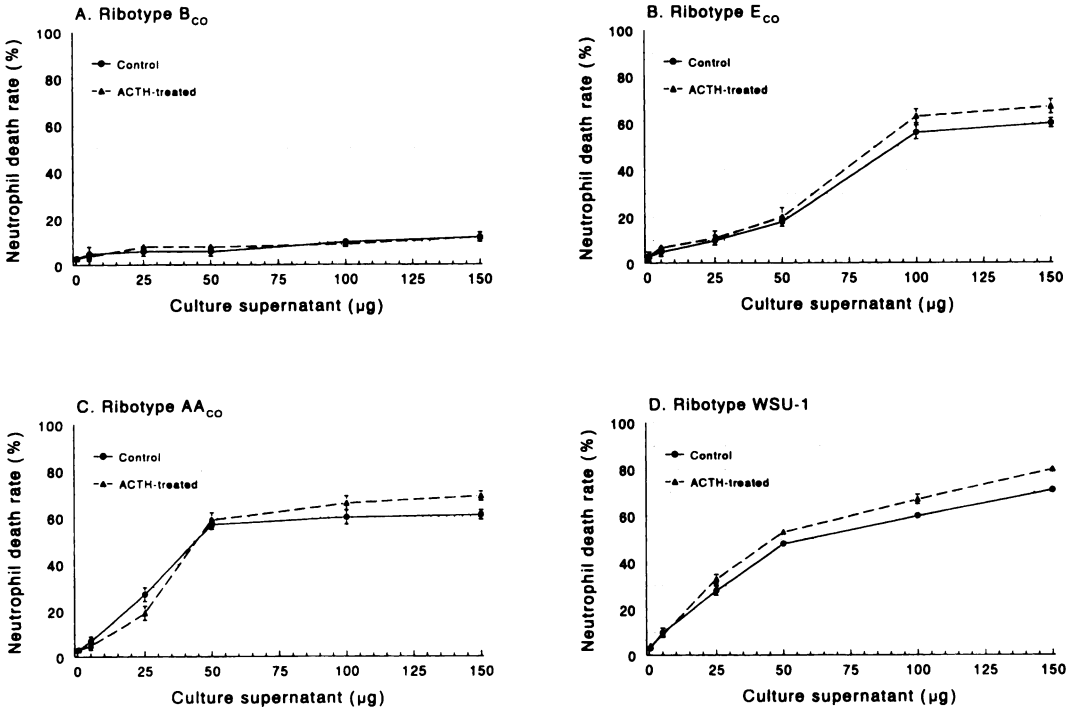

FIGURE 1. A. Culture supernatant from *Pasteurella haemolytica* isolate B_{CO} had virtually no in vitro leukotoxic effects on neutrophils from either control or ACTH-treated bighorn sheep. B, C, D. In contrast, supernatant from the other three *P. haemolytica* isolates (E_{CO}, AA_{CO}, WSU-1) caused marked in vitro leukotoxicity. Moreover, neutrophil death rates at 150 μg/50 μl were about 1.13 times higher ($P = 0.0001$) after bighorn sheep received ACTH, and for two of these (E_{CO}, WSU-1) overall neutrophil death rates were higher ($P \leq 0.001$) in ACTH-treated bighorn sheep. Points represent mean observations; vertical bars represent ± 1 standard error of mean observations.

0.794) between ACTH-treated and control bighorn sheep in neutrophil losses after exposure to supernatant from the fourth isolate (B_{CO}) at any concentration (Fig. 1A).

Thirteen distinct biogroups of *P. haemolytica* were identified from oropharyngeal and nasal swabs (Table 3). None of these profiles matched biogroup profiles of the four isolates used in leukotoxin assays. We detected no differences between isolation rates from ACTH-treated and control bighorns ($P \geq 0.3$).

DISCUSSION

Bighorn sheep neutrophil susceptibility to three of four *P. haemolytica* strains' leukotoxin-mediated lysis was increased by prolonged stress responses stimulated using repositol ACTH. Among the three isolates where neutrophil responses were influenced by treatment (E_{CO}, AA_{CO}, WSU-1), effects tended to be most evident at higher concentrations of supernatant. These three isolates appeared to be inherently leukotoxic (Silflow et al., 1993), with 50% lethal concentrations (LC_{50}) of 39 to 93 μg and maximum killing rates ≥61% in control neutrophils exposed to 150 μg culture supernatant; moreover, at least two of these (E_{CO}, WSU-1) can apparently serve as primary pathogens in bighorn sheep (Foreyt et al., 1994; B. J. Kraabel, unpubl.). We believe the sequence effect seen with isolate AA_{CO} was most likely due to some form of assay error at the 25 μg or 50 μg level (Fig. 1C) because such effects were not evident ($P \geq 0.19$) among the other isolates; this may also explain our failure to demonstrate an overall treatment

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TABLE 3. Biogroup-specific and overall recovery rates for *P. haemolytica* isolated from ACTH-treated and control bighorn sheep.

Biotype	Serotype	Biogroup	Frequency isolated	
			Control	ACTH-treated
A	ut[a]	$1_{(-A)}$	0 (0)[b]	1 (0)
A	1	7	2 (0)	2 (0)
A	11	$9_{(-B,L)}$	1 (0)	1 (1)
A	ut	$10_{(-A)}$	2 (0)	0 (0)
A	ut	16	0 (0)	0 (1)
A	ut	$16_{(-A,E)}$	0 (0)	2 (0)
A	11	$16_{(-A,G)}$	0 (1)	0 (1)
A	1,2,5,6,7,8,9,11	$16_{(-A,B,E)}$	5 (0)	4 (0)
A	ut	$U_{(-A,L)}$	1 (0)	0 (0)
A	1,2,5,6,7,8,9,11	$U_{(-B,E,X)}$	1 (0)	1 (0)
A	1	$U_{(-B,E,L,X)}$	0 (0)	1 (2)
T	3,4,10	2	5 (2)	7 (2)
T	ut	$2_{(-C,D)}$	0 (0)	1 (0)
Total			17 (3)	20 (7)

[a] ut = untypable.

[b] Values represent numbers of isolates recovered from oropharyngeal and (nasal) swabs.

effect for isolate AA_{CO}. The fourth isolate (B_{CO}), where neutrophil susceptibility was not affected by simulated stress at any level, had much lower inherent leukotoxicity (LC_{50} far above 150 μg, maximum killing rate 12% at 150 μg culture supernatant). These observations lend support to hypotheses that inherent differences in leukotoxicity (and potential pathogenicity) among strains of *P. haemolytica*, rather than environmental stressors, may be driving forces in the epizootiology of pasteurellosis in wild bighorns (Miller et al., 1991b; Hobbs and Miller, 1992; Miller et al., 1995).

There are several plausible explanations for why treatment effects were most evident at higher supernatant concentrations and in more leukotoxic strains. The mechanism underlying these effects may be leukotoxin-dose dependent, just as the toxicity of *P. haemolytica* leukotoxin itself is dose dependent (Confer et al., 1990). Alternatively, observed differences in leukotoxicity could have been caused by increased susceptibility to endotoxin rather than leukotoxin, although *P. haemolytica* lipopolysaccharide appears nontoxic to bovine leukocytes in vitro (Confer and Simmons, 1986). Finally, because this assay is relatively insensitive at low concentrations of supernatant (or leukotoxin), subtle differences may have been undetectable.

Although the concentration of leukotoxin released in vivo during *P. haemolytica* replication has not been measured, the highest concentration of supernatant (150 μg/50 μl) used in our study approximated leukotoxin concentrations produced by cultures growing at log-phase (Shewen and Wilkie, 1982). Moreover, alveolar macrophages in bovine lung explants died at about the same rates when explants were seeded with *P. haemolytica* as when explants were treated with leukotoxin concentrations in the range of those used in our study (Wilkie et al., 1990). Consequently, we believe that the supernatant concentrations used here realistically represented the range of leukotoxin concentrations that may occur in lung tissue during *P. haemolytica* proliferation associated with respiratory disease in bighorn sheep.

The observation that ACTH administration and resulting elevated plasma cortisol concentrations increased bighorn sheep neutrophil susceptibility to *P. haemolytica* leukotoxin lends credence to hypotheses

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that stress may play some role in pneumonic pasteurellosis in bighorn sheep (Hudson, 1972; Spraker et al., 1984; Onderka and Wishart, 1984; Festa-Bianchet, 1988; Miller et al., 1991b). In previous accounts of pneumonia epizootics in bighorn populations, workers have sometimes suggested that human or environmental disturbances preceded epizootics (Feuerstein et al., 1980; Spraker et al., 1984; Festa-Bianchet, 1988), although stress-induced pasteurellosis in bighorns appears difficult to reproduce experimentally (Miller et al., 1991b). We believe our experimental treatment simulated at least some of the conditions that occur in chronically stressed bighorns: cortisol levels elicited by repositol ACTH in our study approximated those reported for stressed captive bighorns (Harlow et al., 1987) and for free-ranging bighorns sampled during a stress-related pasteurellosis epizootic (Spraker et al., 1984). Based on our data, bighorn sheep neutrophils were more susceptible to *P. haemolytica* leukotoxin under conditions simulating prolonged stress. This observed increase in susceptibility could be a product of direct cortisol-mediated effects of neutrophils, differential susceptibility of marginal pool neutrophils mobilized by elevated plasma cortisol, or some combination of these. If neutrophils and alveolar macrophages are also more susceptible to leukotoxin in stressed bighorns in vivo, it follows that development or severity of pneumonic pasteurellosis may be more likely in such individuals.

It is possible that stress-mediated effects were even more pronounced than we observed: effects of elevated cortisol on neutrophils could have been waning 10 hr after repositol ACTH administration when we collected samples for in vitro neutrophil assays. In a previous study, Miller et al. (1991a) demonstrated that plasma cortisol concentrations in ACTH-treated adult bighorn sheep (0.5 U/kg) remained elevated $\geq$ 8 hr, but were normalized by 24 hr after administration of ACTH gel. Based on our data, however, the ACTH gel may have had a shorter effect on plasma cortisol concentrations in bighorn lambs than in adults. Despite rather low mean plasma cortisol concentrations observed in lambs 10 hr after treatment, other data supported our assumption that cortisol levels had been elevated by ACTH administration: although ACTH-treated and untreated lambs had essentially equivalent cortisol levels, treated lambs had stress leukograms that were likely a result of ACTH treatment (Table 2).

In vitro analyses of *P. haemolytica* leukotoxin production have increased understanding of bighorns' potential susceptibility to pasteurellosis (Silflow et al., 1993). Comparing leukotoxicities of different *P. haemolytica* isolates has also provided insights into the epizootiology of pasteurellosis in wild bighorn populations (Silflow and Foreyt, 1994; Miller et al., 1995). However, we believe such data should be used with the assay's limitations in mind. If neutrophils from wild or unduly stressed captive bighorn sheep are used, some increase in apparent leukotoxicity can be expected. Moreover, this assay lacks the sensitivity to detect small differences between slightly pathogenic strains. In light of these limitations, it's best use may be a tool for identifying highly leukotoxic and potentially pathogenic strains of *P. haemolytica* among isolates from wild bighorn sheep (Silflow et al., 1994).

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