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Bighorn Sheep (*Ovis canadensis*) with Higher Whole Blood Selenium Levels Have Improved Survival and Altered Immune Responses

Dana Y. Tsuchida,[1,5] Morgan F. Gentzkow,[1,5] Robert S. Spaan,[1,2] Julia Burco,[4] Claire E. Couch,[1,2] Johannie M. Spaan,[3] Clinton W. Epps,[2] and Brianna R. Beechler[1,6] [1]Carlson College of Veterinary Medicine, Oregon State University, 700 SW 30th Street, Corvallis, Oregon 97331, USA; [2]Oregon State University, Department of Fisheries, Wildlife and Conservation Sciences, 2820 SW Campus Way, Corvallis, Oregon 97331, USA; [3]Western University of Health Sciences, 200 Mullins Drive, Lebanon, Oregon 97355, USA; [4]Oregon Department of Fish and Wildlife, Wildlife Health and Population Lab, 7118 NE Vandenberg Avenue, Adair Village, Oregon 97330, USA; [5]These authors contributed equally; [6]Corresponding author (email: brianna.beechler@oregonstate.edu)

ABSTRACT: Bighorn sheep (*Ovis canadensis*) are herbivorous ungulates that live in forage-poor areas of the American west. The trace minerals that herbivores derive from forage are important for immune function. Therefore, identifying trace minerals that affect immune function in bighorn sheep could provide important insights into disease susceptibility and population health in threatened populations. We sought to determine whether trace mineral composition in blood or plasma correlates to survival and determine whether immunologic parameters correlate with any trace minerals that affect survival. We used data collected from 2016 to 2018 as part of a large study on bighorn sheep in southeastern Oregon and northern Nevada, US. We measured the survival of 135 bighorn sheep during the 8-mo monitoring period, including general metrics of immune function and trace mineral levels. We found that animals with higher selenium had improved survival over the monitoring period, with higher peripheral blood mononuclear cell activity (lymphocytes and monocytes) and lower bacterial killing ability in an in vitro assay. This suggests that bighorn sheep may have altered immune function when selenium levels are low, making them more likely to die during the 8-mo monitoring period. Future work should consider whether habitat management strategies that increase selenium intake might improve disease resistance and survival in bighorn sheep in selenium-poor areas.

Key words: Ecoimmunology, micronutrient, nutrition, wildlife.

Bighorn sheep (*Ovis canadensis*) are herbivorous ungulates that depend on seasonally and spatially variable forage for micronutrients (Wagler et al. 2023) and energy acquisition (e.g., Blanchard et al. 2003; Stephenson et al. 2020). Trace minerals are critical for maintaining rumen health, hormone regulation, bone formation, and numerous other physiologic functions. Selenium influences both innate and acquired immunity, although the mechanisms are not fully understood (Arthur et al. 2003). Bighorn sheep are facing severe threat from *Mycoplasma ovipneumoniae* and other pathogens across the range; understanding how forage-derived micronutrients influence disease susceptibility is important for conservation and management efforts.

Selenium concentration in plants is primarily driven by the availability of selenium in the soil (Trippe and Pilon-Smits 2021), which is largely determined by geologic processes (Flueck et al. 2012). Selenium is particularly low in the soils of northwestern US (National Research Council 1983). Therefore, understanding the effects of selenium deficiency on bighorn sheep populations in this region may be important.

We investigated whether blood selenium and other trace minerals predicted survival of bighorn sheep and whether selenium levels correlated to immune function. The study area falls within the Northern Basin and Range, southeastern Oregon and northern Nevada, US (Fig. 1), consisting of high lava plains, dissected high lava plateau and semiarid uplands. Soils in this region often do not support adequate selenium uptake by plants (Poole et al. 1994). Soil chemistry and forage composition vary across our study system (Poole et al. 1994; Spaan et al. 2021); providing an excellent context in which to study the effects of micronutrients on survival and immunity. We studied the relationship between survival and selenium in this system because it is selenium poor, and mortality has been well described. Adults are more likely to suffer mortality when they are male, have lower genetic diversity, and have been exposed to *M. ovipneumoniae* (Spaan 2022), while juvenile survival is decreased by the presence of *M. ovipneumoniae* (Spaan et al. 2021) in the population and is increased by the metabolic condition of the ewe raising the lamb (Laliberte et al. 2023).

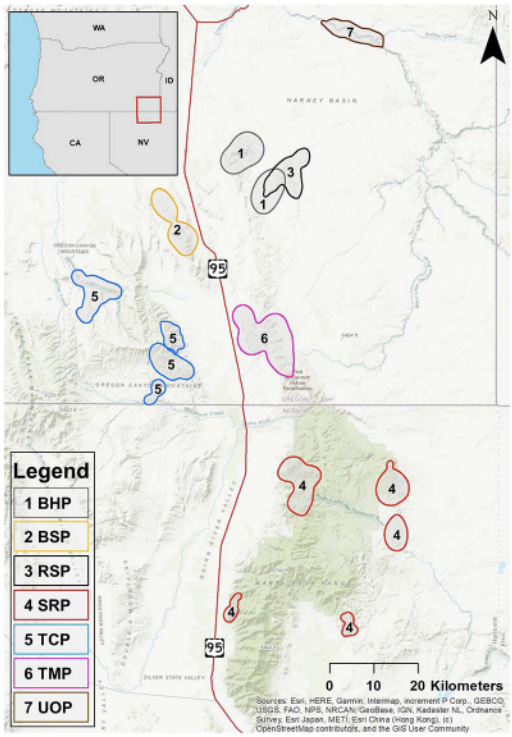

FIGURE 1. Map of the northwestern USA showing the locations of the six study populations of bighorn sheep (*Ovis canadensis*) in southeastern Oregon (OR) and northern Nevada (NV). Polygons in the main map for each population indicate female bighorn sheep 95% summer utilization distributions from Spaan (2022). Bighorn sheep were from the Bowden Hills population (BHP; n=3), Blue Mountain population (BSP; n=10), Rattlesnake population (RSP; n=31), Santa Rosa population (SRP; n=27), Trout Creek population (TCP; n=46), Ten Mile population (TMP; n=10), and the Upper Owyhee population (UOP; n=8).

We focused on six populations of bighorn sheep in the study region, all of which are routinely monitored as part of Oregon Department of Fish and Wildlife (ODFW) and Nevada Department of Wildlife (NDOW) management strategies. Bighorn sheep were captured and sampled during the winter and monitored for survival during the spring and summer, thus allowing us to estimate relationships between micronutrients and immune parameters at the time of capture and survival postcapture.

Between January 2016 and February 2018, ODFW and NDOW captured, collared, and sampled 135 adult bighorn sheep across six populations (Fig. 1) in southeastern Oregon and northern Nevada. Of the 135 individuals, 77 were female (2–11 yr old), and 58 were male (1–10 yr old). Captures were conducted using a net gun fired from a helicopter, with individual bighorn sheep blindfolded and hobbled once captured. The capture and research protocols were reviewed by the Oregon State University Institutional Animal Care and Use Committee and considered exempt from review (protocol no. EFIR16-08) because they were management activities by ODFW and NDOW and no additional animal work was occurring for this research. Captures were performed following standard procedures (Sikes 2016). Captured individuals were brought to a centralized area at the base of the range to be fitted with a telemetry collar and to collect biologic samples. Bighorn captured too far from base camp for rapid transport were processed at the capture location. Each adult was fitted with a satellite collar (Vertex Survey Globalstar collar, Vectronic Aerospace, Berlin, Germany) that was set to report a mortality if stationary for more than 12 h. Age was estimated from horn growth rings (Geist 1966). Blood was collected from each individual by jugular venipuncture with a sterile 18-gauge luer-lock needle attached to a 50-mL syringe and transferred into plain and trace element blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The samples were stored on ice for transport to the field laboratory, and processing occurred within 12 h of collection.

At the field laboratory, whole blood in plain tubes was centrifuged at 5,000 × G for 10 min to separate serum. Both serum and whole blood samples were stored in sterile cryotubes on liquid nitrogen until processing. The trace mineral panel (including cobalt, copper, iron, manganese, molybdenum, selenium, and zinc) was performed at Michigan State University Diagnostic Center for Population and Animal Health (Lansing, Michigan, USA), with serum samples for all parameters, except selenium when whole blood was used. Outliers that were biologically implausible were removed before statistical analysis, resulting in exclusion of one sample for whole blood selenium

(Poppenga et al. 2012). Full mineral data were analyzed for 95 individuals.

Immunoassays were performed at the field site on the day of collection. We used the Leuko-TIC test kit (Medix Corporation, Newbury Park, California, USA) to count total white blood cells (WBC); differential WBC counts were done manually from blood smears (Dugovich et al. 2023). We measured the bacterial killing ability (BKA): the ability of whole blood to kill a laboratory strain of bacteria, reflecting neutrophil and complement activity (Demas et al. 2011). The BKA was performed following methods described in Delgadillo et al. (2021), optimized for use with bighorn sheep whole blood. Briefly, *Escherichia coli* (laboratory strain ATCC 8739, Microbiologics, Minneapolis, Minnesota, USA) were incubated with whole blood diluted 1:128 in CO_2 Independent Medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 h at 37 C in a 96-well cell culture plate. Next, 125 µL of 3% tryptic soy broth (Sigma-Aldrich, St. Louis, Missouri, USA) was added to all wells, and the absorbance was measured at 300 nm using an Epoch spectrophotometer (Agilent Technologies, Santa Clara, California, USA). Plates were incubated at 37 C until a reading was taken at 12 h, and percent killing was calculated as described (Dugovich et al. 2023). We measured the functional ability of peripheral blood mononuclear cells (PBMC; i.e., lymphocytes and monocytes) cultured in vitro to undergo clonal proliferation and activation when stimulated by the pokeweed mitogen (Sigma-Aldrich) in vitro, using the protocol described by Dugovich (2019). Briefly, we cultured isolated PBMC for 48 h with pokeweed, in duplicate, with duplicate control wells that did not contain a mitogen. After 48 h, we added Alamar Blue dye (Sigma-Aldrich) to detect cell metabolic activity and then read the optical density using an Epoch spectrophotometer (Agilent Technologies). We calculated the proportional change in PBMC activity as the following: (absorbance of stimulated−absorbance of control)/absorbance control. Outliers that were biologically implausible were removed prior to statistical analysis, resulting in exclusion of one sample for the WBC parameters and four values for PBMC activity.

Statistical analyses were performed in GraphPad Prism 9 (GraphPad Software 2023) and program R (R Core Team 2022). To assess links between survival and trace minerals, we used a multiple logistic regression with a binomial response variable of survival (did the individual survive 8 mo postcapture: yes or no) and rescaled predictor variables for each trace mineral in the panel, with random intercepts for age and sex. We chose an 8-mo monitoring period to end before winter, when foraging patterns may change. Trace minerals did not show any significant pattern with population (generalized linear model, each mineral on population), so population was not included in the model. None of the trace minerals had a correlation value greater than 0.5 with each other, so we included all of them in the full model (Hosmer and Lemeshow 2000). We did not include interaction terms in the full model, as we had no biologic reason to do so. Model selection was performed based on Akaike information criteron (corrected) statistic (AICc) values using the dredge function in the MuMIn package (Burnham and Anderson 2002; Bartoń 2017), and AICc weights were calculated for each variable across all 128 possible models to evaluate variable importance. The purpose of the AICc-based model selection was to compare the relative predictive ability of different combinations of trace minerals, not to test hypotheses.

We subsequently evaluated correlations (Pearson R correlation) between whole blood selenium and each immune parameter, including WBC differentials (neutrophils, lymphocytes, monocytes, and eosinophils, n=67), BKA (n=58), and PBMC activity (n=51).

Selenium and copper levels varied in the individuals included in the study, with selenium ranging from 0.034 to 0.66 parts per million (ppm; mean 0.1474 ppm; SEM 0.00968 ppm) and copper ranging from 0.5 to 1.55 ppm (mean 0.7323 ppm; SEM 0.01637 ppm). Fifteen mortalities were recorded during the 8-mo monitoring period; of those, 42% died within 1 mo postcapture, 16% died 1–4 mo postcapture,

TABLE 1. Parameter values for the top 10 best fitting logistic regression models, based on Akaike information criterion (corrected) statistic (AICc), of bighorn sheep (*Ovis canadensis*) mortality versus blood mineral levels, as well as cumulative AICc weights, during an 8-mo postcapture monitoring period for bighorn from six populations in southeastern Oregon and northern Nevada, USA.

Model ranking	Cobalt	Copper	Iron	Manganese	Molybdenum	Selenium	Zinc	Δ AICc
1	—[a]	0.444	—	—	—	−1.535	—	0
2	—	—	—	—	—	−1.317	—	0.21
3	—	—	—	—	—	−1.421	0.386	0.73
4	—	0.552	—	−2.047	—	−1.448	—	0.79
5	—	0.554	—	—	−0.416	−1.204	—	1.75
6	—	—	—	−1.909	—	−1.298	0.492	1.79
7	−0.462	—	—	—	—	−1.214	—	1.86
8	−0.346	0.442	—	—	—	−1.411	—	1.86
9	—	—	—	−1.078	—	−1.219	—	1.86
10	—	0.342	—	—	—	−1.541	0.187	2.00
Cumulative parameter AICc weights	0.29	0.49	0.24	0.38	0.32	0.82	0.36	—

[a] — = parameter was not included in the model.

and 42% died 4–8 mo postcapture. The best fit model for survival included only copper (estimate=0.444; P=0.124) and whole blood selenium (estimate=-1.534; P=0.021). There was equivalent support for models that included additional minerals and that did not include copper; however, whole blood selenium was included in all the best fitting models and was negatively associated with mortality (Table 1 and Fig. 2). Blood selenium had a cumulative AICc weight (0.82), far higher than the next best predictor (copper=0.49; Table 1). There was no correlation between whole blood selenium and neutrophils (r=0.0411; P=0.741), monocytes (r=0.0417; P=0.7374), or eosinophils (r=-0.0774; P=0.533). Selenium was marginally negatively correlated with BKA (r=-0.249; P=0.0593) and marginally positively correlated with PBMC activity (r=0.268; P=0.0572).

Overall, we found that whole blood concentration of selenium in bighorn sheep was positively correlated to the survival probability during an 8-mo postsampling monitoring period. In addition, animals with higher selenium levels had stronger lymphocyte and monocyte responses to in vitro stimulation with a mitogen and reduced BKA, suggesting that selenium levels may affect immune function.

Selenium provides essential support for metabolism, antioxidant activity, and immunity in mammals (Gill and Walker 2008), and selenium deficiency influences the health of numerous wild herbivores (Flueck et al. 2012). Previous research in bighorn sheep has suggested that mineral deficiencies, particularly selenium, might play a role in population declines (Dean et al. 2002; Carpenter 2005; Coggins 2006). Our study has identified immunity as a potential mechanism by which this could occur. In humans and model organisms, selenium status is known to influence the function of numerous immune system components, including neutrophil activity, T-cell proliferation and differentiation, and antibody responses (Gill and Walker 2008). Selenium is associated with improved lymphocyte performance in humans, as measured by mitogen-induced proliferation (Kiremidjian-Schumacher and Roy 2001). The immune-enhancing effects of selenium have been demonstrated in domestic sheep (*Ovis aries*), where higher whole blood selenium is associated with improved humoral and innate immune responses and reduced susceptibility to foot rot (Hall et al. 2011). Our study indicates that selenium plays a role in PBMC function in bighorn sheep, supporting the role of selenium as an important environmental driver

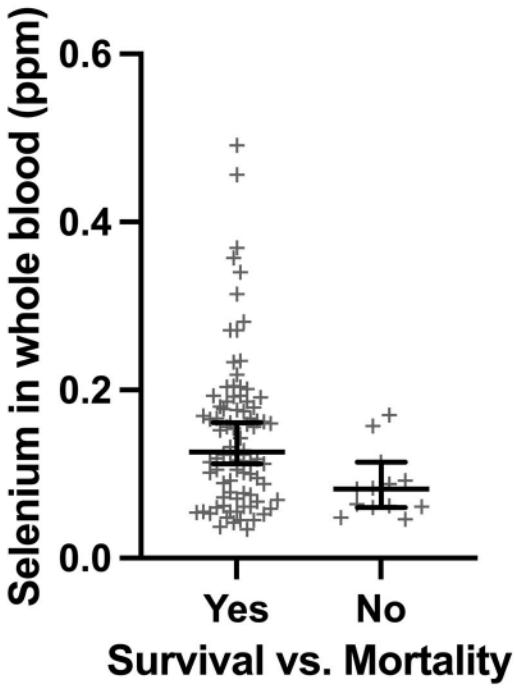

FIGURE 2. Scatter plot showing whole blood selenium levels in bighorn sheep (*Ovis canadensis*) from six populations in southeastern Oregon and northern Nevada, USA, which survived an 8-mo postcapture monitoring period, compared with those who did not survive. The median value in each group is indicated by the solid black line, the 95% confidence intervals are black, and each data point is a gray cross.

of bighorn sheep population health. Therefore, improving selenium uptake of bighorn sheep in selenium-poor regions might improve survival and disease resistance.

Future work should consider selenium availability in forage and how that may vary seasonally and measure long-term intake patterns of forage in bighorn sheep to understand the time frame at which selenium concentration is relevant to mortality risk. Studying the relationships between forage composition and selenium availability might lead to habitat management strategies for indirectly improving selenium uptake, such as improving connectivity to facilitate foraging or managing for growth of forage that concentrates selenium (Flueck et al. 2012). Additional research exploring the role of selenium in the broader context of bighorn sheep immunity and physiology is needed to clarify the importance of this micronutrient for population health, including whether it is relevant to disease susceptibility and severity.

Captures, collaring, and sampling of bighorn sheep were conducted by the Oregon Department of Fish and Wildlife (ODFW) and Nevada Department of Wildlife (NDOW). We thank Phillip Milburn, Scott Torland, and Autumn Larkins of ODFW for helping initiate this project. We also thank Ed Partee and Peregrine Wolff of NDOW for support when working with the bighorn sheep that were located on the Nevada side of the metapopulation. Field technicians Geoff Gerdes, Logan Gmuender, Lindsey Howard, and Colton Padilla were essential in the collection of data.

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