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Effects of Selenium Supplementation and Sample Storage Time on Blood Indices of Selenium Status in Bighorn Sheep

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ABSTRACT: Periodic pneumonia outbreaks cause large-scale die-offs that threaten the viability of bighorn sheep (*Ovis canadensis*) populations. Bighorns are highly susceptible to pneumonia, and in some cases this susceptibility may be exacerbated by trace mineral deficiencies. To evaluate responses to injectable selenium supplementation, eight captive bighorn sheep were treated with either an injectable sodium selenite supplement or a saline control. We collected 6-ml blood aliquots before and at 1, 6, and 12 wk posttreatment. We submitted one set of aliquots immediately to measure selenium (Se) and zinc (Zn) concentrations and glutathione-peroxidase (GSH-Px) activity; additional aliquots were held at about 22 C and then submitted at 1, 3, and 7 days postcollection to assess effects of storage on these measures. Neither Se nor GSH-Px were affected by selenite injections. Both Se and GSH-Px demonstrated small linear decays over the 7-day storage period (0.011 ppm/day [SE=0.0027] and 15.78 mmole/l/sec/day [SE=6.88], respectively); in contrast, Zn concentrations in stored samples increased logarithmically (0.35 ppm/day on the natural log scale). Blood Se and GSH-Px were not correlated in sampled bighorns; however, because all values for both measures were within normal limits, lack of correlation did not affect interpretation of these data in our study.

Key words: Bighorn sheep, glutathione-peroxidase, *Ovis canadensis*, sample handling, selenium.

Bighorn sheep (*Ovis canadensis*) populations have experienced substantial declines since the 1800s (Packard, 1946; Buechner, 1960). Although numerous factors have been linked to these declines, disease has been implicated as playing a major role in the waning bighorn sheep numbers (Mills, 1937; Marsh, 1938; McCann, 1956; Berger, 1990). Periodic pasteurellosis epizootics that cause large-scale die-offs pose the most significant threat to the viability of bighorn sheep populations (Gross et al., 2000; Singer et al., 2000; McClintock and White, 2007). Additionally, postepizootic recruitment is often depressed compared to historical values (McClintock and White, 2007; George et al., 2008), and low lamb recruitment in many bighorn herds limits their ability to recover from epidemics. The extreme susceptibility of bighorn sheep to pasteurellosis is partially explained by the high susceptibility of their neutrophils to *Mannheimia* (*Pasteurella*) *haemolytica* cytotoxin damage; however, this disease process is complex and incompletely understood (Silflow and Foreyt, 1994).

In domestic sheep (*Ovis aries*), selenium (Se), coupled with vitamin E, is important for proper immune function (Rooke et al., 2004). Selenium is a key component of glutathione-peroxidase (GSH-Px), an antioxidant enzyme also used as a marker for Se deficiency (Wilson and Judson, 1976). Of potential relevance to problems seen in bighorn sheep, signs of mild Se deficiency in domestic sheep include poor reproductive success and reduced immune response that can contribute to susceptibility to pneumonia. Severe Se deficiency can cause nutritional muscular dystrophy (NMD) or "white muscle disease" (WMD), as well as sudden death (Radostits et al., 2000). Mineral deficiencies have been documented in bighorn sheep as early as 1946

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(Packard, 1946). Data from a recent study of 11 Colorado bighorn sheep herds suggested deficiencies of trace minerals, including selenium, in several herds based on reference values for domestic sheep (Carpenter and Ramey, 2007). Evidence of potential Se deficiency in some bighorn sheep populations suggests a possible role in recurrent respiratory disease epidemics.

Selenium supplementation with sodium selenite increases blood concentrations of Se and red blood cell GSH-Px activity and prevents NMD in domestic sheep (Andrés et al., 1996). The effects or benefits of Se supplementation by injection have not been demonstrated in bighorn sheep. Additionally, studies have not examined the stability of Se and GSH-Px in bighorn sheep blood samples, which under field conditions might not be processed or analyzed until several days after collection. Studies of GSH-Px activity in ovine blood demonstrated breakdown of the enzyme over time, especially when stored at warmer temperatures (Sheppard and Millar, 1981). With some trace elements such as zinc (Zn), hemolysis can significantly alter results because intracellular concentrations are much higher than serum concentrations (Cornelis et al., 1995). The objectives of this study were to measure the response of bighorn sheep blood Se concentrations and GSH-Px activity to supplementation with sodium selenite, and to assess changes in blood Se and Zn concentrations and GSH-Px activity in stored blood aliquots.

Our study used eight captive bighorn sheep (three 5-yr-old castrated rams, two intact 1-yr-old rams, and three 1-yr-old ewes) between July and October 2007. We fed all study animals a grass/alfalfa hay mix ad libitum and 3 kg/animal/day of a pelleted, high-energy supplement. In addition, sheep grazed on natural forage consisting of cheatgrass (*Bromus tectorum*) and white horehound (*Marrubium vulgare*) at the Colorado Division of Wildlife (CDOW) Foothills Wildlife Research Facility (FWRF) in Fort Collins, Colorado (40°36′N, 105°9′W). Captive bighorns at this facility did not exhibit symptoms of Se deficiency or toxicity prior to starting the experiment. We matched individuals according to age and sex, and randomly assigned each to either a treatment or control group. Four bighorns received 2 ml of a selenium supplement (BO-SE®; Schering-Plough, Union, New Jersey, USA) containing 2.19 mg sodium selenite/ml by subcutaneous (SC) injection; controls received 2 ml sterile saline solution, also by SC injection.

For sampling, we tranquilized sheep with intramuscular (IM) injections of xylazine (50–75 mg; Lloyd Laboratories, Shenandoah, Iowa, USA) or a combination of butorphanol (9–15 mg; Wildlife Pharmaceuticals, Fort Collins, Colorado, USA), azaperone (12–15 mg; Wildlife Pharmaceuticals), and medetomidine (6–10 mg; Wildlife Pharmaceuticals). We collected blood via jugular venipuncture using lithium-heparin plasma separator tubes (Vacutainer®; Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). After pretreatment, blood samples were collected from each sheep; treatment or control injections then were administered SC in the right shoulder. Medetomidine was antagonized with a combination of atipamizole (25 mg; Pfizer, New York City, New York, USA) and tolazoline (100 mg; Lloyd Laboratories). Sheep were bled again 1, 6, and 12 wk posttreatment.

Blood samples were submitted to the Colorado State University Veterinary Diagnostic Laboratories (CSUVDL) for analysis. Selenium was analyzed using hydride generation atomic absorption spectrophotometry (measured in ppm), and glutathione-peroxidase activity was analyzed via an enzymatic assay (measured in mmole/l/sec). Zinc was analyzed using flame atomic absorption spectrophotometry (measured in ppm).

At each sampling, we collected up to four 6-ml aliquots of whole blood from each sheep for analysis. On the day of collection for weeks 0, 1, 6, and 12, one

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aliquot from each sheep was randomly selected and separated by centrifugation, and serum submitted for Se and GSH-Px assay (and, at wk 12, also for Zn assay). In weeks 0 and 1, we also collected extra aliquots and allowed them to separate at about 22 C and then submitted one aliquot from each sheep at 1, 3, and 7 days postcollection for Se and GSH-Px assay; in wk 12, we repeated this process for Zn. We stored aliquots at about 22 C between collection and analysis to simulate field conditions.

Trace mineral reference intervals have not been well-established for bighorn sheep, so determining deficiencies or toxicities of trace minerals are commonly made based on the reference intervals for domestic sheep. Normal domestic sheep values used by CSUVDL range from 0.15–0.50 ppm for Se (Puls, 1994), 250–1000 mmole/l/s for GSH-Px (C. Bedwell, unpubl. data), and 0.80–1.20 ppm for Zn (Puls, 1994). In our samples, values for blood Se concentration ranged from 0.18–0.45 ppm, values for GSH-Px activity ranged from 540–930 mmole/l/sec, and values for serum Zn ranged from 0.82–1.09 ppm. All of these values fall within the normal range for domestic sheep, and are based on data from samples collected and submitted for analysis on the same day.

We performed all analyses in SAS Proc MIXED or Proc REG (SAS Institute, 2006). The effects of selenium supplementation on Se and GSH-Px were analyzed using six linear regression models based on all linear combinations of the effects of treatment, sex, and age nested in sex on blood Se and GSH-Px levels. In addition to these models, we examined a simple intercept model (i.e., no treatment effect). Akaike's information criterion corrected for small sample sizes (AIC_c) was used to select the best model from this suite of models (Burnham and Anderson, 2002).

Selenium supplementation did not appear to increase blood Se concentrations in our captive bighorns. The top explanatory model included only sex (Table 1); this model carried 63% of support for being the actual Kullback-Leibler (K-L) best model in the set. From this model, the effect size on Se levels for males was slightly lower (−0.035; standard error [SE]=0.016) than in females in our study. This model explained about 17% of the variation in data. Because these bighorns had apparently "normal" blood Se concentrations prior to supplementation, homeostatic mechanisms (i.e., GSH-Px in the liver) likely buffered further change in blood Se concentrations (Sunde, 1993). Analyzing liver Se concentrations via biopsy might have yielded evidence of a supplement effect, but we do not consider this a practical approach for evaluating Se status in free-ranging bighorn sheep.

Supplementing with Se also did not cause measurable effects on blood GSH-Px activity. As with blood Se, the top model for GSH-Px included only sex (Table 1) and carried 52% of the weight of evidence as the K-L best model. This model also estimated higher GSH-Px activity in females than in males (−65.35; SE=35.09), and explained about 11% of variation in the data (R^2=0.11). Evidence for sex-related differences in blood Se and GSH-Px levels should be examined further, given the small sample size and relatively close competition with the null model in both analyses (Table 1).

We analyzed the stability of Se and GSH-Px in bighorn sheep blood over time using nine linear regression models. These were based on two models of Se and GSH-Px decay over time: linear decay and exponential decay. These models were also combined with either sex or age nested in sex effects. We also examined a null model (i.e., no decay), and models solely with the sex and age nested in sex effects. Prior to the GSH-Px activity analysis, one influential outlier was removed (DFFITS statistic value of −0.62, DFBeta [intercept] statistic of −0.63, and DFBETA [days] statistic of 0.35 given 1

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TABLE 1. Results of model selection procedure using Akaike's information criterion corrected for small sample sizes (AIC_c) for treatment, sex, and age effects in selenium (Se) and glutathione-peroxidase (GSH-Px) levels of bighorn sheep (*Ovis canadensis*) blood.

Model	AIC_c	ΔAIC_c	Akaike weights
Blood Se			
Sex	−84.1	0.0	0.63
Null model	−82.0	2.1	0.22
Age (sex)	−81.3	2.8	0.15
Treatment, sex	−81.3	2.8	0.15
Treatment	−79.9	4.2	0.08
Treatment, age (sex)	−78.2	5.9	0.03
Blood GSH-Px			
Sex	386.3	0.0	0.52
Null model	387.3	1.0	0.31
Age (sex)	388.5	2.2	0.17
Treatment, sex	388.6	2.3	0.16
Treatment	389.7	3.4	0.09
Treatment, age (sex)	391.1	4.8	0.05

parameter and a sample size of 64); this sample was clotted prior to lab analysis, providing biologic justification for its removal.

Both Se concentration and GSH-Px activity declined slightly in blood stored for 7 days before processing. The linear decay model carried 40% of the weight of evidence (Table 2) as the K-L best model for Se stability in stored bighorn blood (R^2=0.2); however, the magnitude of decay in blood Se concentrations over time estimated from this model was small (−0.011 ppm/day; SE=0.0027). Similarly, the linear decay model carried 44% of the weight of evidence (Table 2) as the K-L best model for GSH-Px stability in stored bighorn blood (R^2=0.08); the magnitude of decay in blood GSH-Px activity over time estimated from this model also was small (−15.78 mmole/l/sec/day; SE=6.88). Based on previous studies in domestic sheep, decreases in GSH-Px activity over time were expected (Sheppard and Millar, 1981). In a recent study of human serum, approximately 10% of selenium was lost by volatilization, and this process occurred more rapidly in serum stored at room temperature (Palacios and Lobinski, 2007). This might explain the slight decrease in Se concentrations seen here. Based on our findings, however, modest delays in processing and analyzing field samples collected from free-ranging bighorns should not affect interpretation of blood Se concentration or GSH-Px activity data provided that samples are held at or near room temperature while stored.

The stability of Zn in bighorn sheep blood over time was analyzed using nine different linear regression models for estimating blood Zn levels over time. Zn levels were transformed using a natural logarithmic transformation. The models were based on two models of Zn change over time: linear increase and logarithmic increase. These models were combined with either sex effects or age nested in sex. We also examined a null model (i.e., no change), and models solely of sex, and age nested in sex effects. One influential outlier was included in the analysis; there was no indication it was an error (DFFits=2.0869, DFBeta [intercept]= −0.5962, DFBeta [log {days}]=1.6732 given 1 parameter and a sample size of 32). Although clearly influential, this observation was retained because we had no biologic justification for removing it.

In contrast to Se concentrations and GSH-Px activity, blood Zn concentrations were clearly affected by delays in sample

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TABLE 2. Results of model selection procedure using Akaike's information criterion corrected for small sample sizes (AIC_c) for decay of blood selenium (Se) and glutathione-peroxidase (GSH-Px) levels in bighorn sheep (*Ovis canadensis*) through time.

Model	AIC_c	ΔAIC_c	Relative likelihood	Akaike weights
Blood Se				
Linear decay	−178.2	0.0	1	0.40
Linear decay, sex	−176.7	1.5	0.47	0.19
Linear decay, age (sex)	−176.3	1.9	0.39	0.15
Exponential decay	−176.2	2.0	0.37	0.15
Exponential decay, sex	−174.6	3.6	0.17	0.07
Exponential decay, age (sex)	−174.2	4.0	0.14	0.05
Null model	−166.0	12.2	0.00	0.00
Sex	−164.4	13.8	0.00	0.00
Age (sex)	−163.7	14.5	0.00	0.00
Blood GSH-Px				
Linear decay	812.0	0.0	1.00	0.44
Linear decay, sex	814.0	2.0	0.37	0.16
Exponential decay	814.4	2.4	0.30	0.13
Null model	815.0	3.0	0.22	0.10
Linear decay, age (sex)	816.2	4.2	0.12	0.05
Exponential decay, sex	816.4	4.4	0.11	0.05
Sex	816.9	4.9	0.09	0.04
Exponential decay, group	818.6	6.6	0.04	0.02
Age (sex)	819.1	7.1	0.03	0.01

processing. The top model for Zn concentrations included logarithmic increase over time (Table 3) and carried 55% of the weight as being the best K-L model; the days postcollection effect estimated from this model was 0.35 (SE=0.063) ppm/day on the natural log scale. There were no competing models and no indications of sex or age effects. The observed increase in Zn concentrations over time probably occurred as Zn sequestered in red blood cells became available in the serum as a result of hemolysis (Cornelis et al., 1995). Our results suggest a potential bias in Zn concentrations could occur if significant time lapses between blood collection and sample analysis, and thus Zn data from free-ranging bighorns should be interpreted with knowledge of the interval sampling and analysis.

We analyzed the ability of GSH-Px to predict Se concentrations, based on the

TABLE 3. Results of model selection procedure using Akaike's information criterion corrected for small sample sizes (AIC_c) for growth of log-transformed blood zinc levels in bighorn sheep (*Ovis canadensis*) through time.

Model	AIC_c	ΔAIC_c	Relative likelihood	Akaike weights
Logarithmic growth	13.2	0.0	1.00	0.55
Logarithmic growth, sex	15.9	2.7	0.26	0.14
Linear growth, age (sex)	17.9	4.7	0.10	0.05
Logarithmic growth, age (sex)	17.9	4.7	0.10	0.05
Linear growth	18.1	4.9	0.09	0.05
Linear growth, sex	20.7	7.5	0.02	0.01
Sex	35.6	22.4	0.00	0.00
Age (sex)	37.8	24.6	0.00	0.00
Null model	47.7	34.5	0.00	0.00

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strong positive relationship between the two demonstrated in other species (Wilson and Judson, 1976). We compared the model using GSH-Px levels as the only predictor of Se with the null model using AIC_c model selection. For this analysis, we considered only those GSH-Px and Se levels from samples submitted for analysis on the same day as collection.

Unlike previous studies in other species, GSH-Px activity did not predict blood Se concentrations in bighorn sheep. The null model carried 100% of the weight of evidence when compared to the model with GSH-Px as the sole independent variable. This lack of a predictive relationship might be because our sample size was small and neither Se nor GSH-Px values extended outside the normal range. In a study of bighorn sheep by Samson et al. (1989), within-herd relationships between Se and GSH-Px were not significant; only when data from multiple herds were pooled along with data from domestic cattle (*Bos taurus*) was a correlation demonstrated. Our data suggest that the reliability of GSH-Px as a predictor of Se concentrations in bighorn sheep is questionable and warrants further investigation.

Given the diversity of habitats occupied by bighorn sheep, it seems plausible that homeostatic mechanisms for maintaining Se and other trace minerals, and perhaps tolerating a wider range of dietary availability have evolved in this species. Consequently, in order to better understand the potential role of trace mineral levels in the bighorn respiratory disease complex, species-specific reference intervals for bighorn sheep are needed. In establishing such values, Se and GSH-Px values from field samples are likely to be robust to necessary delays ($\leq$7 days) between collection and analysis, but Zn and perhaps other trace mineral concentrations from field samples should be interpreted with greater caution. Future research should aim to incorporate a larger and more diverse group of animals to more clearly establish age and sex effects on trace mineral measures in bighorn sheep, as well as to further explore the relationship between Se and GSH-Px.

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