University of Nevada, Reno

A study assessing forage quality and desert bighorn sheep (*Ovis canadensis nelsoni*) diet in central Nevada using microhistology and molecular analysis

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By, Molly J. Bechtel

Dr. David S. Thain/Thesis Advisor

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We recommend that the thesis prepared under our supervision by

MOLLY J. BECHTEL

entitled

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be accepted in partial fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

David S. Thain, D.V.M., Advisor

Meeghan E. Gray, Ph.D., Committee Member

Nathan C. Nieto, Ph.D., Committee Member

Tamzen Stringham, Ph.D., Committee Member

Mariah D.R. Evans Ph.D., Graduate School Representative

Marsha H. Read, Ph. D., Associate Dean, Graduate School

August, 2012
ABSTRACT

Desert bighorn sheep (Ovis canadensis nelsoni) populations in central Nevada have suffered from die-offs although biologists and wildlife managers have struggled to determine the exact cause of these population declines, making population management difficult. An important, often overlooked and under researched element of desert bighorn sheep health is diet and the quality of forage consumed. Using data collected in the Clan Alpine mountain range (a range that supports a population of healthy desert bighorn sheep in central Nevada), this research attempted to first, determine quality of collected forages (grass, shrubs and forbs) collected at five study sites in the range. Forage quality was compared between sites and seasons. Forage types collected were also compared between sites and seasons and correlated to forage quality data. Microhistological analysis and PCR analysis determined the frequency of forage types in desert bighorn sheep fecal samples collected throughout the Clan Alpines. Forage types found in fecal samples were compared seasonally and diversity indices were compared to sites of collection.

In general, nutritive values and, with the exception of Selenium, mineral content met minimum domestic sheep nutrient requirements, although some variation was apparent. Collected forage did not vary between sites, but did vary seasonally. Grasses and forbs were collected more in summer than any season and less forage was collected in winter. Shrubs were collected consistently throughout the year.
Grass occurred more often in fecal samples collected in spring than any other season according to microhistological analyses. Shrubs varied significantly in spring and winter. Not surprisingly, more forbs were observed in fecal samples collected in spring and summer. In order to compare sites of collection, diversity indices were calculated for project sites and for microhistological results. Diversity indices in both sites and microhistological samples declined in fall and winter. More forbs were determined using PCR analysis than any other forage type. The sample size for PCR analysis was small and significant variation between seasons was not quantifiable. However, it is suggested that because of PCR’s ability to identify forage types at a more taxonomically specific level than microhistology (six sequences were identified to species), it was used in combination with microhistological analysis to further determine the diet of desert bighorn sheep. This preliminary data provides a baseline example of nutrition requirements and diet of desert bighorn sheep in the Clan Alpine range. Additionally, the variation in plant collection, nutrient values and mineral quality of forage, as well as the variety of forage types found in bighorn sheep fecal samples reminds managers and biologists alike that a diverse plant community is important for maintaining healthy bighorn sheep herds.
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Background

Bighorn sheep (*Ovis canadensis*) die-offs have occurred in the greater part of the Western United States, drastically affecting the desert bighorn sheep population in Nevada. It is assumed that the historical population of bighorn sheep has now declined to approximately 2% of its past population in North America since the mid to late 19th century (Schwartz et al. 1986).

The first recorded irregular die-offs of bighorn sheep occurred in the late nineteenth century, when Europeans began to settle the West (Buechner 1960). European settlement has been attributed to several causes of bighorn sheep die-offs including, overhunting, habitat loss, inbreeding depression, competition for food and water, predation and disease transmission from livestock (Buechner 1960, Gutierrez-Espeleta et al. 2000). In fact, throughout 1870 - 1877 it was reported that thousands of bighorn sheep were hunted and killed in Yellowstone National Park, effectively causing reductions in the population (Buechner 1960). Furthermore, scabies (*Psoroptes ovis* and *P. cervinu*) killed thousands of sheep in the western North America, a disease that occurred concurrently with the settlement of Europeans (Buechner 1960). Also, studies speculate that bighorn sheep are affected by early stages of range deterioration which predispose populations to disease (Buechner 1960, Dekker 2010). In some parts western North America and Canada, livestock grazing has left several once accessible mountain ranges less accessible to bighorn sheep. Important winter range used by livestock has made high quality forage difficult to attain throughout the year for bighorn sheep in certain parts of North America. High quality winter range is an important aspect for
maintaining healthy body weight in bighorn sheep throughout the year (Beuchner 1960, Oldemeyer et al. 1971, Tilton and Willard 1982). All of these factors gave attributed to population declines in bighorn sheep populations since the late 1800s.

Although the major population declines in bighorn sheep can be attributed to the settlement of the west by Europeans, a literature review of large mammal die-offs conducted by Young (1994) found that most large herbivorous mammal die-offs were related to trophic-level; herbivore die-offs were attributed to forage availability. This is evident in bighorn sheep die-offs that occurred in Canada. Several rocky mountain bighorn sheep populations suffered severe population declines due to lack of available winter forage caused by drought and competition with livestock. Sheep succumbed to disease (i.e. infection lungworm and verminous pneumonia) and high parasite loads due to nutritive stress caused by lack of food (Stelfox 1971).

This trophic-level die-off is also exemplified in some desert bighorn sheep populations. When forage conditions are poor, desert bighorn sheep have low resistance to disease and parasites and populations will decline (Leslie and Douglas 1979). Although little research has been done regarding the die-offs in desert bighorn sheep, population declines have been also been attributed to overstocking livestock, competition with other species and habitat destruction, thereby causing range deterioration and poor forage conditions (Buechner 1960, Brown 1993, Gutierrez-Espeleta et al. 2000). Desert bighorn sheep populations have continued to decline until the advent of modern wildlife conservation and management which has focused on improving the viability of existing populations, (e.g., isolating domestic animals from
bighorn sheep and developing water holes) and reintroducing (translocating) bighorn sheep into previous occupied ranges (Gutierrez-Espeleta et al. 2000, Brown 1993). These efforts have been generally successful; however, die-offs still occur in some desert populations (Gutierrez-Espeleta et al. 2000). This suggests that further investigations regarding the maintenance of bighorn sheep populations should be conducted.

Several desert bighorn sheep populations in Nevada have experienced catastrophic die-offs. In 2007, populations in Slate and Fairview mountains in central Nevada suffered from a pneumonia outbreak in which several sheep were found dead at guzzlers and near paved roads. Furthermore, the lowest lamb:ewe ratios in the state were recorded in nearby Sand Springs mountain range since reintroducing desert bighorn sheep in 1996. Low lamb:ewe ratios are said to be a direct result of poor range conditions; however, the disease outbreak in the neighboring Slate and Fairview mountains may also have impacted lamb survival (Salisbury 2009). Nearby ranges were recorded as having the lowest population numbers of sheep as compared to previous population numbers since 1996 due to drought conditions. However, there are few studies of desert bighorn sheep diet and nutritional requirements as a function of population maintenance.

Nutritional requirements of wild herbivore populations as a limiting factor for health is poorly understood; and evaluations of food resources and nutritional ecology are of central importance in developing predictions for underlying decision making processes in range and wildlife management (Mckinney et al. 2006). Few studies have assessed diet and nutrition and how it can impact species’ health, potentially leading to
population decline. Nutritional information such as, proximate analysis and *micro* and *macro* mineral content in Great Basin plants have not been thoroughly studied. Few studies have assessed how to maintain the range for maintenance of bighorn sheep, although literature exists for pasture utilization of domestic sheep. Data of forage consumption and quality obtained from a healthy population of desert bighorn sheep may serve as an appropriate comparison for populations in adjacent ranges that have suffered declines. Well established nutritional information about domestic sheep can be used to compare to the nutritional requirements of desert bighorn sheep.

Previous studies have investigated nutritional requirements in bighorn sheep in areas in which die-offs have occurred; however these studies were not conducted in Nevada. Wagner and Peek (2006) determined seasonal change in diet quality by analyzing *macro* and *micro* minerals of vegetation browsed by bighorn sheep. This study determined that various plant species reached senescence at different times of the year and resulted in different nutrient contents. Therefore, it was suggested that it is important to manage bighorn sheep habitat for a variety of plants in order to maintain a healthy diet (Wagner and Peek 2006). More recent studies have found vegetation considered low in *micro*-nutrients for domestic sheep, but with perfectly healthy populations of bighorn sheep who forage on the poor quality forage. This further suggests that more information regarding nutrient requirements of bighorn sheep is necessary (Dean et al. 2002).

In addition to forage quality, it is also important to determine diet of desert bighorn sheep. The limited work regarding forage consumption of desert bighorn sheep
in southern and central Nevada includes a study by Brown et al. (1977). Rumen content analyzed from a small sample of rams was collected throughout the hunting season (from early to late fall) in the west central mountain ranges of Nevada. The gut contents were identified as 81% grass as well as a smaller proportion of forbs and shrubs. Similar studies analyzed forage consumed by desert bighorn sheep using various techniques; however, these studies were not conducted in Nevada and found that diet varied based on snow cover (Goodson et al. 1991).

Several methods exist to determine diet in ungulates. Non-invasive field evaluation of wild ungulate diet can be determined by direct observation in the field or identifying plant species in ungulate habitat for signs of browsing and grazing (Raye et al. 2011). These methods provide precise taxonomic evaluation; however, field observations are difficult with elusive animals (including bighorn sheep) and observations may be inaccurate. Furthermore, these techniques are limited to study sites that are easily accessible by researchers and, thus are rarely used (Vaucher 1988, Raye et al. 2011). Evaluation of the rumen content is more accurate than direct observation in determining diet; however, it is invasive and sample size is often biased because samples are collected for analyses following a hunt. Also, identification of forage-type may be difficult at finer taxonomic levels (i.e. genus and species) (Brown et al. 1977, Bertolino et al. 2009, Raye et al. 2011).

More recent methods of diet analyses attempt to create a larger sample size by measuring forage consumption year round and by non-invasive means. Near infrared reflectance spectroscopy (NIRS) is a non-invasive method used with measure forage
quality and has been used in correlation to forage-consumption (Raye et al 2011).
However, this is a non-consumptive method and must be used based on a priori knowledge of the species’ diet (Foley et al. 1998). Similarly, quantification of n-alkanes is a non-invasive method used in experimental settings for assessing forage intake. This method is not conducive for studying animals in the wild that are not feed supplemented because animals are simultaneously dosed with even-chain alkanes in order to obtain estimates of total intake and diet composition (Dove and Mayes 1996).

While both rumen content analysis and NIRS have been used in diet analysis studies, often the biases and difficulty in implementation of these methods prevent their wide acceptance.

Microhistology, is a non-invasive way to quantify diet in ungulates to determine consumed forage in fecal samples by comparing digested plant material in feces with ground plants observed eaten by the animal. With this method, plants are identified to the level of life-form (grass, shrub, or forb) or, if possible, a more specific taxonomic rank. However, microhistological analysis is time consuming and may provide unspecific and context-dependent results. Few labs offer microhistolgical analyses as a method to determine consumed forage because of the associated subjectivity and amount of training involved (Soininen et al. 2009, Raye et al. 2011). This method is used commonly for analysis in numerous species, although it is slowly being replaced by more efficient and less subjective molecular methods.

Recently, the polymerase chain reaction (PCR) in combination with DNA barcoding and sequencing has been used to determine diet in a myriad of species. By
amplifying a short fragment of the chloroplast genome, the P6 loop of the trnL intron, it is possible to sequence plant DNA in feces using PCR. The trnL gene has been used to identify plant species and is a marker used in the molecular taxonomy of plants (Taberlet 1991). DNA sequencing and PCR produce more accurate results than microhistology and is a less biased technique due to specificity of primers used for the highly conserved trnL gene (Soininen et al. 2009, Valentini et al. 2009, Raye et al. 2011). Furthermore, because of success in diet determination, methods sequencing conserved DNA regions, such as the trnL gene, are increasingly used in analysis to determine diet in ecological studies (Valentini et al. 2009).
Using methods of diet determination and forage quality analysis to determine digestible nutrients and *micro* and *macro* mineral content of range plants, this study aims to answer preliminary questions of nutrition and diet as a limiting factor of health in a local bighorn sheep population. Locally, when the sheep population in the Clan Alpine mountain range (a range adjacent to Slate and Fairview mountain range where die-offs occurred) was surveyed in 2007, the lamb ratio of 39 lambs: 100 ewes was considered sufficient to maintain a steady population increase (Salisbury 2009). No abnormal range conditions were reported for the Clan Alpines by NDOW (Salisbury 2009). Although populations of desert bighorn sheep in the Clan Alpines have not been widely studied, even following catastrophic die-offs in adjacent populations, this healthy population of bighorn sheep provides an excellent model for comparing populations that have suffered declines.

Since the 2007 census, population numbers have increased and adjacent sheep populations have almost returned to normal (conditions prior to drought and population declines). Although, when changes occur to the health and survival of local populations, trophic-level interactions that include plant-herbivore relationships in healthy populations may be used to help conserve desert bighorn sheep.

The goals of this study are three-fold. The first objective is to determine if the forage quality of the plants collected (composited into groups of life-form; grass, shrub or forb) in desert bighorn sheep habitat of the Clan Alpine mountain range adequately meet nutritional requirements of desert bighorn sheep, when compared to domestic sheep. The second objective is to determine if the quality of the plants collected varies
between sites and seasons. The third objective is to determine consumed forage of collected fecal samples from a population of desert bighorn sheep in the Clan Alpine mountain range using two methods of diet analyses: *microhistology* and *molecular detection*. Forages found in collected fecal samples will be compared between seasons.

**Thesis Summary**

The first objective of this study was to determine if study sites meet minimum nutritional requirements and to determine how these nutritional requirements change between sites and seasons. Part of this objective was to determine which plant life form group may change between sites and season and how this correlates with quality.

The second objective of the study was to estimate what bighorn sheep are consuming in the Clan Alpine Range of northern Nevada using *microhistological analysis*. Forage type was compared seasonally between summer, fall, winter and spring in order to determine possible seasonal changes in BHS diet. As another possible means of diet determination, PCR and sequencing was used to specifically identify plants that were present in bighorn sheep fecal pellets.

The following questions were answered about desert bighorn sheep in the study area (See Figure 1 for map of study area):

1) Do study sites meet minimum nutritional requirements over seasons for desert bighorn sheep, using domestic sheep nutrition as a guide?

2) Does forage quality change between sites and seasons?
3) Which plant life forms change between sites and seasons and is this correlated to quality?

4) What is the frequency of plant life form groups found in desert bighorn sheep fecal samples?

5) Does plant life form groups found in desert bighorn sheep fecal samples vary by season?

**Literature Cited**


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Chapter 1

Forage Quality in the Clan Alpine Mountain Range of northern Nevada and how it relates to Sheep Nutrition

ABSTRACT

Forage was collected seasonally and across five sites in the Clan Alpine mountain range for forage quality analysis in order to determine if forage met minimum nutrient requirements for desert bighorn sheep survival. A multivariate ANOVA was used to determine variation of forage collected between sites and seasons. Collected forage did not vary between sites, but did vary seasonally. Grass, shrubs and forbs were collected more in summer than any season and less forage was collected in winter. Shrubs, however; remained consistent throughout the five sites forage was collected for analyses. Proximate analyses were measured to determine if forage nutritive values met nutrient requirements according to domestic sheep guidelines. In general, all sites met requirements for nutritive values in domestic sheep with some exceptions. Seasonal variation followed expected trends. Micro and macro minerals were also measured for variation between seasons and sites in collected forages. A GLM was used to determine variation in mineral content. Mineral content varied more erratically between seasons...
and sites than nutritive values, although, with the exception of Se, most minerals met sheep nutrient minimum requirements.

**Introduction**

Populations of desert bighorn sheep in the Clan Alpines have not been widely studied, even following catastrophic die-offs in adjacent populations and thus remains a conservation concern in Nevada. An important, but often overlooked, part of that effort is forage quality and relating that to management of bighorn sheep populations. However, little is known about the diet and nutritional standards of desert bighorn sheep. Forage quality can be measured in desert bighorn sheep habitats and can be compared to nutrition guidelines of domestic sheep to determine if nutrient requirements are satisfied. Information about bighorn sheep diet can help maintain healthy populations and prevent possible future die-offs.

**Site Descriptions**

The project sites were located in central Nevada in the Clan Alpine mountain range. The ecological site descriptions for the research areas are characterized as follows (Table 1):

Deep Canyon is characterized by soils that are excessively shallow to well drained. The soil types vary from stony loam over very cobbly sandy clay loam to cobbly loam over gravelly loam to very gravelly loamy sand, over stratified very gravelly sand to extremely gravelly loamy coarse sand. The dominant soil series is described as Bluewing
(very gravelly loamy sand) with an historical plant community comprised of Bailey greasewood (*Sarcobatus baileyi*), Indian ricegrass (*Achnatherum hymenoides*), desert needlegrass (*Achnatherum speciosum*), a small portion of bud sagebrush (*Artemisia spinescens*), shadscale (*Atriplex confertifolia*), winterfat (*Krascheninnikovia lanata*) and perennial forbs. This site is also composed of the soil series Old Camp-Colbar with rock outcrop association with an historical plant community comprised of Thurber's needlegrass, Wyoming big sagebrush (*Artemisia tridentate*), Indian ricegrass, and small portions of perennial forbs, Nevada jointfir (*Ephedra nevadensis*), sandberg bluegrass (*Poa secunda*), spiny hopsage (*Grayia spinosa*), desert needlegrass, bottlebrush squirreltail (*Elymus elymoides*) and purple sage (*Salvia dorrii*). The project site elevation ranges from 1158 to 2134 meters (Websoilsurvey.gov).

Cow Canyon is characterized by soils that are shallow and well drained. The ecological site description for the project site characterizes the soils as very stony loam over extremely stony clay loam. The dominant soil series is described as Old camp loamy slope with an historical plant community comprised dominantly of Thurber's needlegrass (*Achnatherum thurberianum*), and Wyoming big sagebrush. A smaller portion of the plant community is comprised of spiny hopsage, sandberg bluegrass, Nevada jointfir, Indian ricegrass, desert needlegrass, bottlebrush squirreltail and purple sage. The project site ranges from 1524 to 2134 meters (websoilsurvey.gov).

Horse Creek is characterized by soils that are shallow and well drained. The ecological site description for the research area characterizes the soils as very gravelly sandy loam over very gravelly clay loam. The dominant soil series include Theon-
Mirkwood with Rock outcrop associations. Historical plant communities associated with these two soil series types include: shadscale, Bailey’s greasewood, desert needle grass, and a smaller portion of Indian ricegrass, Bottlebrush squirreltail, bud sagebrush (Artemisia spinescens), spiny hopsage, globemallow (Sphaeralcea sp.), Nevada jointfir, and Anderson’s wolfberry (Lycium andersonii). Elevation at this project site ranges from 1524 to 2134 meters.

Little Angel is characterized by soils that are shallow and somewhat excessively drained. According to the ecological site description, the soil series include: Old Camp-Singatse with rock outcrop association and Theriot-Findout with rock outcrop associations. These soils are typically very stony loam over extremely stony clay loam and very gravelly loam over very gravelly sandy loam, respectively. The historical plant communities associated with Old Camp soil series includes Thurber’s needlegrass, Wyoming big sagebrush and a small portion of Indian ricegrass, Nevada jointfir, Sandberg bluegrass, spiny hopsage. Singatse is associated with shadscale, and a smaller portion of Indian rice grass, bud sagebrush, desert needlegrass, Nevada jointfir, and winterfat.

Bench Creek is characterized with soils that are shallow and well-drained. The soil series for this project site include: Theon-Singatse with rock outcrop association and Barnmot-Bluewing-Badland association. These soils are typically very gravelly loam over very gravelly clay loam and very gravelly sandy loam over very gravelly sandy loam, respectively. The historical plant communities associated with Theon-Singatse include; shadscale, Bailey’s greasewood, Indian ricegrass, and a small portion of desert
needlegrass, bottlebrush squirreltail, Bud sagebrush, winterfat, and Nevada jointfir.

Barnmot-Bluewing and Badland association soil series includes; shadscale, Bailey’s greasewood, Indian ricegrass, and a small portion of desert needlegrass, bud sagebrush, Nevada jointfir and winterfat. The elevation of the project site ranges from 1433 to 1829 meters. (websoilsurvey.gov).

**Forage Quality of Great Basin plants**

**Nutrient content – proximate analysis**

Very little is known about forage quality of Great Basin plants (Ganskopp and Bohnert 2003). Studies that measured crude protein, neutral detergent fiber, and *in vitro* organic matter disappearance in Great Basin grasses noted that forage quality was related to precipitation (Ganskopp and Bohnert 2001). When abundant moisture was available certain cool-season grasses would advance rapidly through maturity and generate an abundance of low quality reproductive stems. On the other hand, less than average moisture would cause grass to sustain a higher plane of nutrition with up to twice as many available days during a growing season. Furthermore, rangelands with a diversity of grasses and soil profiles should provide adequate forage quality for longer time periods. Deep-rooted grass species maintained forage quality longer, while shallow-rooted grasses responded to changes in precipitation (Ganskopp and Bohnert 2001).
Other studies, although not conducted in Nevada, have indicated seasonal changes in nutritional content of forages in bighorn sheep habitat. Wagner and Peek (2006) found protein content of grasses and forbs highest during spring, and declined throughout summer and fall to lowest levels in winter. Crude protein levels increased in late winter and rapidly reached peak levels during spring. Forbs contained significantly more protein than grasses through late summer, fall, winter, and late winter. Protein content of shrubs remained relatively consistent through all seasons. In vitro digestible dry matter (IVDDM) of grasses decreased from spring through late summer-fall, reaching its lowest level during late winter and rapidly increasing in spring. For forbs and browse, IVDDM did not show significant seasonal relationships, but tended to follow patterns similar to grasses (Wagner and Peak 2006).

**Mineral content**

Similar studies found a relationship between soils and forage quality. In nonagricultural soils there is generally a predictable spring nutrient flush and an autumn or winter leaching associated with senescence. In fact, many wild deciduous plants will experience a seasonal decline in nitrogen, phosphorus, and potassium concentrations (Chapin 1980). However, it is important to note that plants adapted to stresses such as drought and salinity, environments common in Nevada, also respond to nutrient stress by maintaining higher tissue nutrient concentrations, in part through reduced growth rate, and may show no visible elemental deficiency symptoms (Chapin 1980).
The scant information available regarding Great Basin plant quality indicates that plant nutrient dynamics are variable throughout the season. However, the only minerals that are deficient in the grasses sampled were copper, zinc and sodium. Phosphorus and potassium were elevated early in the growing season and declined to deficient levels by early July or August. Iron was the only mineral that maintained adequate concentrations in all forages year round (Ganskopp and Bohnert 2003).

Research not conducted in the Great Basin Region, but in western Idaho, also found mineral content to fluctuate in forages. Ahola et al. (2007) found that trace mineral content varied by season and year. Most micro minerals varied annually; however, selenium and manganese tended to vary seasonally. Selenium was found in greater concentrations in the spring than in any other season while manganese was found in greater concentrations in winter when compared to spring (Ahola et al. 2007). This study indicated that mineral concentrations can be extremely variable and this variability does not follow a consistent pattern. Wagner and Peak (2006) found phosphorus, potassium and sulfur content of grasses, forbs and browse to be high in the summer and steadily declined through winter. Zinc content of grasses was highest in summer and spring. Calcium content in grasses and forbs maintained a consistent level throughout the year. The calcium to phosphorous ratio increased during late summer-fall and winter months (Wagner and Peak 2006).

Land and wildlife managers can use an understanding of the nutritional dynamics of forage to sustain adequate growth and reproduction in wildlife. However, nutritional
requirements of bighorn sheep have not been widely studied. Therefore, nutrient requirements for domestic sheep (*Ovis aries*) was used as a guideline for desert bighorn sheep nutrient requirements. In fact, a study on reference intervals for mineral concentrations in whole blood and serum of bighorn sheep (*Ovis canadensis*) in California found reference ranges for minerals in bighorn sheep that were widely accepted guidelines for domestic sheep (Poppenga et al. 2011).

**Proximate analysis and domestic sheep diet**

Nutritious feed is vital for growth and maintenance of sheep and can be used as a guideline (Appendix 1). Energy and digestible nutrients are vital for sheep nutrition. It is important for maintenance of blood pressure and muscle tone; transmission of nerve impulses; ion transport across membranes; reabsorption in the kidneys; synthesis of protein and fat; and secretion of milk. Energy is measured in different ways, but it is important that it is provided in adequate amounts to prevent deficiency (Ensminger 2002).

A proximate analysis is a chemical analysis of feed used to determine nutritive value. A proximate analysis includes measurements of crude fiber, crude protein, crude fat (often measured as ether extract, and ash) a measure of total minerals.

Total digestible nutrients (TDN), are comprised of digestible crude protein, digestible crude fat, digestible nitrogen-free extract and digestible ether extract, and are an important measurement for energy in domestic sheep given that deficiency can lead to death. In general, a lack of TDN in growing animals can cause reduced weight gain
and growth cessation, weight loss, and ultimately death. In reproducing ewes energy deficiency causes reduced conception rate and reduced milk production, with progressively worsening deficiencies causing reproductive failure, cessation of or lack of initiation of lactation, and also death. Similar problems develop in the ram, with an initial reduction and eventual cessation in reproductive activity and performance and finally death (Ensminger 2002).

Total mineral content of forage is measured in ash; however, sheep require a certain quantity of mineral elements in order to carry out normal life processes. Minerals can be divided into two groups based on the amounts required in the diet: *micro* and *macro* minerals. The *micro* minerals important in sheep diet that are needed in relatively small amounts include cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, selenium and zinc. The *macro* minerals that are needed in relatively large amounts in the diet include phosphorus, potassium, sodium, chloride, sulfur and magnesium.

**Mineral content and sheep diet**

*Micro* - minerals are vital for domestic sheep health (Appendix 1). Iodine, iron, molybdenum, copper, cobalt, manganese, zinc and selenium are the eight *micro* minerals considered to be important to sheep health. Diet and overall quality of the forage consumed is an important aspect of desert bighorn sheep health, and most ungulates are sensitive to changes in trace mineral content.
Selenium is an important *micro*- mineral in sheep and ungulate nutrition. For example, cattle accustomed to high levels of selenium in forage will suffer from symptoms of selenium deficiency when forced to suddenly switch to pastures with forages lower in selenium (Dean et al. 2002). Similar studies have suggested that transplanted Rocky Mountain bighorn (*Ovis canadensis canadensis*) sheep may have suffered from such changes in selenium content (Dean et al. 2002). Selenium deficiency causes numerous conditions such as muscle weakness in lambs, immunosuppression, nutritional muscular dystrophy in adults, as well as poor lamb recruitment due to still births and retained placentas in ewes. (Samson et al. 1988). It is important to monitor the range for selenium content in order to prevent deficiency.

Cobalt is essential for the synthesis of vitamin B-12 in the rumen (Ensminger 2002). Cobalt produces an appetite-stimulating function in sheep and hence, deficiency will cause lack of appetite, severe emaciation, weakness, anemia, decreased fertility, and decreased milk production (National research committee council on animal nutrition 2002). In some areas of the U.S. it is important to add cobalt to the salt of domestic sheep rations to prevent deficiency symptoms where it is deficient in the forage (National research committee council on animal nutrition 2002). Little information concerning cobalt deficiency in bighorn sheep species and range plants in the Great Basin exists.

Copper is another *micro*- mineral important to domestic sheep health. Sheep are particularly vulnerable to both copper toxicity and deficiency. Toxicity occurs from
excessive Copper accumulation in the liver. Deficiency is a result of digestive processes in the rumen forming poorly absorbed copper sulphides. This deficiency is exacerbated by interactions with molybdenum (Lee et al. 2002). Again, little research has been done regarding copper deficiency and toxicity and how it relates to bighorn sheep health and possible die-offs. However, it is important to monitor copper and possible interactions with molybdenum to prevent deficiency.

Iodine is necessary for the formation of thyroxin, the iodine containing hormones of the thyroid gland. Areas in which forages are deficient in iodine are widely scattered throughout the U.S. Clinical signs are manifested in lambs born without hair and stillbirths (Ensminger 2002). A study of Rocky Mountain bighorn sheep found that soils containing minerals such as iodine were ingested by bighorn sheep, suggesting that forage may be deficient; however, no further research regarding the deficiency of iodine in bighorn sheep has been conducted (Packard 1946).

Iron is another important micronutrient in domestic sheep nutrition. Clinical signs include anemia, poor growth, lethargy, increased respiration rate, decreased resistance to infection and high mortality rate (Ensminger 2002). Iron deficiency and how it relates to population declines has not been widely studied in bighorn sheep.

The exact requirement of manganese is unknown for domestic sheep and wild sheep; however, it is important for skeletal development and reproduction in domestic sheep. A deficiency would cause impaired growth, skeletal abnormalities and lack of coordination in lambs, as well as depressed reproduction rates (Ensminger 2002). Very little is known about manganese requirements in bighorn sheep.
Molybdenum toxicity causes scouring disease and has been reported in California, Nevada, Oregon, and England, although not in sheep. However, a high molybdenum intake can induce copper deficiency, even when the copper content of the pasture is quite high (Ensminger 2002). Therefore, it is important to monitor forage for copper content and molybdenum content to prevent deficiency symptoms and possible die-offs in bighorn sheep populations. Toxicity symptoms such as scouring and deficiency symptoms associated with molybdenum have not been reported in bighorn sheep.

Zinc is an essential mineral for domestic sheep. It is necessary for growth and normal reproduction. Deficiency will cause impaired growth, excessive salivation, parakeratosis, wool loss, and delayed wound healing. Ram lambs show reduced testicular development and ineffective spermatogenesis. In ewes, all phases of the reproductive process are adversely affected (Ensminger 2002).

The macro-minerals involved in sheep nutrition are sodium, chloride, calcium, phosphorus, magnesium, potassium, and sulfur (Ensminger 2002). Sodium and chloride are important macro-minerals for domestic sheep nutrition. These minerals help maintain osmotic pressure, regulate acid-base balance and control water metabolism in tissues (Nutrition req. of sheep 1985). The functions of sodium and chloride could be important in maintaining a population of bighorn sheep throughout drought situations; however, little research has been done regarding the content of sodium and chloride in range plants and how it relates to bighorn sheep health.
Calcium and phosphorous are important *macro*-minerals for sheep nutrition. Utilization of these minerals is dependent on the presence of vitamin D and magnesium. A 1:1 ratio of calcium to phosphorous in forage is necessary for adequate sheep nutrition. Magnesium deficiency interferes with calcium absorption. Low levels of dietary phosphorus decrease the rate of calcium absorption. High levels of aluminum and iron will increase a need for phosphorus. It should be noted that mature pasture and range forage in North America is almost always deficient in phosphorus (Ensminger 2002). Therefore, careful monitoring by rangeland managers will ensure that the intricate interactions of these *macro*-minerals maintain balance within bighorn sheep populations.

Magnesium is a constituent of bone. It is also necessary in many enzyme systems and the proper functioning of the nervous system. A deficiency of magnesium may result in grass tetany.

Potassium is required for growth in lambs; it affects osmotic pressure and acid-base balance within the cells. It aids in activating several enzyme systems involved in energy transfer and utilization, protein synthesis, and carbohydrate metabolism. The possibility of potassium deficiency is very slight; most forages in the US contain adequate potassium. Deficiency symptoms include listlessness, stiffness, impaired response to sudden disturbances, convulsion and death (Ensminger 2002).

Sulfur is used in the synthesis of sulfur-containing amino acids. Deficiency symptoms include loss of appetite, reduced weight gains and feed conversion efficiency. Excessive salivation and lacrimation is also likely (Ensminger 2002).
Very little research has been conducted on the utilization of range plants by bighorn sheep. However, it is known that optimum utilization of pastures by domestic sheep is very difficult to attain. As pasture forage matures, the protein content declines, fiber increases, and both forage intake and digestibility decline (Jordan and Marten 1968). Therefore, monitoring forage quality for optimum maintenance of bighorn sheep is imperative.

Research Methods
General Description
Methods to assess forage quality were used to determine if plants collected in different study sites across the Clan Alpine mountain range met the minimum nutritional requirements of desert bighorn sheep, using domestic sheep nutrition as a guideline. This information was used to determine if forage quality varied by study site and by season. Plant groups were compared to determine if groups varied by study site and season and if this variation correlated to forage quality.

Data Collection
A multi-stage sampling method was used to collect all data for this study. Three stages of data were collected. The first stage, fecal collection, was selected based on *a priori* knowledge of desert bighorn sheep sightings. This stage determined study sites (eight sites in total: see figure 1). Fecal samples were collected opportunistically at all eight study locations. Stage two data forage collection plots were collected at all eight sites where desert bighorn sheep were sited or where there were recent signs of desert bighorn sheep (fresh fecal samples or tracks). Forage collection plots were defined by
forage collection sites in which eight frames (1 X 1 meter sample point) of forage were collected per plot. One forage collection plot was sampled randomly every month and then seasonally at every study site. The stage three data or eight frames within each forage collection plot were sampled as follows: after the forage collection plot was determined by sited sheep or signs of recent sheep use and the GPS point was noted, a random number generator was used to select a compass bearing and starting at five meters from that GPS point plants were collected within a one by one meter box. This method was repeated eight times at five meter intervals. The GPS point of all eight frames was noted. Enough species of each plant were collected to fill a large paper bag at each plot. A complete plant species list was created at each location; however species were lumped into life-form (grass, shrub, or forb) for forage quality analysis.

**Forage Quality Analysis**

Samples for forage quality analyses were composited by site and season collected and then grouped by life-form (grass, shrub, or forb). Samples were placed in paper bags and air-dried. Portions of the composite samples were analyzed at the University of Nevada, Reno nutritional laboratory for macro-(Ca, P, and Mg) and micro-(Cu, Fe, Mn and Z) minerals as follows.

A dry ashing technique was used to prepare samples for analysis of Ca, Mg, Zn, Fe, Mn, and Cu, following the procedure of Association of Official Analytical Chemists (AOAC, 1975). For selenium analysis, 1 g dried ground (2 mm sieve) plant material was
weighed and placed into a digestion tube. The digestion was done using eight unit digestion block (Foss Tecator\textsuperscript{TM} Digestion System).

The measurement of major minerals and trace minerals were performed using the PerkinElmer AAnalyst\textsuperscript{TM} 800 atomic absorption spectrophotometer (PerkinElmer, Inc., Shelton, CT, USA) equipped with WinLab32\textsuperscript{TM} for AA Version 6.5 software, which features all the tools needed to analyze samples. Before reading the unknown plant digest for all minerals, standard minerals solutions of known concentration were run to establish an absorbency curve.

Proximate analysis and all other macro - and micro - minerals were analyzed by SDK labs (Hutchinson, KS), a commercial lab specializing in feed analyses by the following methods. Moisture and dry matter analysis were determined by oven drying for three hours at 105°C identical to the National Forage Testing Association, Method 2.1.4. Crude protein was determined using the improved Kjeldahl method for nitrate-containing samples (AOAC, 1995). ADF and aNDF were determined using the filter bag technique (Ankom Technology, methods 5 and 6, respectively). Total crude fat was determined by ether extraction and ash content was analyzed by gravimetry as defined by the Association of Analytical Chemists (1995). Macro - minerals Ca, P, K, Mg, and Na that were analyzed by SDK labs were determined by the multi-element method, atomic absorption spectroscopy, established by Association of Analytical Chemists (1995). Once dry samples were digested, the minerals, S, Co, Mo and Se were determined using inductively coupled plasma emission spectroscopy and Cl was determined using titration per the Association of Analytical Chemists (1995).
**Statistical Methods**

In order to determine which vegetation types occurred most often, a frequency distribution was measured on all data and represented graphically.

In order to compare vegetative differences in forage collection data between multiple study locations and seasons an analysis of variance (ANOVA) with season as the dependent variable and vegetation type as the independent variable was used. These tests were conducted with data collected from every study site. If significant differences were found between seasons or study site, a Tukey’s HSD multiple comparison test was used to determine which seasons or sites differed from each other.

To compare forage quality data between seasons an ANOVA was used. To account for seasons with small sample sizes, a generalized linear model (GLM) was used to compare forage quality between seasons. If significant differences were found between seasons or study site, a Tukey’s HSD multiple comparison test was used to determine which season differed from each other. To compare forage quality between project site locations, frequency distributions of mineral content was compared graphically.

**Results**

**Site Descriptions**

Forage was collected for forage quality analysis at five project sites due to sufficient sample size and lack of fecal samples and other visible signs of desert bighorn
sheep at other sites. Deep Canyon was comprised mainly of Cheat grass (*Bromus tectorum*), Sandberg’s bluegrass (*Poa secunda*) and Sage (*Artemisa sp.*). The study site was also comprised of a smaller proportion of shrubs including rabbit brush (Chrysothamnus nauseosus), spiny hopsage (*Grayia spinosa*), golden brush (*Ericameria sp.*), desert gooseberry (*Ribes velutinum*), desert peach (*Prunus andersonii*) and perennial forbs (see figure 2).

Sandberg’s bluegrass and cheat grass comprised most of what was collected at Cow Canyon (figure 3). Other plants included perennial grasses, shrubs such as rabbit brush and perennial forbs.

Horse Creek (Figure 4) was comprised of cheat grass, sage, golden brush, rabbit brush, and squirrel tail as well as a smaller proportion of shrubs and perennial forbs.

Little Angel was comprised of Thurber’s needle grass, Sage, Mormon tea (*Ephedra sp*), and Sandberg’s blue grass and smaller proportion of shrubs and perennial grasses and forbs (see figure 5).

Bench creek was comprised of cheat grass, shadscale (*Atriplex confertifolia*), spiny hopsage, squirrel tail, sandberg’s bluegrass, golden brush, Halogeton (*Halogeton glomeratus*) and prickly phlox (*Leptodactylon californcium*). A smaller portion of shrubs and perennial grass and forbs also comprised the project site (see figure 6).

**Forage Collection**

A general multivariate ANOVA was run to determine if collected forage types (shrubs, grass, and forbs) differed between study locations. The mean number of shrub, forb and grass collected did not differ between study locations (p-value=0.52).
Forage collected between seasons did differ significantly (P-value < 0.001). A Tukey’s HSD pairwise comparison was run to determine differences between seasons (Table 2 for difference in mean collection of forage type season).

Seasonal variation of forage collection for the five sites forage quality was analyzed and represented graphically (Figure 8). Grasses and forbs were collected more often in summer, while shrubs remained relatively consistent throughout all seasons.

**Proximate Analysis**

Forage types (grass, shrub and forb) were grouped together per site and season and analyzed for forage quality. All analyses were based on percent dry matter. Forage quality content varied seasonally and across sites (Figures 9, 10, 11, 12, and 13).

Crude protein (CP) content was highest in summer and lowest in winter. Furthermore, crude protein content was generally higher in shrubs and forbs than it was in grasses.

Acid detergent fiber (ADF) and neutral detergent fiber (aNDF) content varied throughout sites and seasons. ADF and aNDF increased in most forage types in fall and winter across sites. There was a slight decrease in ADF and aNDF in forbs in winter at project sites Cow Canyon and Deep Canyon.

A similar trend was found in grass samples collected at Cow Canyon. Total digestible nutrient (TDN) and non-fiber carbohydrate (NFC) content decreased for grass and shrub in fall and winter. Generally, Fat and Ash content decreased in forage types analyzed in fall and winter as well. Trends in forbs varied across sites and season;
however this was most likely due to small sample size and insufficient quantities to conduct forage quality analyses.

Refer to Appendix 1 for complete forage quality and mineral analysis of composited forage types collected across seasons and sites. Appendix 1 includes recommended nutrient requirements for domestic sheep and notes on which variables were outside recommended ranges.

**Macro- and micro-mineral analysis change between seasons**

Changes between mineral content in grass and season were detected at the 0.05 $\alpha$-level. A multivariate ANOVA was used to determine significant changes between seasonal forage type and *macro* and *micro* mineral content. Variation was detected for *micro* minerals (p-value of 0.043) and *macro* minerals (p-value < 0.001, Table 3).

A generalized linear model was used to determine variation to account for small sample sizes. Variation was found between season and Mg (p-value = 0.031), K (p-value=0.003), and P (p-value = <0.001) (Table 4). Using the GLM, variation was found between season and Zn (p-value < 0.001) and Cl (p-value = 0.029). No variation was found between *micro* mineral content in shrubs and season (Table 5).

**Forage Quality**

**Macro- and micro-mineral content changes between Sites**

*Macro- and micro-mineral* content varied between sites (Figures 14, 15, 16, 17, 18, 19, 20, 21, 22, and 23 and Appendix 1). Co was within the required nutrient requirements for sheep across all sites and showed little variation in all forage types.
sampled. Mo met the required nutrient requirements and showed little variation between sites in grass. Shrub samples collected at Deep Canyon in winter and Horse Creek in fall, had a Mo content of 0.30 ppm and 0.33 ppm, respectively. This is just below the recommended requirement for forage for sheep (0.5 ppm). Forbs showed slightly more variation. Forbs collected at Little Angel in spring and at Deep Canyon in summer and fall were below 0.5 ppm (0.43, 0.43 and 0.30 ppm, respectively).

Se was deficient at all project sites (less than the required 0.1 ppm was reported in most forage). All seasons were deficient except for forbs collected in fall at Bench Creek which had a Se content of 0.09 ppm and shrubs collected in summer and winter at Horse Creek, which had a Se content of 0.1 ppm and 0.15 ppm respectively. All seasons and forages collected at Little Angel were deficient in Se except for shrubs collected in summer (Se = 0.1 ppm). Grass collected at Horse Creek in summer, forbs collected in spring, and shrubs collected in summer and winter were not deficient, with Se content of 0.33, 0.12, 0.10 and 0.15 respectively. All plants collected at Deep Canyon were deficient in Se except for grass collected in summer which had a Se content of 1.44 ppm. All forages collected at Cow Canyon were deficient for Se and below the 0.1 ppm requirement.

Zn showed the most variation across sites. In grass, Zn was deficient across all sites with the exception of Bench Creek. In the summer and winter (Figures 1, 2, 3, 4, and 5) grass was deficient for Zn at Little Angel and Horse Creek. In the winter, grass was deficient for Zn at Deep and Cow Canyon. Zn content in grasses collected at Bench Creek was higher than any other site per season (spring = 90.32 ppm, summer = 50.41 ppm;
fall= 44.77 ppm; winter= 31.43 ppm). Zn content for grass collected at Bench Creek remained in the acceptable range for domestic sheep nutrient requirements.

Forbs varied seasonally and across sites. For all sites, Zn concentrations were highest in forbs collected in fall: Bench Creek, 913.50 ppm; Little Angel, 263.4 ppm; Deep Canyon, 281.0 ppm; Cow Canyon, 170.7 ppm. Forbs were deficient for Zn at Sites Deep Canyon in winter (16.15 ppm), Horse Creek in spring (11.96 ppm) and Little Angel in summer (13.97 ppm). Quantity was insufficient to measure Zn content for forbs collected at Horse Creek in fall.

Zn content in shrubs varied the least between site locations. There was no deficiency in shrubs analyzed at any study site. Zn concentrations were higher in shrubs collected at Bench Creek in summer than at any other site for that season, but remained at an acceptable nutrient range for sheep. Similarly, shrubs collected in fall at Cow Canyon also had higher concentrations of Zn, but remained within the accepted range.

Variation was present in Fe across sites. Fe was deficient in grass samples collected at Little Angel in spring (Fe = 22.29ppm) and Horse Creek in spring (Fe = 17.81ppm). Concentrations were slightly higher than the acceptable range, but not above tolerable levels across all sites. Shrubs were deficient at Deep Canyon in winter (Fe = 8.08ppm), Horse Creek in winter (Fe = 16.68ppm), Little Angel in spring (Fe = 7.74 ppm) and Bench Creek in winter. Again, all sites had shrubs slightly above the recommended range for sheep nutrient requirements, but not above tolerable levels. Forbs varied the least across study sties. The only deficiency appeared in forbs collected
in summer at Horse Creek (Fe = 20.30 ppm). Deep Canyon had the highest concentration
of Fe (Fe = 333.4 ppm). However, no sites other than the ones listed were deficient in Fe.

No deficiencies were observed in Mn. Concentrations across all sites tended to
be higher in spring than in any other season. Several sites did not have sufficient
quantities of plant material to analyze forage quality in spring, so a comparison for all
four seasons is not conducted. Grass maintained the most consistent
concentrations across all sites. The lowest concentration was collected Bench creek in
Fall (Mn = 38.10 ppm), however, this was in the acceptable range of sheep nutrient
requirements. The highest concentration Mn in grass was collected at Little Angel in
spring (Mn = 334.0 ppm). This concentration was not outside the tolerable range for
sheep. Shrubs collected at Bench Creek had the lowest concentrations of Mn at the
study site (spring = 72.53 ppm, summer = 68.32 ppm, fall = 96.70 ppm, winter = 104.70
ppm). However, Little Angel had the highest and the lowest overall concentrations of
Mn for shrubs (spring = 480.50 ppm, summer = 59.10 ppm, fall = 95.40 ppm). Horse
Creek had similar Mn concentrations for shrubs (spring = 460.40 ppm, fall = 92.68 ppm,
winter = 118.60 ppm). Concentrations for shrubs for Deep Canyon were more consistent
throughout seasons (summer = 81.35 ppm, fall = 90.54 ppm, winter = 188.00 ppm). There
was not enough forage available for a spring comparison. Mn concentrations for shrubs
collected at Cow Canyon were highest in summer and decreased in fall (summer =
99.75 ppm, fall = 75.33 ppm).

The highest concentration of Mn for any site or season was observed in forbs
collected in springs at Bench Creek (483.90 ppm). This concentration was above
tolerable levels (1,000 ppm). Mn concentrations in forbs collected at Horse Creek and Cow Canyon were relatively consistent (Horse Creek, spring = 164.20 ppm, summer = 172.20 ppm; Cow Canyon, summer = 111.70 ppm, fall = 159.70 ppm). Concentrations for forbs collected at Deep Canyon increased in winter (summer = 57.42ppm; fall = 65.82 ppm; winter = 131.70 ppm).

Cu varied amongst sites. Cu was deficient in some areas and just above tolerable levels in other study sites. Grass collected at Bench Creek did not vary from season to season and concentrations were in the acceptable range for sheep nutrient requirements (7.0 – 11.0 ppm). Cu concentrations for grass collected at Little Angel were just above tolerable levels in spring (tolerable concentrations for sheep = 25.0 ppm); however, all other seasons remained within the acceptable range (spring = 27.30 ppm; summer = 8.90 ppm; fall = 26.52 ppm; winter = 15.20). Grass samples collected at Horse Creek and Deep Canyon in winter were deficient for Cu (5.9 ppm and 4.2 ppm, respectively). All other grass samples collected across sites showed little variation (Figures 1-5).

Cu varied in shrubs across sites. Similar to grass, shrubs collected at Bench Creek did not vary seasonally and concentrations were in the acceptable range for sheep nutrient requirements. Cu concentrations in shrubs collected at Little Angel did not vary and remained in the acceptable range for sheep nutrient requirements. Shrubs collected in fall at Horse Creek were just over tolerable levels for sheep (Cu = 28.75 ppm). Cu concentration of shrubs collected at Deep Canyon varied compared to other sites. Shrubs collected in summer and fall were above tolerable levels for Cu (summer = 26.75
Likewise, shrubs collected in fall at Cow Canyon were just above tolerable levels (26.91 ppm).

Forbs collected at Bench Creek did not vary considerably between seasons and remained below tolerable levels. Comparatively, forbs collected at Little Angel in summer were just above tolerable levels (27.22 ppm). Forbs collected in spring and summer at Horse Creek (spring = 32.40 ppm; summer = 26.45 ppm, respectively), Deep Canyon (summer = 26.10ppm; fall = 28.28; winter = ppm 33.21 ppm) and Cow Canyon (summer = 26.59 ppm; fall = 29.73 ppm) were just above tolerable levels.

Ca and P varied across sites. Grass samples collected at Bench Creek maintained a ratio of 1:1 or 2:1 with one exception. The 1:1 or 2:1 ratio for Ca and P was not maintained for summer in grass; however, there was sufficient Ca and P concentration of 0.73 % and 0.164 %, respectively (Appendix 1). P was deficient in grass samples collected at Bench Creek in fall (P = 0.036%). Grass samples collected at Little Angel followed a similar trend. The recommended 1:1 or 2:1 Ca:P ratio was not met in summer; however, a sufficient Ca and P concentration was observed. In Fall, P fell below the recommended level (P = 0.061%). Horse Creek had sufficient concentrations of both Ca and P for grass samples with the exception of Ca (0.18%) for grass samples collected in summer. Grass samples collected at Deep Canyon in summer maintained the appropriate Ca to P ratio. Grass samples were slightly deficient in P (0.124%) in fall and deficient in Ca in winter (0.10%). Grasses collected at Cow Canyon maintained appropriate concentrations and ratios for Ca and P with the exception of winter. Ca was observed with a 0.00% concentration in winter.
Shrubs varied in Ca and P content across sites. For shrubs collected at Bench Creek, Ca was deficient in fall and winter (Ca = 0.11 % for both seasons). P was sufficient in shrubs collected at Bench Creek for all seasons. Shrubs were deficient in P collected at Little Angel in fall (P = 0.036 %). Shrubs at Horse Creek were sufficient in Ca and P with the exception of P concentrations in summer (P = 0.053 %). Similarly, shrubs collected at Deep Canyon were sufficient in Ca and P with the exception of P concentrations in summer (P = 0.086 %). These deficiencies affected the recommended 1:1 and 2:1 ratio. Shrubs collected at Cow Canyon were slightly deficient in P in summer (P = 0.140 %). For all other seasons Ca and P were sufficient for shrubs collected.

Ca and P concentrations in forbs also varied between sites. Bench Creek was deficient in Ca in spring for Ca (0.11 %) and in P in summer (0.039 %). Forbs collected at Little Angel were sufficient for Ca and P with the exception of P in summer (0.045 %). Forbs collected at Horse Creek, Deep Canyon and Cow Canyon were also deficient in P in summer (0.045 %, 0.051 %, and 0.052 %, respectively). Forbs collected at Deep Canyon were deficient in Ca in winter with a concentration of 0.16 %.

Mg varied across sites. No trends between sites were apparent. Bench Creek was deficient in Mg for all forage types in winter (grass = 0.02 %, shrubs = 0.03 %, and forbs = 0.01 %). Shrubs and forbs collected at Bench Creek were also deficient in spring (0.01% and 0.11%, respectively). Shrubs and forbs collected at Little Angel were deficient for Mg except in spring and winter. Shrubs collected in the spring had a Mg content of 0.03% and forbs collected in winter had a Mg content of 0.00%. Mg content in grasses collected at Horse Creek was only deficient in winter (0.02%). However, shrubs were
deficient in the spring, summer and fall with concentrations of 0.09%, 0.44%, and 0.00% respectively. Shrubs collected at Deep Canyon were deficient for Mg in all seasons collected with concentrations of 0.64% in summer, 0.05 % in fall and 0.06% in winter. All other forage types and seasons for Deep Canyon were sufficient for Mg. Forage types collected at Cow Canyon were sufficient in Mg throughout all seasons.

All sites were sufficient in K with the exception of Horse Creek, Deep Canyon and Cow Canyon. Grass samples collected at these three sites were deficient for K in winter. Horse Creek had a K concentration of 0.23%, Deep Canyon, 0.14% and Cow Canyon had a concentration 0.18%. All other sites and seasons were in the range for sheep nutrients.

Na concentration varied between sites and forage types. Deficiencies commonly occurred in grasses in winter. Grass samples collected at Bench Creek were deficient in fall and winter (0.06% and 0.03% respectively). Forbs collected at Bench Creek were deficient in winter (0.02%). Grasses and forbs collected at Little Angel were also deficient for Na; spring and winter grass was 0.04% and 0.01%, respectively. Forbs collected in winter were deficient with a concentration of 0.03%. Horse creek had trends in grass samples similar to Little Angel. Grass samples in spring and winter were deficient with concentrations of 0.03 % and 0.05 %, respectively. Shrubs samples collected in winter at Horse Creek were also deficient in Na (Na = 0.07 %). Grass and forbs were deficient in Na at Deep Canyon in summer, fall and winter. Grass collected in the fall and winter had Na concentrations of 0.06 % and 0.02 %, respectively. Forbs collected in the summer, fall and winter had Na concentrations of 0.06 %, 0.06 % and
0.02% respectively. Cow Canyon was sufficient for Na concentration across all seasons
and forage with the exception of grass in fall and winter (fall = 0.06% and winter =
0.01%).

S varied across sites and between forage types. Generally, deficiencies were in
grain samples. Bench Creek maintained sufficient S concentrations between forage
types and seasons. Little Angel was slightly deficient in S in grass samples collected in
spring (S = 0.13%). Similarly, Horse Creek was deficient for S in grass samples collected in
spring and winter (S = 0.13 and 0.05 respectively). Grass samples collected in Deep
Canyon were also deficient for S in winter (S = 0.05). Forbs collected in fall and shrubs
collected in winter at Deep Canyon were deficient with concentrations of 0.12% and
0.05%, respectively. Grass samples collected in Cow Canyon followed a similar trend
with low S concentrations in winter (S = 0.05%). All other forage types sampled were
sufficient for S content.

Discussion

Site Descriptions and Forage Collection

Grass was the dominant species collected at all project sites. More grasses and
forbs were collected in spring and summer than any other season (Table 2). The
abundance of grass and forbs in the spring and summer may be important for bighorn
sheep, especially ewes in the latter stages of gestation, parturition and then lactation
(Robbins 1993). These events in the life of an adult ewe correspond to the period of
abundant nutrients found in grass during spring and summer (Wagner and Peek 2006).
Shrubs, which were collected consistently throughout the year, may be important for
maintaining protein levels in sheep diet. Protein obtained from shrubs, when grasses and forbs are not as readily available, may help maintain a healthy rumen environment for microbes when grasses are low in crude protein (Wagner and Peek 2006). Shrubs were collected consistently throughout the year and may provide vital nutrients for sheep when other forage types are unavailable.

Proximate analyses of forage type across sites and season differed. However, previous studies have suggested that a variety of species and forage types are important to maintain a healthy bighorn sheep population throughout the year (Wagner and Peek 2006, Wikeem and Pitt 1992, Geist 1971). As demonstrated by the fluctuation in forage quality between forage types, desert bighorn sheep may be reliant on this variety of vegetation as quality changes between plants and throughout the seasons.

**Forage Quality**

**Proximate analysis**

Generally, proximate analyses followed previously determined trends. Wagner and Peek (2006) found protein content of grasses and forbs highest during spring, and declined throughout summer and fall to the lowest levels in winter. The crude protein requirement for grazing ungulates is 4.5%. All sites met this requirement with the exception of grass samples collected at Horse Creek in winter and fall (CP content = 3.54% and 3.57%, respectively) and shrubs collected at Deep Canyon in winter (CP =
3.28). However, other forage types collected in the same seasons met the CP requirements.

Neutral detergent fiber (aNDF) concentration is comprised of cellulose, hemicellulose, and lignin which are cell wall components that require microbial fermentation for digestion. The remaining cell contents are up to 98% digestible and readily available to the animal. NDF is often used as a more accurate measure of forage quality than acid detergent fiber (ADF) because ADF places hemicellulose in the completely digested fraction. However, this may be erroneous as hemicellulose is actually less digestible than cellulose in some plant species (Meyer and Brown 1985). The decrease in ADF and aNDF content for samples collected at Cow and Deep Canyon could be due to the collection time, which coincided with green-up (new growth period) of some perennial forbs and grasses. A reduction of fiber content is typical in plants with new growth (Meyer and Brown 1985).

TDN requirements for maintenance of sheep are approximately 55% of total dry matter (Nutrient Requirements of Sheep, 1985). TDN are comprised of digestible crude protein, digestible crude fat, digestible nitrogen-free extract and digestible ether extract. All sites, across all seasons met the nutritional requirement for sheep for TDN. Forbs collected in summer at Cow Canyon were below 40% TDN although other forage types met the 55% requirement. Shrubs collected in all seasons at Bench Creek were below 50% TDN, but above 40% TDN. All other forage types collected at Bench Creek met the 55% TDN requirement.
Fat, measured as ether extract, is important for energy. Previous studies have found fat to vary seasonally and decrease as plants senesce (Hundly 1959). Although fat content did decrease in fall and winter, TDN contents of all forage types throughout seasons and sites met a sustainable level required for sheep nutritional maintenance.

As expected, ash content, a measure of mineral content, decreased as plants senesced. A more thorough overview of mineral content is included in the analyses of the micro and macro minerals.

**Micro- and macro- mineral content change between seasons**

It is well known that macro and micro nutrients vary between seasons. However, because of limited sample sizes collected over one season it is possible that some minerals may actually fluctuate between seasons, although the data indicates they do not (Brownlee 1965). To further prove the fluctuation of mineral content between seasons in forage at the Clan Alpine range, a larger sample size collected over several seasons is suggested. However, changes over season can be shown graphically (Figures 1, 2, 3, 4, 5, and 6). Furthermore, this is consistent with previous research that has studied mineral variation across seasons (Wagner and Peek 2006, Barnes et al. 1990). Ultimately, moisture, temperature and soil nutrients are what affect plant growth and nutritive value (Barnes et al. 1990).

**Micro- and macro- mineral content changes between sites**
Mineral content varied slightly between sites was slight, in most cases, no trends were visible. This could be due to soil type and plant communities collected and composited between sites (Table 1 and Appendix 1). For example, at Bench Creek, which generally had the highest mineral content in forage than any other project site (Ash = 26.08 % for grass in fall) the dominant soil type is a calcareous type clay soil. These clay-like soils have increased dispersivity of Na particles and break up aggregates more easily than a sandy-like soil, making soil organic matter more available for decomposition (Bronick and Lal 2005). In general, minerals followed the same seasonal trend as in the proximate analysis; however, some sites showed no trends and mineral content was erratic across sites. Cheat grass was a dominant plant at some study sites such as Deep Canyon, Horse Creek, and Bench Creek. Cheat grass is a winter annual. Germination generally occurs in late winter and early spring (though rarely occurs in fall), and it completes its life cycle before soil moisture is exhausted. Emergence following winter dormancy may be slow in spring, spring-germinated plants are often smaller, less numerous, and less vigorous than cheat grass which began germination in winter (Hull et al. 1975). Therefore, the early green-up in cheat grass could account for high mineral content in grass samples in winter (i.e. P, Co and Zn concentrations in grass samples collected at Deep Canyon, and Fe and Mo concentrations in grass samples collected at Horse Creek).

Ca and P did not always maintain a 2:1 or a 1:1 ratio. However, in most cases when Ca was deficient, P concentrations were high, but only in one forage type. A low Ca: high P ratio may cause urinary calculi in sheep, but not a Ca deficiency (Ensminger
2002). Furthermore, when Ca or P was low in one forage type collected for that season it was sufficient for another forage type collected in that same study site. The only exception was grass collected at Cow Canyon which had a Ca concentration of 0.00; no other forage types were analyzed for Ca for that season due to insufficient quantity. Deficiencies in minerals were common across all sites. In most cases these deficiencies related to plant senescence. Where some forage types were deficient in minerals, other samples collected at that same study site and season met requirements for sheep, suggesting that bighorn sheep may conform to Geist’s suggestion that they are extremely selective foragers (Geist 1971).

Cu was found to be deficient in forages collected at several sites. However, it is important to note that an excess of Cu was not reported in the forages analyzed at any of these sites, as sheep are sensitive to Cu toxicity. With the exception of Deep Canyon shrubs collected in the fall (Cu = 160.10 ppm). However, Mo, S, and Zn levels were sufficient in forages collected at Deep Canyon that same year, suggesting that the high Cu levels were interacting with other minerals and preventing toxicity (Ensminger 2002). Furthermore, toxicity and deficiency in wild animals may be more difficult to determine than in domestic animals, as they have evolved under different selection pressures. For example, desert bighorn sheep have evolved to live in drought and desert conditions with little vegetation, domestic sheep were bred from mouflan species of sheep and were provided water and fed forages on cultivated pastures. (Robbins 1993).

The trace mineral Se was found below detectable levels in most plants collected. Suggesting deficiencies could be possible in bighorn sheep in this range. These results
were similar to study by Wagner and Peek (2006) in which Se was also deficient.

However, although Se deficiency may result in white muscle disease in domestic sheep, it may not be a problem in wildlife. Wild animals have entirely different selection pressures and would not be expected to have similar nutrient requirements as domestic sheep, even though domestic sheep provide established guidelines (Robbins 1993). Therefore, it is difficult to evaluate whether bighorn sheep may suffer from a lack of these nutrients by comparing nutrient requirements for domestic sheep (Wagner and Peek 2006, Robbins 1993). Furthermore, deficiencies in bighorn sheep are more difficult to detect and toxicity due to accumulation of mineral elements is likely rare (Poppenga 2011).
Literature Cited


Figure Legends

Figure 1: Map of project sites and recent fires. The most recent fire occurred in 2005 (four years prior to study start). Also note, eight project sites were visited only viable data were collected from six: Deep Canyon, Cow Canyon, Horse Creek, Lauderback Hills, Little Angel and Bench Creek. Only enough bulk plant material was sufficient to conduct analyses from five sites: Deep Canyon, Cow Canyon, Horse Creek, Little Angel and Bench Creek. Statistical methods were adjusted appropriately.

Figure 2: Number of plants collected at all study sites per sample point per species (n = 308 sample points).

Figure 3: Number of plants collected at Deep Canyon (n = 32 sample points).

Figure 4: Number of plants collected at Cow Canyon (n = 40 sample points).

Figure 5: Number of plants collected at Little Angel (n = 72 sample points).

Figure 6: Number of plants collected at Horse Creek (n = 48 sample points).

Figure 7. Number of plants collected at Bench Creek (n = 56 sample points).

Figure 8. Seasonal average number of plants collected across all sites over seasons

Figure 9. Percent nutrient content (CP – crude protein, ADF – acid detergent fiber, aNDF – neutral detergent fiber, NFC – non fibrous carbohydrates, TDN – total digestible nutrients, Fat (EE), and Ash) for composited grass, shrub and forb samples collected at Deep Canyon.

Figure 10. Percent nutrient content (CP – crude protein, ADF – acid detergent fiber, aNDF – neutral detergent fiber, NFC – non fibrous carbohydrates, TDN – total digestible nutrients, Fat (EE), and Ash) for composited grass, shrub and forb samples collected at Cow Canyon.

Figure 11. Percent nutrient content (CP – crude protein, ADF – acid detergent fiber, aNDF – neutral detergent fiber, NFC – non fibrous carbohydrates, TDN – total digestible nutrients, Fat (EE), and Ash) for composited grass, shrub and forb samples collected at Horse Creek.
Figure 12. Percent nutrient content (CP – crude protein, ADF – acid detergent fiber, aNDF – neutral detergent fiber, NFC – non fibrous carbohydrates, TDN – total digestible nutrients, Fat (EE), and Ash) for composited shrub, grass and forb samples collected at Little Angel.

Figure 13. Percent nutrient content (CP – crude protein, ADF – acid detergent fiber, aNDF – neutral detergent fiber, NFC – non fibrous carbohydrates, TDN – total digestible nutrients, Fat (EE), and Ash) for composited grass, shrub and forb samples collected at Bench Creek.

Figure 14. Micro mineral content for composited grass, shrub and forb samples collected at Deep Canyon.

Figure 15. Micro mineral content for composited grass, shrub and forb samples collected at Cow Canyon.

Figure 16. Micro mineral content for composited grass, shrub and forb samples collected at Horse Creek.

Figure 17. Micro mineral content for composited grass, shrub and forb samples collected at Little Angel.

Figure 18. Micro mineral content for composited grass, shrub and forb samples collected at Bench Creek.

Figure 19. Macro mineral content for composited grass, shrub and forb samples collected at Deep Canyon.

Figure 20. Macro mineral content for composited grass, shrub and forb samples collected at Cow Canyon.

Figure 21. Macro mineral content for composited grass, shrub and forb samples collected at Horse Creek.

Figure 22. Macro mineral content for composited grass, shrub and forb samples collected at Little Angel.

Figure 23. Macro mineral content for composited grass, shrub and forb samples collected at Bench Creek.
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Table 1. Ecological site descriptions. Websoilsurvey.gov
### Table 2. Results of Tukey HSD pairwise comparison test for collected forage type versus season. Treatments not sharing like letters differ significantly

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<th>Shrub</th>
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<td>5.2 a</td>
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### Table 3. Macro and micro content of collected grass samples compared seasons. Treatments not sharing like letters differ significantly

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<th>K%</th>
<th>Na%</th>
<th>Fe ppm</th>
<th>Mn ppm</th>
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<td>0.48 a</td>
<td>0.91 ab</td>
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### Table 4. Mean mineral content of collected shrubs compared to seasons. Treatments not sharing like letters differ significantly.

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<tr>
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<td>Mean Micro Mineral Content</td>
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Table 5. Mean mineral content of collected forbs compared to seasons. Treatments not sharing like letters differ significantly.
Figure 1.

Legend
- Red: Clan Alpine Wildfire
- Yellow: Forage Collection Plots

0 3 6 12 Kilometers
1 cm = 6 km
Figure 2.
Figure 3.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.
Figure 13.
Figure 14.
Figure 15.
Figure 16.
Figure 17.
Figure 18.
Figure 19.
Figure 20.
Figure 21.
Figure 22.
Figure 23.
Chapter 2

Methods of Diet Determination in Desert Bighorn Sheep

Abstract

Microhistology, PCR and sequencing were used to determine the frequency of forage types in desert bighorn sheep fecal samples collected in the Clan Alpine mountain range of central Nevada. Microhistological analysis found significantly more grass in desert bighorn sheep fecal samples than any other forage type. A multivariate ANOVA was used to determine variation in forage type frequency found in microhistological analysis between seasons. Frequency of forages types varied between seasons. Grass was found significantly more in spring than any other season. Shrubs varied significantly in spring and winter. More shrubs were observed in samples collected in spring than in winter. Not surprisingly, more forbs were observed fecal samples collected in spring and summer. In order to compare sites, diversity indices were calculated for forage collected at sites and for frequencies of forage types found in microhistological analyses. Diversity indices at both sites and microhistological samples declined in fall and winter. PCR analysis identified samples to family, genus and species. More forbs were determined using PCR analysis than any other forage type. The sample size for PCR analysis was small and significant variation between seasons was unable to be determined. However, it is suggested that because of PCR’s ability to identify forage types at a taxonomic specific level, it can be used in combination with microhistology to help determine diet in desert bighorn sheep.
**Introduction**

Studies quantifying desert bighorn sheep diet have been conducted using microhistology, although in different regions in the west. To date, no studies have determined diet in desert bighorn sheep using molecular techniques; however, Raye et al. (2011) determined diet in Chamois using PCR which provides a foundation for our research.

Miller and Gaud (1989) studied composition and variability in desert bighorn sheep diets in the Sonoran Desert of Arizona using plant transects and microhistology. Availability of forage was compared spatially and seasonally among three neighboring habitats for eight seasons. Lamb and adult diets were also compared (Miller and Gaud 1989). Microhistological analysis was used to determine diet in bighorn sheep in Nevada in a study by Brown et al. (1971). It was concluded that sheep consume mainly grass (Brown et al. 1971). Although, the study was conducted only in the fall and winter (Brown et al. 1971).

Wikeem and Pitt (1992) studied California bighorn sheep diet in relation to availability and forage quality in British Columbia. Diet was determined using microhistology and forage quality was determined based on the concentration of nitrogen, acid detergent fiber, calcium, and phosphorous in selected forage species. The study concluded a variety of forage – grasses, forbs and browse – are important for a high-quality diet, as the amount of each forage consumed changed seasonally and selection did not necessarily depend on the quality of the forage (Wikeem and Pitt 1992).
Methods of Diet Determination

Microhistology

Sparks and Malechek (1968) first reported microhistology as an accurate method to determine the dry-weight composition of stomach, esophageal and rumen samples. However, analyzing samples of this nature is invasive and not conducive to studying threatened or elusive wildlife. Later studies used microhistological analysis to quantify the consumed forage in fecal samples. Digested plant material in feces was compared to ground plants observed being eaten by the animal reported by other researchers.

Microhistiolography has been used extensively in range studies for diet analysis. For example, Shrestha and Wegge (2006) compared diet composition of herbivores using microhistology. Analysis of fecal samples determined diet to the level of life-form (shrub, grass, forb) in domestic sheep. However, the authors note that microhistology is less accurate when the diet consists more of shrubs and forbs than of grasses (Shrestha and Wegge 2006). Furthermore, other studies have reported that when bite count and microhistology analyses were compared, forbs are overestimated (Alipayo et al. 1992). In general, results varied between microhistological studies. This is most likely due to differential digestibility of plant species, presence of woody material in the diet, observer errors, procedures used in calculating the botanical composition and sample preparation (Alipayo et al. 1992). However, microhistology remains a popular, non-invasive method in diet determination today.

Molecular Methods: PCR and Sequencing
Previous diet analysis studies have used PCR in combination with sequencing and DNA barcoding successfully in many herbivorous species including voles, kangaroos, frugivorous non-human primates, and chamois (Bradley et al. 2007, Ho et al. 2009, Soininen et al. 2009, Raye et al. 2011). These studies created DNA barcoding systems and used conserved universal primers that amplified a variable region of the plant chloroplast gene to determine diet, in extinct or extant animals or humans.

Previously, part of the rbcL gene of the chloroplast genome was used as a plant marker to determine diet. For example, a study by Bradley et al. (2007) collected fresh feces from four endangered primate species and chloroplast DNA was amplified and sequenced from approximately 300 rbcL clones. A minimum of 16 different plants were identified to the levels of subclass and family in individual fecal samples (Bradley et al. 2007). Other studies have used the rbcL gene to determine diet using paleofecal remains of ancient Native American tribes (Poinar et al. 2001). Using methods to amplify ancient DNA, it was determined that native people historically consumed four to eight different plant species; however, plant sequences were only identified to the level of family (Poinar et al. 2001). The rbcL gene encodes a large subunit of the chloroplast genome; however, the barcoding system using the rbcL gene has had most success at the level of family and is too ambiguous to distinguish between species (Gielly and Taberlet 1994). Although the rbcL gene has had some success for amplifying degraded DNA; this ambiguity helped fuel research for a more genetically conserved gene for use in phylogenetic plant studies. Taberlet et al. (1991, 2007) helped develop the trnL intron
of the chloroplast genome, a highly conserved gene, with some variation that allowed for more taxonomic specificity and greater success at amplifying degraded fragments of DNA (Taberlet et al. 2007).

In fact, the chloroplast trnL (UAA) intron was used to determine diet in herbivorous species such as chamois because of its ability to amplify degraded and fragmented DNA (Raye et al. 2011). Also, sequences of this gene fragment have been used in phylogenetic analysis of closely related species and for identifying plant species (Taberlet et al. 2011).

Furthermore, the evolution of the trnL (UAA) intron has been thoroughly analyzed and is well understood. For example, this region is the only Group I intron in chloroplast DNA. Introns are the non-coding region of a gene that is removed by RNA splicing before the final mature RNA product of a gene is produced (Griffiths et al. 1996). Furthermore, this Group I intron has a conserved secondary structure with alternation of conserved and variable regions which allows for the design of new versatile primers embedded in conserved regions and amplification of short variable regions in between (Taberlet et al. 2006). In fact, universal primers for this region were designed more than 15 years ago (Taberlet et al. 1991) and have been used extensively and proven to work well in phylogenetic studies among closely related genera and species and in diet determination studies (Taberlet et al. 2006). The forward primer “C” and reverse primer “D” universal primer set used for this study, developed by Taberlet et al. (1991) has been used with great success in diet determination studies that
amplified degraded DNA from fecal samples in species such as kangaroos, chamois, and voles (Ho et al. 2010,).

**Research Methods**

**General Description**

Methods to determine forage consumption were used to determine if plants found in analyzed fecal samples varied by site and by season and if forage type found in those samples compared to forage type of collected plants. Diet was quantified using microhistology, PCR and sequencing. A set of universal primers developed by Taberlet et al. (1991) and (2007) were used to amplify the trnL chloroplast gene from fresh plant material and desert bighorn sheep fecal samples.

**Plant Sampling**

As described previously, a multi-stage sampling method was used to collect all data for this study. Three stages of data were collected. The first stage, fecal collection, was selected based on a priori knowledge of bighorn sheep sightings. This stage determined study sites (Figure 1). Fecal samples were collected opportunistically at all eight study locations. Stage two data or forage collection plots were collected at all eight sites where bighorn sheep were sited or where there were recent signs of bighorn sheep (fresh fecal samples or tracks). Forage collection plots were defined as forage collection sites in which eight frames (1m X 1m sample point) of forage were collected per plot. One forage collection plot was sampled randomly every month and then seasonally at every study site. The stage three data or eight frames within each forage collection plot were sampled as follows: after the forage collection plot was determined
by sited sheep or signs of recent sheep use and the GPS point was noted, a random
number generator was used to select a compass bearing and starting at five meters
from that GPS point plants were collected within a one by one meter box. This method
was repeated eight times at five meter intervals. The GPS point of all eight frames was
noted. Enough species of each plant were collected to fill a large paper bag at each plot.
A complete plant species list was created at each location; however species were
lumped into life-form (grass, shrub, or forb) for microhistological analysis.

**Fecal Collection**

Fecal samples were collected opportunistically at least three times a week where
bighorn sheep were known to browse. Samples were collected with a plastic bag and
kept in a cooler until they could be stored in a freezer at -20° C.

**Microhistology**

Plants were collected randomly as in methods previously described (McKinney
and Smith 2007). Place, time, elevation, season of collection, and plant species were
recorded. Collected plants were sorted by species and allowed to dry. The groups
created were washed over a 0.2 mm and 1.0 mm mesh sieve. Plant fragments were
soaked in nitric acid for five minutes to remove color. This mixture was diluted with
95mL of distilled water and using a plastic dropper, several drops of the solution were
mounted on a gridded microscope slide for analysis (Sparks and Malechek 1968,
Erickson et al. 2005). Ground up plants were identified according to epidermal and
cuticle characteristics (Cain et al. 2008) under a compound binocular microscope (Sparks
and Malechek 1968). The slides of the plant species also served as a reference for the microhistological analysis of the fecal samples.

The reference collection of ground plant samples was used to familiarize the user with shape, size, cellular arrangement and other distinguishing characteristics of plant cells. Once the observer became familiar with plant species collected at sites where bighorn sheep frequent a series of tests was performed using the reference collection to determine the accuracy at which the observer could identify plant cells. For each test, the observer was shown a random series of unmarked slides of the species in the reference collection. The plant specimen on each slide was identified based on the first fragment seen in the field of view so that the context of other fragments on the slide could not be used as reference when identifying a plant sample. Any unrecognizable fragments were recorded as “unidentified.” The observer’s rate of accuracy was then determined by calculating the proportion of times a fragment was correctly identified. Once a desired accuracy rate of 80% was achieved, fecal pellet samples were examined for diet estimation. All fragments were identified to forage type.

Fecal samples were kept in a cooler after collection in the field and later stored frozen at -20°C until dried for microhistological analysis. Fecal samples were oven-dried at 65°C. All fecal samples were ground and mixed using the method described above for plant material and as outlined in Sparks and Malechek (1968) and Erickson et al. (2005). Samples were mounted on slides per group and plant fragments were identified using
characteristics of the epidermis and cuticle (Cain et al. 2008). Frequencies of identified fragments were counted per grid per slide.

**PCR analysis**

DNA was extracted from fecal samples using the QiaGen QIAamp mini stool kit following the manufactures instructions with any necessary minor modifications. DNA was extracted from a set of plants determined to be most abundant in study locations using the Qiagen mini plant kit. These plants served as positive controls for future analysis. For thoroughness, the *trnL* chloroplast gene was amplified using two different universal primer-pairs developed by Taberlet et al. 1991 and 2007 (Table 2). DNA amplification was carried out in a final reaction volume of 50 µL using 20 µL of DNA extract as template. For both primer pairs, the reaction formulation was as follows: 25 µL of Qiagen Master Mix, 2.5 µL of 10 µMol concentration forward and reverse primers and 2 µL of 10 ng/mL concentration BSA. Except for differing melting-temperatures, the cycling conditions were the same for each primer-pair and followed a gradual stepdown program (Tables 3 - 4). All reactions for both primer pairs were run three times on all fecal samples. Positive PCR products were purified using the QIAquick PCR purification kit and QIAquick gel extraction kit for products that resulted with more than one band. Positive sequences resulting from fecal samples were compared to sequences obtained from plants that served as positive controls and those already created and saved on Genbank from previous research (www.ncbi.nlm.nih.gov/genbank). Once sequences
were compared to Genbank, they were aligned using ClustalX to further limit the possibility of sequence errors.

**Statistical Methods**

In order to compare differences between forage types found in fecal samples determined by microhistological analysis between seasons an analysis of variance (ANOVA) was used with season as the dependent variable and vegetation type as the independent variable. If significant differences were found between seasons, a Tukey’s HSD pairwise comparison test was used to determine which seasons differed from each other. Frequencies and descriptive statistics were used to describe the prevalence of genus and species in sequences found in fecal samples using PCR analyses. In order to determine which vegetation types occurred most often, frequency distributions were measured on all data and represented graphically. These data were compared to forage collection data.

**Results**

**Microhistology**

A total of 133 individual pellet groups were analyzed using microhistology. According to microhistological analyses, grass comprised consistently high proportion of the samples analyzed throughout the year. Grass was identified an average of 2.75 times out of the 133 samples analyzed (Figure 1). In spring, grass was identified an average of 7.25 times in the samples. In summer, grass was found an average of 2.2 times in the samples and in fall and winter grass was found an average of 2.8 and 1.7
times, respectively (Figure 2). A general multivariate ANOVA was used to determine variation between forage types found in fecal pellets with microhistology and seasons. The ANOVA returned a p-value <0.001, α = 0.05, proving that forage types differed between seasons. A Tukey’s HSD pairwise comparison test was used to determine if grass varied between seasons. Spring proved to vary significantly from all other seasons (Table 1). In fact, more grass fragments were observed in samples collected in spring than in samples collected in any other season (Figure 2).

Microhistological analysis determined forbs at an average frequency of 0.76 in the desert bighorn sheep pellet groups analyzed (Figure 1). An average frequency of 1.63, 0.84, 0.61 and 0.1 of forbs were found in bighorn sheep fecal pellets collected in spring, summer, fall and winter, respectively (Figure 2). A general multivariate ANOVA was used to determine significant differences between seasons. A P-value < 0.00, α = 0.05, proved significant differences between seasons. A Tukey’s HSD pairwise comparison test proved that samples collected in spring and summer varied significantly from samples collected in fall and winter (Table 1). On average, more forbs were observed in spring than in fall and winter, also more forbs were observed in summer than in winter (Figure 2).

Shrubs were observed an average of 0.79 times in the desert bighorn sheep fecal samples analyzed (Figure 1). Shrub frequency was found to vary slightly across seasons, with average frequencies of 2.13, 0.53, 0.92 and 1.2 in spring, summer, fall and winter.
respectively (Figure 2). A Tukey’s HSD pairwise comparison test determined shrubs to vary significantly in spring and winter (Table 1).

Diversity indices were used to compare microhistology results to forage collection across project sites (Table 5). Both microhistology analysis and forage collection data followed similar trends; forage collected at project sites and microhistological data grouped into sites of collection lost diversity in fall and winter.

**PCR analysis**

A total of 133 fecal samples were analyzed using PCR and of those 33 samples were sequenced to forage type (Figure 7), 33 to family (Figure 9), 29 to genus (Figure 11) and six to species using the c-d primer pair. No positive results were returned with the g-h primer pair due to small product of less than 100 base pairs.

When sequences were aligned it was determined that some variation existed between species, genera, and families of plants. However, because the trnL gene is so conserved, similar taxa did align after being run through an alignment program.

PCR analysis found forbs at a much higher frequency than any other vegetation type. Twenty-four sequences resulted in forbs, seven in grass, one sample was sequenced to shrub, and one was sequenced to tree. Thirty-three forage types were identified to 4 families: Asteraceae, Brassicaceae, Cupressaceae and Poaceae (Figure 9). When these families were compared to seasons it was found that Asteraceae, Brassicaceae, and Cupressaceae were observed more in fecal samples collected in summer. Poaceae was found in fecal samples collected in fall and winter only. Although
frequencies of sequences obtained to family and forage type in desert bighorn sheep diet varied seasonally (Figures 8 and 10).

Twenty-nine sequences were obtained to the level of genus. Genera varied across season. Genera associated with forbs were identified in spring and summer. *Crepis* and *Lactuca* were observed in spring and summer. *Tetrydimia*, a species of shrub, was observed in the summer. Grasses (*Hesperostipa, Achnatherum*, and *Thinopyrum*) were found in fecal samples collected in summer. *Bromus* was found in fecal samples collected in winter. One sequence was identified *Juniperus* in summer (Figure 12).

Six sequences were identified to species. *Descurainia sophia* was observed twice in different fecal pellet groups (both collected in summer), *Stephanomeria virgata, Lactuca serriola*, and *Tertydimia filifolia* were observed in fecal samples collected in the summer. *Bromus tectorum* was observed in a fecal sample collected in winter.

**Discussion**

**Microhistology**

Grass samples were identified in fecal pellets collected in spring more than they were in summer fall, or winter. This could be because as grasses green-up in the spring they have more nutritive value than they do in fall and winter months when they begin to senesce (Chapin 1980). In fact, when seasonal values for proximate analysis in composited grasses were averaged for all project sites values were found to be higher in spring than any other season, CP = 7.86% and TDN = 58.59%. (Figure 4).
Corresponding to the mean frequency determined by microhistological analysis, more shrubs were found in fecal samples in spring and winter than in summer and fall. Furthermore, according to forage collection data, shrubs are consistently available through the year (Figure 2). This consistency could make them a reliable source of protein for sheep throughout the year, but especially in winter when grass and forbs are dead or covered by snow. Shrubs identified in bighorn sheep pellet samples varied from season to season. More shrubs were identified in spring and winter than in summer or fall. This differs from previous research which found shrub consumption to be heaviest in late-summer through fall (Wagner and Peek 2006, Brown 1977). Although, more shrub fragments were observed in pellets collected in fall than in summer, the difference was not significant. Interestingly, minerals, in particular P, were found to be higher in shrubs collected in spring than in any other season (Figure 6). The average P content of shrubs in spring was, 0.79267\%, in forbs, 0.443 \%, and in grass, 0.567\%.

Sheep may be eating more shrubs in spring to account for low P levels in other forage types. Although more were found in fecal pellets in spring than in any other season, it is important to note that more shrubs were identified in pellets collected in winter than in fall or summer. According to previous research, protein content of shrubs remain high throughout later months, which is consistent with the forage quality analysis conducted in the project sites in the Clan Alpines (Wagner and Peek 2006).

More forbs were observed in fecal pellets collected in spring and summer than in any other season (Figure 2). According to forage collection data, more forbs were collected on average in spring and summer. Forbs were higher in mineral content
(Figures 5 - 6) in spring and summer than nearly every other forage type (although several proximate analyses and mineral parameters were unable to be determined due to insufficient plant material). Also, it should be noted that microhistological analysis is less accurate at determining forbs than any other forage type (Shrestha and Wegge 2006). Therefore, the amount of forbs may be underestimated, especially in spring when mineral and protein content is high (Wagner and Peek 2006).

Interestingly, when diversity indices of microhistological analyses and forage collection were compared to site, both followed the same pattern. All project site forage collection was less diverse in fall and winter. These same observations were made in microhistological analyses when analyzed for diversity and grouped into sites of collection (Table 5). The mean transit rate of digestion in sheep is approximately fifty-seven hours (Uden et al. 1981). Animals on this study were not collared with GPS units and it could not be determined if forage types observed in fecal samples collected at one project site, were also consumed there. Therefore, in order to more appropriately compare forage type in diet between study locations, future studies might include studying animals with GPS units. Also, variation between sites may not be as much factor as variation between seasons, as statistical analyses determined no significant difference between forage collected between sites. The importance of plants that grow and senesce seasonally and those that grow throughout the year and maintain relatively consistent nutrient levels may be important for maintaining populations as plant diversity decreases in winter.
Due to changing nutrient levels and varying availability of forages, a diverse plant community is important to maintain healthy bighorn sheep populations (Wagner and Peek 2006, Miller and Gaud 1989). For example, bighorn sheep may be selecting certain more nutrient forage types over others, for example shrubs that have a higher P concentration in spring than grasses. An opportunity to choose from a variety of forage types throughout the year may keep bighorn sheep populations healthy.

**PCR**

PCR is an extremely robust assay and methods such as PCR and sequencing may yield more taxonomically accurate results for use in determining diet. The PCR analyses for this research were, in fact, more taxonomically accurate than microhistological analysis, resulting in six sequences identified to species. However, these results were not quantitative and yielded only qualitative results that may add to the more quantitative of microhistology results. Furthermore, with such a small sample size it should be noted that the sequences found in bighorn sheep fecal samples provide a basis for future research, and have helped determined that these plant families, genera and species are in fact present in the diet.

The PCR results at the level of forage type versus season were compared to microhistological results. Although no statistical significance was determined, more forbs were observed per season in PCR analysis than in microhistological analysis (Figures 3, 5). As noted in previous research, forbs are difficult to determine using
microhistological analysis (Shrestha and Wegge 2006), and PCR may be used as a tool to
determine plant fragments that are not identified using microhistology.

Moreover, it might be possible to use PCR, with more optimization, to determine
which genera or species bighorn sheep are selecting in summer. As mentioned
previously, nutrient contents may be higher in winter due to the early green-up of some
winter and fall annuls such as cheat grass, it may be possible to determine if bighorn
sheep are selecting these grasses by using PCR and sequencing in concert with
microhistology. As determined by the research for this study, cheat grass was
sequenced from fecal samples collected in winter. Also, Brassicaceae, the mustard
family, is a family that was observed in fecal samples less frequently than Asteraceae.
Brassicaceae may have been observed less frequently because of toxic compounds; it is
being selected less frequently than other forbs such as Lactuca seriola (Allred 2006). In
fact, as determined from the sequences identified to the level of genera, more
sequences belonging to the genus Lactuca, a member of the asteraceae family, were
identified than to Lepidium, a member of the Brassicaceae family (Figure 11).

Previous research conducted using both microhistology, PCR and sequencing has
returned similar results, PCR and sequencing resulted in more taxonomically detailed
products (Soininen et al. 2009). However, more recent studies have adopted next
generation sequencing and quantitative PCR for diet analysis using fecal samples. Raye
et al. (2011) identified over 100 species in the diet of Chamois in one season using this
method. Furthermore, because of detailed and abundant data obtained from
pyrosequencing, a clear diet shift was determined between the two months that fecal pellets were sampled (Raye et al. 2011). Pyrosequencing is a powerful ecological tool and can read products that are under 100 base-pair. However, this is an expensive assay and can cost upwards of $1,000 a sample (Schuster 2008). Until resources, such as high-throughput pyrosequencers are more readily available, using methods such as PCR and traditional Sanger-sequencing combined with microhistology may be a more viable option for determining diet in elusive wildlife.

LITERATURE CITED


Allred K.W. 2006. An annotated checklist of poisonous or injurious range plants of New Mexico. Circular 636. New Mexico State University, College of Agriculture, Consumer and Environmental Science.


Erickson, Marcy. Influence of balsam fir (Abies balsamea) abundance on moose (Alces alces) utilization in Isle Royale National Park, USA.


Figure Legends

Figure 1: Average frequency of forage types found in desert bighorn sheep pellets collected from spring 2010 through winter 2011 with Microhistological analysis. A total of 133 individual fecal samples analyzed.

Figure 2: Average seasonal frequency of forage type found in desert bighorn sheep fecal samples using microhistological analysis. A total of 133 individual fecal samples analyzed.

Figure 3: Seasonal average of forage types (grass, shrub and forb) collected.

Figure 4: Seasonal nutritive value for composited grass, forb and shrub samples collected across project sites in the Clan Alpine range.

Figure 5: Seasonal micro mineral content (Co=cobalt, Mo=molybdenum, Se=selenium, Zn=zinc, Fe=iron, Mn=Manganese Cu=copper) of composted grass, shrubs and forbs collected across sites in the Clan Alpine range.

Figure 6: Seasonal macro mineral content (Ca=calcium, Mg=magnesium, P=phosphorous, K=potassium, Na=sodium, Cl=Chloride, S=sulfur) of composted grass, shrubs and forbs collected across sites in the Clan Alpine range.

Figure 7: Number of sequences obtained to forage type in desert bighorn sheep fecal pellets collected from spring 2010 thru winter 2011. For the analysis of 133 individual fecal samples a total of 33 sequences corresponding to the level of forage type were obtained.

Figure 8: Comparison of sequenced forage types in desert bighorn sheep fecal pellets between seasons. For the analysis of 133 individual fecal samples a total of 33 sequences corresponding to the level of forage type were obtained.

Figure 9: Number of sequenced plant families in desert bighorn sheep fecal samples collected from spring 2010 thru winter 2011. For the analysis of 133 individual fecal samples a total of 33 sequences corresponding to the level of family were obtained.

Figure 10: Comparison of sequenced plant families in desert bighorn sheep fecal samples. For the analysis of 133 individual fecal samples a total of 33 sequences corresponding to the level of family were obtained.

Figure 11: Frequency of sequenced plant genera observed in desert bighorn sheep fecal samples. For the analysis of 133 individual fecal samples a total of 29 sequences corresponding to the level of genera were obtained.
Figure 12. Comparison of sequenced genera in desert bighorn sheep fecal pellets between seasons. For the analysis of 133 individual fecal samples a total of 29 sequences corresponding to the level of genera were obtained.
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Table 1. Results of Tukey’s HSD pairwise comparison test for forage type identified by microhistology versus season. Treatments not sharing like letters differ significantly.

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Table 2. Sequences of the Universal primers for the trnL chloroplast gene used to amplify plant fragments in fecal samples.

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<td>back to 7</td>
</tr>
<tr>
<td>11</td>
<td>68</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3. Thermocycler program for g-h primer set

<table>
<thead>
<tr>
<th>c-d primer pair Step down program</th>
<th>67C to 53.8 C</th>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>-0.4C</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>back to 2</td>
</tr>
<tr>
<td>6</td>
<td>back to 7</td>
</tr>
<tr>
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<td>95</td>
</tr>
<tr>
<td>8</td>
<td>53.8</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
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<tr>
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</tr>
<tr>
<td>11</td>
<td>68</td>
</tr>
<tr>
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<td>8</td>
</tr>
</tbody>
</table>

Table 4. Thermocycler program for c-d primer set
Table 5. Diversity indices for forage collection and forage types observed from microhistological analysis from bighorn fecal samples collected from project sites.

<table>
<thead>
<tr>
<th>Site</th>
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<tbody>
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<td>Horse Creek</td>
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<tr>
<td>Lauderback Hills</td>
<td>N/A</td>
</tr>
<tr>
<td>Cow Canyon</td>
<td>N/A</td>
</tr>
<tr>
<td>Little Angel</td>
<td>0.18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site</th>
<th>Simpson Diversity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td>Bench Creek</td>
<td>N/A</td>
</tr>
<tr>
<td>Deep Canyon</td>
<td>N/A</td>
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<tr>
<td>Horse Creek</td>
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</tr>
<tr>
<td>Lauderback Hills</td>
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</tr>
<tr>
<td>Cow Canyon</td>
<td>N/A</td>
</tr>
<tr>
<td>Little Angel</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.
Figure 12.
Conclusions

In conclusion, with some exceptions, study sites met minimum nutrition requirements over seasons for desert bighorn sheep as compared to domestic sheep nutrition guidelines. Zn content was high for plants collected Bench Creek. This was most likely due to the calcareous type clay soil. These clay-like soils have increased dispersivity of Na particles and break up aggregates more easily than a sandy-like soil, making soil organic matter (such minerals like Zn) more available for decomposition. Se was consistently deficient in all forage types and throughout all seasons, suggesting that Se deficiencies may exist in bighorn sheep populations in the Clan Alpines. However, other research has argued that any deficiency may be difficult to determine in wild animals that have adapted to different selection pressures than domestic animals (Robbins 1993). Furthermore, case studies that fed Rocky Mountain bighorn sheep feed low in Se and considered deficient in Se for domestic sheep did not result in clinical signs of Se deficiency (Dean et al. 2002). Markers for trace minerals are difficult to determine in wild animals (Robbins 1993, Dean et al 2002, Poppenga 2011). Moreover, sources of variation in measuring intake and interaction with other trace minerals and nutrients combined with environmental stresses and adaption differences may compound this variation (Dean et al. 2002). Therefore, further research may be needed to determine if forage in the Clan Alpine range is not adequately meeting Se requirements for desert bighorn sheep. Furthermore, ongoing investigations in adjacent ranges, Fairview Peak and Sandsprings Mountain ranges, that experienced catastrophic die-offs in 2007 may contribute to our understanding of Se forage deficiency in desert bighorn sheep.
As mentioned, forage quality varied between seasons and sites. Mineral concentrations did not follow any real trends; however, proximate analyses generally followed previously determined seasonal trends (Chapin 1980). In fact, nutrients such as CP and TDN were higher in spring than in winter. Microhistological analysis determined grasses as the dominant species in bighorn sheep fecal pellets. This data compares to previous diet analysis studies in which grass was determined as the most dominant forage type (Oldemeyer et al. 1971, Brown 1977, Wagner and Peek 2006). Interestingly, grass was collected more across seasons and sites than any other forage type. Grass maintained a high level of TDN and CP in spring and summer, an important time of year for ewes, coinciding with parturition and lactation in spring and breeding in late summer (Robbins 1993). However, shrubs and forbs, although, not found at a high frequency in fecal samples, are most likely just as important to diet as grasses.

For example, more shrubs were identified in spring and winter than in summer or fall. Interestingly, minerals, in particular P, were found to be higher in shrubs collected in spring than in any other season. The average P content of shrubs in spring was higher than any other forage type. Sheep may be eating more shrubs in spring to account for low P levels in other forage types.

Furthermore, according to forage collection data, more forbs were collected on average in spring and summer. Forbs were higher in mineral content in spring and summer than nearly every other forage type (although several proximate analyses and mineral parameters were unable to be determined to do insufficient plant material).
Also, it should be noted that microhistological analysis is less accurate at determining forbs than any other forage type (Shrestha and Wegge 2006). Therefore, the amount of forbs may be underestimated, especially in spring when mineral and protein content is high (Wagner and Peek 2006). Despite the lack of forb frequency in microhistology data, information obtained from PCR analyses can help determine if forbs are being consumed. PCR analyses found more forbs in fecal samples than any other forage type. Therefore, forbs are being consumed by bighorn sheep in the Clan Alpine range. It might be possible to use PCR, with more optimization, to determine which genera or species bighorn sheep are selecting in summer. As mentioned previously, nutrient contents may be higher in winter due to the early green-up of some winter and fall annuls such as cheat grass, it may be possible to determine if bighorn sheep are selecting these grasses by using PCR and sequencing in concert with microhistology.

The data from this research is preliminary. A larger sample size collected over several years would be needed to determine if forage from the Clan Alpine range is deficient in nutrients as well as further PCR optimization to determine diet from microhistological analysis. In fact, techniques such as pyrosequencing and qPCR may be explored for future research. Also collaring bighorn sheep in the range with GPS to better determine where sheep are foraging and make it possible to capture animals for blood and hair sampling is a possibility for future research. However, this data provides an excellent baseline example of nutrition requirements and diet of desert bighorn sheep in the Clan Alpine range for future research endeavors and reminds land
managers that diverse plant community is important for maintaining healthy sheep populations.

**Suggestions for future research**

Desert bighorn sheep are landscape animals that are capable of covering several acres in one day (Geist 1971). Although the information obtained from this study did determine diet in fecal pellets and forage quality on a small scale, for future studies, it is suggested that different methods are adopted in order to determine diet and forage quality for such large habitats for management purposes. By comparing historical ecological site data to sampled data, researchers could determine what forages desert bighorn sheep are using.

For example, installing permanent plant sampling plots determined by differing ecological site descriptions throughout the Clan Alpine range and comparing collected forage to data obtained from collected fecal samples will determine what forages desert bighorn sheep are using based on what forage is available. Soil samples can be compared from historical data to help determine changes in forage quality. Preference indices can be calculated using information obtained from microhistological analyses and information obtained from plant sampling. Therefore, managers and biologists can more accurately manage forages that desert bighorn sheep are consuming throughout the year.

Furthermore, collaring desert bighorn sheep with GPS collars will help with fecal collection and also determine what part of the range desert bighorn sheep are using most, which will also aid in management practices. Finally, although techniques such as
qPCR and pyrosequencing should be explored for future diet analysis research; PCR and traditional Sanger-sequencing in combination with microhistology will help determine taxonomic specific forage desert bighorn sheep are selecting. These changes will allow wildlife managers and biologists to more easily manage the range for desert bighorn sheep also allow for easier sampling throughout the year both important for objectives for the maintenance of desert bighorn sheep health.

**Literature Cited**


Appendix 1