Role of selective foraging and cecal microflora in sage-grouse nutritional ecology

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THE ROLE OF SELECTIVE FORAGING AND CECAL MICROFLORA IN SAGE-GROUSE NUTRITIONAL ECOLOGY

by

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Avian folivory is relatively uncommon in nature because leaves are generally difficult to digest, low in nutrients, and defended by plant secondary metabolites. Food quality is especially important to avian herbivores since they are constrained by body size and weight limits for flight. However, herbivorous birds may be able to overcome these constraints by selectively feeding on the highest quality forage. Greater sage-grouse (*Centrocercus urophasianus*) are strict sagebrush (*Artemisia* spp.) specialists in the winter. I evaluated sage-grouse winter feeding site selection to determine if plant nutrients and chemical profiles affect feeding site distribution at the landscape level. Sage-grouse feeding sites were best characterized as flat sites that were higher in sagebrush crude protein and sagebrush cover than what was available on average across the landscape. Selection for sagebrush higher in protein may aid in alleviating high costs of detoxifying plant secondary compounds and may result in increased clutch sizes and nest success.

Selective foraging may assure that the best possible forage is being consumed, but it is still a major physiological challenge to digest nearly whole leaves while remaining light enough for flight. In addition to behavioral adaptations for consuming sagebrush, ceca may aid in digestion and recycling of nutrients. Since the ceca contain tremendous numbers of bacteria, most of which are anaerobic and difficult to culture, the other focus of my research was to use molecular techniques to identify the microflora present in the ceca and to study the changes in community composition in relation to season and diet. There was no seasonal difference in cecal microbial community composition however there was marked animal-to-animal variation. Partial 16s rDNA sequence analysis revealed some similarities of the sage-grouse cecal microflora to chicken gastrointestinal tracts and cow rumen, but sage-grouse cecal microflora is largely unique and unknown. It was not possible to make inferences about ceca function due to the unknown bacteria present in the ceca.
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Introduction

Greater sage-grouse (*Centrocercus urophasianus*, hereafter referred to as “sage-grouse”) are associated with sagebrush (*Artemisia* spp.) throughout the year for both cover and food, but dietary specialization is the main reason that sage-grouse survival is so closely linked to available sagebrush habitat. Sage-grouse are completely dependent on sagebrush for survival since it constitutes almost 100% of their diet for as much as eight months of the year (October to May) (Wallestad 1975).

Sagebrush is a widely distributed range plant in western North America and is an available winter food resource when other plants may be leafless, covered with snow, or of poor nutritional quality (Nagy and Tengerdy 1968). Compared to alternate winter forages, sagebrush leaves are relatively high in crude protein (Striby et al. 1987) and low in fiber (Remington 1989). However, sagebrush is also highly defended by compounds such as the volatile oils that give sagebrush its distinctive smell. Unlike other grouse, sage-grouse lack a grinding gizzard, which may be an adaptation to limit the release of plant defensive compounds that are stored in glandular trichomes on the leaf surface (Remington and Braun 1985). Leaves are generally less digestible and contain lower concentrations of nutrients compared to animal tissue or fruit (Robbins 1993). Unlike ruminant mammals, sage-grouse are constrained by body size and weight requirements for flight, and so lack large digestive structures to efficiently digest leaves (Dudley and Vermeiji 1992). The lack of mechanical disruption of leaves in the gizzard may further reduce digestive efficiency. Yet, despite these challenges, sage-grouse juveniles continue to grow over the winter and adults maintain body mass and fat reserves even in areas known for harsh conditions (Remington and Braun 1988).
How do they do it while eating only sagebrush? Sagebrush chemistry is known to vary seasonally and spatially. It may be that sage-grouse do so well on a strict sagebrush diet by simply choosing to feed at sites with nutritionally higher quality sagebrush. A major focus of my research was to determine if sage-grouse winter feeding site selection was influenced by the nutritional and chemical characteristics of sagebrush. Specifically, I investigated if sage-grouse were more likely to feed in areas where sagebrush has higher nutritional quality and lower levels of defensive compounds.

Selective foraging may assure that the best possible forage is being consumed, but it is still a major physiological challenge to digest nearly whole leaves while remaining light enough for flight. In addition to behavioral adaptations for consuming sagebrush, ceca may aid in digestion and recycling of nutrients. Sage-grouse have very well developed ceca, which are paired microbe-filled chambers that are filled with liquid matter from the large and small intestines (McLelland 1989). Their size and the fact that they elongate seasonally with changes in diet implies that, in grouse, the ceca are functional organs (Fenna and Boag 1974); however the function is not well understood. Since the ceca contain tremendous numbers of bacteria (~10^{11}/g wet weight) (Mead 1989), most of which are anaerobic and difficult to culture, the other focus of my research was to use molecular techniques to identify the microflora present in the ceca and to study the changes in community composition in relation to season and diet. Presumably, the bacterial community plays an important role in the function of the ceca. By identifying the bacteria present and perhaps their metabolic capabilities, it may be possible to infer more about the function of the ceca and whether it increases digestive efficiency by helping to recover nitrogen and water.
Sage-grouse are declining throughout their range. The primary cause of their decline is habitat alteration and destruction. For a species whose reliance on sagebrush is tied to diet, it is of critical importance to understand how plant chemistry influences sage-grouse selection of winter feeding sites. In ruffed grouse, maternal dietary protein is also associated with increases in egg weights, hatching success, chick weight at hatching, and chick survival (Beckerton and Middleton 1982). Thus, behavioral adaptations that correlate to consuming food that is more nutritious and physiological adaptations that correlate to greater digestive efficiency can have significant impacts on survival and fitness.
Chapter 1

Sage-grouse Winter Feeding Site Selection: Does Plant Chemistry Matter?

Abstract
Avian folivory is relatively uncommon in nature because leaves are generally difficult to digest, low in nutrients, and defended by plant secondary metabolites. Food quality is especially important to avian herbivores since they are constrained by body size and weight limits for flight. However, herbivorous birds may be able to overcome these constraints by selectively feeding on the highest quality forage. Greater sage-grouse (Centrocercus urophasianus) are strict specialist herbivores in the winter. Sage-grouse winter feeding site selection was evaluated to determine if plant nutrients and chemical profiles affect feeding site distribution at the landscape level. Sage-grouse feeding sites (n = 54) and available sites (n = 54) were surveyed in southern Phillips County, Montana from October 2004 to March 2005. At each site slope and sagebrush (Artemisia spp.) structure was measured and the nutritional quality of sagebrush leaves was assessed. Univariate analysis revealed significant differences in slope (P = 0.001), crude protein (P < 0.001), cover (P < 0.001), fiber (P = 0.004), and the monoterpenes p-cymene (P = 0.044) between feeding and available sites, with feeding sites being flat sites that were higher in sagebrush protein content and cover and lower in sagebrush fiber and monoterpene content. In a logistic regression model, crude protein (P = 0.016) and cover (P = 0.031) were found to be significant predictors of sage-grouse feeding sites. Sage-grouse feeding sites were best characterized as flat sites that were higher in sagebrush crude protein and sagebrush cover than what was available on average across the landscape. Selection for sagebrush higher in protein may aid in alleviating high costs of detoxifying plant secondary compounds and may result in increased clutch sizes and nest success.

Introduction
Leaves are generally less digestible (higher in fiber) and contain lower concentrations of nutrients compared to animal tissue or fruit (Robbins 1993).

Additionally, plant leaves are often defended by a wide array of toxic secondary compounds (McNab 2002). Food quality is especially important to avian herbivores since they are constrained by body size and weight limits for flight. Thus, they are limited in their ability to consume more food in order to compensate for low nutrient content (Sedinger 1997), and they cannot support large, complex digestive structures such as those found in ruminate mammals (Dudley and Vermeij 1992). However, herbivorous
birds may be able to overcome lower nutrient levels by selectively foraging on plants with the highest nutrient concentrations.

Greater sage-grouse (*Centrocercus urophasianus*, hereafter “sage-grouse”) are associated with sagebrush (*Artemisia* spp.) throughout the year for both cover and food but dietary specialization is the main reason that sage-grouse survival is so closely linked to available sagebrush habitat. The diet of both juvenile and adult sage-grouse is dominated by sagebrush, and in Montana, big sagebrush (*A. tridentata*) constitutes up to 60% of the summer diet and almost 100% of the winter diet (Wallestad et al. 1975.)

Sagebrush is a widely distributed range plant in western North America and is an available winter food resource when other plants may be leafless, covered with snow, or of poor nutritional quality (Nagy and Tengerdy 1968). Compared to alternate winter forages, such as dried grasses, sagebrush leaves are relatively high in crude protein (Striby et al. 1987) and low in fiber (Remington 1989). However, sagebrush leaves are also high in secondary compounds including monoterpenes, sesquiterpene lactones, coumarins, and flavonoids. Many of these compounds are biologically active and could act as feeding deterrents (Kelsey et al. 1982).

Unlike other grouse, sage-grouse lack a grinding gizzard\(^1\) (Remington and Braun 1985, Welch et al. 1988), which may be an adaptation to limit the release of plant defensive compounds that are stored in glandular trichomes on the leaf surface (Kelsey et al. 1982). However, having the leaves pass through the gizzard without complete physical disruption could result in reduced digestive efficiency. Yet, despite these challenges,

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\(^1\) Welch et al. disagree with Remington and Braun in characterizing the gizzard as non-grinding. They prefer to characterize it as a nongrit grinding gizzard that is not capable of breaking down seeds. While some leaves are certainly ground into fragments, I have observed whole and nearly-whole sagebrush leaves in sage-grouse intestinal (woody) droppings that were collected in the winter from roosts.
sage-grouse juveniles continue to grow over the winter and adults maintain their body mass and fat reserves even in areas known for harsh conditions (Remington and Braun 1988).

Sagebrush nutrient content and concentrations of defensive compounds can vary seasonally (Kelsey et al. 1982) as well as among individual plants (Personius et al. 1987), subspecies (Welch and McArthur 1981), and species (Nagy and Regelin 1977). It may be that sage-grouse are able to continue to grow as juveniles and maintain weight as adults on a strict winter diet of sagebrush by selectively foraging in areas where plants have higher nutritional quality and lower concentrations of defensive compounds.

Selective foraging based on plant chemistry and nutrient content has been demonstrated in other grouse. For example, spruce grouse (*Dendragapus canadensis*) have demonstrated selection for lodgepole pine (*Pinus contorta*) leaves that have higher protein content (Hohf et al. 1987). Ruffed grouse (*Bonasa umbellus*) feeding preference is best predicted by both crude protein content and coniferyl benzoate (a plant secondary metabolite) content of aspen buds (Jakubus et al. 1989). Thus, feeding preferences may be based on nutrient content, plant secondary compounds, or a combination of several chemical and nutritional characteristics.

Sage-grouse have repeatedly exhibited differential feeding preferences both in the field (Remington and Braun 1985, Welch et al. 1988) and in captive feeding trials (Welch et al. 1991). However, the relationship between feeding preference and plant nutritional quality is unclear. In Colorado, sage-grouse prefer Wyoming big sagebrush (*A. tridentata wyomingensis*) over mountain big sagebrush (*A. tridentata vaseyana*), presumably because of the lower concentrations of monoterpenes in Wyoming big sagebrush. In
addition, crude protein content was higher in browsed plants compared to random plants within a subspecies (Remington and Braun 1985). In contrast, in Utah there was no difference in crude protein or monoterpane content between browsed and unbrowsed plants. Yet, there was a significant difference in *in vitro* digestibility, with browsed Wyoming big sagebrush plants being more digestible (Welch et al. 1988). These conflicting results may be due to differences in experimental design or simply geographic differences in plant chemistry. Clearly the relationship between sage-grouse feeding behavior and plant nutrient and plant secondary metabolite concentrations needs further resolution.

The objective of this study was to determine whether sage-grouse feeding is related to sagebrush nutrient and plant secondary metabolite (PSM) composition at the landscape scale and to determine the factors that best predict sage-grouse winter feeding sites. While sage-grouse depend on sagebrush as a sole winter food source, they also depend on sagebrush for other important functions such as thermal cover and cover from predators. To account for these other requirements, I measured a range of variables, in addition to measures of sagebrush forage quality, including slope, sagebrush cover, sagebrush height, and snow depth.

**Study Area**

The study area was located on the Charles M. Russell National Wildlife Refuge (CMR) and in the Sun Prairie area (north of CMR) of Phillips County, Montana. The Sun Prairie area is a mosaic of private ranches, state, and Bureau of Land Management (BLM) lands. The majority of the area is rolling shrub-steppe although the southern boundary of Phillips County is forested Missouri River “breaks” habitat. The dominant

**Methods**

**Site Selection**

Sage-grouse hens were trapped near leks by either rocket nets at dawn or handheld nets while spotlighting at night in the spring (late March to early April) and late summer (August to mid-September) of 2004 (Geisen et al. 1982). A total of 80 trapped hens were fitted with a necklace radio transmitter (model A4060, Advanced Telemetry Systems, Inc., Insanti, MN) (Moynahan et al. 2006). Trapping and handling protocols were approved by the Institutional Animal Care and Use Committee at the University of Montana. Additionally, trapping and special-use permits were issued by the Montana Department of Fish, Wildlife, and Parks (FWP), the US Fish & Wildlife Service (USFWS), and the Bureau of Land Management (BLM).

To find feeding sites, I located randomly selected radio collared hens each month from October 2004 to March 2005. At sites with low shrub cover, near roads, or when snow was present, sage grouse could be directly observed from a distance, using a vehicle as an observation point. However, this method of observation could only be used occasionally since off-road vehicle use is not permitted on the CMR and is discouraged on BLM lands. In areas with denser shrub cover it was difficult to observe sage-grouse
feeding from a distance, and individuals had to be approached on foot. This, however, was problematic as birds often flushed when approached. To circumvent these problems, I was able to determine where sage-grouse fed based on characteristic damage to sagebrush leaves. Fed upon leaves have a diagnostic contrast in color between the outside leaf surface and the inside cut surface (Figure 1) (Remington and Braun 1985). I classified a location as a feeding site if I flushed birds from the site and found evidence of fresh droppings and fresh feeding on sagebrush leaves (Hupp and Braun 1989). If, after several attempts, I was not able to locate one of the selected birds, I located an alternate randomly-selected bird to follow.

I compared physical characteristics of feeding sites and characteristics of plants utilized for feeding by sage-grouse to an equal number of randomly selected sites and plants at those sites to determine if sage-grouse fed selectively. Randomly selected sites were separated from each other by at least 500 m and contained at least one big sagebrush plant within a 25 m radius of the center point. These sites were selected with the aid of a geographic information system (ArcGIS). Sites along the Missouri River in “breaks” habitat with either ponderosa pine (Pinus ponderosa) or Douglas-fir (Pseudotsuga menziesii) present were not considered. All available sites were within sage-grouse winter habitat use area.

Vegetation Sampling

At all sites, I measured the percent cover and height of big sagebrush (and, if present, silver sage [Artemisia cana]) along a 50 m line transect (Canfield 1941, Connelly et al. 2003). At feeding sites, the transect line center point was a fed-on plant. At available sites, the center point was the random coordinates. From the center point, all
transects were oriented at a random bearing. I measured sagebrush height as the tallest branch of the plant with live leaves that did not include any inflorescences. Plants that intercepted the transect line were also sampled for further chemical analysis. Since there was only one silver sagebrush plant that intercepted the transect line at a single feeding site, only big sagebrush leaf samples were included in the chemical analyses. I clipped branches from all over the plant with pruning shears and pooled samples from all plants along the transect (hereafter referred to as composite site samples) prior to chemical analysis. Plant samples were placed in airtight plastic bags, transported on ice/snow back to the field camp, and stored frozen. (Since monoterpenes are volatile, it would be best to immediately freeze samples on dry ice; however this was not possible due to logistical constraints.) I recorded the slope of each site (from the center point) using a clinometer and snow depth along the transect line every 5 m. At feeding sites, I recorded whether individual plants had been fed on by sage-grouse.

I measured cover with snow present and with snow absent. There are important biological and management reasons for considering cover both ways. It is important to consider what the conditions were when the grouse were actually present at the site. However, snow depth can be a rapidly changing and ephemeral variable to capture. It may be logistically impractical for managers to assess sagebrush cover for an entire area in a brief-enough time interval that all of the measurements were recorded under similar snow conditions. It may be easier to standardize cover measurements by simply measuring cover when no snow is present. Although sagebrush will have additional leaves and new growth in the summer compared to winter, it is unlikely that the relative cover between feeding and available sites will significantly change.
In order to assess variation in plant chemistry among individual plants within a site, I kept clipped branches from individual shrubs separate, in addition to pooling samples at half of the sites in December and February. In order to have enough leaf tissue to analyze for fiber, crude protein, and monoterpene content, I selected the five largest, most vigorous plants along each transect line. These samples were treated the same as the composite sample, with the exception that they were kept separated as individual plants for the chemical analyses.

_Sagebrush Chemical Analysis_

To assess sagebrush nutritional quality, I measured the water content, fiber, crude protein, and monoterpenene content of sagebrush leaves. Water content has not been previously considered. While sage-grouse are known to drink from reservoirs and eat snow, there was the potential for winter conditions in which there was no snow and all available water had frozen, in which case all water would have to be supplied by the diet. Fiber was considered since, in Utah, digestibility was considered to help discriminate between browsed and unbrowsed Wyoming big sagebrush plants (Welch et al. 1988). Crude protein was considered because sage-grouse as well as other grouse species demonstrate preferences for forages containing relatively high protein (Remington and Braun 1985). Finally, monoterpenes were measured because they have been shown previously to influence sage-grouse preferences among sagebrush subspecies and because a single plant secondary metabolite was found to be important in ruffed grouse winter diet selection (Jakubus and Gullion 1991).
Sample Preparation and Water Content

I analyzed only sagebrush leaves since sage-grouse typically eat only this part of shrubs. In the laboratory, plant samples were processed in a 4°C cold room to limit the volatilization of monoterpenes. I clipped leaves with a pair of dissecting scissors and these leaves were mixed well and split into two groups. One group was used for monoterpenene analysis and was stored frozen at -20°C. The other group was dried at 60°C oven for 48 hours to determine water content.

Fiber and Crude Protein Content

After drying, I ground leaves in a Wiley mill (1mm mesh size) and a 2-3 g subsample was analyzed for neutral detergent fiber (NDF) at the Washington State University Wildlife Habitat Nutrition Laboratory (Pullman WA, USA) according to methods of Van Soest and Ankom (Goering and Van Soest 1970, Komarek 1993). NDF includes hemicellulose, cellulose, and lignin (Komarek 1993). The remaining subsample of leaves was dried at 80°C for 24 hours before being pulverized. Approximately 100 mg of dried leaves were placed in a 2 ml centrifuge tube with a 5 mm stainless steel grinding bead. The tubes were then shaken in a Geno/Grinder 2000 at a rate of 700 strokes/min for one minute. Total nitrogen content was measured at the University of Montana Environmental Biogeochemistry Laboratory using an elemental analyzer (CE Instruments Model 1110). The instrument was calibrated using standards purchased from the manufacturer. Crude protein was estimated by multiplying total nitrogen by 6.25.

Monoterpene Content

Leaf samples were shipped on dry ice to the University of Western Ontario for monoterpenene analysis. Wet leaves were ground in liquid nitrogen using a pre-cooled
mortar and pestle (Welch and McArthur 1981). After grinding, approximately 100 mg of tissue was immediately put in a microcentrifuge tube and 1 ml of hexane spiked with an internal standard (1 µl carvone [CAS#99-49-0]) was added. Monoterpenes were extracted by incubating the sample on a horizontal shaker at 24° C for 24 hours (modified from Wallin and Raffa 1999). Tubes were centrifuged at high speed (10,000 x g) for 5 min and the supernatant was removed using glass Pasteur pipettes. Extracts were dried over calcium chloride and centrifuged at high speed for 1 min. The supernatant was removed and filtered through glass wool into autosampler vials with 200 µl inserts. The leaf tissue in the original microcentrifuge tube was air dried in a fume hood overnight and then dried at 60 ° C for 24 hours to determine dry weight.

Monoterpenes were separated and measured on a CX3400 gas chromatograph with a FID detector. The stationary phase was a WCOT Fused Silica column (30 x 0.25 mm), coated with CPWAX 52CB (DF=0.25 µm) (Chrompack, Netherlands). I modified the temperature program of Raffa and Steffeck (1988) to an initial temperature of 60° C for 7.5 min, then increasing 10° C per min until reaching 180° C, and then held at 180° C for 2.5 min. The injector and detector temperatures were 220° C. The make-up gas was nitrogen and the carrier gas was helium.

Prior to analyzing samples, I verified that the internal standard carvone was not naturally present in our samples (Welch and McArthur 1981). I choose standards based on monoterpenes found in big sagebrush by Welch and McArthur (1981). To check for any drift in retention times, I ran a standard mix (1 µl of each α-pinene [CAS# 2437-95-8], 2-carene [CAS# 4497-92-1], α-phellandrene [CAS# 4221-98-1], eucalyptol [CAS# 470-82-6], p-cymene [CAS# 99-87-6], α-thujone [CAS# 546-80-5], α-terpineol [CAS#
10482-56-1], and carvone in 992 µl of hexane) at the start of every group run. All monoterpene standards were supplied by ChromaDex (Santa Ana, California). Additionally, I ran a “blank control” at the end of every group run to check for any contaminants. The blank control was 1 ml of the hexane/carvone extraction solvent that went through all the same steps of the process as the samples.

Monoterpene retention times and peak areas were calculated using Saturn GC/MS Workstation version 5.41. Monoterpenes were identified by relative retention time (retention time of the peak in question divided by the retention time of carvone). To be sure of correct identifications, whenever possible, I verified the relative retention time of a compound by co-chromatography with a known standard. All other peaks were identified by running example samples through the same column and temperature program on a 3800 Gas Chromatograph with Saturn 2000 Mass Detector (ion trap mass detector with a m/z limit of 650 amu). Mass spectral data generated for unidentified peaks was compared to a published library of mass spectra for essential oils (Adams 1989).

Since I did not have standards for all the compounds, I was not able to correct individual peak areas by the response factors for each compound. Since my aim was to compare feeding sites relative to available sites, I used a relative measure of concentration by expressing concentrations in carvone equivalents. Monoterpene amounts were expressed as percent dry matter. For each compound, the following calculations were made to determine the concentration of the compound in sagebrush leaf tissue. First, relative peak areas were calculated as the peak area divided by the area of the carvone peak. Relative area was then multiplied by the density of carvone (0.9646 mg/ml) and then multiplied by the amount of tissue extracted (mg).
Data Analysis

Composite Samples – Feeding Site Selection

I performed both univariate and multivariate analyses to investigate whether sage-grouse were selecting feeding sites based on plant chemistry or on site characteristics. Univariate analyses do not account for possible interactions between variables and it is certainly possible that there may be a combination of variables that are preferred. Since I was able to identify several different monoterpenes, the number of potential variables for a logistic regression increased beyond what was appropriate for my sample size (Hosmer and Lemeshow 2000). For sage-grouse, there is no previous information that implies that one particular monoterpenene is more important than another so I did not want to arbitrarily exclude compounds. To screen for the most important variables, I performed several univariate tests in which I tested each continuous variable separately for differences between feeding and available sites using either a t-test or a Mann-Whitney test, depending on the distribution. Slope measurements were collapsed into two categories: flat (areas of ≤ 5° slope) or sloped (areas of > 5° slope) (Hupp and Braun 1989). A Chi-Square was performed to determine if there were significant differences in slope between feeding and available sites. All variables significant at P<0.10 were retained for further analysis in a logistic regression (Manly et al. 2002). Variables were removed from the logistic regression model until only variables that were statistically significant remained (Hosmer and Lemeshow 2000). (Although see Keating and Cherry 2004 for arguments against using logistic regression in use-availability studies.)
Individual Plant Samples – Within and Between Site Variation

Since plant chemistry is known to vary among individual plants, I assessed if there was more variation among plants within a site than among sites. I tested each variable separately using a random-effects (Model II) analysis of variance (ANOVA). Most tests were conducted using SPSS v14.0, except for the random-effects ANOVA tests which were conducted using S-Plus.

Results

Snow Depth

Over a six-month period, I sampled 54 feeding sites and 54 random sites (Figure 2). While there was a light dusting of snow in December (transect mean snow depth 0-1 cm), the only month with significant snowfall was January. I recorded the “actual cover” as the cover available under the conditions in which I observed the grouse at the site. Sagebrush covered by snow was not included in the cover measurement and so “actual cover” may be much less than what is there when there is no snow. I went back to all of the January sites and recorded a second measure of cover after the snow melted, which I refer to as “absolute cover”. The absolute cover measurements for the January sites were at the same site but not along the original transect since the sagebrush had been clipped for chemical analysis. There was no difference in snow depth between feeding and available sites (t = 0.533, P = 0.590), with average snow depth at feeding sites 15 cm and 14 cm at available sites. There was also no difference in actual cover above snow (t = 1.674, P = 0.132) or absolute cover (no snow) (t = 1.735, P = 0.108) between feeding sites and available sites in January (n = 14). However, snow depth in January was enough
to result in a difference between cover measurements when snow was present and after it had melted ($t = 4.926$, $P < 0.001$).

**Monoterpene Content**

Using co-chromatography and mass spectroscopy, I was able to identify 10 monoterpenes. Two peaks, though clearly resolved at retention times of 4.284 and 4.994 minutes, appeared to both be forms of camphene. The mass spectra for these peaks are practically identical to each other and a good match to a published mass spectrum of camphene. For the univariate tests, I tested them both as separate compounds as well as by summing the two peaks into a combined camphene variable. There was no significant difference between feeding sites and available sites for either camphene peak measured individually (camphene1 $U = 1347.50$, $P = 0.603$; camphene2 $U = 1363.0$, $P = 0.672$) or for “combined camphene” ($U = 1321.0$, $P = 0.493$). Four other peaks could not be identified by co-chromatography. Artemiseole and borneol were identified by mass spectra. The other two peaks show mass spectra with patterns similar to other monoterpenes but I was unable to confidently determine a match to published mass spectra. Retention times for all the compounds detected in the sample as well as standards used in the standard mix, including the internal standard, are reported in Table 1. While the mean concentration of both individual monoterpenes (Figure 3) and total monoterpene content (Figure 4) between feeding and random sites composite samples is nearly identical, it is interesting to note that not all of the compounds were found in every sample and there can be a considerable range in concentrations between samples (Table 2).
Composite Samples – Feeding Site Selection

Univariate Analysis

There was a significant difference in slope ($\chi^2 = 10.565, P = 0.001$) between feeding and available sites; 96% of feeding sites had a slope < 5° whereas this was only the case for 74% of available sites. Both absolute cover (no snow) ($t = -3.673, P < 0.001$) and actual cover above snow ($t = -3.114, P = 0.002$) were significantly higher at feeding sites than at available sites (Table 3). In terms of plant chemistry, crude protein ($t = -3.767, P < 0.001$) and $\rho$-cymene ($U = 1109.00, P = 0.044$) were significantly higher at feeding sites and NDF ($U = 991.50, P = 0.004$) was significantly lower at feeding sites compared to available sites (Tables 3 and 4). In addition to the above significant variables, $\alpha$-pinene was also retained for further multivariate analysis ($t = -0.1803, P = 0.074$).

Multivariate Analysis

Since there was a significant difference between absolute cover (no snow) and actual cover above snow, I chose to include each in separate logistic regression models. Other variables included were slope, crude protein, NDF, and the monoterpenes $\rho$-cymene and $\alpha$-pinene. I also included a date variable which was used to make sure that site survey date (and thus any seasonal variation in plant chemistry) was not significantly influencing results since sites were surveyed over a six-month period.

The results for both logistic regression models, with snow absent and snow present, are the same. Sagebrush cover and crude protein content were significantly higher at feeding sites compared to available sites (Table 5). When statistically insignificant variables are removed one-at-a-time, sagebrush cover ($P = 0.009$) and crude
protein content ($P = 0.014$) remain the only significant feeding site predictor variables when snow is absent. However, when variables are removed from the snow present model, in addition to sagebrush cover ($P = 0.030$) and crude protein content ($P = 0.017$) being significant, slope also becomes a significant predictor variable ($P = 0.045$) with flat sites being more likely to be used as feeding sites (Table 6). Date of site survey is not significant in any model, suggesting that any potential variation in plant chemistry over the sampling period is not a result of temporal changes in leaf chemistry.

*Individual Plant Samples – Within and Between Site Variation*

Plant chemistry variables were considered for 97 individual plant samples collected at 10 feeding and 10 available sites. There was significantly more variation among sites than among individual plants within a site plants for 8 of the 14 variables considered (Table 7).

**Discussion**

*Sagebrush Canopy Cover and Leaf Crude Protein Content*

The objective of this study was to determine if sage-grouse feeding site selection is related to sagebrush nutritional quality at the landscape scale and to determine the characteristics that best predict sage-grouse winter feeding sites. Sagebrush nutritional quality is an important indicator of sage-grouse winter feeding sites, with feeding sites being significantly higher in crude protein than randomly-selected available sites. Other important characteristics of sage-grouse winter feeding sites are slope and sagebrush cover. Sage-grouse winter feeding sites were typically flat and had significantly more sagebrush cover than available sites. While sage-grouse use sagebrush for both cover and food throughout the year, their dependence on sagebrush for survival is driven by dietary
specialization. Since sage-grouse consume a strict diet of sagebrush during the winter, it
is critical to consider feeding site selection in regards to winter habitat use.

Connelly et al. (2000) recommend managing sage-grouse winter habitat so that
there is 10 – 30% sagebrush canopy cover. Feeding sites in my study had a mean
sagebrush canopy of 12.5% when no snow was present and 11.1% above snow. While
Eng and Schladweiler (1972) found sage-grouse winter-use sites in Montana were
characterized by greater than 20% sagebrush cover, only 15% of winter feeding sites in
this study were characterized by that much cover (either with snow present or absent). A
little more than a third of the feeding sites (35%) had less than 10% sagebrush canopy
cover. However, most of the 2004-2005 winter was mild with the exception of notably
cold temperatures and persistent snow cover occurring only in January.

Plants at sage-grouse winter feeding sites were also significantly higher in crude
protein than available sites, which supports previous observations in Colorado
(Remington and Braun 1985). However, mean crude protein content at feeding sites and
available sites was very similar at 14.7% and 13.8%, respectively. While Wambolt (2004)
found a significant difference in crude protein content between young and mature
Wyoming big sagebrush plants, he concluded that a 1.2% difference in crude protein was
not a meaningful difference for ungulate herbivores especially since even mature plants
had a crude protein content of 11.3% which is well above the 7.5% maintenance
requirement for deer. The dietary concentration of protein necessary for winter
maintenance may vary depending on sex, age, and environmental conditions and is not
known (to my knowledge) for sage-grouse. It is difficult to determine specific nutritional
requirements from captive birds since gut morphology is known to change in grouse kept
in captivity. Additionally, this change in gut morphology leads to significant changes in how well the birds digest natural foods, with it being common for captive grouse fed natural diets to lose weight (Moss and Hanssen 1980). However, the effects of dietary protein and reproductive success in grouse have been studied and it may be that improved reproductive performance is the most important fitness consequence of selectively foraging on sagebrush high in protein.

Sage-grouse hens begin laying early in the spring when the availability of other foods such as herbs and insects may be limited. Even after the emergence of forbs, sagebrush comprised 50 to 82% of the diet of pre-laying hens in Oregon (Barnett and Crawford 1994) and in Montana, sagebrush was found to account for over 80% of the crop volume as late as May (Wallestad et al. 1975). It is unknown whether sage-grouse rely on spring diet or endogenous reserves for egg production. However, since sagebrush is an important dietary component both in the months and weeks preceding laying, it should be considered an important component of maternal nutrition. Selectively foraging on sagebrush with more crude protein may have significant impacts on fitness. For example, for ruffed grouse there is a significant positive linear relationship between dietary crude protein content (from 7.6% up to 20.1%) and clutch size, clutch weight, hatching success, chick weight at hatching, and chick survival to nine weeks of age (Beckerton and Middleton 1982). Since it can be difficult to assess dietary intake of wild birds, blood plasma protein levels can be used as an indicator of dietary protein. For sage-grouse, it has been suggested that a relatively small increase in blood plasma protein (0.1 g/dl) would approximately double the likelihood of at least one chick from a clutch
surviving until late summer (Dunbar et al. 2005). If true, even seemingly small increases in dietary crude protein could have significant biological consequences.

Anecdotal observations also support the notion that crude protein differences between sites are more than simple statistical artifact. First, my experimental design resulted in conservative estimates of differences in protein content since crude protein levels were compared between feeding sites and available sites and did not consider whether or not individual plants within a site were preferred over others. Other studies (Remington and Braun 1985, Welch et al. 1988) have shown that sage-grouse do not browse on all sagebrush plants within a site. Remington and Braun (1985) found significant differences in crude protein content of browsed and unbrowsed plants within a feeding site. My estimates of the difference in crude protein content between feeding sites and available sites are likely conservative since not all plants at a feeding site are browsed. Also, available sites, by definition, are not limited to unused sites. In fact, sage-grouse were flushed from an available site during the survey and later analysis showed that leaf samples from that site had a crude protein content of 16.05%. I also made an interesting observation of sage-grouse feeding on a black-tailed prairie dog (Cynomys ludovicianus) town in January. The prairie dogs had clipped the sagebrush so that mean height was only 9 cm and thus sagebrush cover above snow was only 1%. Yet, a group of 15 sage-grouse were feeding on the sagebrush by pecking away at the snow to uncover it. Crude protein content of the sagebrush at that site was 15.75%. Sage-grouse have been known to uncover sagebrush that is covered in snow and they are capable of doing such in snow up to 30 cm deep (Beck 1977). However, it is intriguing that they would do so on
the prairie dog town since there was sagebrush available above the snow at the edge of
the prairie dog town (less than 100 meters away).

Slope

There were mixed results for the importance of slope in feeding site selection. In a
multivariate analysis, slope was only a significant predictor of feeding sites when snow
was present. Since there was no significant difference in snow depth between site types
and all sites in January (only time when snow was present) were flat, it may be that slope
is correlated with another unmeasured variable that drives feeding site preference. While
all variables were checked for multicollinearity prior to the logistic regression, there is a
trend for flat sites to be higher in cover and crude protein than sloped sites. Univariate
analysis resulted in a highly significant difference in slope between feeding and available
sites. Slope may still be an important variable that should be considered locally in the
context of snowfall in evaluating sage-grouse winter habitat. While sage-grouse prefer
flat sites in both northern Colorado (Beck 1977) and eastern Montana (Eng and
Schladweiler 1972), Hupp and Braun (1989) found sage-grouse were unable to use flat
sites due to snow depth in the Gunnison Basin (Colorado)^2. They suggest that snow
depths of greater than 30 cm were likely to result in sagebrush being buried and thus
unavailable at flat sites. Median height of sagebrush at feeding sites in my study was 24
cm. While sage-grouse used predominately flat sites in this study, it was not a severe
winter. Sloped sites should be considered potential winter feeding sites during severe
winters when snow accumulation may bury sagebrush at flat sites.

^Sage-grouse present in the Gunnison Basin have subsequently become known as Gunnison sage-grouse
(Centrocercus minimus).

2
While slope is an important variable to consider, it is important to note that the relationship between slope and crude protein content is not responsible for crude protein content being significant in either the univariate or multivariate analysis. When only flat sites were analyzed (n = 92), crude protein and sagebrush cover continue to be significant predictor variables of sage-grouse feeding sites.

Monoterpene Content and Fiber

Two monoterpenes (α-pinene and p-cymene) and fiber were variables that were significant in univariate tests but were not significant predictors of feeding sites when considered in conjunction with other variables. Since I violated an assumption of logistic regression in my analysis (feeding sites and available sites are not mutually exclusive groups\(^3\)), it is important to evaluate variables that univariate tests concluded were significantly different between site types.

Only two monoterpenes, α-pinene and p-cymene, were included as potentially important predictor variables in the logistic regression analysis. While there is a difference in the compounds between site types, the difference is very small (Figure 3). Additionally, the overall concentration of both of the compounds in composite leaf samples is very small (less than 0.1% dry matter). Finally, there is slightly more of each compound found in composite leaf samples from feeding sites than in samples from available sites, so it does not appear that monoterpenes act as feeding deterrents.

Monoterpenes isolated from big sagebrush have been shown to have antimicrobial properties that can change both the overall abundance of and community composition of bacteria in mule deer (*Odocoileus hemionus*) rumen (Nagy and Tendergy 1968). Due to

\(^3\) It is unreasonable to attempt to define sites as feeding sites and non-feeding sites since observing evidence of sage-grouse browsing requires examination of individual leaves on plants. It would be highly likely that some “non-feeding” sites would be misclassified.
the tremendous numbers of bacteria present in both a rumen and in sage-grouse ceca, it is tempting to draw parallels. Remington and Braun (1985) found a difference in monoterpenic content between subspecies of sagebrush and hypothesized that sage-grouse needed to forage on sagebrush low in monoterpenes to protect the cecal microflora. However, a critical difference between a rumen and the ceca are their location in the digestive tract. The ceca are a pair of blind sacs at the junction of the small and large intestine. Welch et al. (1989) found that while monoterpenes were detectable in the gizzard, they were not present in detectable levels in the ceca. Thus, while there is overlap between rumen and ceca microflora (see Chapter 2), the antimicrobial properties of monoterpenes are not important since they are dealt with prior to reaching the ceca. If plant secondary metabolites are not degraded by symbiotic gastrointestinal bacteria, then they must undergo biotransformation before they can be excreted. Guglielmo et al. (1996) found that detoxification costs are high for ruffed grouse. Conjugation of coniferyl benzoate with ornithine (an amino acid derived from the essential amino acid arginine) increased nitrogen requirements by 68 to 90%. Ruffed grouse minimize detoxification costs by selectively feeding on aspen buds lower in coniferyl benzoate.

While there was quite a range in both concentration and composition of monoterpenes in sagebrush leaves between feeding sites and available sites, there is no evidence of sage-grouse selectively choosing feeding sites based on sagebrush monoterpenic content. Because sage-grouse do not appear to reduce monoterpenes in their diet through selective foraging, and because these birds do not have foregut microbial populations (i.e. a rumen) capable of degrading these compounds, sage-grouse must
conjugate the compounds, which requires both high levels of nitrogen, as well as the essential amino acid arginine (Guglielmo et al. 1996).

Although Welch et al. (1988) found a significant difference in *in vitro* digestibility between browsed and unbrowsed Wyoming big sagebrush, I have mixed results regarding the significance of fiber content in the diet. Univariate analysis suggest that there is a significantly less NDF at feeding sites compared to available sites (*P* = 0.004), however when considered in context with other variables in a multivariate analysis it is not a significant predictor variable. As mentioned previously, there is some overlap in microbial communities between the rumen and sage-grouse ceca; however it is unlikely that much fiber digestion occurs in the ceca because microvilli at the opening of the ceca prevent large particles from entering (McLelland 1989, Remington 1989). While it makes sense that sage-grouse would prefer low fiber plants (as suggested by the univariate analysis) since they do not efficiently digest fiber, it may be that cover from predators and crude protein for detoxification are more important predictors of feed site selection than is low fiber.

*Management Implications and Recommendations for Future Research*

Moynahan et al. (2006) noted variation in clutch sizes and encouraged enhancement of winter habitat in an effort to improve the conditions of hens and increase clutch sizes. While dietary protein intake can certainly influence body condition, clutch size, hatching success, chick weight at hatching, and chick survival, it may be difficult to manage sagebrush to increase protein content. Remington and Braun (1985) suggested that applying a nitrogen fertilizer would increase sagebrush protein content. Attempts to improve sagebrush forage quality through nitrogen fertilization appear to either be
ineffective or short-lived. Sneva et al. (1983) found no significant difference in essential oil content of sagebrush that had been fertilized. Although Myers (1992) found a 30-52% increase in foliar protein content following nitrogen fertilization, the effect was short-lived. Managing for a particular age class does not look promising either. Wambolt (2004) concluded that managing for early successional stages of sagebrush would not result in stands of sagebrush with a biologically significant higher protein content.

Since crude protein content in sagebrush appears under genetic control and sagebrush is a long-lived plant, the best strategy may be to simply identify high-quality patches of sagebrush as critical winter feeding areas. While there is within site variation, it would be possible to identify sites that are composed of a higher proportion of high-quality sagebrush. It may be possible in the future to map large areas of sagebrush in terms of forage quality by using near infrared reflectance spectroscopy (NIRS). NIRS has been commonly used to identify nitrogen content of grains and a hand-held battery operated instrument has been used to measure nitrogen of rice leaves (Foley et al. 1998). However, I believe efforts to map and prioritize sagebrush habitat in terms of forage quality would be premature at this point since we do not know a minimum target amount of crude protein for management purposes.

The results of this study suggest that sage-grouse are selecting winter feeding sites based on sagebrush cover and crude protein content. My results also bolster previous studies showing (Welch and McArthur 1979) variation in crude protein content in individual plants. It is not known if differential forage selection translates into differential reproductive success of sage-grouse. Mean crude protein content at feeding sites in this study was 14.71%. However, there is a wide range in protein content of sagebrush at
sage-grouse feeding sites, from a high of over 17% in Wyoming big sagebrush in Colorado to a low of 9.5% in basin big sagebrush in Utah (Remington and Braun 1985, Welch et al. 1988). Before considering possible management strategies, it is critical to determine if there is a significant relationship between sagebrush crude protein content and fitness, and if so, at what level.
Figure 1. Enlarged photograph of big sagebrush leaves with arrows highlighting contrasting colors inside and outside of the cut leaf surface after having been fed on by sage-grouse.

Figure 2. Locations of Greater sage-grouse (Centrocercus urophasianus) feeding and available sites in southern Phillips County, Montana. The southern boundary is the Missouri River. Major roads are included for reference.
Table 1. Retention times of monoterpenes detected in the samples of Wyoming big sagebrush (*Artemisia tridentata wyomingensis*) leaves and standards used in the standard mix.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene^2</td>
<td>4.202</td>
</tr>
<tr>
<td>Camphene 1</td>
<td>4.284</td>
</tr>
<tr>
<td>Camphene 2</td>
<td>4.994</td>
</tr>
<tr>
<td>2-Carene^2,3</td>
<td>6.767</td>
</tr>
<tr>
<td>α-Phellandrene^2,3</td>
<td>7.936</td>
</tr>
<tr>
<td>Artemiseole</td>
<td>8.371</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>9.207</td>
</tr>
<tr>
<td>ρ-Cymene^2</td>
<td>11.013</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>12.541</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>14.322</td>
</tr>
<tr>
<td>α-Thujone^2,3</td>
<td>14.465</td>
</tr>
<tr>
<td>Camphor</td>
<td>16.137</td>
</tr>
<tr>
<td>α-Terpineol^2,3</td>
<td>18.746</td>
</tr>
<tr>
<td>Borneol</td>
<td>18.799</td>
</tr>
<tr>
<td>Carvone^1</td>
<td>19.330</td>
</tr>
</tbody>
</table>

^1Internal standard
^2Other standards
^3Compound not detected in samples
Figure 3. Mean concentrations of individual monoterpenes for composite leaf samples of Wyoming big sagebrush (Artemisia tridentata wyomingensis) collected at greater sage-grouse (Centrocercus urophasianus) feeding sites and available sites in southern Phillips County, Montana from October 2004 through March 2005. Error bars represent 95% confidence intervals.
Figure 4. Box-plots of total monoterpane content of composite Wyoming big sagebrush (*Artemisia tridentata wyomingensis*) leaf samples collected at greater sage-grouse (*Centrocercus urophasianus*) feeding and available sites in southern Phillips County, Montana from October 2004 through March 2005. Center lines represent the median, boxes represent the interquartile range (IQR), whiskers represent samples within ±1.5x the IQR, and circle is an outliers.
Table 2. Monoterpene composition and concentration (% dry matter) of composite Wyoming big sagebrush (*Artemisia tridentata wyomingensis*) leaf samples collected at greater sage-grouse (*Centrocercus urophasianus*) feeding and available sites in southern Phillips County, Montana from October 2004 through March 2005.

<table>
<thead>
<tr>
<th>Monoterpene</th>
<th>Feeding Sites</th>
<th>Available Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detected¹</td>
<td>Mean</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>67% 0.015</td>
<td>0 - 0.045</td>
</tr>
<tr>
<td>Camphene1</td>
<td>91% 0.038</td>
<td>0 - 0.087</td>
</tr>
<tr>
<td>Camphene2</td>
<td>100% 0.196</td>
<td>0.090 - 0.339</td>
</tr>
<tr>
<td>Artemiseole</td>
<td>98% 0.402</td>
<td>0 - 0.639</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>98% 0.590</td>
<td>0 - 0.926</td>
</tr>
<tr>
<td>ρ-Cymene</td>
<td>89% 0.028</td>
<td>0 - 0.045</td>
</tr>
<tr>
<td>Unknown1</td>
<td>70% 0.017</td>
<td>0 - 0.078</td>
</tr>
<tr>
<td>Unknown2</td>
<td>100% 0.116</td>
<td>0.078 - 0.186</td>
</tr>
<tr>
<td>Camphor</td>
<td>100% 0.901</td>
<td>0.452 - 1.263</td>
</tr>
<tr>
<td>Borneol</td>
<td>98% 0.056</td>
<td>0 - 0.119</td>
</tr>
<tr>
<td>Total</td>
<td>-- 2.359</td>
<td>1.35 - 3.53</td>
</tr>
</tbody>
</table>

¹Percent of samples in which the compound was detected.
Table 3. Comparison of Wyoming big sagebrush (*Artemisia tridentata wyomingensis*) cover and plant chemistry at greater sage-grouse (*Centrocercus urophasianus*) winter feeding sites and available sites in southern Phillips County, Montana. Variables significant at \( P = 0.10 \) were included in the multivariate analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>Mean</th>
<th>SE</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein(^1)</td>
<td>108</td>
<td>14.71</td>
<td>0.163</td>
<td>13.78</td>
<td>0.186</td>
<td>-3.767</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cover (Above Snow)(^2)</td>
<td>108</td>
<td>11.13</td>
<td>0.851</td>
<td>7.39</td>
<td>0.848</td>
<td>-3.114</td>
<td>0.002</td>
</tr>
<tr>
<td>Cover (No Snow)(^2)</td>
<td>108</td>
<td>12.57</td>
<td>0.801</td>
<td>8.28</td>
<td>0.852</td>
<td>-3.673</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total Monoterpenes(^1)</td>
<td>107</td>
<td>2.359</td>
<td>0.064</td>
<td>2.301</td>
<td>0.068</td>
<td>-0.616</td>
<td>0.539</td>
</tr>
<tr>
<td>(\alpha)-Pinene(^1)</td>
<td>107</td>
<td>0.015</td>
<td>0.002</td>
<td>0.011</td>
<td>0.001</td>
<td>-0.180</td>
<td>0.074</td>
</tr>
<tr>
<td>Eucalyptol(^1)</td>
<td>107</td>
<td>0.590</td>
<td>0.023</td>
<td>0.544</td>
<td>0.023</td>
<td>-1.408</td>
<td>0.162</td>
</tr>
<tr>
<td>Unknown 2(^1)</td>
<td>107</td>
<td>0.116</td>
<td>0.003</td>
<td>0.117</td>
<td>0.004</td>
<td>0.084</td>
<td>0.933</td>
</tr>
<tr>
<td>Camphor(^1)</td>
<td>107</td>
<td>0.901</td>
<td>0.026</td>
<td>0.890</td>
<td>0.036</td>
<td>-0.227</td>
<td>0.821</td>
</tr>
</tbody>
</table>

\(^1\)Percent dry matter.

\(^2\)Cover above snow omitted sagebrush completely buried by snow. Cover (no snow) was an additional measure of cover that was taken again after the snow had melted at the same site (but not along the original transect line since sagebrush branches were cut for chemical analysis).
Table 4. Comparison of Wyoming big sagebrush (*Artemisia tridentata wyomingensis*) height and plant chemistry at Greater sage-grouse (*Centrocercus urophasianus*) winter feeding sites and available sites in southern Phillips County, Montana. Variables significant at $P = 0.10$ were included in the multivariate analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Median Used</th>
<th>Median Available</th>
<th>U-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDF$^1$</td>
<td>108</td>
<td>32.00</td>
<td>33.99</td>
<td>991.50</td>
<td>0.004</td>
</tr>
<tr>
<td>Water$^2$</td>
<td>90</td>
<td>43.80</td>
<td>43.90</td>
<td>966.00</td>
<td>0.710</td>
</tr>
<tr>
<td>Height</td>
<td>108</td>
<td>24.0</td>
<td>25.0</td>
<td>1383.00</td>
<td>0.644</td>
</tr>
<tr>
<td>Monoterpenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camphene$^1_1$</td>
<td>107</td>
<td>0.039</td>
<td>0.041</td>
<td>1347.50</td>
<td>0.603</td>
</tr>
<tr>
<td>Camphene$^1_2$</td>
<td>107</td>
<td>0.186</td>
<td>0.196</td>
<td>1363.00</td>
<td>0.672</td>
</tr>
<tr>
<td>Artemiseole$^1$</td>
<td>107</td>
<td>0.387</td>
<td>0.411</td>
<td>1314.00</td>
<td>0.466</td>
</tr>
<tr>
<td>Cymene$^1$</td>
<td>107</td>
<td>0.030</td>
<td>0.027</td>
<td>1109.00</td>
<td>0.044</td>
</tr>
<tr>
<td>Unknown$^1$</td>
<td>107</td>
<td>0.014</td>
<td>0.010</td>
<td>1234.00</td>
<td>0.208</td>
</tr>
<tr>
<td>Borneol$^1$</td>
<td>107</td>
<td>0.057</td>
<td>0.055</td>
<td>1262.50</td>
<td>0.294</td>
</tr>
</tbody>
</table>

$^1$Percent dry matter.

$^2$Sites where plants were covered in frost or snow were not included.
Table 5. Logistic regression model summaries including all variables that were significant at $P = 0.10$ in univariate tests. There are separate models for when snow is present and snow is absent to account for significant differences in sagebrush cover (both models include all sites). A date variable is also included to account for any seasonal variation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Snow Absent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cover (No Snow)</td>
<td>0.088</td>
<td>0.041</td>
<td>0.031</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>0.464</td>
<td>0.193</td>
<td>0.016</td>
</tr>
<tr>
<td>Slope</td>
<td>1.246</td>
<td>0.865</td>
<td>0.150</td>
</tr>
<tr>
<td>NDF</td>
<td>-0.118</td>
<td>0.090</td>
<td>0.193</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>14.252</td>
<td>22.956</td>
<td>0.535</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>16.363</td>
<td>19.892</td>
<td>0.411</td>
</tr>
<tr>
<td>Date</td>
<td>-0.006</td>
<td>0.005</td>
<td>0.225</td>
</tr>
<tr>
<td>Constant</td>
<td>-4.805</td>
<td>4.319</td>
<td>---</td>
</tr>
<tr>
<td><strong>Snow Present</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cover (Above Snow)</td>
<td>0.075</td>
<td>0.038</td>
<td>0.050</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>0.442</td>
<td>0.191</td>
<td>0.021</td>
</tr>
<tr>
<td>Slope</td>
<td>1.353</td>
<td>0.858</td>
<td>0.115</td>
</tr>
<tr>
<td>NDF</td>
<td>-0.135</td>
<td>0.090</td>
<td>0.134</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>10.190</td>
<td>22.686</td>
<td>0.653</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>23.688</td>
<td>19.800</td>
<td>0.232</td>
</tr>
<tr>
<td>Date</td>
<td>-0.006</td>
<td>0.005</td>
<td>0.270</td>
</tr>
<tr>
<td>Constant</td>
<td>-3.975</td>
<td>4.236</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 6. Logistic regression model summaries that include only variables that are biologically or statistically significant. There are separate models for when snow is present and snow is absent to account for significant differences in sagebrush cover (both models include all sites). A date variable is also included to account for any seasonal variation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Snow Absent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cover (No Snow)</td>
<td>0.101</td>
<td>0.039</td>
<td>0.009</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>0.467</td>
<td>0.189</td>
<td>0.014</td>
</tr>
<tr>
<td>Slope</td>
<td>1.464</td>
<td>0.837</td>
<td>0.080</td>
</tr>
<tr>
<td>Date</td>
<td>-0.005</td>
<td>0.005</td>
<td>0.279</td>
</tr>
<tr>
<td>Constant</td>
<td>-8.602</td>
<td>2.722</td>
<td>---</td>
</tr>
<tr>
<td><strong>Snow Present</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cover (Above Snow)</td>
<td>0.079</td>
<td>0.036</td>
<td>0.030</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>0.436</td>
<td>0.183</td>
<td>0.017</td>
</tr>
<tr>
<td>Slope</td>
<td>1.654</td>
<td>0.825</td>
<td>0.045</td>
</tr>
<tr>
<td>Date</td>
<td>-0.004</td>
<td>0.005</td>
<td>0.361</td>
</tr>
<tr>
<td>Constant</td>
<td>-8.057</td>
<td>2.607</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 7. Random-effects (Model II) ANOVA results from individual plant samples used to determine if there is more variation within a site (between individual plants) or between sites.

<table>
<thead>
<tr>
<th>Variable</th>
<th>F</th>
<th>P-value</th>
<th>More Variation Between Sites?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>1.221</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>Fiber (NDF)</td>
<td>4.487</td>
<td>&lt;0.001</td>
<td>yes</td>
</tr>
<tr>
<td>Water</td>
<td>5.214</td>
<td>&lt;0.001</td>
<td>yes</td>
</tr>
<tr>
<td>Total Monoterpenes</td>
<td>2.428</td>
<td>&lt;0.01</td>
<td>yes</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>1.384</td>
<td>&gt;0.10</td>
<td></td>
</tr>
<tr>
<td>Camphene 1</td>
<td>2.585</td>
<td>&lt;0.005</td>
<td>yes</td>
</tr>
<tr>
<td>Camphene 2</td>
<td>1.693</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Artemiseole</td>
<td>2.421</td>
<td>&lt;0.01</td>
<td>yes</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>1.256</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>ρ-Cymene</td>
<td>1.842</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Unknown 1</td>
<td>0.983</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>Unknown 2</td>
<td>2.199</td>
<td>&lt;0.025</td>
<td>yes</td>
</tr>
<tr>
<td>Camphor</td>
<td>1.908</td>
<td>&lt;0.05</td>
<td>yes</td>
</tr>
<tr>
<td>Borneol</td>
<td>2.205</td>
<td>&lt;0.025</td>
<td>yes</td>
</tr>
</tbody>
</table>
Chapter 2

Sage-Grouse Cecal Microflora Does Not Change Seasonally with Diet

Abstract

The ceca of birds are a pair of blind sacs found at the junction of the small and large intestine that contain large numbers of bacteria (~10^{11}g wet weight). Their function is poorly understood, but their size and the fact that they elongate seasonally suggest that, in grouse, they are functional organs. If ceca function changes with diet, then microbial community composition would presumably also change with diet. Greater sage-grouse (Centrocercus urophasianus) are ideal for studying cecal microflora in wild birds because they have large ceca and dramatic seasonal dietary shifts from a strict winter diet of sagebrush (Artemisia spp.) to a varied summer diet. The purpose of this study was to determine if there are seasonal changes in the community composition of sage-grouse cecal microflora and to identify some of the bacteria present in the ceca. Denaturing gradient gel electrophoresis (DGGE) was used to compare the microbial community composition of 40 cecal samples collected in the summer, fall, winter, and spring. Ninety of the DGGE bands were excised and the resulting 221 clones were sequenced. There was no seasonal difference in cecal microbial community composition however there was marked animal-to-animal variation. Partial 16s rDNA sequence analysis revealed some similarities of the sage-grouse cecal microflora to chicken gastrointestinal tracts and cow rumen, but sage-grouse cecal microflora is largely unique and unknown. It was not possible to make inferences about ceca function due to the unknown bacteria present in the ceca.

Introduction

In birds, ceca are a pair of blind, intestinal sacs that are found at the junction of the small and large intestines (McLelland 1989). They are present in a variety of groups but are most developed in the Tetraonidae (Robbins 1993). In Greater sage-grouse (Centrocercus urophasianus, hereafter “sage-grouse”), the paired ceca may be as large as the small intestine (Obst and Diamond 1989). Their size and the fact that they elongate seasonally with changes in diet implies that, in grouse, the ceca are functional organs (Fenna and Boag 1974); however the function is not well understood. Proposed functions include fiber digestion, recovery of dietary nitrogen, and recycling of urinary nitrogen and water (Obst and Diamond 1989).
Since the ceca enlarge in winter as grouse diets typically shift to higher fiber foods and because the ceca contain tremendous numbers of bacteria ($\sim 10^{11}/g$ wet weight) (Mead 1989), it was hypothesized that the major function might be fiber digestion by bacteria (similar to a rumen) (Remington 1989). Although grouse can digest cellulose (Moss and Hanssen 1980), it is currently believed that fiber digestion is not the primary function of the ceca. Coarse intestinal material is excluded from the ceca by villi at the base of each cecum and so very little fibrous material may actually reach the ceca. In addition, it has been demonstrated that the ceca lengthens in response to the quantity of food intake rather than the quality (or fiber content) of the diet (Fenna and Boag 1974, Remington 1989). Finally, bacterial strains capable of degrading cellulose may be absent in the ceca or present in very low numbers ($\sim 100/g$) (Mead 1989).

An alternative hypothesis is that the ceca aid in maintaining a positive nitrogen balance by recovering nitrogen from dietary nitrogenous compounds. The ceca receive dietary compounds from the intestine when they are filled by waves of contractions in the small and large intestines (Fenna and Boag 1974). Reverse peristaltic contractions of the large intestine also fill the ceca with urine from the cloaca (Robbins 1993). The liquid fraction and small particles of the digesta that enter the ceca may contain dietary amino acids and urea that have not been absorbed in the intestine. Urine also contributes urea and amino acids, along with uric acid (Karasawa 1989). Bacteria capable of degrading uric acid, urea, and amino acids have been found in the ceca (Barnes and Impey 1974, Karasawa 1989, Mead 1989). Ammonia is produced from the breakdown of these compounds and may be absorbed directly by the ceca (Karasawa 1989) or used by bacteria to synthesize amino acids (Mortensen and Tindall 1981). Microbial protein
would become available to the bird as a result of bacterial autolysis and subsequent absorption of the amino acids by the ceca (Obst and Diamond 1989). Thus, the bacteria in the ceca may aid in recovering nitrogen that would have otherwise been wasted.

Any attempt to understand the function of the avian ceca must consider the metabolic activity of the large microbial population present in the ceca. In Montana, sage-grouse undergo major diet shifts in May and October (Wallestad et al. 1975). Sage-grouse consume a varied diet of sagebrush (*Artemisia* spp.), forbs, and insects in the summer and then switch to a strict diet of only sagebrush throughout the winter. If ceca function changes with diet, then microbial community composition would presumably also change with diet. The purpose of this study was to determine if there are seasonal changes in the community composition of sage-grouse cecal microflora and to identify some of the bacteria present in the ceca.

**Methods**

**Sample Collection**

Sage-grouse hens were captured by either spotlighting or rocket net methods (Giesen et al. 1982) and fitted with a necklace radio collar (Advanced Telemetry Systems) in the spring and late-summer of 2004. To collect fresh cecal and fecal droppings, I used radio-marked birds to find roost sites after dark. Exact positions of roosting birds were found by using a spotlight and binoculars to locate the birds by eye-shine and taking compass bearings towards the birds from two different spots. I would return early the next morning after the birds had left of their own accord to collect the samples. Cecal droppings were collected from an individual’s roost and placed in sterile 50 ml conical tubes. Care was taken not to contaminate the cecal droppings with either
fecal droppings or soil. All samples were frozen after collection and stored frozen until needed. All samples were collected on the Charles M. Russell National Wildlife Refuge (CMR) in southern Phillips County, Montana between August 2004 and April 2005.

I collected cecal droppings from 10 individuals each month from August 2004 through April 2004. Since the timing of the diet shifts in Montana is typically in May and October (Wallestad et al. 1975), I was able to collect samples from birds that were on strictly sagebrush winter diets as well as samples from birds that were on more varied summer diets.

Whenever possible, I tried to collect samples from individuals within the same flock (although this was not always possible in late spring and during the breeding season, when flocks broke up). By collecting samples from individuals within the same flock that experienced similar feeding conditions immediately before capture, I attempted to minimize large differences between individuals. One disadvantage of this approach, however, was that I was unable to determine the age or sex of the individuals collected from flocks. Since only one or a few birds in a flock typically had a radio-collar, I was also unable to determine if the same (unmarked) bird was sampled in more than one month. I am confident, however, that the ten samples collected within a month are from ten different individuals.

**DNA Extraction and Amplification**

I used molecular techniques to obtain a more complete assessment of the cecal microbial community composition since only 10 – 60 % of cecal bacteria grow in culture (Zhu et al. 2002). Prior to lysing the bacteria and extracting DNA, I washed samples in a buffer to help remove polysaccharides and other compounds that are present in cecal
droppings that will inhibit amplification via polymerase chain reaction (PCR). Using a modification of Apajalahti’s (1998) washing process, 0.25 g of cecal samples were suspended in 30 ml of wash buffer (50mM sodium phosphate buffer [pH 8], 0.1% Tween 80) and then shaken on a reciprocating horizontal platform shaker for 10 min on high speed at room temperature. The suspension was then centrifuged at 30,000 x g for 15 min. The supernatant was carefully decanted and the pelleted bacterial fraction was resuspended in 30 ml of fresh wash buffer. This process was repeated until each sample had been through the wash, shake, centrifuge process four times.

After washing, the DNA in the resulting pelleted bacterial fraction was extracted using the UltraClean Fecal DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA), with a modification of the lysis method. To lyse the bacteria cells, I substituted bead beating with a Geno/Grinder 2000 for vortexing. Bead tubes were shaken for a total of ten minutes at 1,700 strokes/min, with a samples being put on ice for 2.5 min intervals between each 2.5 min beating session.

From the extracted DNA, I amplified partial 16S rRNA genes for denaturing gradient gel electrophoresis (DGGE) via PCR using Platinum Taq (Invitrogen) and universally conserved primers 536f (5'-CAGCMGCCGCGGGTAATWC-3') and 907r (5'-CCGTCAATTCMTTTRAGTTT-3'). The forward primer had a 40-base GC clamp added (536fC). For each 50 µl reaction, the master mix consisted of 35.0 µl UV-sterilized H₂O, 5.0 µl 10x Platinum Taq buffer, 0.25 µl bovine serum albumin (BSA), 1.5 µl MgCl₂, 2.0 µl dNTP 5mM (equal proportions of each base), 2.5 µl 536fC (20 picomol/µl), 2.5 µl 907r (20 picomol/µl), 0.25 µl Platinum Taq enzyme, and 1.0µl sample template. The reactions were then amplified using a thermocycler program which
consisted of 10 min at 94° C followed by 25 cycles of denaturing for 1.5 min at 94° C, annealing for 1.5 min at 55° C, and extending for 1.5 min at 72° C. Next, samples were held at 72° C for 10 min before being held at 4° C until retrieved. All PCR reactions included both a positive control, *Micrococcus luteus*, and a negative control, UV sterilized H₂O.

*PCR Amplicon Precipitation and Quantification*

Multiple reactions were combined and precipitated in 100 % ethanol (2.5x the volume of the combined reactions) with 1µl glycogen (20mg/mL) overnight at -20° C. The DNA was pelleted by centrifugation for 30 min at 10,000 × g at 4° C. The ethanol was carefully decanted by vacuum aspiration and the pellet was washed in ice-cold 70 % ethanol (2.5x the original volume of the combined reactions). After vortexing to resuspend the pellet, the DNA was again pelleted by centrifugation for 15 min. The ethanol was decanted and the 70 % ethanol wash was repeated with fresh ethanol. Precipitated DNA was then allowed to dry in open tubes on the bench top for one hour before being resuspended in 30 µl 10mM Tris 1mM EDTA.

*Denaturing gradient gel electrophoresis (DGGE)*

For quality control, each gel contained three ladders (located on each end and in the center lane) and a test sample (PCR amplicons from a single sample) (Figure 1). Additionally, each sample was spiked with 60 ng of an internal standard (ladder band “L1”). The ladder was made from isolates amplified according to the same specifications as the samples (Figure 2). The isolates used were *Clostridium perfringens*, *Curtobacterium flaccumfaciens*, *Bifidobacterium breve*, *Micrococcus luteus*. Additionally, I also used two bands that were cut from DGGE optimization trials. Bands
L1 and L7 were unknown bacteria that most closely matched *Slackia faecicanis* (S_{ab} score 0.693) and *Ruminococcus lactaris* (S_{ab} score 0.758), respectively.

I randomly assigned the forty samples (ten from summer diet [August 2004], winter diet [February 2005], and spring [April 2005] and fall [October 2004] transition periods) to four different denaturing gels. Approximately 750 ng of sample DNA was loaded into each well of a 6% acrylamide gel with a 40% to 65% linear denaturing gradient (100% denaturant was 7M urea and 40% formamide). A Bio-Rad Gradient Former was used to pour the gels. Electrophoresis was performed at 60° C and 130V for 5.5 hours. Gels were stained using 10 µl of SyberGold stain (10,000X concentration) in 40 ml of 1X TAE buffer. Gels were stained for one hour at 37° C. Gel images were captured using Gel Doc software (Bio-Rad Laboratories) and analyzed using BioNumerics software (Applied Maths). Using BioNumerics, I analyzed the similarity between different samples by cluster analysis. I used Dice similarity coefficient and set the optimization (2 %) and position tolerance (2.5 %) settings so that the test samples on different gels were identical.

**Sequencing**

Ninety bands were excised from the denaturing gels using a flame-sterilized razor blade and placed in individual microcentrifuge tubes with 50 µl DGGE extraction buffer (50mM KCl, 10mM TRIS, 0.1% Triton pH 9.0) overnight to extract the DNA from the gel. The tubes were centrifuged at 10,000 x g for 5 min. The resulting supernatant was used as a template in PCR using the above mentioned master mix, except that the forward primer (536f) was not clamped. Two microliters of the PCR product was cloned using the Novagen Perfectly Blunt Cloning Kit using the pT7Blue-3 vector. Transformed
competent cells were grown on LB (Luria-Bertani) agar with kanamycin and tetracycline (antibiotics to inhibit growth of other bacteria) and IPTG and X-Gal (to screen for blue or white colonies). Positive colonies (white) were picked and grown overnight in LB (Luria-Bertani) broth with kanamycin before the plasmids were purified using a QIAprep Spin Miniprep Kit (using vacuum manifold). Plasmid DNA was sequenced by Polymorphic DNA Technologies, Inc. (Alameda, CA).

I compared sequences to a public sequence database, the Ribosomal Database Project II (RDP). A similarity score that compares sequences based on short oligos, $S_{ab}$ score, was used to compare sage-grouse cecal bacterial sequences to those in the library. A $S_{ab}$ score $\geq 0.95$ was considered to be a match at the species level. A sequence was matched to a known genus if $0.70 \leq S_{ab} < 0.95$. Any sequences with a $S_{ab} < 0.70$ were considered to be from unknown genus (Apajalahti et al. 2004).

Results

Seasonal Gel Comparisons

Cecal microbial community composition did not cluster by diet. There was no obvious difference in the cecal microbial community composition between birds on a varied summer diet and a strict sagebrush winter diet (Figure 3). There was also no difference in the community composition when the two dietary transition time points (spring and fall) were compared (Figure 4). The trend did not change when samples were compared chronologically (summer vs fall [Figure 5] or winter vs spring [Figure 6]). There appeared to be a great deal of animal-to-animal variation within a sampling period.
Sequence Data

Ninety excised bands from the DGGE successfully produced at least one cloned sequence resulting in 221 clone sequences. Average sequence length was 359 base pairs. Of those sequences, the majority could be assigned to a known genus but less than 2% could be assigned to a previously known, cultured species (Table 1). Over a third of the sequences could not be matched to any previously known genera. For the 84 bands where multiple clones where sequenced, 46.4% did not have all clones match the same genus.

When RDP was searched for previously cultured bacteria (either isolates or type strains), the following genera were found to match sage-grouse cecal bacteria sequences with a similarity score of at least 70%: *Acetanaerobacterium*, *Actinomyces*, *Anaerotruncus*, *Anaerovorax*, *Atopobium*, *Bacteriodes*, *Bifidobacterium*, *Clostridium*, *Collinsella*, *Escherichia* *coli*, *Eubacterium*, *Megamonas*, *Olsenella*, *Pectinatus*, *Pseudobutyrivibrio*, *Roseburia*, *Ruminococcus*, *Shigella*, *Slackia*, and *Syntrophococcus*. *Escherichia coli* and *Shigella flexneri* were the only previously cultured bacteria found to match sage-grouse cecal bacteria sequences at the species level (*S_\text{ab} \geq 0.95*).

Of the 18 clones that matched uncultured bacteria sequences found in RDP at the species level (*S_\text{ab} \geq 0.95*), most matched bacteria previously found in gastrointestinal tracts from humans, chickens, and Thompson’s gazelle (*Gazella rufifrons*). The remaining clones whose sequences matched either known or unknown (uncultured) bacteria at the genus level (*S_\text{ab} \geq 0.70*) matched bacteria that were isolated from either freshwater sediment or the gastrointestinal tracts (including oral microflora) of humans, mice, pigs, cattle (rumen), cats, and dogs.
Since DGGE bands were not randomly selected for sequencing and due to potential PCR bias, it is not possible to describe which of the above genera or species are most abundant in sage-grouse ceca.

**Discussion**

Due to the tremendous numbers of bacteria found in avian ceca, it is likely that the metabolic capabilities of those bacteria directly relate to the function of the ceca. Sage-grouse are ideal candidates to study cecal microflora in wild birds because they have large ceca that change in size in response to seasonal shifts in diet.

**Community Composition and Diet**

I expected to find seasonal changes in the community composition of the cecal microflora that corresponded to shifts between a varied summer diet and a strict winter diet of sagebrush. Diet of host animals strongly influences bacterial community composition in the gastrointestinal tract because it is the primary source of substrates for metabolism. Since bacteria differ in their ability to use various substrates, changes in diet can cause the composition of the microbial community to change as different bacterial groups are able to out-compete other groups (Apajalahti et al. 2004). Indeed, feed was shown to be the most important factor in determining the microbial community composition in the ceca of chickens (Apajalahti et al. 2001). DGGE analysis has also shown differences in the bacterial populations of the gastrointestinal tract of pigs on different diets (Simpson et al. 1999). In this study, sage-grouse cecal microbial composition did not cluster by diet or season. Individual samples collected in the summer were no more similar to each other than they were to samples collected in the winter.
There was a lot of animal-to-animal variation in the cecal microbial community composition, regardless of season or diet.

There are several possible explanations to account for why diet was not a strong determinant in the cecal microbial community composition for wild sage-grouse. The diet may not be different enough between summer and winter to affect the cecal microflora. While sage-grouse have a varied summer diet, sagebrush is a consistent food item throughout the year and the summer diet may contain up to 60% sagebrush (Wallestad et al. 1975). However, the cecal microflora of chickens fed a whole wheat amendment and commercial feed was significantly different from that of chickens fed only commercial feed (Apajalahti et al. 2004). Therefore, it appears that diets do not have to be completely different to illicit changes in the cecal microflora. Additionally, consumption of some sagebrush in the summer also does not account for the large amount of animal-to-animal variation within a sampling period.

The environment can affect the microbial populations in the gastrointestinal tract by supplying a source of bacteria and by affecting the physical condition of the host birds (Apajalahti et al. 2004). Sage-grouse winter flocks are fluid and birds frequently change membership from one flock to another. Introducing new animals to a group could certainly serve as a new source of bacteria for the group. Additionally, sage-grouse can move over 13 km when flushed (Beck 1977). Large movements may impact the cecal microflora by not only by providing new sources of inoculant but also by changing the quality of the diet. Even on a strict sagebrush diet, the quality of the diet can vary significantly across the landscape (see Chapter 1).
A lack of a clear relationship between diet and cecal microflora composition could also be due to experimental design. In retrospect, it would have been more appropriate to compare changes in bacterial functional groups rather than changes in individual bacterial species composition.

**Bacteria Present in Sage-Grouse Cecal Samples**

Most of the sequences recovered from sage-grouse cecal samples represent unknown bacteria. While most of the clones matched bacteria that had been previously recovered from gastrointestinal tracts of other animals, less than 2% matched sequences for previously cultured isolates or type strains at the species level. Over a third of all clones could not be matched to any previously cultured bacteria. A significant number, 13.6%, of the clones were not closely related to any sequence, from either cultured or unknown bacteria, previously deposited in RDP.

There is some overlap between sage-grouse cecal microflora and the well-studied microflora of chicken ceca and cow rumen. *Clostridium, Ruminococcus, Eubacterium, Escherichia, and Bacteroides* have been isolated from both chickens and sage-grouse (Gong et al. 2002, Zhu et al. 2002, Lu et al. 2003). Thirty clones (out of 221) matched unidentified rumen microflora. Overall, sage-grouse cecal microflora appears to be unique and largely unknown.

It is impossible to make strong inferences about ceca function with my current data. Many of the bacterial genera, including *Acetanaerobacterium, Actinomyces, Bacteroides, Clostridium, Eubacterium, Megamona, Ruminococcus,* and *Shigella,* ferment carbohydrates (Holt et al. 1994). Others, such as *Bacteroides, Clostridium,* and *Eubacterium* are capable of decomposing uric acid (Barnes and Impey 1974). However,
again, over a third of the sequences could not be matched to a known genus. Even with a match at the genus level, it requires broad generalizations to infer the role of bacteria in sage-grouse ceca. For example, *Clostridium* is present in the ceca but it is a genus that includes around 100 species and is known to be metabolically a very diverse group (Holt et al. 1994).

*Recommendations for Future Research*

Instead of focusing on differences in individual species, I think it would be better to focus on functional groups. For DGGE, using specific primers rather than universal primers would reduce the complexity of the community being examined which would allow for more resolution on gels. An attempt to look at functional groups, rather than individual species, may also reduce the complexity enough so that it is possible to examine dietary differences without focusing on animal-to-animal variation. Since many of the bacteria are unknown, using sequence data to attempt to identify bacteria present is not the best approach to learn about metabolic capabilities of bacteria in the ceca. While searching for the presence of a specific gene does not guarantee that it is expressed in the environment, at least it would focus the research more on metabolic capabilities rather than simple identities.
Figure 1. A typical DGGE used to analyze Greater sage-grouse (*Centrocercus urophasianus*) cecal microbial community composition. Individual samples are run on the lanes in between the ladders. A test sample was included to aid in alignment of multiple gels (the test sample is distorted due to its position in the last lane of the gel).
Figure 2. The relative positions of the isolates used in the ladder. The isolates were amplified using the same PCR protocol as the samples. Variation in the denaturing gradient can occur within a single gel and between gels. A ladder that responds to changes in the gradient in the same manner as the samples is of utmost importance if it is to align the individual lanes within a gel and as well as multiple gels.

Clostridium perfringens (lower end of the denaturing gradient)

DGGE Band L7

Curtobacterium flaccumfaciens

Micrococcus luteus

Bifidobacterium breve

DGGE Band L1

1 Closest match for band L7 was Ruminococcus lactaris, $S_{ab} = 0.758$

2 Closest match for band L1 was Slackia faecicanis, $S_{ab} = 0.693$
Figure 3. Cluster analysis using Dice similarity coefficient comparing sage-grouse summer and winter cecal samples. All samples include an internal standard (last band on right). An aliquot of a test sample was run on each gel.
Figure 4. Cluster analysis using Dice similarity coefficient comparing sage-grouse fall and spring cecal samples. All samples include an internal standard (last band on right). An aliquot of a test sample was run on each gel.
Figure 5. Cluster analysis using Dice similarity coefficient comparing sage-grouse summer and fall cecal samples. All samples include an internal standard (last band on right). An aliquot of a test sample was run on each gel.
Figure 6. Cluster analysis using Dice similarity coefficient comparing sage-grouse winter and spring cecal samples. All samples include an internal standard (last band on right). An aliquot of a test sample was run on each gel.
**Table 1.** The percentage of clones (n = 221) sequenced from DGGE bands of sage-grouse cecal samples that matched bacteria sequences in RDP.

<table>
<thead>
<tr>
<th>Similarity Score</th>
<th>Taxonomic Level Represented</th>
<th>Best Overall Match</th>
<th>Best Isolate Match</th>
<th>Best Type Strain Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{ab} &lt; 0.70$</td>
<td>Unknown Genus</td>
<td>13.6%</td>
<td>35.7%</td>
<td>39.4%</td>
</tr>
<tr>
<td>$0.70 \leq S_{ab} &lt; 0.95$</td>
<td>Genus</td>
<td>77.4%</td>
<td>62.4%</td>
<td>59.3%</td>
</tr>
<tr>
<td>$S_{ab} \geq 0.95$</td>
<td>Species</td>
<td>9.0%</td>
<td>1.8%</td>
<td>1.4%</td>
</tr>
</tbody>
</table>
Conclusions and Recommendations

In this thesis, I compared site characteristics and the nutritional quality of sagebrush at sage-grouse feeding sites and available sites across the landscape. I found that sage-grouse feeding sites in southern Phillips County, Montana were best described as flat sites with higher levels of sagebrush cover and crude protein content than what was randomly available.

There are many possible reasons why high protein content in sagebrush could have important fitness consequences for sage-grouse; however no one has explicitly examined them. For example, there are other compounds besides ornithine that can be used to conjugate plant secondary metabolites. While it has been demonstrated in ruffed grouse that detoxification of plant secondary metabolites is a very nitrogen-costly process, this has not been examined for sage-grouse.

There is also a need for more work regarding pre-laying nutrition and reproductive success. It is not known whether sage-grouse rely on spring diet or endogenous reserves for reproduction. Dunbar et al. (2005) used plasma protein levels to infer that dietary nitrogen influenced nest success and chick survival. However, it is not known if the increase in blood plasma protein levels is the result of selectively foraging on sagebrush high in protein or the result of consuming nitrogen-rich forbs. Barnett and Crawford (1994) suggest that consuming forbs in the spring increases the overall dietary protein intake for sage-grouse. However, they were unable to link reproductive success of individual birds with diet choices since diet composition was based on crop contents of birds harvested five weeks prior to laying. There are also conditions in which grouse begin to nest and forbs are not available either due to snow
cover or drought (Barnett and Crawford 1994, Moynahan et al. 2006). It is important to focus not only on how winter diet influences the condition of hens going into the breeding season, but it is also critical to examine how dietary protein content influences reproductive success. I think the best way to investigate the effects of dietary protein is to focus on more proximate measures such as the rate and duration of egg-laying, egg shell defects, clutch size, and hatch weight; all of which ultimately influence nest success and chick survival.

The other objective of this thesis was to determine if sage-grouse cecal microflora community composition changed with seasonal shifts in diet. Since cecal bacteria are strict anaerobes and difficult to culture, I used molecular techniques to assess the communities. Denaturing gradient gels can be important tools to use in the study of microbial communities. However, there are certain important quality control steps that must be included in their use. Initially, I found that sage-grouse cecal microbial communities were grouping according to season. Upon closer inspection, I noticed that the seasonal groupings were simply artifact. The samples were actually grouping by the gel that they were run on. Slight variation in denaturing gradient was found both within single gels and between gels, even when meticulously poured every time by the same person. Unless corrected, this variation in denaturant causes samples to migrate through the gel at different rates.

After much effort, I believe we established a set of quality controls that allows for confidence in the analysis of multiple denaturing gels. First, it is critical that the ladder responds to the denaturant similar to the samples. Originally I used a 100bp ladder that only separates based on size (not sequence) and so it took me a while to
realize there was variation in the gradient between gels. Ultimately, I was not able to correct the differences in the actual gradient between gels, but I was able to account for it by using a ladder that responded to the gradient. I was then able to use the ladder as a correct alignment of gel position both from one side of a single gel to the other as well as between multiple gels. Secondly, I included an aliquot of a test sample on each gel. It was important to use an aliquot instead of individual amplifications to avoid any possible discrepancies between PCR cycles. By including an identical test sample on each gel, I was able to test the alignment of the gels. Lastly, it is critical to place the samples in random order across the gels to further minimize any bias caused by an individual gel. If I had not changed my protocol to run the samples randomly, I never would have noticed the now obvious differences in the denaturing gradients and would have come to completely different (and erroneous) conclusions.
Literature Cited


