THESIS

FUNCTION OF THE CECAL MICROFLORA IN SAGE GROUSE NUTRITION

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY THOMAS AMOS BARBER ENTITLED FUNCTION OF THE CECAL MICROFLORA IN SAGE GROUSE NUTRITION BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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Permission to publish this thesis or any part of it must be obtained from the Dean of the Graduate School.

ABSTRACT OF THESIS

FUNCTION OF THE CECAL MICROFLORA IN SAGE GROUSE NUTRITION

An investigation of the cecal bacteria of sage grouse (Centrocercus urophasianus) was undertaken in an effort to obtain information on the function of the cecal microflora in the nutrition of this species. The project included experiments in three main areas:

(1) isolation and characterization of cecal bacteria;

(2) gas chromatographic analysis of cecal contents for VFA produced by bacterial fermentation;

(3) chemical analysis of sage grouse feed and droppings.

Cultures of bacteria from the cecal contents of sage grouse showed a large bacterial population to be present. Predominant organisms in cecal contents were similar to normal avian enteric types. Organisms resembling Actinomyces bifidus and a streptococcus-like form were the most numerous types in the ceca.

Solid media including the supernate of centrifuged cecal contents (CFAM) and clarified rumen fluid (RFAM) proved to be superior to all other media tested for total counts of cecal bacteria;

the use of these media was a necessity for the culture of the two predominant types of cecal organisms. Total culture counts of cecal bacteria in winter-killed birds showed a mean of 18×10^9 organisms per gram of wet cecal contents as compared to 30×10^9 for those collected in summer. Only insignificant numbers of cellulose-digesting organisms were cultured despite use of several enrichment media.

Large amounts of volatile fatty acids (VFA) were produced by the cecal microbial fermentations. Higher levels of VFA were present in ceca in winter when the birds were on total sagebrush leaf diets and lower total numbers of cecal bacteria were present.

Analysis of cecal contents showed that they contained only about 3% fiber (of a 10-16% dry weight). The high concentration of fiber in rectal droppings accounts for nearly the total amount ingested in sagebrush leaves.

Essential oils of sagebrush are present in greater amounts in the Type A-3 sagebrush leaves selected by the grouse examined as summer food. Crops of winter-killed birds contain a mixture of all three subtypes of sagebrush; total oil levels in the digestive tract at this season were higher than in the summer when forbs comprised a portion of the diet.

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CHAPTER I

INTRODUCTION

Need for Study

Wildlife biologists in the United States are presently faced with the problem of intensive management of wildlife and its rapidly diminishing range for an increasing human population which has more free time to enjoy recreational pursuits. In the Western United States the eradication of sagebrush will very probably adversely affect wildlife species, particularly the sage grouse (Centrocercus urophasianus). Field studies of this bird have led to detailed knowledge of daily and seasonal movements, distribution, sex and age ratios, and other life history information, but the present state of knowledge of sage grouse nutrition is so superficial that even the coefficients of digestion of the various fractions of sagebrush for this species are unknown.

Food habits studies (Semonov-Tyan-Shanskii, 1960; Moss, 1966; West and Meng, 1966) have shown that the winter food supply of many of the tetraonids consists of only one or two plant species; for two-thirds of the year the sage grouse feeds almost wholly on the leaves and flowers of sagebrush (Artemesia tridentata).

The ability of sage grouse to survive deep snow and cold while utilizing the leaves of this plant as food enables them to occupy a niche in which seed-eating gallinaceous birds could not exist.

The existence of symbiotic cellulose digesting microflora in ruminants, ungulates, and some rodents which have high fiber diets has led to speculation that microbial cellulose digestion also takes place in the ceca of wild gallinaceous birds with browsing food habits. Microbial decomposition of the fiber portion of sagebrush leaves in the ceca of sage grouse has been suggested as the main reason for the bird's efficient utilization of this food (Girard, 1937). The value of the fiber portion of feeds is quite high for ruminants, and it is difficult to understand the existence of birds on leaf diets without some digestion of fiber. The determination of the function of the cecal microflora of sage grouse in the digestion of its atypical diet is a necessary step prior to further nutrition studies.

Previous Findings

Barber (1967)[1] reviewed and summarized the results of previous research into the function of avian cecal microflora. This review of literature is presented under a separate cover; a portion

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[1] Review of Literature Pertinent to the Function of the Ceca in Sage Grouse Nutrition. Dept. of Fishery and Wildl. Bio., Colorado State Univ.

of the research reviewed which has particular bearing on the present study is briefly mentioned below.

Present Views

Coates and Jayne-Williams (1966) summarized present views on the function of the gut flora of the domestic fowl and reached a number of conclusions:

(1) the cecal flora generates significant amounts of enzymes;

(2) the cecal flora is responsible for some binding and destruction of vitamin A;

(3) normal domestic fowl contain cecal bacteria which synthesize B-complex vitamins;

(4) volatile fatty acids (VFA) are generated in the ceca;

(5) the cecal flora governs the catabolism of cholesterol and bile;

(6) growth depressing effects may be exerted by some of the intestinal bacteria.

Symbiotic Cellulose Digestion

The digestive systems of the domestic fowl and turkey, because of economic importance, have been thoroughly investigated. Although these birds are seed-eaters and their digestion may function somewhat differently than that of grouse, this research provides the only available basis for studies of wild gallinaceous birds.

Early experiments with cecectomy, or cecal abligation, involving the above species (Durant, 1929; Schlotthauer et al.,

1933) showed that their digestive systems functioned well without ceca. A detailed study of digestion coefficients for various feed components by Hunter et al. (1930) showed digestion of the various food portions to be almost equal for cecectomized and normal domestic fowl with a slight difference in wheat fiber digestion in favor of the normal birds. Digestion of barley fiber was unaffected by the operation.

Suomalainen and Arhimo (1945) cultured bacteria from the ceca of several species of grouse (willow ptarmigan, rock ptarmigan, and capercaillie) and observed cellulose decomposition in vitro. A lack of quantitative data in this report makes any attempt at analysis of the efficiency of cecal microbial digestion of cellulose impossible.

Leopold (1953) speculated that enlarged ceca in grouse and quail ingesting a low quality bulk diet containing a high proportion of green food provided a site for microbial digestion of cellulose. Assumptions made without the laboratory investigation necessary to reinforce his theory lessens the value of this report. Semenov-Tyan-Shanskii (1960), in a useful treatise on grouse nutrition, remarked that the "textbook" explanation of cecal fiber digestion in grouse had not been adequately proven.

Moss (1966), in an experiment to test the digestibility of the fiber of heather (Calluna sp.) for red grouse, placed the birds in

cages over heather and allowed them free access to natural food. Proximate analyses were done on droppings, crop contents, and the parts of the heather plants selected by the grouse as food. Forty percent of the plant's alpha-cellulose and lignin were found to be digested. A further interesting observation was the high overall efficiency of the grouse's digestion of other food components (up to 35% of crude fat). In contradiction to the theory that large ceca result from a high fiber diet, red grouse on a diet of green, new-growth heather, high in nitrogen and phosphorus, were found to have larger ceca than those on a diet of older plants with a much higher fiber content. This investigator unfortunately gave no detailed information on his methods of chemical analysis; no comparison of his results could be made with results of work with domestic fowl.

Moss's examination of the ceca of these birds disclosed only viscous matter; particles of the highly fibrous heather on which some of the birds fed were never found. This finding was similar to those of Browne (1922) who believed that the backward-facing arrangement of the cecal valves of the domestic fowl prevented any solid particles of other than very small size from entering the ceca.

Digestion of Less Complex Carbohydrates

Experimentation with cecal digestion of carbohydrates by domestic fowl (Thornburn and Wilcox, 1964a) provided an insight into this process in the ceca. After cecectomy cockerels showed reduced dry matter in their feces and a reduced overall digestibility of dry matter in food. Digestibility of crude fiber was generally lowered but the effect was highly variable between birds, indicating a bacterial rather than glandular action. Starch and pentosan digestion was unaffected by cecectomy.

An in vivo investigation by the dacron bag technique (Thornburn and Wilcox, 1964b) showed cecal digestion of hay components to be variable; up to 3% of the cellulose was digested, but starch and pentosan were generally unaffected. The incubation of other carbohydrate substrates (starch, xylan, glucose) in beakers with cecal material led to their breakdown into simple carbohydrates and acidic products. Results of in vivo experimentation with cannulated ceca closely paralleled these results.

Protein Digestion

Maumus (1902) examined the action of cecal juices on various substrates and detected the presence of a proteolytic enzyme thought to be similar to trypsin. Cecal juice of a chicken fed on an exclusively carnivorous diet had no effect on carbohydrates but was twice as efficient in proteolysis as that from fowl on a normal diet.

Whether or not the origin of these enzymes was bacterial was not investigated.

In feeding antibiotics to chicks and determining the effect of protein digestion, Biely et al. (1952) found that rations well balanced in amino acids were best utilized by antibiotic fed chicks. Payne (1967) reported increased proteolytic activity in ceca of chicks grown on raw soybean flakes over those grown on a diet with heated soybean meal. Cecectomized adult male White Rocks showed reduced digestibility of low grade proteins over normal fowl.

The Problem

The real significance of feed-feces analyses, digestion coefficients, and other nutrition information for sage grouse will be unknown until data are available on the contribution of the cecal microflora to the nutrition of this species.

Problem Analysis

Specific objectives were:

(1) to characterize the predominant groups of sage grouse cecal bacteria;

(2) to determine the relative abundance of each of the predominant groups of sage grouse cecal bacteria;

(3) to attempt to culture cellulose decomposing bacteria from sage grouse cecal contents;

(4) to define the major contributions of sage grouse cecal bacteria to the nutrition of the bird.

Areas of Interest

The project included experiments in three main areas:

(1) isolation and characterization of cecal bacteria;

(2) analysis of cecal contents for VFA produced by bacterial fermentation;

(3) chemical analysis of sage grouse feed and droppings.

CHAPTER II

THE SAGE GROUSE CECAL MICROFLORA

Introduction

General Considerations

The enteric bacterial populations of gallinaceous birds are delicately balanced ecosystems; in the ceca, as in the bovine rumen, many complex interactions doubtless exist between bacterial species. The less fastidious cecal organisms are commonly enumerated and identified in studies of domestic fowl but it is likely that an unusually high percentage of the organisms observed are never cultured. McBee and West (1968) found that up to 70% of the normal number of viable cecal organisms could not be grown even though a number of different media were used.

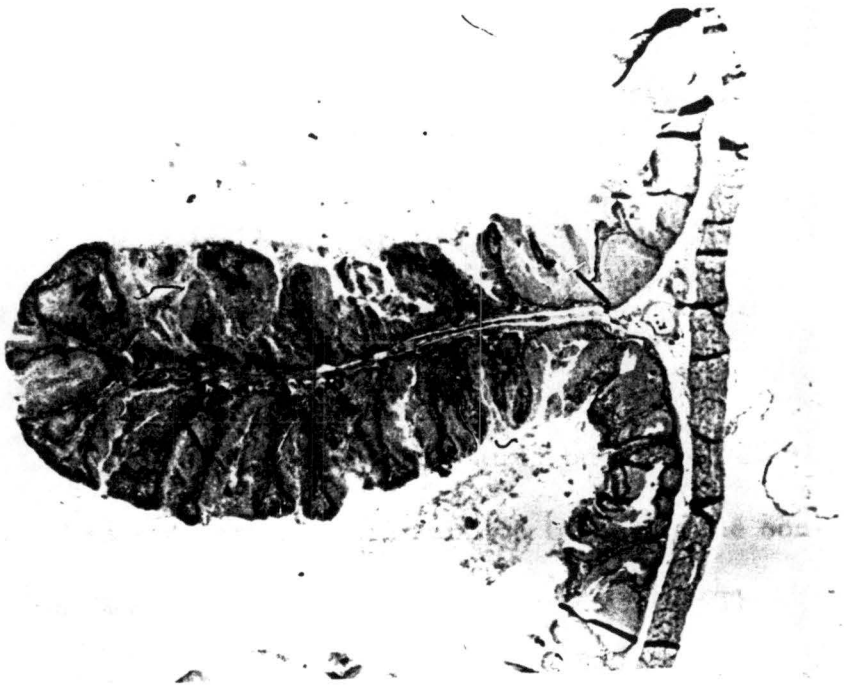
Cecal Anatomy of the Sage Grouse

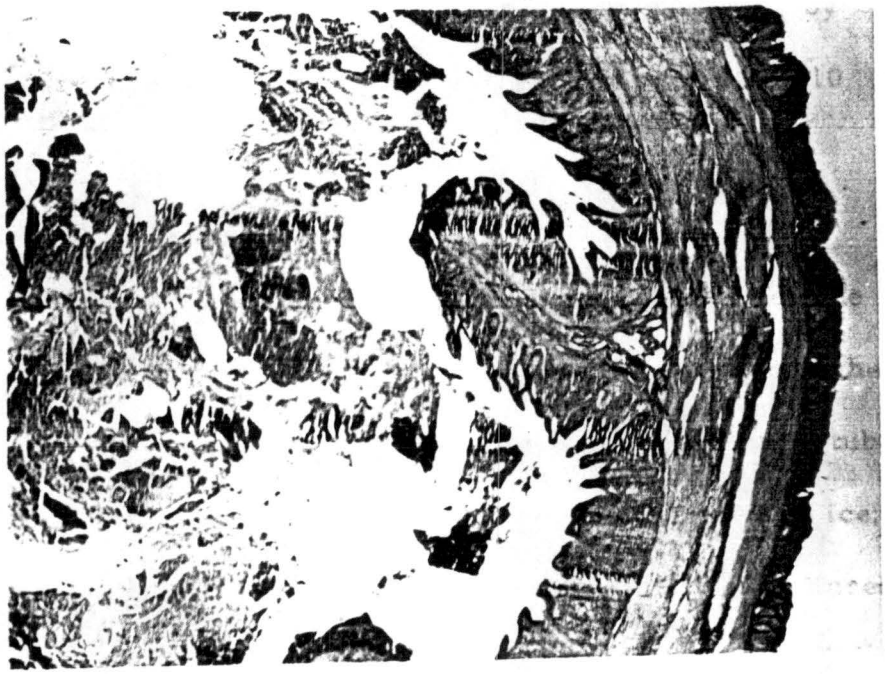
The ceca of sage grouse are well developed, each one being of slightly larger diameter than and of nearly as great a length as the small intestine. The total capacity of the two ceca is considerably larger than that of the small intestine. The cecal tubes are attached by a muscular "metering" portion to the gut at the juncture of the small and large intestine. The body or sacular

portion of the cecum is lined with parallel rows of outfolded mucosa layer which are well supplied with blood vessels; this apparatus for absorption is probably as efficient as that of the small intestine, where villi occur over much of the inner surface. The large size of these folds of villous tissue is obvious if a cross-section of a grouse cecum is compared with one of a cecum of a chicken (Figs. 1 and 2). This structure must serve to increase capacity for absorption; its function in the uptake of bacterial metabolic products is undetermined.

FIGURE 1. Cross-section of villous tissue from sage grouse cecum (X 250).

FIGURE 2. Cross-section of villous tissue from domestic fowl cecum (X 250).





Materials and Methods

Obtaining of Cecal Material

Sage grouse were collected in the North Park area of northern Colorado with a .22 caliber rifle. Additional viscera were contributed by hunters passing through a Colorado Game, Fish, and Parks Department check station at Walden, Colorado, on September 9 and 10, 1967. Immediately after being collected, birds were weighed and pertinent information including the conditions, location, and time of collection was recorded. The visceral cavity was then opened and the ceca removed, weighed, measured, and placed in plastic bags on shaved ice in a styrofoam cooler for the return trip to Fort Collins. Hydrogen ion concentration of cecal contents was determined at this time by use of a battery-operated Beckman 610 pH meter with a No. 10 pencil electrode.

The remainder of the viscera and the carcasses of the collected birds were also placed in plastic bags and iced for the return to the laboratory where they were frozen. For five of the seven cultures made, a 1:10 dilution of cecal contents in test tubes of thioglycollate broth was made prior to placing the ceca on ice; these tubes were cooled on ice during the return trip and cultured for comparison of counts with those obtained from contents of the iced ceca. Time elapsed between collection and culturing ranged

from 5 to 12 hours. Upon returning to the laboratory the cecal contents were extruded into a sterile beaker, weighed, and prepared for bacterial culturing, gas chromatography, and chemical analyses as described in the following chapters.

Preparation of Inoculum

Serial dulutions of cecal contents through 10^{-15} were made in the anaerobic diluting fluid of Bryant and Robinson (1961). The 1:10 dilutions which had been made in Fluid Thioglycollate Medium from freshly killed birds served as the first dilution for another series of inocula. Cecal contents not used for inoculation served as an additive in media or were frozen for later chemical analysis and gas chromatography.

Sterilization

All media, bottles, test tubes, flasks, and stoppers used in bacterial culturing were autoclaved at 120 C for 15 minutes. Clarified rumen fluid and cecal fluid were autoclaved after being added to media. Pipettes were sterilized by heating in a hot-air oven for 12 to 14 hours at 180 C. Sugars used in the medium of Bryant and Burkey (1952) for determination of fermentation reactions were sterilized by filtering through a HA 0.45 u plain Millipore membrane.

Media

A number of commercial dehydrated media and laboratory prepared media were used during the course of the study (Table 1). The Rumen Fluid Cellulose Broth (RFCB) medium of Bryant and Robinson (1961) was used in attempts to isolate cellulose digesters as was the Yeast Extract Cellulose Broth (YECB) of Suomalainen and Arhimo (1945). The RFCB with clarified grouse cecal fluid in place of clarified rumen fluid (CFCB) proved to be the best medium for cellulose digesters. The Improved Rumen Fluid Agar Medium (RFAM) of Bryant and Robinson (1961) and a similar medium made by adding clarified grouse cecal fluid instead of rumen fluid (CFAM) were used for total counts and enumeration of fastidious cecal organisms. The anaerobic diluting solution of Bryant and Burkey (1952) was used for all dilutions of cecal matter prior to inoculation.

Commercial media[1] used consisted of Streptococcus faecalis (S F) medium for the enumeration and isolation of fecal streptococci, Violet Red Bile Agar for the enumeration and isolation of coliforms, Tomato Juice Agar Special for aerobic and facultative lactobacilli, and Trypticase Soy Agar for culture

[1] All commercially prepared media used were the products of Difco Laboratories, Detroit, Michigan.

TABLE 1. Steps in culture of sage grouse cecal bacteria

Cecal Contents

Serial Dilution Through 10^{-15}

Undiluted

I. Roll tubes (total counts)

a. RFAM
b. CFAM
c. Brain-Heart Infusion Agar

II. RFCB and CFCB

Measurement of cellulose digestion by gravimetric methods

YECB

(2.0 g to 0.1 g inoculum) Measurement of cellulose digestion as in II.

III. Differential media

a. Lactobacilli
   Aerobic -- Tomato Juice Agar Special
   Anaerobic -- CFAM
b. Streptococci
   Aerobic -- S F Agar
   Anaerobic -- CFAM
c. Coliforms
   Violet Red Bile Agar
   E M B Agar
d. Gram-positive nonspore-forming rods -- T-Soy Agar
e. *Proteus* sp.
   Urea Agar Base
f. *Salmonella* sp.
   S S Agar

of aerobic sporeformers and gram-positive nonsporeforming rods other than lactobacilli.

Anaerobic isolation and enumeration were carried out with RFAM, CFAM, and Blood Agar (Clostridium). Anaerobic lactobacilli and a fastidious anaerobic streptococcus-like organism, the most numerous cecal organisms, could only be grown on RFAM and CFAM. Total anaerobe counts were made from initial cultures on these two media; total aerobe counts were arrived at by calculating the total number of organisms growing in aerobic cultures.

Culture Methods

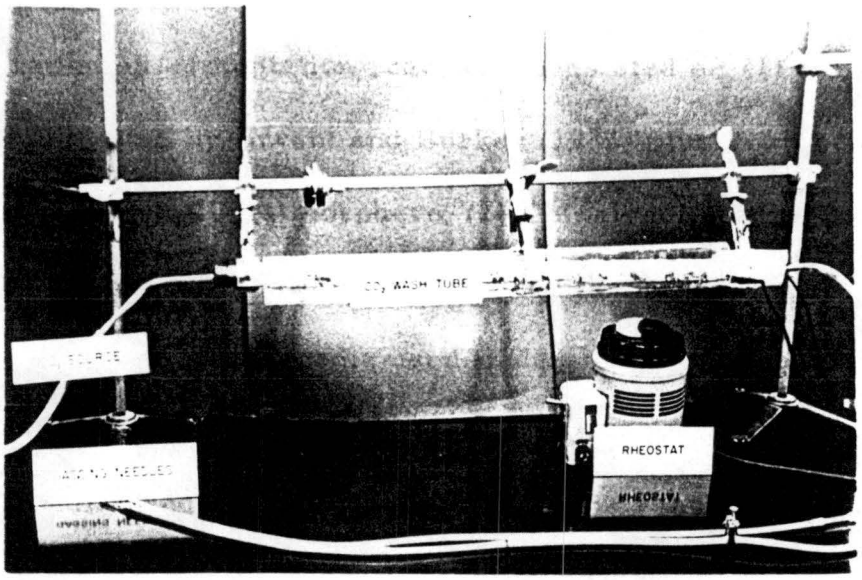
Anaerobic cultures were made in rubber stoppered roll tubes with an atmosphere of washed CO_2. Anaerobiosis was obtained by the technique of Hungate (1966). Oxygen-free CO_2 was obtained by washing CO_2 through a class tube containing copper powder and turnings heated to 200 C by a rheostat controlled electrical element (Fig. 3). All media were prepared and autoclaved in a stoppered 500 ml round bottomed flask fitted with a wire collar to retain the stopper during autoclaving. The CFAM, RFAM, CFCB, and RFCB were cooled to 60 C prior to the addition of Na_2CO_3 and Cysteine-HCl. Flasks containing the media were gassed as the media was boiled to drive off any remaining oxygen; the media were then cooled and used immediately.

Anaerobic media were dispensed in 9 ml amounts from continuously gassed boiling flasks into 16 X 160 mm test tubes by means of a 10 ml pipette attached to a section of rubber tubing and aspirated by mouth; tubes were gassed with CO_2 during filling and inoculation by means of a 2 inch 20-gauge hypodermic needle bent so that about three-fourths of its length extended into the tube. Gas flow was adjusted so that currents at the mouth of the tube were at a minimum.

After filling, tubes of media were stoppered with sterile No. 2 black rubber stoppers, the stoppers being gradually inserted as the gassing needle was removed (Fig. 4). The tubed medium was then cooled in a water bath to 47 C and inoculated with 1 ml quantities of serially diluted cecal contents from sterile 1 ml pipettes. The tubes of media were gassed during inoculation and for several seconds prior to inserting stoppers to prevent the entrance of air. It was found to be necessary to keep the tubes stoppered under CO_2 during cooling of the media to prevent any oxidation and a resultant failure of the more fastidious organisms to grow. When dispensing the CFCB, RFCB, and YECB media, all of which contained small particles of cellulose, it was necessary to use an automatic stirrer to keep the cellulose in suspension and a wide mouthed pipette for dispensing them into tubes. Magnetic stirrer bars were sterilized by autoclaving in the media.

FIGURE 3. Carbon dioxide washing apparatus.

FIGURE 4. Method of obtaining anaerobiosis with tubed media.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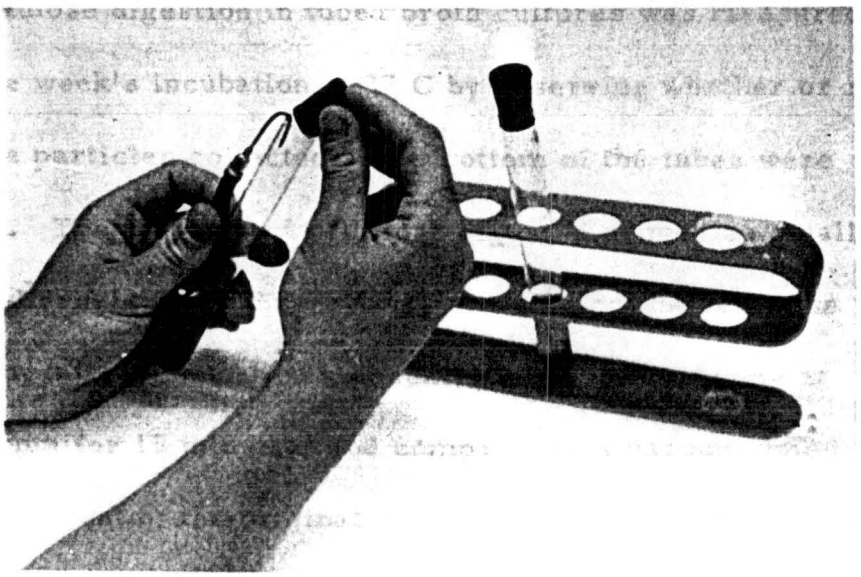


All aerobic cultures were made by the pour-plate dilution method with plastic disposable petri dishes. Media used for determination of fermentation reactions consisted of: (1) the basal medium suggested by Bryant and Burkey (1952) plus 0.5% of the test sugar under a CO_2 atmosphere; (2) Thioglycollate Sugar media; and (3) Nutrient Broth including the tested sugars at 0.5% concentration. All media used in determining fermentation reactions were adjusted to pH 7 prior to use. The three different types of media used were necessary due to the requirements of the fastidious organisms encountered. Hydrogen ion concentration of cultures was determined by reading at the end of one week's incubation of 37 C with a Beckman No. 10 pencil electrode inserted directly into the tubes.

Cellulose digestion in tubed broth cultures was measured after one week's incubation at 37 C by observing whether or not cellulose particles collected at the bottom of the tubes were digested. The amounts of cellulose digested proved so small that an alternate method consisting of weighing the cellulose remaining after incubation (concentrated by centrifuging at 10,000 rpm for 15 minutes) and comparing its vacuum dried weight with that of the original amount of cellulose in the tubes was adopted.

Clarified rumen fluid for addition to media was obtained by straining rumen fluid from a fistulated steer through cheesecloth

and centrifuging it at 15,000 rpm for 45 minutes in a Servall SS-3 centrifuge. Clarified cecal fluid was obtained from cecal matter diluted 1:2 with distilled water, suspended with a Waring Blender and centrifuged at the same speed and time as the rumen fluid.

Special media used during the project in the identification of organisms included Iron Milk, Litmus Milk, Mannitol Salt Agar, Cooked Meat, Tryptone Glucose Extract Agar, MR-VP Broth, SIM, Simmons Citrate, and Urea Agar. Direct counts of suitable dilutions of cecal contents were made by means of a Petroff-Hauser counting chamber. Culture counts were made by multiplying the numbers of organisms grown by the reciprocal of the dilution factor. Cultures were stored in a refrigerator at 5 C for 30 day periods at the end of which time they were subcultured.

Results

No attempt was made to classify any organisms other than members of the Eubacteriales although numerous protozoans (coccidia and trichomonads) and roundworms were encountered. Wet mounts of 10^{-2} dilutions were observed at 450X using a dark field condenser; gram stains were later made from the same slide by removing the cover glass and allowing the smear to air dry. Isolates of the predominant cecal bacteria were given letter type and subtype designations (A, B-1, B-2, C-1, C-2, C-3, D, E, and F) for identification.

An extremely log rod, 50 to 300 u in length, was observed in six of the seven cecal samples. This organism was only grown in pure culture twice, in both cases on RFAM. As this organism was similar to the aerobic or microaerophilic genus Lineola reported by Pringsheim and Robinow (1947), the anaerobic procedure used in cultivation may have prevented its growth. A large sarcina-like organism similar to Lampropedia of the bovine rumen (Hungate, 1966) was noted in wet mounts of four of the cecal samples but was not cultured. Neither of these organisms were numerous in the ceca but both were conspicuous due to their large size. Spirochetes and vibrio-like organisms were obvious in wet mounts taken from all cecal samples. These latter two organisms were not cultured.

TABLE 2. Counts of the predominant types of cecal bacteria (X 10^{-9})

Culture Number	Sex and age class of specimen	Anaerobic lactobacilli	Gram-pos., nonspore-forming rods other than lactobacilli	Anaerobic streptococci	Facultative anaerobic lactobacilli	Gram-neg. rods	Facultative streptococci	Total Counts	
								Culture	Direct
Summer									
1	Ad. M.	11.0	3.0	10.0	0.6	0.005	0.03	24.6	51.2
2	Imm. M.	1.0	1.0	11.0	0.01	0.006	0.02	13.0	27.2
3	Ad. M.	29.0	1.9	5.0	0.001	0.007	0.10	36.1	39.2
4	Ad. F.	15.0	1.0	7.0	0.006	0.002	0.03	23.0	47.2
5	Ad. F.	9.0	1.6	15.0	0.030	0.001	11.00	36.6	66.2
Winter									
6	Ad. F.	1.0	1.1	15.0	0.030	--	1.00	18.1	29.6
7	Ad. M.	1.0	1.0	13.0	0.0002	0.004	2.00	17.0	49.1
Mean Counts		9.6	1.5	10.9	0.097	0.003	2.02	24.1	44.2
Percent of Mean Total Count (culture)		39.7	6.2	45.4	0.40	0.013	8.85		

Anaerobic roll tubes with CFAM gave the highest total counts. Individual counts for the predominant organisms are given in Table 2; total anaerobic counts averaged 20.5 billion organisms per gram of wet cecal contents and total aerobe counts averaged 3.6 billion. The use of RFAM media resulted in total counts nearly as high as on CFAM and due to the easy availability of rumen fluid, RFAM was used for subculturing. Total anaerobe counts on Brain Heart Agar were far lower than counts on the above media, averaging 73 million organisms per gram; VFA from rumen or cecal fluid in media was probably necessary for growth of the large majority of the cecal bacteria. Aerobic cultures with Tomato Juice Agar Special averaged 400 million organisms per gram of wet cecal contents. Gram-positive nonsporeforming organisms other than lactobacilli (types C_1 to C_3) on Trypticase Soy Agar amounted to an average of 1.5 billion organisms. Low numbers of staphylococci and Salmonella sp. growing on selective media and the low numbers of anaerobic sporeformers found in heat-shocked inocula indicated that few potential pathogens were present in the ceca of normal birds.

Cultures on S F agar (facultative streptococci) averaged 2 billion organisms and coliforms averaged 3 million organisms per gram of wet contents. Proteus organisms cultured on Urea Agar always averaged less than 10 thousand organisms per gram as did

clostridia. Mannitol Salt and S S medium aerobic streak plates showed only incidental staphylococci and non-lactose fermenters.

It must be kept in mind in any consideration of the above results that each count category has the limitation of being a count of organisms growing on a particular type of selective medium and not necessarily a certain type of organism. In the case of the non-sporeforming rods other than the lactobacilli, which were identified for enumeration largely by colonial morphology on low dilution plates, some confusion in counting probably occurred.

Organisms chosen for characterization were subcultured on various media after being picked with an inoculating needle from plates or by removing an entire colony from roll tubes with a bent Pasteur pipette. Pure growth from subcultures was then used to inoculate differential media and sugar fermentation tubes. Types A and B, the most numerous organisms in cecal matter, would not grow on commercially prepared media; in these cases RFAM was used for subcultures. Whenever roll tube stock cultures or subcultures were opened they were gassed prior to closing for storage. Temperature ranges were determined by incubating cultures at room temperature, 37 C, 41 C, and 45 C. Types A and B showed optimum growth at 41 C, all other cultures grew best at 37 C.

Attempts to culture cellulose decomposers with CFNB, RFNB, and YECB aerobically and anaerobically resulted in little

cellulose decomposition although tubes of these liquid media often became turbid with growth after 24 hours' incubation. Measurable amounts of cellulose digestion occurred only when large (0.1 to 2.0 g) amounts of cecal contents were used to inoculate CFNB, and these cultures grown anaerobically (Table 3). Total amounts of cellulose digested after incubation of 0.15 g amounts of cellulose in these cultures at 37 C for one week ranged from no measurable decomposition to 0.05 g; such a slow rate of cellulose digestion would hardly be useful to the sage grouse with its rapid movement of ingested food. Cultures of rumen cellulolytic organisms in RFCB completely liquify cellulose within three days (Bryant and Burkey, 1952).

The results shown in Table 3 may indicate that the original proportion of cellulose digesters in ceca contents was small and that a large inoculum was necessary to prevent their being overgrown by other cecal organisms. Comparison of total counts by direct and cultural methods showed that approximately 60% of the cecal organisms present were being cultured. No reason can presently be suggested for the lower winter counts.

In Table 4 physiological characteristics of predominant cecal organisms are listed and their formation of acid from several carbohydrate substrates is given by pH value. Initial pH of all sugar media was adjusted to neutral. It should be emphasized

TABLE 3. Cellulose decomposition by cecal bacteria in CFNB.

Inoculum (undiluted cecal contents)	Medium (CFNB)	Weight fiber digested Sample number: 1	3	7
2.0 ml	8.0 ml	0.05 g	0.03 g	0.01 g
1.0	9.0	0.02	0.01	0.01
0.5	9.5	0.01	0.00	0.00
0.25	9.75	0.00	0.00	0.00

TABLE 4. Fermentation reactions of predominant types of bacteria from sage grouse ceca

Isolate Type	Gram Morphology	O_2 Reaction	Fermentation Reactions (pH)									
			Mal.	Ara.	Xyl.	Cell.	Suc.	Glu.	Gel.	Gly.	Man.	Lac.
A	G+ often branched rods	an	4.2	6.9	4.2	6.7	6.1	5.2	+	6.7	5.0	6.8
B_2	long chains of pleo. G+ cocci	an	4.6	6.7	6.8	6.8	5.2	5.3	-	6.9	5.7	4.9
C_1	G+ long rods, pairs	an	5.3	5.1	7.1	7.0	5.7	5.8	-	6.9	6.8	5.4
C_2	G+ pleo. rods, pairs		5.2	5.6	7.0	6.9	5.4	5.9	-	6.9	6.8	4.1
C_3	G+ short rods, slight. pleo.	Mic fac an	6.8	6.9	7.1	7.0	7.2	6.9	-	7.1	7.1	5.8
D	G+ med. rods, paired, single	fac an	4.9	6.8	6.9	7.0	5.2	5.7	-	6.1	5.8	4.1
E	G+ cocci pairs, short chains	fac an	5.0	6.8	7.0	6.9	5.1	4.7	-	6.8	6.7	4.7
F	G- short fat rods	fac an	5.9	5.3	7.1	6.9	6.0	5.9	-	6.3	6.2	5.7

Abbreviations used:

Mal. = maltose Xyl. = xylose Suc. = sucrose Gel. = gelatin Man. = mannitol
Ara. = arabinose Cell. = cellobiose Glu. = glucose Gly. = glycerol Lac. = lactose
An = anaerobic Mic = microaerophilic Fac = facultative

that a failure to cause acid production cannot be taken as a failure of an organism to grow in a certain medium. In several cases, particularly those involving type B-1 organisms, sugar media became turbid with growth although pH values remained relatively unchanged.

Predominant Organisms

Type A -- Obligately anaerobic, branched chain-forming bacilli similar to Actinomyces bifidus[1] (Fig. 5).

In the ceca of fall and summer-killed birds this was the most numerous organism. These organisms were strict anaerobes, failing to grow on aerobic plates or CFAM and RFAM which had been oxidized. New cultures of this organism showed the branching form of growth characteristic of A. bifidus. All cultures of this organism fermented lactose, maltose, sucrose, and xylose. This organism grew poorly after being subcultured.

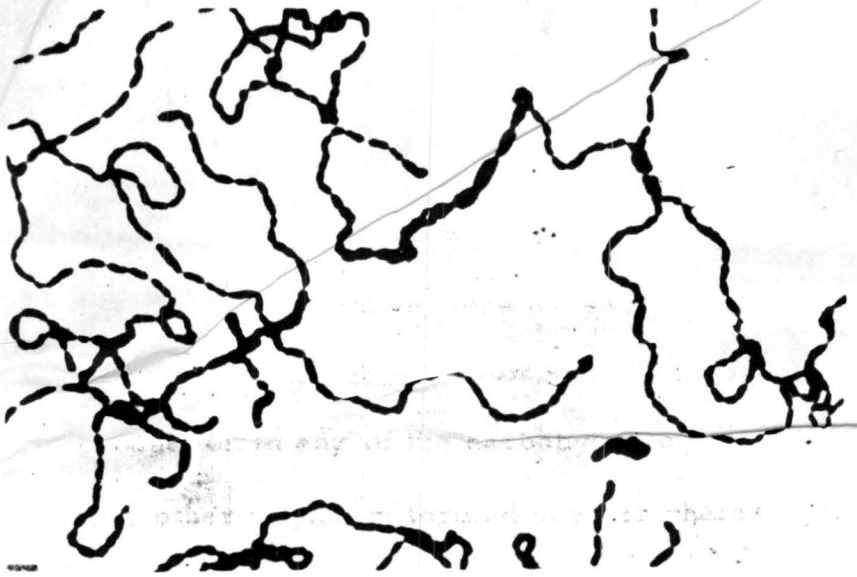
Types B-1, B-2 -- Anaerobic streptococcus-like organisms (Fig. 6).

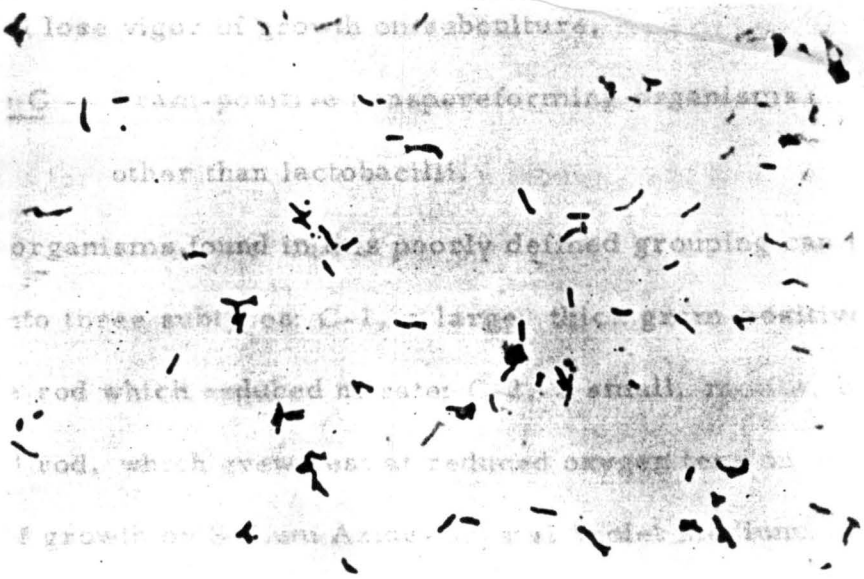
Whether these organisms or type A, the anaerobic lactobacillus-like organisms, were more numerous in cecal contents

[1] According to the proposed reclassification of Lactobacillus bifidus by Rosebury (1962).

FIGURE 5. Type A organism from sage grouse cecal contents (X 1400).

FIGURE 6. Type B-1 organism from sage grouse cecal contents (X 1400).





was dependent on season. Type B organisms were highly pleomorphic and formed medium to very long chains. Culture was only successful on RFAM and CFAM media and on subculturing shorter chains were formed. The designation streptococcus is descriptive; these organisms may possibly belong in another genus.

Two subtypes of this organism were present in the ceca. The first formed extremely long chains, grew well only on CFAM, and gave no acid production in any of the carbohydrate media used on the project. The other organism formed shorter chains, grew fairly well on both CFAM and RFAM, formed acid in Bryant and Robinson's fermentation medium with maltose, glucose, sucrose, and lactose, but failed to grow in other carbohydrate media. Both subtypes, designated B-1 and B-2 respectively, tended to form short chains and lose vigor of growth on subculture.

Type C -- Gram-positive nonsporeforming organisms other than lactobacilli.

The organisms found in this poorly defined grouping can be divided into three subtypes: C-1, a large, thick gram-positive, nonmotile rod which reduced nitrate; C-2, a small, motile, bent or curved rod, which grew best at reduced oxygen tension and was capable of growth on Sodium Azide-Crystal Violet Medium; and C-3, a small catalase positive rod. The first and last subtypes mentioned could very possibly be lactobacilli. Since all of these

organisms were counted at low dilutions on Trypticase Soy Agar plates with many other organisms present, allowance for error due to double counting or incorrect identification must be made.

Type D -- Facultative lactobacilli.

These lactobacilli grew well on aerobic pour plates of Tomato Juice Agar Special; best growth was subsurface or under reduced oxygen tension. Plate counts were made from aerobic plates. This organism was hardier than the Type A chain-forming rods, but was never cultured in significant numbers.

Type E -- Facultative streptococci (enterococci).

The organisms of this group exhibit the cultural and physiological characteristics of most of the enterococci. This type of organism was present in all cecal samples cultured. The majority of cultures of this type examined appeared to be a strain of Streptococcus faecalis. None were hemolytic, all reduced litmus milk, most fermented glycerol but not mannitol, and none liquified gelatin. A few colonies of this type, apparently of a different strain, liquified gelatin and fermented both mannitol and glycerol.

Type F -- Coliforms

Coliforms and a few colonies of Paracolobactrum sp. grew on media used in the project; counts of organisms of this type were obtained on Violet Red Bile Agar. Individual isolates were IMViC tested to differentiate Aerobacter sp. and Escherichia coli.

Relatively low counts of coliforms (3 million mean) indicate that these organisms are of relatively little importance in the sage grouse ceca.

Summary

With the exception of the very long rod forms and sarcina-like organisms, enteric bacteria cultured from sage grouse ceca were of normal avian enteric types. An organism very similar to Actinomyces bifidus and a streptococcus-like form predominated. The majority of organisms in the cecal flora were of a few types.

Total counts were only slightly lower in winter-killed birds; a difference between summer and winter counts of the magnitude noted is within the range of error of the roll tube counting method used. The most noticeable cultural differences were the increase of the anaerobic streptococcus-like organism from summer to winter and a slight winter decrease in the numbers of the anaerobic actinomycete. The principal role of the predominant cecal organisms is probably the production of the significant amounts of VFA present in the ceca (Chapter III).

Facultatively anaerobic streptococci were the most variable organisms in total number, fluctuating from a low of 20 million per gram of cecal contents to a high of 11 billion. Coliforms were generally low in number, the average count on Violet Red Bile Agar being 3 million. Staphylococci, hemolytic streptococci, clostridia, and a salmonella-like organism occurred only irregularly in concentrations of less than a thousand per gram of wet cecal

contents. None of the organisms isolated from cecal contents produced significant amounts of gas on carbohydrate media.

CHAPTER III

VOLATILE FATTY ACID PRODUCTION BY SAGE GROUSE CECAL BACTERIA

Introduction

General Considerations

The end products of rumen microbial fermentations of plant cellulose and less complex carbohydrates are predominantly acetic, propionic, and butyric acids and carbon dioxide and methane. If feed types and varying microbial flora are taken into proper consideration, VFA production can serve as a general indicator of metabolism in the rumen (Hungate et al., 1961).

Fermentation processes similar to those of the rumen have been found to occur in the ceca of porcupines and beavers; Johnson and McBee (1967) found that up to 83% of the VFA generated in the porcupine cecum was directly absorbed into the animal's bloodstream, making a significant contribution to energy requirements.

Hill et al. (1965) found significant amounts of VFA in the ceca of normal domestic fowl, while only very small quantities were present in the ceca of germ-free birds. Variation of blood VFA content between the two groups of birds was not significant, leading

Hill to conclude that VFA in the blood of the fowl was not of microbial origin. More recent work by Chung et al. (1967), indicated that the gut of normal chickens absorbed highly variable amounts of the non-volatile fatty acids in feeds. McBee and West (1968) found that sufficient amounts of VFA were generated in willow ptarmigan ceca to provide an appreciable contribution to the energy requirements of the bird.

Purpose

The purpose of the analysis of cecal contents for VFA was the determination of the total amounts of VFA produced by the cecal microflora, the relative proportions of each VFA present, and whether the total amounts of ceca-produced VFA varied with time of day and season.

Materials and Methods

Preparation of Cecal Fluid

Cecal contents not needed for inocula or for addition to media (extracted and stored as in Chapter II) were prepared for gas chromatography by thawing, diluting 1:5 in distilled water, blending in a Waring Blender at high speed for three minutes to suspend and break up the larger particles, and centrifuging the suspension at 15,000 rpm for 45 minutes. The supernate was acidified with a 25% metaphosphoric acid solution (2 ml to 10 ml supernate) and was either analyzed immediately or frozen for later use.

Chromatographic Apparatus Used

An Aerograph 600 H-Fi gas chromatograph with hydrogen flame detector was used for analyses. Essential information was as follows: column -- 5' X 1/8", 10% LAC on 2% terephthalic acid, HMOS 80/100w; oven temperature -- 160 C; injection port temperature -- 200 C; gas flow rates -- hydrogen 30 ml/min.; carrier (nitrogen) -- 20 ml/min.; attenuation -- varied, output 10^9 millivolts; recorder -- Honeywell-Brown with disc integrator.

Results

Results presented in Table 5 are the mean of triplicate runs of analysis on cecal contents from the two birds in each sample. Although it is felt by this investigator that insufficient numbers of samples were taken to allow firm conclusions as to VFA production and utilization in sage grouse, these data show some interesting general trends.

These data indicate that birds taken in summer have lower total amounts of cecal VFA than those taken in winter, and that morning-killed birds have lower total amounts of VFA present in both winter and summer than those killed in the afternoon. Total quantities of VFA present in winter samples compare favorably to the amounts present in the rumen fluid of cattle on a non-concentrate diet, although the relative proportions of each acid are slightly different.

Lower total VFA content in samples collected in the morning may indicate a loss of VFA either by absorption or by leakage from the ceca during the night when the birds are not feeding. The lower content of VFA in the ceca of morning-killed birds may also be due to dilution of the cecal contents by liquid from the intestine after the cecal droppings are voided. Further studies of the time necessary for food to pass from the esophagus to the entraces of the cecal tubes will be necessary to clarify this question.

TABLE 5. Concentration and relative proportion of VFA in cecal contents

Sample Number	Age of Bird	Season Killed	Time * Killed	Percentage Distribution of: Acetic	Propionic + Isobutyric	Butyric	Total VFA (m moles/l)
1	Ad. F.	Summer	A. M.	51.2	48.1	.7	47.2
2	Juv. M.	Summer	P. M.	69.9	27.7	1.4	31.6
3	Ad. M.	Summer	P. M.	78.6	20.3	1.1	133.7
4	Ad. F.	Summer	A. M.	47.6	52.3	.1	58.0
5	Ad. M.	Summer	P. M.	71.5	26.3	2.2	160.5
6	Ad. M.	Winter	A. M.	74.6	25.0	.4	48.4
7	Ad. M.	Winter	P. M.	68.4	29.2	.4	205.5

*A. M. = Before 9 A. M.

P. M. = After 3 P. M.

Summary

Bacteria generate significant amounts of VFA in sage grouse ceca. Amounts of VFA present increase during the day, due probably to the filling of the ceca and an increase in microbial fermentation, and decrease during the night. Levels of VFA in the ceca of winter-killed grouse were consistently higher than the amounts present in the ceca of grouse killed during the summer and early fall.

CHAPTER IV

CHEMICAL ANALYSES OF SAGE GROUSE FOOD AND CECAL AND RECTAL DROPPINGS

Introduction

General Considerations

It was felt by this investigator that a comparison of results of analyses of cecal and rectal droppings of sage grouse with data from analyses of sagebrush leaves from the crops of the birds and from plants in areas where they had been feeding would facilitate a preliminary discussion of the bird's nutrition. Data on the chemical composition of the feed and feces of sage grouse, in addition to indicating whether or not significant amounts of fiber are digested, would be useful in determining preferred types of sagebrush leaves and the utilization of sagebrush essential oils by sage grouse.

Three distinct types of sagebrush were described by Keller et al. (1941): A-1, less than 5 inches in height; A-2, 5 to 10 inches in height; and A-3, greater than 10 inches in height. Gill (1966) and Rogers (1964) have more recently commented that during summer and early fall sagebrush plants utilized by sage grouse for

food are almost exclusively Types A-1 and Types A-2 and that Type A-3 serves as nesting and loafing cover.

A sense of taste in domestic fowl has been demonstrated by the use of sugar solutions (Sturkie, 1965). The selection of different types of sagebrush leaves by sage grouse has been postulated to depend on the oil content of the leaves, Type A-1 leaves of low oil content being preferred (Gill, 1966). Nagy and Tengerdy (1968) determined that the volatile oils of several species of sagebrush exerted an inhibitory effect on the growth of rumen microorganisms in vivo and in vitro and decreased rumen motility. It appeared that volatile oils of sagebrush might possibly exert similar effects on the bacteria of the sage grouse ceca or on the ceca themselves.

Purpose

The purpose of this portion of the study was to determine whether "feed-feces" checmial analyses would:

(1) give some indication of the role of the cecal microflora in nutrition of the sage grouse;

(2) allow a chemical identification of the type of sagebrush leaf most commonly found in the crops of sage grouse during the summer and early fall;

(3) indicate to what extent the volatile oils of sagebrush are utilized by sage grouse.

Materials and Methods

General Considerations

The procedure for the Weende or proximate analysis of feeds, feces, plants, and meat and other agricultural products is well known and will not be described here. Chemical analyses of the various components of sagebrush leaves and sage grouse droppings were by A. O. A. C. standard methods (1965) with the exception of the substitution of the Acid Detergent Fiber method (ADF) of Van Soest (1967) for the crude fiber determination. All percentages were placed on a dry weight basis.

Cecal contents for chemical analysis were taken as described in Chapter II from viscera of collected birds and that donated by hunters. This cecal matter was dried in a vacuum oven at 80 C for 12 hours in standard size glass petri dishes. After drying, contents were scraped from the dishes, weighed, and analyzed. Cecal and rectal droppings analyzed were obtained in the same areas from which birds furnishing viscera were collected or shot. Both types of droppings were vacuum dried as above prior to analysis.

Leaves of types A-1, A-2, and A-3 sagebrush analyzed were taken from plants in the immediate areas from which sample birds came. Current annual growth was clipped and placed in plastic bags on which the location of collection, type of plant, and a number

to identify the leaf sample with a bird or viscera sample was inscribed. Leaves were separated from stems by freezing at -25 C for 48 hours and then agitating sample stems together rapidly by hand. This process resulted in a quick and efficient separation of the leaves.

Twig particles, sand, and dirt were separated from leaves by shaking over a series of screens, the mesh of which varied from 10 mm to 2 mm in size. Leaves were vacuum dried prior to analysis for the same time and at the same temperature as the cecal contents and cecal and rectal droppings. Clumps of sagebrush leaves taken from the crops of birds were broken apart and the few leaves of forbs usually present were picked out. Observations as to the morphology of the type of sagebrush leaves present in crops was made at this time.

Quantitative analysis of the volatile oils present in the leaves of the sagebrush plants in areas used by grouse and in droppings and crop contents was accomplished by boiling 100 g amounts (wet weight) of each material in a 5 liter capacity round bottom distilling flask with a calibrated oil trap and a 24 cm long condenser. The flask was half-filled with water; the period of boiling was 6 hours in duration. After distillation oils were drained into a separate trap, measured, and stored in screw-capped vials in a refrigerator.

Results

Table 7 contains the results of chemical analyses run during this phase of the project. Values for leaves and droppings are from duplicate runs of six samples; those for crop and cecal contents were obtained from duplicate analyses with material from six adult birds taken during late summer and early fall. Crop samples taken from birds killed during the project consisted almost entirely of leaves similar in chemical composition to those of A-3 type plants. This finding indicates that, contrary to the results of some field studies, type A-3 sagebrush is sometimes selected for food at this season. Selected A-3 sagebrush leaves were considerably higher in essential oil content than those of A-1 sagebrush, containing up to 4.9 ml per 100 g of wet leaves compared with a high of 2.1 ml per 100 g of type A-1 leaves.

The 2.2 ml per 100 g essential oil content of rectal droppings was quite high compared to that of cecal droppings and cecal contents which usually contained less than .1 ml of essential oils per 100 g wet weight. Selective prevention of essential oils or other liquids from entering the ceca is unlikely (Browne, 1922), but some concentration of oils in the rectal feces may be caused by slow leakage from the ceca into the cloaca. Neither ether extract nor essential oil concentration in the rectal droppings was

sufficient to account for all of the oils ingested; some absorption of the oils by the bird or their degradation by its cecal microflora probably occurs.

Cecal contents had very low fiber levels in comparison to rectal feces. Maximum ADF value was only 3.9% of a 10 to 16% dry weight. NFE levels were high in cecal contents, possibly accounting for the source of VFA produced. Microscopic examination of cecal contents showed that only isolated minute fiber particles were present, hardly enough to furnish substrate for significant cellulose degradation. An interesting alteration of lignin-cellulose ratios occurred between cecal contents and cecal droppings. Further study will be necessary to determine whether lignin is a useable marker substance for digestibility studies involving grouse. Although it is improbable that cellulose digestion is totally absent in cecal contents, the amount occurring appears to be insignificant.

Sagebrush of the species analyzed (A. tridentata), although not nearly as concentrated a food source as the seeds eaten by many gallinaceous birds, proved to be of high theoretical nutritive value in comparison to many other plant foods. Type A-3 plants contain crude protein and nitrogen-free extract equal to and in some cases surpassing good alfalfa meal. This finding will remain relatively useless until marker studies are done to determine the digestibility of the leaf components.

Apart from the disadvantages caused by a small sample taken during one season of the year, a number of other opportunities for error in this portion of the study are evident. It is possible that sage grouse do not actually feed on the type A-3 sagebrush to any great extent; leaves found in crops of specimens may merely have been selected from type A-1 or A-2 plants which for some reason had unusually large leaves with high essential oil content. Changes in moisture received in a single year are sufficient to cause such a change in some cases. Droppings analyzed were unfortunately often more than one day old and their soluble component and volatile oil content may have been considerably lowered. Studies of food preferences during the winter season when types A-2 and A-3 plants are usually covered with snow must also be postponed until pen study methods have been worked out. The necessity for determining which type of sagebrush leaves constitute the preferred food of sage grouse is obvious; chemical analyses of crop contents will provide the best possible basis for selective eradication of sagebrush types.

TABLE 6. Percentage chemical composition of sagebrush leaves and sage grouse crop and cecal contents and cecal and rectal droppings.[1]

	Leaves		Crop	Cecal	Rectal	Cecal
	A-1	A-3	Contents	Contents	Droppings	Droppings
Dry Weight	45.0%	39.1%	37.5%	12.1%	66.2%	17.2%
Ash	7.9	5.5	5.3	11.1	5.2	13.6
Crude Protein	13.7	12.8	12.5	5.7	19.2	4.7
Nitrogen-free Extract	39.7	45.2	44.2	76.5	23.9	76.3
Ether Extract	2.6	5.9	5.9	3.1	10.1	2.3
Acid Detergent Fiber	37.1	30.6	32.1	3.6	41.6	3.1
Cellulose	27.3	22.7	22.0	1.5	30.1	1.7
Lignin	9.8	7.9	10.1	2.1	11.5	1.4
Essential Oils (ml/100 g wet wt)	1.9 (ml/100g dry) 4.2	4.9 12.6	4.7 12.5	0.1 0.83	3.2 4.8	0.1 0.58

[1] Dry weight basis.

Summary

Chemical analyses of sage grouse food and cecal and rectal droppings showed that at least in some areas Type A-3 sagebruch leaves are the preferred food of sage grouse. Crop contents of grouse collected in early fall were composed almost entirely of sagebrush leaves. Essential oil content of rectal droppings was much higher (3.2 ml per 100 g) than that of cecal droppings (0.1 ml per 100 g); the high ether extract content of rectal droppings (10.1%) may account for the remaining volatile oils ingested. A concentration of plant fiber occurs in the rectal droppings, but the determination of digestibility of the fiber can only be made after a suitable marker substance is discovered.

CHAPTER V

DISCUSSION

Bacterial Flora

Cultures of bacteria from the cecal contents of sage grouse showed a large bacterial population to be present. Predominant organisms in cecal contents were similar to normal avian enteric types. Organisms resembling Actinomyces bifidus and a streptococcus-like form were the most numerous types in the ceca.

Solid media including the supernate of centrifuged cecal contents (CFAM) and clarified rumen fluid (RFAM) proved to be superior to all other media tested for total counts of cecal bacteria; the use of these media was a necessity for the culture of the two predominant types of cecal organisms. Total culture counts of cecal bacteria in winter-killed birds showed a mean of 18×10^9 organisms per gram of wet cecal contents as compared to 30×10^9 for those collected in summer. Only insignificant numbers of cellulose-digesting organisms were cultured despite use of several enrichment media. Low initial numbers of these organisms in cecal contents and their being outgrown in poorly suited media by less fastidious organisms were probably responsible.

Slight inhibition of bacterial growth by the essential oils of sagebrush may account for the lower winter counts which occurred when the birds were on a pure sagebrush diet. Only very low numbers of pathogenic bacteria were ever found in cecal contents; the cecal microflora may serve as a buffer against infection by competition with pathogens.

VFA Production

The large amounts of VFA produced by the cecal microbial fermentations are most likely products of NFE breakdown. Higher levels of VFA were present in ceca in winter when the birds were on total sagebrush leaf diets and lower total numbers of cecal bacteria were present.

Further work, involving a nutrition study of penned grouse on a natural diet, will be necessary to determine the relationship of cecal and blood levels of VFA; a situation similar to that in chickens, which do not require ceca-produced VFA, may exist. Low morning levels of VFA possibly result from the loss of VFA at night due to absorption by the bird, dilution, or a gradual leakage from the ceca. Cecal fermentation rates probably rise during the day as the ceca fill during feeding and bacteria increase in number.

Chemical Analyses of Feed and Feces

Analysis of cecal contents showed that they contained only about 3% fiber (of a 10-16% dry weight). The high concentration of fiber in rectal droppings accounts for nearly the total amount ingested in sagebrush leaves. It will be necessary to calculate digestibility coefficients of the fiber portion of sagebrush leaves, probably with penned grouse, before authoritative information is available on this subject. An obvious difficulty in this analysis is the necessity of combining cecal and rectal droppings in the proper proportions to get a valid estimate of the chemical makeup of the soild wastes. Use of an indigestible marker substance other than lignin may be necessary.

Essential oils of sagebrush are present in greater amounts in the Type A-3 sagebrush leaves selected by the grouse examined as summer food. Crops of winter-killed birds contain a mixture of all three subtypes of sagebrush; total oil levels in the digestive tract at this season were higher than in the summer when forbs comprised a portion of the diet.

Further Study

The project investigation made obvious several area in which co-ordinated studies should be undertaken:

(1) additional work is necessary to fully characterize the predominant cecal bacteria and assign them roles in the cecal fermentation;

(2) the substrates for cecal VFA production should be determined;

(3) the utilization of ceca-produced VFA by the bird should be measured;

(4) detailed studies of the nutrition of penned sage grouse should be undertaken to determine digestibility of the various fractions of sagebrush leaves and the manner in which the leaves are selected by the birds.

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