EFFECTS OF TANNINS ON PROTEIN DIGESTIBILITY AND DETOXIFICATION ACTIVITY IN GRAY SQUIRRELS (Sciurus carolinensis)

by

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Thesis submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in
Fisheries and Wildlife Sciences
(Wildlife Science)

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(ABSTRACT)

Captive gray squirrels were fed acorns or diets containing commercial tannins to determine the effects of tannins on digestion and detoxification (glucuronidation) activity. In the Acorn experiment, Northern red oak acorns (Quercus rubra), white oak acorns (Q. alba), or rat chow were fed to squirrels. Levels of phenols, condensed tannins, and hydrolyzable tannins were higher in red oak acorns than white oak acorns and were likely responsible for the lower dry matter intake, lower apparent protein digestibility, lower digestible protein and energy intakes, and higher glucuronidation activity observed in squirrels fed red oak acorns. Although apparent protein digestibility and digestible protein intakes were reduced on a white oak acorn diet, this diet did not continuously suppress dry matter intake or stimulate glucuronidation. It appears that gray squirrels may not be able to subsist on red oak acorns alone, but may require other foods to dilute tannin intake and provide additional nutrients.

In the Tannin experiment, squirrels were fed rat chow containing no tannins, 4% or 8% tannic acid (hydrolyzable tannin), or 3% or 6% quebracho (condensed tannin). Apparent protein and energy digestibilities of tannic acid-containing diets were lower than the control. These reductions were likely due to the formation of strong complexes between protein and high molecular weight gallotannins. These complexes may have simultaneously protected these gallotannins from hydrolysis and

allowed tannic acid to reduce digestive efficiency. Apparent protein and energy digestibilities of quebracho-containing diets were reduced, indicating protein complexing by these nonhydrolyzable tannins. Consistent with the hypothesis that hydrolyzable tannins are more likely to be broken down and absorbed internally than condensed tannins, only the 8% tannic acid diet tended to increase glucuronide excretion.

ACKNOWLEDGEMENTS

I owe thanks to many people for the success and speed with which my project was conducted. First and foremost, I would like to thank my graduate advisor and committee chair, Dr. Roy L. Kirkpatrick, for his guidance, support, and confidence in my abilities despite his skepticism that squirrels were suitable subjects for my experiments. My thanks also go to Dr. Dean Stauffer and Dr. Ken E. Webb, Jr. for their helpful advice and criticism regarding the technical aspects of this project. In addition, special thanks go to Dr. Ann E. Hagerman of Miami University, Oxford, OH for conducting the tannin analyses and for her help in the design of the project and interpretation of results. I am also indebted to Dave Hewitt, who provided assistance in my experiments as well as helpful input in the design, execution, and interpretation of my project. Much of my lab analyses would not have been possible without the diligent and reliable help of my work-study student, Llora Krupinski. Most of all, I owe thanks to my husband, Ian, who provided support and encouragement as well as assistance in many aspects of my project, and who has been patient and loving throughout my academic pursuits. This project was funded by a Pratt Animal Nutrition Assistantship.

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INTRODUCTION

Mast is an important fall and winter food for many wildlife species. Acorns, hickories, and other nuts provide a concentrated source of energy during seasons when other valuable foods are absent. Mast species vary in the degree to which they are protected from herbivores by internally produced plant chemicals. Acorns may contain considerable amounts of tannins, a group of secondary plant compounds that deter herbivory by reducing palatability, dietary protein availability, and digestive efficiency, by damaging the gastrointestinal tract and vital organs, and by affecting growth, survival, and reproduction in many animals (Salunkhe et al. 1990). The total tannin content of acorns varies with the species of oak. However, the red oak subgenus, Erythrobalanus, is generally higher in tannins than the white oak subgenus, Lepidobalanus (Ofcarcik and Burns 1971).

Differences in total tannins between acorn species have been proposed as the basis for certain food preferences, burying behaviors, and foraging strategies in squirrels (Smith and Follmer 1972, Havera and Smith 1979, Lewis 1982, Smallwood and Peters 1986). However, little research has been conducted on the physiological response of consumers to acorn tannins. Of specific interest are digestive effects and detoxification response to ingested tannins, variables that represent effects of nonabsorbed and internally absorbed tannins, respectively.

The overall effects of tannins are a composite of effects that results from the action of two types of tannins, condensed and hydrolyzable. Because these two groups of tannins differ in structural and physical properties, they may exert different effects on the consumer. Nonetheless, specific effects of each type of tannin and the condensed and hydrolyzable tannin content in acorns are not clear. Questions pertaining to the differences in effects of condensed and hydrolyzable tannins, the content of these tannins in acorns, the physiological effects of tannins on gray squirrels (Sciurus carolinensis), and the nutritive value of acorns were addressed by this project.

LITERATURE REVIEW

Tannins as a Plant Defense

Years of coevolution between plants and herbivores have resulted in a diverse array of plant defenses and herbivore counterdefenses. Many known antiherbivore defenses are physical or mechanical in nature (e.g. silica in horsetails, thorns, pubescence). However, chemicals as antiherbivorous agents, although proposed as early as the late 1800's, only began to gain attention in the 1950's (Rhoades 1979).

Secondary plant compounds are chemicals that have little or no known metabolic function in the plant, are not essential to the life of the plant, and may serve as deterrents to phytopathogens or vertebrate and invertebrate herbivores (Singleton and Kratzer 1969, Rhoades 1979). Tannins, a diverse group of polar, high molecular weight, polyphenolic compounds found in many vascular plants, especially woody perennials, are considered to be secondary plant chemicals (Rhoades 1979, Swain 1979). Tannins protect plant tissues from degradation by microbes or fungi, protect tissues from consumption by deterring herbivory, and in some plants, protect the overwintering seed and delay the onset of germination (McLeod 1974, Swain 1979). Because tannins have been characterized primarily by their ability to precipitate protein, their major effects on vertebrate consumers have been thought to be a reduction in protein digestibility. Nonetheless, tannins may cause numerous other detrimental effects to the consumer, including intestinal erosion, bone deformations, and liver and kidney toxicity (McLeod 1974, Mitjavila et al. 1977, Elkin et al. 1978).

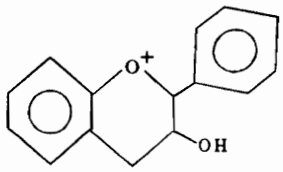
Tannin Structure and Classification

Vegetable tannins have been defined as water-soluble, polyphenolic compounds with a molecular weight between 500 and 3000 that contain sufficient numbers of phenolic hydroxyl groups to effectively complex with protein and other

macromolecules (Salunkhe et al. 1990). Not all phenols contained in plant tissues are capable of cross-linking with protein. Thus tannins may constitute only a portion of the plant's total phenols (Salunkhe et al. 1990).

On the basis of chemical composition and susceptibility to hydrolysis, tannins are divided into two categories, hydrolyzable and condensed (or nonhydrolyzable) tannins (Hagerman and Klucher 1986). Condensed tannins are polymers of flavan-3-ol and flavan-3,4-diol monomers (Figure 1) and are resistant to hydrolysis by acids, bases, or enzymes (Price and Butler 1980, Hagerman and Butler 1989, Salunkhe et al. 1990). Because condensed tannins are more complex than and less regular in structure than hydrolyzable tannins, many of their structures are yet to be determined (Zucker 1983, Salunkhe et al. 1990). Hydrolyzable tannins are composed of a polyhydroxylalcohol (typically glucose) esterified partially or wholly to polyphenolic acids such as gallic acid, digallic or trigallic acid (gallotannins), or ellagic acid (ellagitannins) (Figure 2). As their name implies, hydrolyzable tannins are readily hydrolyzed into their components by acids, bases, or enzymes (Price and Butler 1980, Hagerman and Klucher 1986, Salunkhe et al. 1990).

Hydrolyzable tannins occur primarily in the dicotyledonous plants of the angiosperms. They are found in seed pods, bark, wood, leaves, and galls (Swain 1979, Hagerman and Klucher 1986). Most studies investigating the effects of hydrolyzable tannins have used tannic acid (a mixture of gallotannins originally isolated from the Turkish or Chinese nutgall [Merck Index 1983]). Condensed tannins are found in a wider spectrum of plants, from primitive vascular plants (e.g. ferns) to gymnosperms and angiosperms (Swain 1979, Salunkhe et al. 1990). Some plants (e.g. Quercus spp.) contain both types of tannins (Feeny 1968). Sorghum grain is the most commonly used natural source of condensed tannins for biological studies. Quebracho, the most commonly used commercial preparation of condensed tannins, is an extract of the dried bark of Aspidosperma quebracho-blanco, a species native to Argentina (Merck Index 1983).



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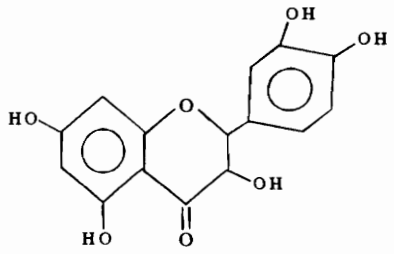

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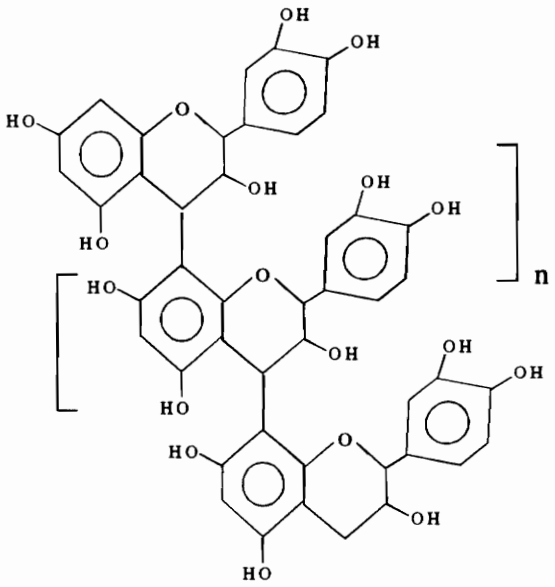

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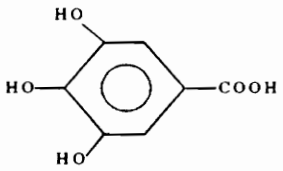
Figure 1. Representative structures of condensed tannins: **1** is flavan-3-ol; **2** is flavan-3,4-diol; **3** is the condensed tannin in *Sorghum* (from Salunkhe et al. 1990).



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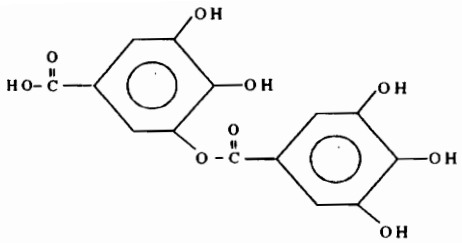

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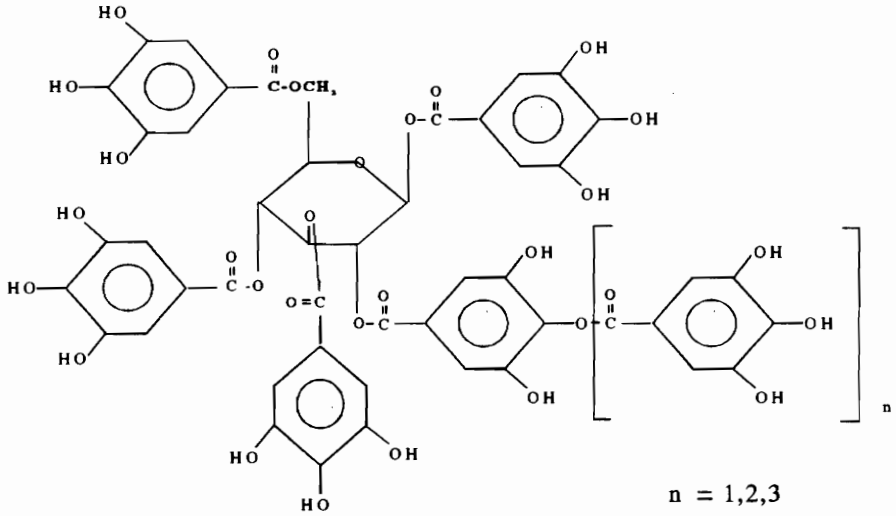

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Figure 2. Representative structures of hydrolyzable tannins: **1** is the subunit gallic acid; **2** is the subunit m-digallic acid; **3** is Chinese gallotannin (from Hagerman and Butler 1989).

Physiological Effects of Tannins

Plant tannins may affect many aspects of the consumer's physiology, nutrition, and metabolism, including 1) dietary intake, 2) dietary protein availability, 3) digestive enzyme activity, 4) endogenous protein excretion, 5) integrity of the intestinal mucosa, 6) detoxification activity, 7) integrity of the kidney and liver, 8) postabsorptive metabolism, and 9) growth and performance.

Effects of tannins on dietary intake

Tannins impart an astringent taste by binding salivary proteins and the mucous epithelium of the mouth (McLeod 1974, Salunkhe et al. 1990). Thus tannins may decrease palatability and intake of a particular plant material or diet. As reviewed by Mole and Waterman (1987a), various tannin-containing plants such as savannah vegetation, subarctic browse, blackbrush (Coleogyne spp.), and lespedeza (Sericea lespedeza) reduced intake in buffalo (Syncerus caffer), snowshoe hares (Lepus americanus), goats, and sheep, respectively. Chicks and rats fed sorghum grain or tannic acid-containing diets reduced intake (Joslyn and Glick 1969, Price and Butler 1980). Ruffed grouse fed tannin-containing acorns also reduced dry matter intake (Servello and Kirkpatrick 1989). Low molecular weight phenols associated with plant tannins may be partially responsible for reducing intake by causing internal toxicity, altering physiological processes, and causing additional energy costs for detoxification (Robbins et al. 1987b). Although most studies have shown tannins to decrease intake if any effect occurs at all, two studies have shown that tannic acid-containing diets may have increased intake slightly in laboratory rats and prairie voles (Microtus ochrogaster) (Mitjavila et al. 1971, Lindroth and Batzli 1984). In these situations, intakes may have been increased to make up for nutritional losses due to tannins. Some researchers have attributed the main effects of tannin to its ability to reduce intake. The deaths of prairie voles consuming quebracho-containing diets were attributed to the severe and prolonged reduction in intake (Lindroth and Batzli 1984).

Growth suppression in rats consuming tannic acid-containing diets was largely attributed to intake reduction (Joslyn and Glick 1969). Although evidence seems to indicate that tannins affect growth rate mainly by reducing intake, animals deterred by tannin-containing diets in preference trials did not necessarily experience negative effects on performance when fed tannin-containing diets alone (Meyer 1989). Thus, one may ask of tannins, as did Mole and Waterman (1987a), whether "their bark is worse than their bite".

Effects of tannins on protein availability and digestive enzymes

The ability to precipitate protein in vitro is a defining characteristic of tannins (Salunkhe et al. 1990). Hydrogen bonding between phenolic hydroxyls of the tannin polymer and amide carbonyls of the protein polypeptide may be the primary binding mechanism between the two entities (Figure 3). The result of this interaction is a large, crosslinked, and insoluble complex that may precipitate from solution (Hagerman and Klucher 1986).

A commonly observed effect of tannins on consumers is an increase in fecal nitrogen. Traditionally, the increased excretion of nitrogen has been attributed to a reduction in protein digestibility due to complexing of dietary protein by tannins (Rhoades 1979, Butler et al. 1986). Mule deer (Odocoileus hemionus hemionus) fed tannin-containing forages exhibited decreasing protein digestibility as tannin content increased (Robbins et al. 1987b). Apparent protein digestibility was reduced in prairie voles, singing voles (Microtus miurus), Mus musculus, mule deer, sheep, and black bears fed quebracho-containing diets (Lindroth and Batzli 1984, Robbins et al. 1991, Hagerman et al. 1992, Meyer 1989). Tannic acid-containing diets reduced apparent protein digestibilities in prairie voles, singing voles, and Mus musculus, but not in snowshoe hares, New Zealand white laboratory rabbits (Oryctolagus cunniculus), mule deer, or sheep (Lindroth and Batzli 1984, Hagerman et al. 1992, Meyer 1989). Ruffed grouse fed tannin-containing acorns exhibited lower nitrogen

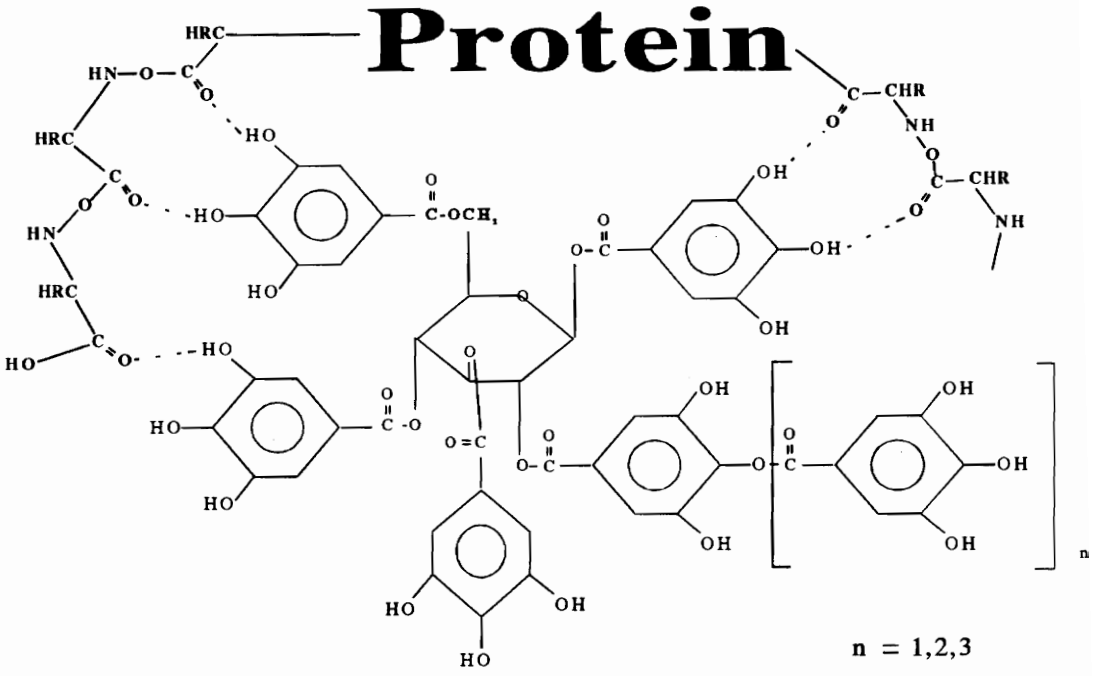

Figure 3. Hydrogen bonding between gallotannin and **protein.**

balances (Servello and Kirkpatrick 1989). Additional studies with rats, chicks, hens, and cattle determined that tannin consumption was associated with decreases in protein and dry matter digestibilities (Butler et al. 1986).

Effects of tannins on endogenous protein excretion

The impression that tannins reduce protein digestibility has developed mainly from in vitro studies that have demonstrated the binding of tannins to added proteins (Hagerman and Klucher 1986, Mole and Waterman 1987a, Butler and Rogler 1992). Consistent with their ability to bind protein, tannins may also bind digestive enzymes in vitro (Tamir and Alumot 1969). The inhibition of digestive enzymes may reduce digestive efficiency, thus explaining the decreases in metabolizable energy of animals fed tannin diets (Salunkhe et al. 1990).

Sources of nitrogen cannot be determined from fecal nitrogen. Thus, decreases in apparent protein digestibility may be due to tannin complexes with dietary protein, digestive enzymes, or other endogenous proteins. Only indirect in vivo evidence exists to support the binding of dietary protein by tannins. The addition of protein to tannin-containing diets alleviated or overcame tannin-induced growth suppression in chicks, rats, and prairie voles presumably by the binding of additional dietary protein to tannins (Lindroth and Batzli 1984, Rogler et al. 1985). Based on in vitro studies, Mole and Waterman (1987b) confirmed that substrate deprivation, not enzyme inhibition, was the cause of reduced protein digestibility in vivo. However, several reviews and studies have cited endogenous protein/tannin complexes as the source of increased fecal nitrogen (Glick and Joslyn 1970b, Freeland et al. 1985, Mole and Waterman 1987a, Mole et al. 1990b, Butler and Rogler 1992, Clausen et al. 1992). When rats were fed radiolabeled proteins (^{14}C-labeled casein or ^{15}N-labeled yeast) and tannin-containing diets (hydrolyzable or condensed), more endogenous protein was excreted in the feces than dietary protein (Glick and Joslyn 1970b, Mole et al. 1990b).

The endogenous proteins bound by tannins may be digestive enzymes, mucoproteins, intestinal wall debris, salivary proteins, or any combination of these. Intestinal proteolytic enzyme activity increased 3-4 times in tannin-fed rats. Thus, digestive enzymes were likely to have constituted a portion of the increased fecal nitrogen (Glick and Joslyn 1970b). In sufficient quantities, tannins may impair the gastrointestinal tract. Freeland et al. (1985) proposed that the toxic effects of tannins in mice were due to intestinal erosion, not reduced protein digestibility. Lab mice fed 5% tannic acid diets exhibited increasing numbers of goblet cells in the upper intestinal mucosa as days of the trial progressed, indicating an increase in mucous secretion. Hypersecretion by the mucosal (goblet) cells is likely to increase the metabolic costs of tannin ingestion (Salunkhe et al. 1990). By the ninth day of the trial, the intestinal mucosa of all mice were severely eroded, indicating that increased mucous secretions did not provide sufficient protection from tannins (Freeland et al. 1985). Impaired nutrient absorption is likely to result from erosion of the intestinal mucosa (Salunkhe et al. 1990). Mucosal erosion has also been observed in rats consuming tannic acid diets, but not in rats, chicks, and laying hens consuming sorghum (condensed tannin) diets (Mitjavila et al. 1977, Sell et al. 1985).

Proline-rich proteins (PRP) in the saliva of many herbivores are thought to be a defensive mechanism against tannins in the diet (Mole et al. 1990a, Robbins et al. 1991). Because proline rich proteins have a higher affinity for tannins, salivary PRP may maximize the amount of tannin bound per unit protein (Hagerman and Klucher 1986, Robbins et al. 1991). Binding of tannins by salivary PRP may reduce the amount of tannin available for absorption, thus reducing the potential for toxic internal effects (Robbins et al. 1991). In addition, because salivary PRP are relatively low in essential amino acids, these proteins of relatively low nutritional value may be sacrificed to protect proteins of potentially higher nutritional value (Mole et al. 1990a). Mule deer and black bears, species that may commonly encounter tannin-containing foods, can produce salivary PRP (Robbins et al. 1991). These species lost

These species lost less fecal nitrogen per unit tannin and absorbed less tannins than prairie voles and domestic sheep, species that did not or were not likely to produce salivary PRP because of their relatively low level of tannin exposure (Robbins et al. 1991). The enlarged parotid glands of lab mice fed 5% tannic acid diets (Freeland et al. 1985) may have indicated salivary PRP production. A high fecal content of proline provided direct evidence of the presence of PRP in the feces of tannin-fed animals (Butler and Rogler 1992).

Thus, substantial evidence seems to exist for the complexing and subsequent excretion of both dietary and endogenous protein by tannin-consuming animals. The biological activity of tannins may depend highly upon the chemistry of each individual tannin (Clausen et al. 1990, Hagerman et al. 1992). Due to the diversity of tannins and the dependence of effects on characteristics of the tannin and the consumer, generalizations about the effects of tannins should not be made (Robbins et al. 1991, Hagerman et al. 1992). Instead each of these variables should be evaluated individually when addressing the effects of tannins on a particular species.

Are tannins absorbed?

Evidence exists, however, that some tannins may not act by reducing protein digestibility, and arguments have been made that the major effects of tannins occur after absorption from the digestive tract. If the main effects of tannins were the formation of dietary protein complexes or the inhibition of digestive enzymes, dietary supplementation with free amino acids should have improved animal performance. However, supplementation of a high tannin sorghum diet with amino acids did not overcome growth suppression in chicks and rats. Thus, growth suppression was probably caused by some mechanism other than reduced protein digestibility (Rogler et al. 1985). Evidence exists for the absorption and toxic action of tannins internally. Only 61% of ^{125}I-labeled tannins fed to rats were recovered in feces. The recovery of 20% from the urine and the remaining 9% from the serum, liver, and kidney

indicated that tannins had been absorbed (Butler et al. 1986). Because prairie voles fed tannic acid diets had the same digestible protein intake as voles on control diets, Lindroth and Batzli (1984) concluded that toxic internal effects, not reduced protein digestibility, had caused the observed growth depression.

Although intact molecules of condensed and hydrolyzable tannins may not be absorbed by a normally functioning intestine (Singleton and Kratzer 1969), constituent phenolic acids may be absorbed. Hydrolyzable tannins are easily degraded in the gut to their constituent phenolic acids. Thus, they are more likely to be absorbed than the relatively resistant condensed tannins (Hagerman et al. 1992). Excretion of gallic acid metabolites in the urine of rats fed tannic acid indicated that hydrolyzable tannins had been absorbed (Booth et al. 1959). However, the absorption of gallic acid and digallic acid, but not gallotannins, by everted rat intestine demonstrated that constituents of hydrolyzable tannins, but not intact molecules, were absorbed (Glick and Joslyn 1970a).

Condensed tannins are not expected to be absorbed because of their resistance to hydrolysis (Hagerman et al. 1992). Accordingly, almost all (98%) of the condensed tannins (quebracho) consumed by mule deer and black bears were recovered in the feces (Robbins et al. 1991). However, Clausen et al. (1990) proposed that condensed tannins may be depolymerized and absorbed by the digestive tract, thus exerting major toxic effects internally. Butler and Rogler (1992) provided evidence against this hypothesis by determining that polymeric ^{14}C-labeled condensed tannins were not absorbed by chicks. However, only 75% of the condensed tannins consumed by sheep were recovered in the feces, providing evidence that condensed tannins had been assimilated (Robbins et al. 1991). The absorption of low molecular weight phenolics associated with condensed tannins has been demonstrated by Butler and Rogler (1992) and may explain the toxic internal effects observed in animals consuming these tannins (Robbins et al. 1987b). Alternatively, the absorption of whole condensed tannin molecules may occur when the integrity of the intestinal wall

has been compromised (Salunkhe et al. 1990).

Effects of absorbed tannins

Tannins that are absorbed through the intestines may have serious internal effects. Ingestion of tannic acid or tannin-containing plants has been associated with the deterioration of liver integrity and function and renal tubular necrosis (McLeod 1974, Govindwar and Dalvi 1990). Acorn tannins caused death in cattle by damaging renal tubules of the kidney to the extent that proper filtration of the blood could not occur (Govindwar and Dalvi 1990). The absorption of tannins and subsequent interference in the proper formation of the organic matrix of bone may have resulted in the high incidence of leg abnormalities in broiler chicks fed high tannin sorghum (Elkin et al. 1978).

The absorption of tannins may also lead to increases in detoxification activity and increases in metabolic requirements. Increased activity of UDP-glucuronyltransferases and increased output of uronic acids have been observed in chicks fed sorghum grain and prairie voles fed tannic acid, respectively (Lindroth and Batzli 1983, Sell and Rogler 1983). Increased liver microsomal activities were observed in rats that had received intraperitoneal injections of acorn extracts (Govindwar and Dalvi 1990). Basal metabolic rates of meadow voles (Microtus pennsylvanicus) fed 6% gallic acid diets increased 14-23%. These increased rates may have been due to greater energy expenditures on detoxification activities and tissue repair processes (Thomas et al. 1988).

The most significant postabsorptive effect of tannins may be the inhibition of postdigestive metabolism. Mole et al. (1990c) demonstrated that the major effect of sorghum tannins on rats was not the reduction in nutrient digestibility, but the reduction in efficiency with which digested and absorbed nutrients were converted to bodily materials (efficiency of conversion of digested nutrients [ECD]). High tannin sorghum diets reduced digestibilities of dry matter and nitrogen from 88% to 78%

and 75% to 45%, respectively. However, ECD's were reduced even more dramatically. ECD(dry matter) was reduced from 7 to 0.7%, and ECD(N) was reduced from 30 to 0%. Mole et al. (1990c) proposed that inhibition of key metabolic pathways or diversion of energy into detoxification activities may have caused the large reductions in ECD's. However, the alternative explanation offered was that reduced intakes and digestibilities may have resulted in nitrogen starvation and inefficient utilization of nonnitrogenous nutrients, thus decreasing ECD's. Because experiments have shown that condensed tannins are not absorbed (Butler and Rogler 1992), it was proposed that the lower MW phenols associated with condensed tannins in sorghum may have been responsible for the observed effects.

Detoxification

Compounds are absorbed through the intestines by mediated transport or passive diffusion. Lipophilic substances may passively migrate across the lipid bilayer membrane of cells lining the intestine. Small polar molecules may cross the membranes of cells lining the gastrointestinal tract by passing through aqueous channels. Most xenobiotics are absorbed passively and not by mediated transport (Klaasen 1986). Because of their large size and polarity, intact tannin molecules are unlikely candidates for passive absorption. As mentioned above, however, constituents of hydrolyzable tannins and low molecular weight phenolics associated with tannins may be absorbed, and condensed tannins may be absorbed after sufficient erosion of the intestinal wall.

Once in the body, an absorbed toxicant may 1) cause direct toxicity to certain tissues, 2) impose energetic costs on the animal by the processes of detoxification and excretion, especially if special moities must be conjugated to the toxicant before excretion, or 3) cause the animal to forego nutrients if food intake and digestibility are reduced and nutrient absorption is impaired (Thomas et al. 1988, Remington 1990).

Biotransformation is the process of polarizing a foreign compound such that it may be excreted by the kidneys into the urine or by the liver into the bile. Thus urine and bile are the major routes of xenobiotic excretion. Biotransformation occurs by Phase I and/or Phase II reactions. Phase I reactions simply expose or add a polar functional group (i.e. -OH, $-NH_2$, -COOH) and occur mainly in the liver. The Phase I metabolite may either be excreted directly or passed on to the Phase II reactions. Phase II reactions conjugate certain moities (glucuronic acid, sulfur, amino acids) to the xenobiotic, thus increasing polarity (Sipes and Gandolfi 1986). Most xenobiotics can enter more than one detoxification pathway. The particular conjugation mechanism depends on concentration of xenobiotic administered, energy status, and other factors (Klaasen 1986).

Amino acid conjugation, sulfation, and glucuronidation are conjugation pathways by which phenolic compounds may be biotransformed. Amino acids may be conjugated to aromatic carboxylic acids for subsequent excretion in the urine. The amino acid conjugation pathway has a high affinity for xenobiotics, but a low to medium capacity (Klaasen 1986). Once saturated, the pathway must pass additional xenobiotics to other conjugation pathways. Sulfation primarily involves the transfer of a sulfur group to a phenolic hydroxyl group. Although the sulfation process has a high affinity for phenols, it has a low capacity. At low doses of phenols, sulfate esters predominate. However, at higher doses of phenols, less sulfate esters and more glucuronides appear in the urine (Klaasen 1986).

Of the several conjugation reactions that exist, glucuronidation is by far the dominant Phase II reaction in mammals. Glucuronidation is the most important conjugation reaction because of the system's high capacity, broad substrate affinity, and ability to continue the phase II detoxification process after other low capacity conjugation systems have become saturated (Dutton 1980). Glucuronosyltransferases, enzymes located on the endoplasmic reticulum of liver cells, conjugate glucuronic acid to the toxicant. Glucuronidation is less demanding of the body's resources than

amino acid conjugation and sulfation because glucuronic acid is readily obtained from glucose and glycogen (Dutton 1980). End products under 350 daltons can be excreted in the urine. End products greater than 250 daltons can be excreted in the bile (Sipes and Gandolfi 1986). The glucuronidation process accounts for most of the conjugation products found in urine and bile (Dutton 1980).

Relative levels of glucuronidation products in the urine may be used as a general indicator of detoxification activity. Aryl-O-glucuronides, which result from glucuronidation of phenolics, are particularly stable because of the ether linkage between the phenolic and glucuronic acid. Thus, quantitative isolation and determination of these glucuronides are facilitated (Dutton 1980). Lindroth and Batzli (1983) found that uronic acid excretion in urine was a simple and reliable index of the detoxification load in prairie voles fed different phenolic compounds. Voles fed diets containing 3% or 6% tannic acid increased uronic acid output by 8 and 31 times the control, respectively.

Variation in Consumer Response to Tannins

Specific effects of tannins on the consumer and the degree to which they occur depend on the primary diet of the species and the degree of exposure to tannins in that diet (McLeod 1974, Lindroth and Batzli 1983, Robbins et al. 1991). Tannins may be most toxic to animals that are least exposed to them (e.g. carnivores and insectivores) and least toxic to animals that are customarily exposed (e.g. herbivores) (Singleton and Kratzer 1969, Salunkhe et al. 1990). As a result of co-evolution with plant tannins, some herbivorous species (e.g. deer, domestic goats, insects) have developed digestive or metabolic adaptations that reduce the effects of tannins. Some species of insects may raise gut pH to reduce tannin-protein interactions. Other animals may produce salivary PRP that minimize tannin interaction with other endogenous or dietary protein (Robbins et al. 1987b). The probability that a species has evolved digestive adaptations and the degree of the effects on the animal may primarily

depend on the proportion of tannin-containing plants or plant parts consumed in the natural diet (Robbins et al. 1987a).

Acorns and the Gray Squirrel

Acorns are a valuable food for many species of wildlife, including deer, bears, raccoons, squirrels, mice, songbirds, and gamebirds (Martin et al. 1951, Fleck and Layne 1990). Despite their importance to these species, acorns contain significant quantities of tannins, a property that may reduce their nutritive value and detrimentally affect the consumer. Acorns vary widely in tannin content according to species. Generally, acorns of the Erythrobalanus subgenus (red oaks) contain higher levels of tannins than the Lepidobalanus subgenus (white oaks). White oak species ranged from 1-10% tannins, and red oak species ranged from 6-13% tannins (Wainio and Forbes 1941, Ofcarcik and Burns 1971). Little research has investigated the nutritive value of tannin-containing acorns to acorn consumers.

The principal diet of the gray squirrel is primarily seeds and nuts, which may constitute approximately 50-95% of the year round diet (Moller 1983). Acorns alone can constitute approximately 25-50% of the diet of the Eastern gray squirrel in any season (Martin et al. 1951). The current study, which investigates the nutritional value of acorns to gray squirrels, was conducted in Montgomery County, Virginia. In this locale, acorns provided 30-50% of the gray squirrel diet in fall, winter, and spring seasons (Dudderar 1967). Because acorns are a large part of their diet, gray squirrels have had a high level of exposure to tannins in their natural history and thus may be more tolerant to high levels of tannins.

Although studies exist that have examined squirrel preferences and energy and nutrient digestibilities of different species of mast, no study has yet examined the apparent digestibilities and physiological effects of acorns on gray squirrels with a specific emphasis on tannin content. Tannin levels may vary within and between acorn species (Ofcarcik and Burns 1971). Thus it is impractical to use previously

reported values for acorn tannin content to interpret effects observed in separate studies of digestibility. This study is the first to examine the tannin content of acorns (red oak, Quercus rubra and white oak, Q. alba) and physiological effects of acorn consumption together. Because a major activity of tannins in the digestive tract may be the formation of dietary protein complexes and the inhibition of digestive enzymes, effects of acorn tannins were interpreted by examining apparent protein, dry matter, and energy digestibilities and intakes. In addition, detoxification activity was measured as an indicator of the levels of toxins absorbed and the potential for toxic internal effects.

Acorns contain both condensed and hydrolyzable tannins (Koenig and Heck 1988, Koenig 1991). Because the two tannin types may act differently within the animal, the proportions of each type of tannin in acorns may influence the types of effects on the animal. In Coast live oak (Quercus agrifolia), Canyon live oak (Q. chrysolepis), and Valley oak (Q. lobata) acorns, condensed tannins constituted 0.84 to 1.27% of the dry mass, and hydrolyzable tannins constituted 9.96 to 19.00% of the dry mass (Koenig and Heck 1988). Aside from this study, no other information is available regarding the condensed and hydrolyzable tannin content of other acorn species. Thus, condensed and hydrolyzable tannin contents of red and white oak acorns used in the present study were determined and used in the interpretation of observed effects.

Experiments that investigate the effects of tannins on consumers may use commercial or natural sources of tannins. The use of natural tannins involves the feeding of whole plants, plant parts, or plant extracts. The effects seen in animals consuming natural tannins are more ecologically applicable because the biological activity of tannins may depend largely on the specific tannin(s) contained in the plant (Hagerman et al. 1992). However, different plants, plant parts, and extracts may have widely different nutritional, tannin, and phenolic compositions. Thus, it is difficult to determine which variable(s) caused the effects observed and why. The

addition of commercially produced tannins to a basal diet allows control of the nutritional and chemical compositions of treatment diets. Observed effects are more attributable to the added tannins. Thus, the mechanisms by which tannins (condensed or hydrolyzable) act may be elucidated. However, commercial tannins may not have the same structures or structural diversity found in natural tannins. Thus, the use of commercial tannins may be less ecologically applicable.

OBJECTIVES

In this study, gray squirrels were fed tannins from both natural and commercial sources to achieve the following **objectives**:

1. To examine apparent protein digestibility and detoxification activity as indicators of the effects of tannins on gray squirrels fed 100% red or white oak acorns.

2. To determine and compare the effects of commercially produced condensed and hydrolyzable tannins on protein digestibility and detoxification activity in gray squirrels.

3. To determine which effects observed in squirrels consuming acorns may be explained by their condensed tannin, hydrolyzable tannin, and overall phenolic content.

MATERIALS AND METHODS

Acorn Experiment

Experimental animals

The 30 gray squirrels used in these experiments were caught in wooden box traps during the second week of January 1992 from President's Hill on the Virginia Tech campus. The male:female ratio of captured animals was 50:50.

A maximum of 4 squirrels was housed in each of 8 outdoor squirrel pens (1.8m W x 1.8m H x 2.7m L) at the Department of Fisheries and Wildlife Sciences' Center Woods Facility (Figure 4). Three to four wooden nest boxes were suspended on the walls of each pen. Squirrels were tagged in both ears with colored enamel coated, size 1 Monel ear tags (National Band and Tag Company, Newport, KY). For quick identification, squirrels within each pen were marked with black commercial hair dye behind the neck and shoulders, on the mid-back, on the rump, or not at all. Squirrels were maintained on Agway PROLAB Rat/Mouse/Hamster 3000 ad libitum. The guaranteed composition of the rat chow was 22.0% protein (min.), 5.0% fat (min.), 5.0% fiber (max.), 6.0% ash (max.), and 11.0% moisture (max.).

Acorn diets

Northern red oak (Quercus rubra) acorns and white oak (Quercus alba) acorns were collected in September and October 1991 from President's Hill and the Center Woods Facility on the Virginia Tech campus and frozen at -13°C. Treatment diets consisted of 100% red oak acorns, 100% white oak acorns, or 100% commercial rat chow (Agway PROLAB Rat/Mouse/Hamster 3000). Acorns were shelled before feeding to prevent shells from contaminating food, feces, and urine. Rotten and larva-infested acorns were discarded.

Kjeldahl analyses were performed with a Kjeltac Analyzer at the Forage

Figure 4. Outdoor pens (top) and metabolic cages (bottom) used to house squirrels

Testing Lab at Virginia Tech to determine the crude protein content of each diet. Crude fat contents were determined by ether extraction in a Soxhlet apparatus (Horwitz 1975). Energy content of each diet was determined with a Parr Adiabatic Bomb Calorimeter (Model 1241) in the Swine Nutrition Lab of Virginia Tech (Principal investigator: E. T. Kornegay). Samples of the three diets were sent to the Department of Chemistry, Miami University, Oxford, Ohio (Principal investigator: A. E. Hagerman) for determination of the total phenol, condensed tannin, and hydrolyzable tannin content. Total phenols were determined by the Prussian Blue assay (Price and Butler 1977). Phenol content was determined using a gallic acid standard curve and is thus reported as mg gallic acid per 100 mg diet. Condensed tannin content was determined by the acid butanol assay (Porter et al. 1986) and was reported as mg quebracho per 100 mg diet. Hydrolyzable tannin content was determined by the gallotannin assay with rhodanine (Inoue and Hagerman 1988) and was reported as mg gallic acid per 100 mg diet. High performance liquid chromatography was used to determine the proportions of different molecular weight gallotannins in the tannic acid.

Feeding trials

During the feeding trials, squirrels were housed individually in metabolic cages (29.9cm H, 26.0cm W, 42.4cm L) within the squirrel pens at the Center Woods facility. The floor of each cage was a 1.3cm by 1.3cm mesh that allowed feces to pass down onto a collection screen. Screen door mesh was placed under the collection screen to prevent small food particles and feces from passing into the urine. A stainless steel funnel directed urine into a glass collection jar below. Cages were wired to the walls of the pen approximately 20cm above the floor. Slanted wooden roofs were placed over each cage to prevent rain from entering the cages (Figure 4). An open-ended and open-bottom shelter (9.5cm H, 14.0cm W, 17.8cm L) made of sheet metal was installed in each cage to provide a retreat for the squirrel. Metabolic

cages were placed inside the squirrel pens before the trials to allow squirrels to become familiar with cages, thus reducing the effects of stress during trials. Because a limited number of metabolic cages were available, the experiment was conducted in 2 feeding trials. The first feeding trial included the 8 males and 7 females in the first 4 adjacent holding pens, and the second trial included the 7 males and 8 females in the last 4 pens. During each feeding trial, 5 animals were assigned to each diet (red oak, white oak, or control). Overall, there were 10 animals per treatment. Treatments were randomly assigned to the metabolic cages in a pen. Squirrels were then placed in the cages in the order they were captured. All squirrels remained in their original pens. No distinctions were made between males and females when treatments were assigned.

Because squirrels were extremely wasteful with their food, much of the food fell through the floor of the cage and became inaccessible to the animal. Thus, control of intake was impossible and diets were fed ad libitum. In the case of prolonged and/or severe reduction in food intake, the animal was removed from the treatment diet. Each morning, a quantity of food was weighed out for each animal. In addition, a standard quantity was added to a running composite of each diet for later determination of percent dry matter. Feeding trials lasted for 10 days. Animals were allowed to acclimate to the diet during the first 5 days (pretrial period). Remaining food, feces, and urine were collected from the 6th through the 10th days (trial period) and analyzed. Individual body weights were measured when animals were placed in the cages on day 1 and when animals were removed from cages on day 10.

Remaining food and feces were collected from the screens below each cage bottom on a daily basis. These mixtures were dried at 65°C and were later separated into food and feces and weighed to determine total feces output and total feed intake for each animal over the 5-day collection period.

To prevent ammonia volatilization and microbial degradation of the urine,

urine pH was kept below 3 by adding sulfuric acid to the collection jar after removal of the previous day's sample. In many cases, water leaked from the water bottle into the urine collection pan, resulting in large volumes of dilute urine. These samples were lyophilized in a freeze dryer, reconstituted to a standard volume, and stored frozen at -15°C until analysis. Urine was pooled for the pretrial and trial periods.

The first and second feeding trials were conducted from 17 June - 26 June 1992 and from 3 July - 12 July 1992, respectively. Animals used for this experiment also were used for the subsequent experiment with commercial tannin diets. Thus, sufficient time between experiments was allowed (3-4 weeks) to minimize carryover effects. Feeding trials with 100% acorn diets were conducted first because the natural acorn diets were believed to be less likely to cause serious carryover effects.

Chemical analyses

Each feces composite was analyzed in duplicate by Kjeldahl analysis at the Forage Testing Lab to determine crude protein content. Total protein intake was determined by multiplying total feed intake by the crude protein content of the diet. Total protein excreted was determined by multiplying total feces output by crude protein content of the feces. Apparent protein digestibility (APD) was calculated by the following equation:

$$\text{APD (\%)} = \frac{\text{total protein intake - total protein excreted}}{\text{total protein intake}} \times 100$$

Digestible protein intake was calculated by multiplying total protein intake by APD.

Single samples of feces and diets were analyzed by bomb calorimetry to determine energy content (in calories). Total energy intake was determined by multiplying total feed intake by energy content of the feed. Total energy excretion was determined by multiplying total feces output by energy content of the feces. Apparent energy digestibility (AED) was calculated by the following equation:

$$\text{AED (\%)} = \frac{\text{total energy intake - total energy excreted}}{\text{total energy intake}} \times 100$$

Digestible energy intake was calculated by multiplying total energy intake by AED.

Although a uronic acid assay was used by Lindroth and Batzli (1984) to determine glucuronidation activity, the assay did not distinguish between free glucuronic acids, conjugated glucuronides, and other glucuronic acid cycle metabolites. Thus, for this study, conjugated glucuronic acids in urine were determined by the method of Mazzuchin et al. (1971). The glucuronic acid assay is a colorimetric assay that reacts glucuronic acids with a modified napthoresorcinol reagent (MNR) to form a colored product that absorbs at 564 nm. After removing other compounds that react with MNR, conjugated glucuronide concentrations are determined by comparing the absorbance of the colored products to a standard curve of known concentrations of glucuronic acid. (See Appendix A for details). Total conjugated glucuronide excretion was determined for the entire trial period. All samples were analyzed in triplicate and in one single assay to avoid interassay variation.

Statistical analyses

Bartlett's test was used to test the hypothesis of equal variances for each variable (Zar 1984). Because most variances were unequal, typical parametric procedures were not used to make multiple comparisons. Data from the 2 trials were combined for analysis by the Smith-Satterthwaite procedure (Gaylor 1988). By this procedure, approximate t-tests were used to make pairwise comparisons between treatment groups. It was difficult to predict what effects acorns would have on digestibilities, intakes, and weight changes relative to the control because of the many differences in nutrient composition between the diets. Thus, 2-tailed t-tests were appropriate when testing for differences between treatments.

The upper limit of experimentwise type I error considered acceptable was $\alpha = 0.15$. Thus, each of the 3 pairwise comparisons was judged at $\alpha/3 = 0.05$, as in Bonferroni's multiple comparison procedure. Because this procedure controls the upper limit of, but not the exact, experimentwise type I error, differences that may have been significant had exact experimentwise error been controlled may have been overlooked. The higher experimentwise error of $\alpha = 0.15$ was considered acceptable because this more conservative approach to multiple comparisons was used. However, P-values represent a continuum from highly nonsignificant to highly significant differences. Thus, marginal P-values (i.e. $0.05 < P < 0.10$) that do not qualify as 'significant' may still be worth noting and may warrant further investigation. Marginal P-values are noted in the text.

Tannin Experiment

Experimental animals

The 30 gray squirrels used in the Acorn experiment were used also in the Tannin experiment. As mentioned previously, 3-4 weeks were allowed between the Acorn and Tannin experiments to prevent carryover effects between trials. During this interval, rat chow was fed ad libitum so that squirrels could regain any lost weight. Midway through this interval, animals were switched from Agway PROLAB Rat/Mouse/Hamster 3000 (RMH 3000) to Agway PROLAB Rat/Mouse/Hamster 1000 (RMH 1000), the basal chow to be used in the Tannin experiment. RMH 1000 was of similar ingredients, texture, and form as RMH 3000, but had a different guaranteed composition of 14.0% protein (min.), 6.0% fat (min.), 4.5% fiber (max.), 8.0% ash (max.), and 11.0% moisture (max.)

Condensed and hydrolyzable tannin diets

Different levels of tannins were added to a basal diet of RMH 1000. Based on

levels of tannins used in previous research and literature regarding the natural exposure of squirrels to tannins, quebracho was added to the diet at lower levels (3% and 6%) than the tannic acid (4% and 8%). Treatment diets were mixed and tableted into 1 gram pellets by P. J. Noyes Co. (Lancaster, New Hampshire). Agway RMH 1000 was ground and prepared for a tablet press prior to mixing with the tannins. To reduce the potential for tannin-protein interactions in the diet prior to consumption by the animal, the preparation process added no water once tannins had been mixed into the diet. The control diet consisted of ground and repelleted chow only. Crude tannic acid (Sigma Chemical, St. Louis, MO) and quebracho (Tannin Corp., Peabody, MA) were added to the diets because the costs of purification or semipurification were prohibitive.

Each of the 5 diets was analyzed in duplicate by Kjeldahl analysis and ether extraction to determine protein and fat contents, respectively. A single sample of each diet was analyzed by bomb calorimetry to determine energy content. Samples of the tannic acid were sent to the Department of Chemistry, Miami University, Oxford, Ohio (Principal investigator: A. E. Hagerman) for analyses of gallotannin composition. Because commercial preparations of quebracho can contain different levels of nontannin phenolics, samples of quebracho were analyzed for purity at the Hagerman lab.

Feeding trials

Two feeding trials were conducted in the same manner as described in the Acorn experiment. As mentioned previously, the wasteful behavior of the squirrels and the cage structure made control of intake extremely difficult. Thus, all diets were fed ad libitum. In each trial, 3 animals were assigned to each of the 5 treatment diets. A total of 6 animals received each treatment diet.

The first and second feeding trials were conducted from 27 July - 4 August 1992 and from 6 August - 15 August 1992, respectively. Trials lasted 10 days each.

Urine was collected and combined for the last 5 days. However, due to a problem in the collection process, food and feces collections were combined for only the last 4 days. Collections and analyses were conducted as described in the Acorn experiment.

Chemical analyses

Determinations of crude protein, energy content, apparent protein digestibility, apparent digestible energy, and conjugated glucuronides were performed as described previously.

Statistical analyses

Most analyses were conducted as described for Acorn experiment. However, if biological support exists to indicate that the effect of a treatment is likely to be 1-sided, 1-tailed t-tests are a valid approach for analysis that may increase the power to detect differences (Sokal and Rohlf 1981). Because previous research has quite consistently shown that tannins decrease nutrient digestibility, 1-tailed t-tests were used to detect decreases in digestibilities. Literature regarding effects of tannins on intake and weight gain are more controversial, and information on effects of tannins on detoxification activity is sparse. Thus, 2-tailed t-tests were used in the analysis of these variables.

Three pairwise comparisons (control vs. 4%, control vs. 8%, 4% vs. 8%) were made between the control and tannic acid groups. The upper limit of experimentwise type I error considered acceptable was $\alpha = 0.15$. Thus, each of the three comparisons was judged at $\alpha/3 = 0.05$. Three pairwise comparisons (control vs. 3%, control vs. 6%, 3% vs. 6%) were made between the control and quebracho groups. Each pairwise comparison was also judged at $\alpha = 0.05$ to achieve an upper limit on experimentwise type I error rate of $\alpha = 0.15$. As mentioned previously, marginal P-values will be noted as well.

RESULTS

Acorn Experiment

Protein contents of red and white oak acorns were approximately one-third the protein in the control diet (Table 1). Crude fat was highest in the red oak acorns and lowest in the control diet. Red oak acorns contained a higher level of gross energy than the control and white oak acorn diets (Table 1). Levels of total phenols, condensed tannins, and hydrolyzable tannins were relatively low in white oak acorns (1.37%, 0.07%, and 0.07%, respectively). Red oak acorns contained much higher levels of phenols, condensed tannins, and hydrolyzable tannins (3.8x, 25x, 28x) than white oak acorns (Table 1). Because different assays were used to evaluate these parameters, cross comparisons could not be made between levels of phenols and tannin types. Approximately 78% of the gallotannins in white oak acorns and 63% of the gallotannins in tannic acid contained 5 to 7 galloyl groups (Table 2). Red oaks contained $<15\%$ gallotannins with 5 to 7 galloyl groups and $>50\%$ with 9 to 10 galloyl groups (Table 2). Gallotannins with >10 galloyl groups were not detected in the red oak acorns, white oak acorns, or tannic acid.

Average initial squirrel weights were not significantly different between diets (Table 3). Dry matter intakes (DMI) for all squirrels increased through the pretrial period (days 1-4) and stabilized through the trial period (days 5-9) (Figure 5). During the pretrial period, significant differences existed in DMI between all diets (Table 3). DMI's on the control diet were highest, and DMI's on the red oak diet were the lowest. However, during the trial period, squirrels on the red oak diet consumed significantly less than the other groups (Table 3). Weight loss occurred for animals on all treatments, but the degree of loss did not differ between treatment diets (Table 3).

Table 1. Protein, gross energy, phenol, and tannin content of control and acorn diets in the Acorn experiment, Blacksburg, Virginia.

Diet	Protein[1] (%)	Fat[1] (%)	Energy[1] (kcal/g dry matter)	Total Phenols[2] (mg gallic acid/ 100 mg diet	Tannins	
					Condensed[3] (mg quebracho /100 mg diet)	Hydrolyzable[4] (mg gallic acid /100 mg diet)
Control	23.8	5.3	4.48	0.06	0.02	NA[5]
White oak	7.2	8.8	4.51	1.37	0.07	0.07
Red oak	6.9	20.2	5.29	5.22	1.73	2.03

[1] Protein: Kjeldahl analysis. Energy: bomb calorimetry. Fat: ether extraction.

[2] Prussian blue assay (Price and Butler 1977). Performed at Miami University.

[3] Acid butanol assay (Porter et al. 1986). Performed at Miami University.

[4] Gallotannin assay (Inoue and Hagerman 1988). Performed at Miami University.

[5] Because almost no phenols were detected in the control diet, this assay was not performed.

Table 2. Gallotannin profile for red and white oak acorns and tannic acid.

# galloyl groups	White oak (%)[1,2]	Red oak (%)[1,2]	Tannic Acid (%)[1,2]
1	3.1	9.3	2.7
2	6.9	6.1	6.7
3	3.9	6.3	14.5
4	8.5	10.0	21.1
5	16.7	8.1	25.1
6	39.3	5.2	17.0
7	21.8	ND	8.6
8	ND	1.6	4.2
9	ND	17.8	ND
10	ND	33.3	ND

[1] Numbers represent the percent of gallotannins with the designated number of galloyl groups. The sum of all gallotannins with up to 10 galloyl groups = 100%.

[2] ND = none detected

Table 3. Average pretrial and trial dry matter intakes (DMI), initial weight, and weight loss for squirrels in the Acorn experiment, Blacksburg, Virginia, June-July 1992.

		Pretrial DMI[1,2] (g/d/kgBW$^{.75}$)		Trial DMI[1,2] (g/d/kgBW$^{.75}$)		Initial weight[2] (g)		Weight loss[2] (g)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	10	41.1[a]	2.7	53.3[a]	4.2	561.6[a]	13.9	68.3[a]	12.8
White oak	10	31.0[b]	2.7	47.5[a]	2.7	590.7[a]	16.2	[3]59.1[a]	10.6
Red oak	10	21.1[c]	1.5	30.7[b]	1.6	591.0[a]	19.4	84.7[a]	12.3

[1] grams dry matter consumed/ day/ kg initial body weight$^{.75}$.

[2] Means in the same column with different letters are significantly different. For each pairwise comparison, α = 0.05. P-values are reported in Appendix B.

[3] n = 9.

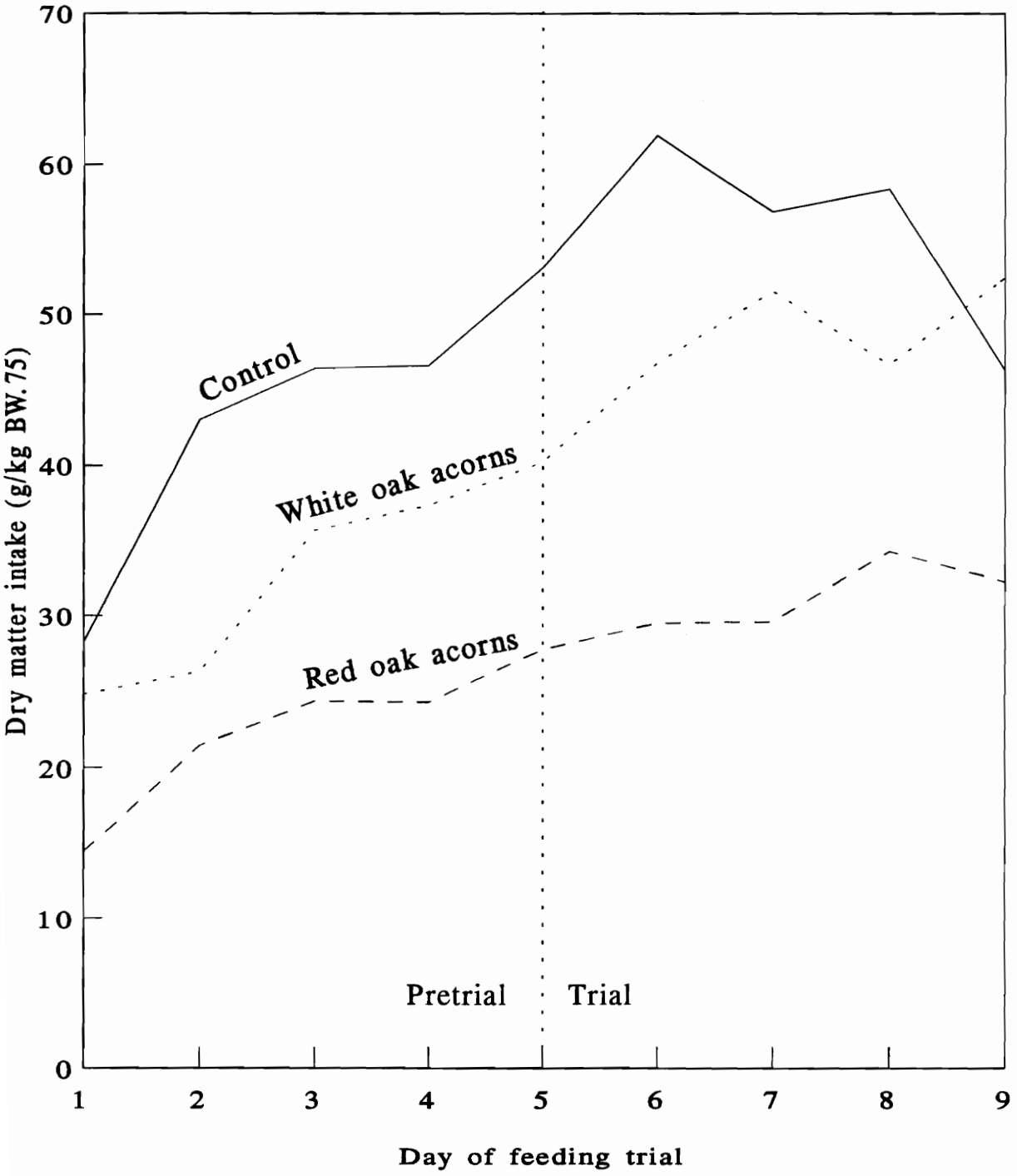

Figure 5. Average daily dry matter intakes for the Acorn experiment.

Gross energy intake by squirrels on the red oak diet also was significantly lower than the other diets (Table 4). Apparent energy digestibilities of red and white oak acorns were significantly higher than the control (Table 4). Apparent energy digestibility of the red oak may have been lower than the white oak ($P = 0.083$). The red oak group showed significantly lower digestible energy intakes than the other groups (Table 4).

Significant differences existed between all three treatment groups with respect to average protein intake, apparent protein digestibility, and digestible protein intake (Table 5). Squirrels on the red oak diet averaged the lowest protein intake, apparent protein digestibility, and digestible protein intake. Squirrels on the control diet averaged the highest values of these variables.

Total conjugated glucuronide excretion in the urine of squirrels fed red oak acorns was significantly higher than that by squirrels fed the control diet or white oak acorns (Table 6). Glucuronide output by squirrels fed white oak acorns may have been higher than squirrels fed the control diet ($P = 0.096$).

Table 4. Average gross energy intake, apparent energy digestibility (AED), and digestible energy intake for squirrels in the Acorn experiment, Blacksburg, Virginia, June-July 1992.

		Gross energy intake[1,2] (kcal/d/kgBW$^{.75}$)		AED[2] (%)		Digestible energy intake[1,2] (kcal/d/kgBW$^{.75}$)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	10	247.6[a]	18.8	78.1[a]	0.8	193.0[a]	14.4
White oak	10	214.2[a]	12.1	87.7[b]	0.3	187.9[a]	10.9
Red oak	10	162.2[b]	8.7	85.4[b]	1.2	138.4[b]	7.1

[1] kcal Energy consumed/ day/ kg initial body weight$^{.75}$

[2] Means in the same column with different letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix B.

Table 5. Average protein intake, apparent protein digestibility (APD), and digestible protein intake for squirrels in the Acorn experiment, Blacksburg, Virginia, June-July 1992.

		Protein intake[1,2] (g/d/kgBW$^{.75}$)		APD[2] (%)		Digestible protein intake[1,2] (g/d/kgBW$^{.75}$)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	10	13.2[a]	1.0	73.4[a]	1.7	9.6[a]	0.7
White oak	10	3.4[b]	0.3	42.0[b]	3.1	1.5[b]	0.2
Red oak	10	2.1[c]	0.1	10.2[c]	5.7	0.2[c]	0.1

[1] grams protein consumed/ day/ kg initial body weight$^{.75}$

[2] Means in the same column with different letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix B.

Table 6. Conjugated glucuronide excretion in urine of squirrels in the Acorn experiment, Blacksburg, Virginia, June-July 1992.

		mmol conjugated glucuronides excreted[1]	
Diet	n	$\bar{x}$	SE
Control	10	1.3^{A}	0.2
White oak	10	1.7^{A}	0.2
Red oak	10	13.2^{B}	1.6

[1] Means in the same column with different letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix B.

Tannin Experiment

Protein, fat, and energy content of the diets in this experiment ranged from 19.6% to 17.7%, from 5.4% to 4.6%, and from 4.43 to 4.35 kcal/g, respectively (Table 7). These levels generally decreased as tannins were added to the basal diet (RMH 1000). The gallotannin composition of the tannic acid is reported in Table 2. Quebracho was approximately 82% pure, containing approximately 18% nontannin phenolic compounds.

The average initial body weights of the tannic acid groups and the quebracho groups were not significantly different from the control (Table 8). One animal in the 8% tannic acid group was removed from the experiment because its intake was extremely low. Initially, dry matter intakes (DMI) were low for all diets (Figure 6). Generally, DMI increased during the first 5 days and stabilized for the last 5 days. During the pretrial period (days 1-5), average DMI for animals on the tannic acid diets were significantly lower than the control (Table 8). However, average pretrial DMI of the quebracho diets were not different from the control. During the trial period (days 6-10), average DMI for squirrels on tannic acid and quebracho diets were not significantly different from the control (Table 8). As in the Acorn experiment, all animals lost weight. However, weight losses for the tannic acid and quebracho groups were not significantly different from the control group (Table 8).

Gross energy intake by animals on tannic acid and quebracho diets were not significantly different than the control (Table 9). Average apparent energy digestibilities (AED) of 4% and 8% tannic acid were significantly lower than the control (Table 9). However the 4% and 8% tannic acid diets were not significantly different from each other (note that $P = 0.085$). Similarly, AED of the quebracho diets were significantly lower than the control diet, but were not significantly different from each other (Table 9). Despite any differences in energy digestibility between treatment diets and the control, digestible energy intake of squirrels on treatment diets did not significantly differ from the control animals (Table 9).

Table 7. Protein, fat, and gross energy content of control and tannin-containing diets in the Tannin experiment, Blacksburg, Virginia.

Diet	Protein[1] (%)	Fat[1] (%)	Gross energy[2] (kcal/ g dry matter)
Control	19.6	5.4	4.43
4% Tannic Acid	19.0	4.7	4.3
8% Tannic Acid	18.3	4.6	4.35
3% Quebracho	18.1	5.1	4.41
6% Quebracho	17.7	4.8	4.40

[1] Determined by Kjeldahl analysis. Forage Testing Lab of Virginia Tech.

[2] Determined by bomb calorimetry.

[3] Determined by ether extraction.

Table 8. Average pretrial and trial dry matter intakes (DMI), initial weights, and weight loss for squirrels in the Tannin experiment, Blacksburg, Virginia, July-August 1992.

		Pretrial DMI[1,2] (g/day/kgBW$^{.75}$)		Trial DMI[1,2] (g/day/kgBW$^{.75}$)		Initial weight[2] (g)		Weight loss[2] (g)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	6	$^{A}51.6^{a}$	6.7	$^{A}60.6^{a}$	6.1	$^{A}592.9^{a}$	39.2	$^{A}28.9^{a}$	13.8
4% Tannic Acid	6	$^{B}25.0$	7.7	$^{A}56.1$	2.6	$^{A}543.7$	16.4	$^{A}61.0$	17.4
8% Tannic Acid	6[3]	$^{B}29.5$	3.9	$^{A}60.5$	5.7	$^{A}590.8$	5.7	$^{A}65.2$	15.5
3% Quebracho	6	57.4^{a}	2.3	64.3^{a}	4.2	588.2^{a}	15.9	29.9^{a}	8.2
6% Quebracho	6	36.4^{a}	7.9	58.4^{a}	2.9	565.9^{a}	7.5	40.6^{a}	7.3

[1] grams dry matter consumed/ day/ kg initial body weight$^{.75}$.

[2] Comparisons were made between squirrels on control and tannic acid diets or control and quebracho diets. Means in the same column with different upper case letters are significantly different. Means in the same column with different lower case letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix C.

[3] Because an animal was removed from the trial on the 6th day, n = 6 for pretrial DMI and Initial weight, but n = 5 for trial DMI and Weight loss.

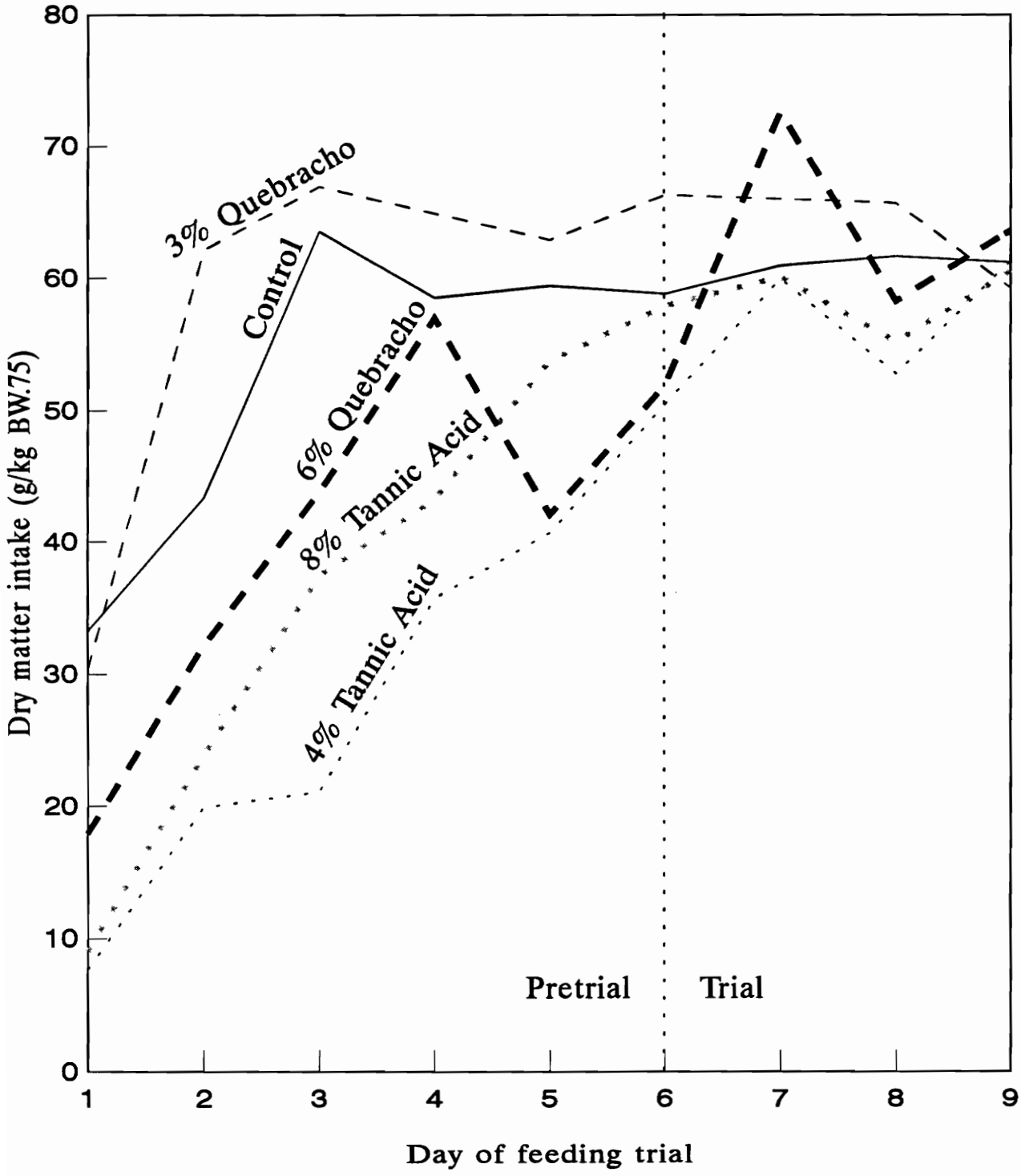

Figure 6. Average daily dry matter intakes during the Tannin experiment.

Table 9. Average gross energy intake, apparent energy digestibility (AED), and digestible energy intake for squirrels in the Tannin experiment, Blacksburg, Virginia, July-August 1992.

		Gross energy intake[1,2] (kcal/d/kgBW$^{.75}$)		AED[2] (%)		Digestible energy intake[1,2] (kcal/d/kgBW$^{.75}$)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	6	$^{A}268.4^{a}$	26.8	$^{A}78.9^{a}$	0.8	$^{A}212.2^{a}$	22.4
4% Tannic Acid	6	$^{A}246.2$	11.5	$^{B}76.4$	1.0	$^{A}187.8$	8.4
8% Tannic Acid	5	$^{A}262.1$	24.7	$^{B}72.3$	2.0	$^{A}189.3$	18.3
3% Quebracho	6	283.4^{a}	18.4	71.0^{b}	1.4	201.6^{a}	14.2
6% Quebracho	6	256.7^{a}	12.7	71.3^{b}	1.9	182.4^{a}	8.3

[1] kcal Energy consumed during trial/ day/ kg initial body weight$^{.75}$

[2] Comparisons were made between squirrels on control and tannic acid diets or control and quebracho diets. Means in the same column with different upper case letters are significantly different. Means in the same column with different lower case letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix C.

Protein intakes of squirrels on tannic acid or quebracho diets were not significantly different than the control (Table 10). However, average apparent protein digestibility (APD) decreased as tannic acid content increased (Table 10). APD was highest for animals on the control diet and was lowest for animals on 8% tannic acid. Average digestible protein intake for animals on the 8% tannic acid diet was significantly lower than the control group, but was not significantly different from the 4% tannic acid group (Table 10).

APD of the 3% and 6% quebracho diets were significantly lower than the control group, but were not different from each other. No significant difference in digestible protein intake existed between the 3% quebracho and control diets. However, the digestible protein intake of the 6% quebracho diet may have been lower than the control diet ($P = 0.069$) and the 3% quebracho diet ($P = 0.062$).

No significant differences in total conjugated glucuronide excretion existed between treatment groups and the control (Table 11). However, the comparison between the control and 8% tannic acid groups had a P-value = 0.087.

Table 10. Average protein intake, apparent protein digestibility (APD), and digestible protein intake for squirrels in the Tannin experiment, Blacksburg, Virginia, July-August 1992.

		Protein intake[1,2] (g/d/kgBW$^{.75}$)		APD[2] (%)		Digestible protein intake[1,2] (g/d/kgBW$^{.75}$)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	6	$^{A}11.9^{a}$	1.2	$^{A}71.2^{a}$	1.3	$^{A}8.5^{a}$	1.0
4% Tannic Acid	6	$^{A}10.6$	0.5	$^{B}60.2$	1.4	$^{AB}6.4$	0.3
8% Tannic Acid	5	$^{A}11.0$	1.0	$^{C}49.0$	3.5	$^{B}5.4$	0.6
3% Quebracho	6	11.6^{a}	0.8	59.3^{b}	1.2	6.9^{a}	0.4
6% Quebracho	6	10.3^{a}	0.5	57.8^{b}	3.9	5.9^{a}	0.4

[1] grams protein consumed/ day/ kg initial body weight$^{.75}$

[2] Comparisons were made between squirrels on control and tannic acid diets or control and quebracho diets. Means in the same column with different upper case letters are significantly different. Means in the same column with different lower case letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix C.

Table 11. Conjugated glucuronide excretion in urine of squirrels in the Tannin experiment, Blacksburg, Virginia, July-August 1992.

		mmol conjugated glucuronides excreted[1]	
Diet	n	$\bar{x}$	SE
Control	6	$^{A}2.0^{a}$	0.1
4% Tannic Acid	6	$^{A}5.1$	1.9
8% Tannic Acid	5	$^{A}23.2$	7.3
3% Quebracho	6	2.2^{a}	0.2
6% Quebracho	6	2.1^{a}	0.3

[1] Comparisons were made between squirrels on control and tannic acid diets or control and quebracho diets. Means in the same column with different upper case letters are significantly different. Means in the same column with different lower case letters are significantly different. For each pairwise comparison, $\alpha = 0.05$. P-values are reported in Appendix C.

DISCUSSION

Diet Composition

Acorn Experiment

Crude protein, crude fat, and gross energy content of red and white oak acorns used in this study were similar to values determined in previous studies (Table 12). Protein and fat contents were most similar to values determined for the same species in Blacksburg, VA by Servello and Kirkpatrick (1989).

Consistent with previous research, this study found that red oak acorns contained higher levels of tannins than white oak acorns. The impression that red oak acorns contain higher levels of tannins has been largely based on their bitter taste. However, actual information regarding tannin content of acorns is sparse. The research that has been conducted has occurred sporadically over the last 50 years. Although the tannin content of various mast species was first examined by Wainio and Forbes in 1941, interest in this area did not resume until the 1970's. In addition, because each researcher used a different method of tannin analysis and analyzed different species of acorns, comparisons between studies are difficult. However, support does exist that acorns of the red oak subgenus, Erythrobalanus, contain higher levels of tannins than the white oak subgenus, Lepidobalanus. Wainio and Forbes (1941) determined that red oak (Q. rubra) and white oak (Q. alba) acorns contained 9.8 and 5.6% tannins, respectively. Using a different method of analysis, Ofcarcik and Burns (1971) determined that 5 species of white oaks (Q. macrocarpa, Q. virginia, Q. lyrata, Q. stellata, and Q. michauxii) and 5 species of red oaks (Q. marilandica, Q. incana, Q. shumardii, Q. falcata, and Q. nigra) contained 0.6 to 2.1% and 5.7 to 8.8% tannins, respectively. Fleck and Layne (1990) measured the protein precipitating activity of tannins in 7 different acorn species and stated that their results were consistent with the hypothesis that Erythrobalanus acorns contain

Table 12. Crude protein, crude fat, and gross energy contents of Northern red (Q. rubra) and white oak (Q. alba) acorns.

Acorn	Crude protein (%)	Crude fat (%)	Gross energy (kcal/g)	Reference
White oak	3.31	3.33	-	Wainio and Forbes (1941)
	5.92	4.41	-	Baumgras (1944)
	4.6	5.8	-	Short (1976)
	7.3	8.8	-	Servello and Kirkpatrick (1989)
	7.00	-	4.46	Havera and Smith (1979)
	7.2	8.8	4.51	This study
Red oak	4.6	12.87	-	Wainio and Forbes (1941)
	5.92	16.05	-	Baumgras (1944)
	4.9	14.0	-	Short (1976)
	6.3	23.0	-	Servello and Kirkpatrick (1989)
	6.25	-	4.92	Havera and Smith (1979)
	6.9	20.2	5.29	This study

higher tannins than Lepidobalanus acorns.

The total phenolic contents of red and white oak acorns used in the present study were 5.22 and 1.37%, respectively, as determined by the Prussian blue assay (Price and Butler 1977). Red and white oak acorns from the same area (Blacksburg, VA) were reported to contain 13 and 3.3% total phenols, respectively (Servello and Kirkpatrick 1989). The difference in values between the two studies probably resulted from the use of two different methods to estimate total phenols. Because the chemistry of each tannin/phenol assay is different, no single assay can report the 'true' tannin level of an acorn, and values reported should be used more in a relative sense (red oak vs. white oak) than an absolute sense (Hagerman, pers. comm.). Only one other study was found that examined the different types of tannins contained in acorns. Acorns of Q. agrifolia, a member of the Erythrobalanus subgenus, contained greater levels of condensed and hydrolyzable tannins (1.27% and 19.00%, respectively) than acorns of Q. lobata, a member of the subgenus Lepidobalanus (0.89% and 9.96%, respectively) (Koenig and Heck 1988). A different set of assays was used to determine the condensed and hydrolyzable tannin contents of acorns in the present study. Condensed and hydrolyzable tannin contents of the white oak acorns in this study were 0.07% and 0.07%. Similar to the Koenig and Heck (1988) study, red oak acorns (Q. rubra) contained higher levels of phenolics (4x), condensed tannins (25x), and hydrolyzable tannins (28x) than the white oak acorns (Q. alba).

Tannin Experiment

Tannic acid was unlikely to have contained phenols other than galloyl esters of glucose (gallotannins) (Hagerman, pers. comm.). A majority (78%) of the gallotannins in the tannic acid used contained 3 to 6 galloyl groups (Table 2). Gallotannins with 5 or more galloyl groups can precipitate proteins in vitro (Hagerman et al. 1992) and constituted 55% of the tannic acid used in this study. Purity of the quebracho added to the diets was approximately 82%. The remaining

18% was nontannin phenols. Thus, 3% and 6% quebracho diets actually contained 2.46% and 4.92% quebracho. Depending on the source, quebracho purity can be as low as 50% (Robbins et al. 1991). In the small mammal studies cited elsewhere in this thesis, source and/or purity of quebracho were not mentioned. Thus, comparisons between studies regarding the effects of different levels of quebracho are difficult to make. Future studies should always mention purity of the quebracho to allow determination of actual condensed tannin administration in the diet.

Dry Matter and Energy Intake

Acorn Experiment

Dietary intake can be influenced by several factors, including previous exposure to a diet, palatability, tannin and phenolic content, time of year, energy content, and energy requirements. Animals accustomed to the foods found within their habitat may be cautious of new foods (Freeland and Janzen 1974, Robbins 1983) and thus may decrease intake when first exposed. Fox squirrels (Sciurus niger) and white-footed mice (Peromyscus leucopus) ate lesser quantities of acorns from species of oaks found outside their native origin (Ofcarcik et al. 1973, Briggs and Smith 1989). White-footed mice from white oak forests preferred white oak acorns, and mice from red oak forests preferred red oak acorns. However, the mice from acorn-free environments (no oaks in forest) preferred the lower tannin white oak acorns (Briggs and Smith 1989). In laboratory experiments, squirrels consumed low tannin, white oak acorns in greater quantities than the high tannin, red oak acorns (Baumgrass 1944, Short 1976, Havera and Smith 1979). Diets containing commercial tannins also may decrease intake (Glick and Joslyn 1970a, Lindroth and Batzli 1984) and cause toxic internal effects (Salunkhe 1990). Time of year may affect intake by determining whether a particular acorn species is consumed or buried (Smallwood and Peters 1986). In addition, animals usually regulate intake to meet energy requirements

(Robbins 1983). Thus, as nutrient digestibility or content increases, intake may decrease. Smith and Follmer (1972) proposed that fox squirrels selected and consumed greater quantities of foods that would maximize the net energy benefit. An animal's intake of a particular food may be determined by any combination of the factors above and probably depends on the situation (i.e. captive vs. free-ranging, choice of diets vs. no choice, time of year, etc.).

At the start of the feeding trial and throughout the pretrial period, all squirrels showed lower levels of intake (Figure 5). Because control animals were consuming the same food as when they were uncaged, initial depressions in intake must have been partly due to the stress of confinement.

During the pretrial period, DMI on white and red oak acorn diets were significantly more depressed than the control. Although DMI of the white oak diet was significantly reduced relative to the control diet during the pretrial period, it was not significantly different from the control during the trial period. The dry matter intake of white oak acorns during the pretrial period may have been reduced due to the low levels of tannin and phenolics in the acorns. However, these lower levels may have been unlikely to suppress intake for an extended period of time. Thus, dry matter intake increased to levels indistinguishable from the control by the trial period. Animals on white oak diets would be expected to consume similar quantities as those on control diets because the control and white oak diets had very similar gross energy contents (4.48 and 4.51 kcal/g, respectively). Although apparent energy digestibility of white oak acorns was significantly higher than the control, it was not high enough to increase the digestible energy intake of white oak acorns relative to the control. Thus, if squirrels were regulating DMI based on digestible energy requirements, DMI would not have to be altered on the white oak diet relative to the control.

DMI of red oak acorns was lower than the white oak and control diets throughout the pretrial and trial periods. The higher gross energy content of red oak acorns (5.29 kcal/g) probably resulted from their high levels of fat. Red oak acorns

contained 20.2% crude fat, but white oak acorns contained only 8.8% crude fat. It would seem that the high fat content of the red oak acorns would explain the lower DMI of this diet. However, despite the significantly higher gross energy content and AED of red oak acorns relative to the control, digestible energy intake of squirrels consuming the red oak diet was significantly lower than those on the control and white oak diets during the trial. If squirrels were regulating DMI solely on digestible energy requirements, DMI would not have decreased so much. Because of the differences in nutrient composition between the 3 diets, the cause for the significant decrease in DMI of red oak acorns that resulted in lower digestible energy intake is difficult to pinpoint. However, it is likely that the higher tannin and phenolic content of the red oak acorns were responsible for the depressed intake throughout the pretrial and trial periods. Fox squirrels and ruffed grouse (Bonasa umbellus) fed red oak acorns also exhibited decreases in DMI and metabolizable energy intake relative to a white oak acorn diet (Havera and Smith 1979, Servello and Kirkpatrick 1989). The significant decrease in DMI was attributed to the low palatability of red oak acorns (Servello and Kirkpatrick 1989). When offered a diet of a single species of acorn, fox squirrels consistently consumed greater quantities of white oak acorns than red oak (Baumgrass 1944, Short 1976, Havera and Smith 1979). I believe that researchers who have conducted the single choice trials have misinterpreted the greater consumption of white oak acorns as a preference for white oak acorns. The relative consumption of one diet versus another when no choice is available is more likely to reflect the diet's palatability and the animal's response to subsistence on that single diet. The amount of a food containing secondary plant compounds eaten may be limited by the consumer's rate of internal detoxification (Freeland and Janzen 1974). Based on previous single choice trials, it is difficult to determine whether greater quantities of white oak acorns were required to meet energy requirements or whether lesser quantities of red oaks were consumed due to the high tannin or fat content. In the present study, based on the lower digestible energy intake of animals

on the red oak diet relative to the similar digestible energy intakes of animals consuming the tannin-free control and white oak diets, it appears that the high tannin and phenolic content was responsible for the depressed intake of red oak acorns relative to the other diets.

Squirrels presented with a choice of diet may respond differently than those offered a single diet. When gray squirrels and fox squirrels were given a choice between acorn species, they consumed more of the red oak species than the white oak species or they consumed disproportionately more red oak acorns relative to their availability (Smith and Follmer 1972, Ofcarcik et al. 1973, Lewis 1982). These results seem contradictory to the depressed intake of animals fed a diet of red oak acorns alone. However, this discrepancy may be explained by the fact that squirrels in preference trials (and in the wild) are able to balance their intake of high tannin-containing red oak acorns with other lower tannin foods. The amount of a particular food consumed is in part limited by its toxin content (Freeland and Janzen 1974). Thus, herbivores may be forced to eat other foods to dilute the toxin, to compensate for nutrients lost due to the toxin (i.e. protein), and to provide for the additional costs of internal detoxification processes. Squirrels consuming high levels of red oak acorns and low levels of white oak acorns also consumed large quantities of hickory nuts (Lewis 1982). The higher protein content of hickory nuts (11-16% [Wainio and Forbes 1941, Havera and Smith 1979]) would provide an excellent counterbalance to the protein precipitating activity of tannins. Thus, in the present study, squirrels may have significantly reduced intake of red oak acorns because they could not dilute their tannin consumption and balance their nutritional requirements with other foods. Despite the higher tannin content of red oak acorns, wild squirrels may prefer red oaks when other foods are available to dilute tannin intake and counteract physiological effects of tannins.

Tannin Experiment

Previous research on small mammals has reported a variety of intake responses to diets containing commercial tannins (Table 13). Laboratory rats fed 8% tannic acid diets initially restricted their intake severely. After several days on the diet, intakes increased but remained significantly below the control group, and growth was restricted (Glick and Joslyn 1970a). In a study conducted in our lab, white-footed mice (Peromyscus leucopus) increased DMI on a 2%, but not on a 4% or 6% tannic acid diet (Appendix D). Tannin type may also determine the effects on palatability and intake. Although quebracho-containing diets (2% and 3%) severely decreased intake and weight in prairie voles, tannic acid-containing diets (4% and 6%) did not affect or tended to increase intake (Lindroth and Batzli 1984, Meyer 1989). In addition to factors affecting intake mentioned previously, it appears that intake response also may depend on the animal species, the type of tannin, and the degree of adaptation or acclimation to the tannin-containing diets.

As in the Acorn experiment, dry matter intakes of all diets were initially low, but increased throughout the pretrial period (Figure 6, Table 8). Because pretrial DMI of the control diet was also low, it was likely that the intake depression partially resulted from the stress of confinement. As animals became accustomed to confinement, intake increased to the relatively stable levels of the trial period. Diets containing tannic acid (4% and 8%) further depressed DMI relative to the control diet during the pretrial period (Figure 6). However, after the pretrial period, tannic acid in the diet no longer suppressed intake relative to the control. In a study of squirrel diet preferences, free-ranging gray squirrels reduced the amount of time spent eating acorn doughballs containing 2% or 6% tannic acid relative to tannin-free doughballs (Smallwood and Peters 1986). This situation may be analagous to the pretrial period in the present study. Animals may be cautious of new and different foods (Robbins 1983), and the astringency caused by added tannins may have initially deterred consumption in the free-ranging squirrels of Smallwood and Peters (1986) and in the

Table 13. Effects of tannic acid (TA) and quebracho (Q) on dry matter intake (DMI) of various small mammals.

Species	Added tannins		Effect on DMI	Reference
	TA	Q		
Sciurus carolinensis	4%TA, 8%TA		pretrial - decrease trial - none	This study
		3%Q, 6%Q	pretrial - none trial - none	This study
Laboratory rats	8%TA		decrease	Glick and Joslyn (1970a)
Microtus ochrogaster	4%TA		none	Meyer (1989)
	6%TA		increase	Lindroth and Batzli (1984)
		2%Q	decrease	Meyer (1989)
		3%Q	decrease	Lindroth and Batzli (1984)
Microtus miurus	4%TA		decrease	Meyer (1989)
		2%Q	decrease	Meyer (1989)
Mus musculus	4%TA		none	Meyer (1989)
		2%Q	none	Meyer (1989)
Lepus americanus	4%TA		none	Meyer (1989)
Oryctolagus cunniculus	4%TA		none	Meyer (1989)
Peromyscus leucopus	2%TA		increase	Appendix D
	4%TA, 6%TA		none	Appendix D

captive squirrels of this study. The free-ranging squirrels had the option to stop feeding, which they subsequently did. Because the squirrels in this study had no other source of food, they were forced to acclimatize to the diet. Thus, squirrels on the tannic acid diets increased intake to levels indistinguishable from the control diet by the last 5 days of the trial. Gray squirrels may be expected to acclimatize to astringent diets to a greater degree and in a more rapid manner than other species because of their regular exposure to high levels of acorn tannins in the fall and winter. In the herbivorous prairie vole, tannin-containing diets continuously depressed intakes to the point of death (Lindroth and Batzli 1984). Prairie voles do not commonly consume tanniniferous foods (Cole and Batzli 1979) and therefore would not be expected to acclimate well to high tannin diets. The omnivorous Mus musculus, however, was more capable of maintaining mass balance on tannin-containing diets than were prairie voles (Meyer 1989).

Previous research has shown quebracho to decrease intake if any effect occurs at all (Table 13). In the present study, the presence of quebracho in the diet had no effect on DMI of squirrels in the pretrial or trial periods. This result reflects the variation between species in the response of intake to quebracho and tannins in general. Apparently, the presence of quebracho (a condensed tannin) in the diet did not affect palatability as much as tannic acid (a hydrolyzable tannin). These results are consistent with the statement by Butler and Rogler (1992) that hydrolyzable tannins may be more effective at reducing dietary intake than condensed tannins.

The question may be asked as to why squirrels were able to acclimate to a tannic acid-containing diet and why intake was not affected by quebracho when dry matter intake was consistently reduced by natural tannin-containing red oak acorns. In the tannin trial, the only major nutritional difference between the diets was the added tannins (either a condensed or hydrolyzable tannin). However, the nutrient and chemical compositions of the diets in the Acorn experiment undoubtedly differed in

many other respects, many of which may have affected palatability. Analyses determined that red oak acorns contained higher levels of both hydrolyzable and condensed tannins and phenolics relative to the white oak acorns. Aside from the gallotannin profile, the actual tannin and phenolic composition of red oak acorns remains largely undefined. Robbins et al. (1987b) hypothesized that high levels of nontannin phenolics ($\geq$ 87% of total phenols) in plants fed to deer may have reduced intake by creating internal toxicity, by imposing an additional energy burden due to increased detoxification activity, or by altering physiological processes. In our study, the percentage of tannin and nontannin phenolics in acorns could not be determined because such an assay has not yet been developed (Hagerman, pers. comm.). Yet, it is likely that a significant amount of nontannin phenolics may have been found in red oak acorns and may have been a contributing factor to the reduced intake. In addition, years of plant-herbivore coevolution probably have resulted in acorns that contain tannins with a greater potential for biological activity and greater specificity for the herbivore than commercially produced tannins. (Although the commercially produced tannin, quebracho, originates from plant bark, it is likely that quebracho may be intended to deter phytopathogens more than mammalian herbivores.) It is likely that a high degree of tannin specificity, the presence of nontannin phenolics, the possible synergistic effects of the different types of tannins and phenols, and the differences in nutrient composition, size, shape, and texture of the diets ultimately resulted in the reduced intake of red oak acorns. Although quebracho also contained nontannin phenolics, these levels were relatively low (18%). Thus, the quebracho-containing diets may not have been unpalatable enough and may not have contained enough nontannin phenols to suppress intake in squirrels accustomed to consuming bitter tasting foods. In addition, commercial tannin-containing diets may have been similar enough to the control diets (base ingredients, texture, size, protein, energy, etc.) that added tannins did not change palatability and other qualities enough to cause an intake reduction (quebracho-containing diets) or to cause a sustained intake

reduction (tannic acid-containing diets).

Weight Loss

A majority of the weight loss experienced by all animals in both experiments probably occurred during the pretrial period when dry matter intakes on all diets were low. In both experiments, weight losses of animals on treatment diets were not significantly different from the control groups (Tables 3 and 8). Due to the high variability in weight loss within each diet, the power to detect differences in weight loss was reduced. However, certain relationships are worth noting.

Relative to the control and white oak groups, the red oak acorn group had significantly reduced digestible energy intakes. Thus, a higher degree of weight loss by animals on the red oak diet than white oak diet would have been conceivable and may be reflected by a marginal P-value ($P = 0.126$). The greater weight loss of animals on the red oak diet would support the hypothesis that squirrels require other foods to dilute tannins and fulfill their nutritional requirements. Other nonsignificant, but notable P-values were those for the control-4% tannic acid and control-8% tannic acid comparisons ($P = 0.151, 0.110$, respectively). Mean weight losses on the control, 4%, and 8% tannic acid diets were 28.9, 61.0, and 65.2 g, respectively.

Nutrient Digestibility and Digestible Intake

Acorn Experiment

Apparent energy digestibilities of the white oak and red oak acorns were significantly higher than the control diet and were probably due to the higher fat and starch content of the acorns. As the main nutrient in acorns, starch may compose over 50% of the nut (Ofcarcik and Burns 1971). The crude fat contents for the control diet, white oak acorns, and red oak acorns were 5.3%, 8.8%, and 20.2%, respectively.

Apparent energy digestibility of red oak acorns tended to be lower than that of white oak acorns (P= 0.083). Based on their high fat and neutral detergent soluble contents (Servello and Kirkpatrick 1989), red oak acorns might be expected to have a higher energy digestibility. Smith and Follmer (1972) found that digestive efficiency of gray squirrels was correlated with the fat content of the mast species. In addition, digestive efficiency is expected to increase with the percentage of neutral detergent solubles, the fraction of a detergent fiber analysis reflecting highly digestible nutrients in a food (Goering and Van Soest 1970). Extrapolating from data in Servello et al. (1987), the average neutral detergent solubles (NDS) of red and white oak acorns from Blacksburg, Virginia were 91.4% and 82.8%, respectively.

Despite the higher fat and NDS content of red oak acorns, AED tended to be lower than that of white oak acorns. The combination of reduced dry matter intake and a lower AED of red oak acorns resulted in significantly lower digestible energy intake as well. Although these results were not predictable based on NDS and fat content, the lower energy digestibility of red oak acorns was predictable due to their higher tannin content. Tannins are defined by their ability to bind protein and digestive enzymes in vitro (Salunkhe et al. 1990). The condensed tannins of carob (Ceratonia siliqua) and the hydrolyzable tannin, m-digallic acid, inhibited activities of the digestive enzymes, alpha-amylase, lipase, and trypsin in vitro (Tamir and Alumot 1969). By impairing the enzymatic function of digestive enzymes, the high tannin content of red oak acorns may have prevented proper breakdown of dietary nutrients and thus may have reduced energy and protein digestibility of the higher tannin acorns. The discrepancy between the findings of this study and those of Smith and Follmer (1972) probably lies in the tannin content of the acorns fed. Because Smith and Follmer (1972) did not examine tannin content or report gross energy intake, the discrepancy cannot be resolved.

Although high fecal protein or energy contents may indicate an apparent reduction in digestibility, they do not necessarily indicate the excretion of dietary

nutrients. The excretion of endogenous protein (e.g. salivary proteins, intestinal mucous) in feces may also result in an apparent, but not true, reduction in digestibility. In this study, exogenous (dietary) and endogenous losses were both likely to have occurred in animals fed tannin-containing diets, thus resulting in an apparent and true reduction in digestibilities. However, the degree to which each contributed to the reduction in energy and protein digestibility could not be determined.

The apparent protein digestibilities (APD) of the white and red oak acorns (42.0% and 10.2%, respectively) were significantly lower than the control diet (73.4%). The higher APD of the control diet may be in part due to the higher protein intake of these squirrels. Because the control diet was of a different nutritional composition than the acorns and because the control diet probably contained different proteins than the acorns, comparisons of APD between the control and acorn diets are of lesser value than comparisons between acorn species.

APD of the red oak diet was significantly lower than the white oak diet. Because only a small difference in protein intake existed between the white and red oak diets, the difference in protein intake was not likely the cause of the reduced APD of red oak acorns. However, the higher tannin content of the red oak acorns was more likely responsible. Smallwood and Peters (1986) suggested that tannin content had no effect on protein digestibility, using as evidence the lack of correlation between tannin contents of several species of acorns determined by Ofcarcik and Burns (1971) and the protein digestibilities of those acorn species determined by Short (1976). Because the tannin contents and APD's were not determined in the same study, support for their "lack of correlation" conclusion is weak.

It is highly likely that the higher levels of tannins in red oak acorns caused the observed reductions in apparent protein digestibility. Unlike hydrolyzable tannins, condensed tannins are not likely to be degraded in the digestive tract and are thus capable of binding protein in vivo (Hagerman and Klucher 1986, Hagerman et al.

1992). Thus, the condensed tannins of the red oak acorns may have been primarily responsible for the large reduction in apparent protein digestibility of red oak acorns relative to white oak acorns.

Although hydrolyzable tannins were not originally believed to interact with protein in the digestive tract, some of the gallotannins in red oak acorns may have contributed to the largely reduced APD of red oak acorns. Although hydrolyzable tannins are capable of binding protein in vitro (Hagerman and Klucher 1986), they may be degraded to their smaller constituents in the gastrointestinal tract. Thus, hydrolyzable tannins would subsequently lose their ability to interact with protein in vivo (Hagerman et al. 1992). However, the particular composition of hydrolyzable tannins may ultimately determine whether protein is bound in vivo or not (Hagerman et al. 1992). Digestion trials with mule deer (Odocoileus hemionus hemionus) and Suffolk sheep found that the primarily hydrolyzable tannins of fireweed flowers (Epilobium angustifolium) reduced protein digestibility, whereas commercial tannic acid did not (Hagerman et al. 1992). The presence of mainly high molecular weight (MW) gallotannins in fireweed flowers (8 to 9 galloyl groups) relative to the low MW gallotannins (mainly 3 to 4 galloyl groups) in tannic acid (Mallinckrodt Chemical Co., St. Louis, MO) may have explained why protein digestibility was reduced by fireweed flowers, but not tannic acid (Hagerman et al. 1992). Generally, the ability of tannins (condensed and hydrolyzable) to interact with protein in vitro increases with the molecular weight and the number of reactive groups on the tannin (Hagerman 1989, Salunkhe et al. 1990). The high MW of the fireweed tannins may have resulted in strong complexs between tannins and dietary or endogenous protein. Bound tannins may have been protected from hydrolysis, thus allowing high MW hydrolyzable tannins to reduce protein digestibility (Hagerman et al. 1992).

The high content (>50%) of high MW gallotannins (8 to 10 galloyl groups) in red oak acorns was likely to have contributed to the reduced protein digestibility in addition to reductions caused by condensed tannins. Because relatively small

quantities of high MW gallotannins are required to precipitate protein in vitro (Hagerman et al. 1992), red oak acorns probably contained sufficient levels to affect protein digestibility. However, the white oak acorns contained a majority (78%) of moderate MW gallotannins (5 to 7 galloyl groups) and no high MW gallotannins (8 to 10 galloyl groups). Larger quantities of moderately sized gallotannins are required to precipitate protein (Hagerman et al. 1992). Although the percentage of moderate MW gallotannins in white oak acorns was high, the total quantity (78% of 0.07% = 0.06% moderate MW gallotannins) may not have been enough to significantly contribute to the APD reduction. The larger quantity of high MW hydrolyzable tannins in red oak acorns (53% of 2.03% = 1.07% high MW gallotannins) may have led to a higher degree of protein interaction and digestibility reduction.

As mentioned earlier, the quantity of hydrolyzable tannins in the red (and white) oak acorns seemed low relative to other studies and one might question the ability of such low percentages of tannins to exert effects. First, it is important to remember that tannin values reported in other studies were analyzed by different assays. Because of the unique chemistry of each assay and the use of different standards, different values are bound to result for the same sample analyzed by different methods. Thus, value comparisons should not be made between assays, but only within one assay type and perhaps within a single lab. Tannin analyses of diets in the present study were conducted in the same lab and by the same methods as those used to determine the tannin content of fireweed flowers in the study by Hagerman et al. (1992). The hydrolyzable tannin content of 3.9% in fireweed flowers was sufficient to reduce protein digestibility. Thus, it is conceivable that the hydrolyzable tannins (2.03%) in red oak acorns used in this study contributed to the reduction in apparent protein digestibility.

A great deal of variability existed in the protein digestibilities of individual animals on the red oak diets relative to the white oak diets. Protein digestibilities of individuals on the control, white oak, and red oak diets ranged from 78.6 to 61.6%,

55.6 to 25.7%, and 32.8 to -18.1%, respectively. Such variability in the responses of animals on the red oak diet could not be explained by differences in dry matter intake, digestible energy intake, or digestible protein intakes. Also, no correlations existed between reduction in protein digestibility and detoxification activity. Age may have been a factor affecting response. However, age was not determined for squirrels in this study. Response also may have depended on the degree of prior exposure to acorn tannins, which would depend entirely on the individual foraging strategies of each squirrel. In addition, the genetics and physiology of wild animals are likely to be more variable than the laboratory animals upon which much tannin research has been based.

Tannin Experiment

The addition of 4% and 8% tannic acid significantly reduced apparent energy digestibilities relative to the control. Also, AED of the 8% tannic acid diet tended to be lower than that of the 4% tannic acid diet ($P = 0.085$). However, digestible energy intakes of animals on tannic acid diets were not significantly different from the control despite the reductions in AED. The addition of 4% or 8% tannic acid to diets significantly reduced apparent protein digestibilities (APD) from 71% to 60% and 49%, respectively. Based on the hypothesis that hydrolyzable tannins may be degraded in the digestive tract (thus rendering them incapable of interacting with protein), these reductions in digestive efficiencies were not expected. However, these results are consistent with new evidence that higher MW gallotannins may be protected from hydrolysis by complexing protein and the concept that effects must be interpreted based on tannin structure (Hagerman et al. 1992). Approximately 51% of the tannic acid was comprised of gallotannins with 5 to 7 galloyl groups (moderate MW). The tannic acid preparation contained only a low percentage (4%) of high MW gallotannins ($\geq$8 galloyl groups). However, the moderate MW gallotannins were probably present in sufficiently large quantities (2% of 4% tannic acid diets and

4% of 8% tannic acid diets) to cause a reduction in apparent protein digestibility. Again, the actions (intestinal erosion or binding of digestive enzymes or dietary protein) by which tannins reduced APD could not be determined. Despite reductions in APD by both tannic acid-containing diets, only the 8% diet affected APD enough to reduce digestible protein intake. Because total protein intake of the 8% tannic acid diet was not significantly different from the control, the reduced digestible protein intake was attributed to the reduction in APD.

Previous research on the effects of tannic acid on digestive efficiency in different species has yielded a variety of results. Diets containing 4% tannic acid reduced APD and dry matter digestibility in Mus musculus and Microtus miurus, but not Lepus americanus or Microtus ochrogaster (Meyer 1989). However, Lindroth and Batzli (1984) determined that a 6% tannic acid diet reduced APD in Microtus ochrogaster. Tannic acid fed to rats at 2% to 8% increased fecal nitrogen excretion as well (Glick and Joslyn 1970b). It is likely that high MW gallotannins in the tannic acid diets of these studies resulted in the observed reductions in protein digestibility. The content of high MW gallotannins in the 4% tannic acid diet of the Meyer (1989) study may have been sufficient to reduce APD in Mus musculus and Microtus miurus, but may have been too low to exert effects on APD in Lepus americanus and Microtus ochrogaster, thus supporting the suggested species differences in tolerance to tannins. Although gallotannin profile and composition may explain the effects of hydrolyzable tannins (or lack thereof), previous studies have not examined this aspect. In a recent study, tannic acid fed to mule deer and domestic sheep did not affect APD (Hagerman et al. 1992). The lack of effect was attributed to the low MW of the gallotannins added to the diets. In the present study, the high content of moderate to high MW gallotannins in the tannic acid diets may have caused the observed reductions in apparent protein digestibility.

As expected, the addition of quebracho to diets significantly reduced AED and APD relative to the control. The reduction in AED and APD by quebracho is

consistent with previous studies and the hypothesis that condensed tannins act mainly in the digestive tract because they cannot be hydrolyzed. However, no significant differences existed between AED or APD of 3% and 6% quebracho diets. The lack of a dosage dependent response may indicate that quebracho exerted its maximal effects at the lower percentage added (3%). For example, all of the high affinity binding sites on digestive enzymes may have been saturated at 3% quebracho. Alternatively, defensive mechanisms (i.e. secretion of proline-rich salivary proteins, changes in pH of the digestive tract) may have been stimulated at levels above 3%, thus preventing further effects of tannins in the digestive tract.

Despite the reduction of AED and APD by quebracho-containing diets, no significant differences in digestible energy and protein intake existed relative to the control diet. However, digestible protein intake on the 6% quebracho diet tended to be lower than the control ($P = 0.062$) and the 3% quebracho diet ($P = 0.069$). The effects of quebracho on APD in previous research have been more consistent than the effects of tannic acid. Quebracho-containing diets (2-3%) have been shown to reduce APD in prairie voles, Mus musculus, Microtus miurus, mule deer, domestic sheep, and black bears (Lindroth and Batzli 1984, Meyer 1989, Robbins et al. 1991, Hagerman et al. 1992). The consistency in the effect of quebracho may be due to its nonhydrolyzable nature and different structural characteristics. Condensed tannins have been shown to be slightly more effective in precipitating proteins in vitro than hydrolyzable tannins of similar MW (Hagerman and Klucher 1986). In addition, the ability of condensed tannins to interact with protein also increases with their molecular weight (Salunkhe et al. 1990, Hagerman et al. 1992). However, because molecular weights of condensed tannins are not easily assessed (Hagerman, pers. comm.), no such analyses of the quebracho used in this study were performed.

Upon comparing the results of the Acorn and Tannin experiments, it is notable that commercial tannin-containing diets did not reduce APD as much as the white oak and red oak acorn diets. The most severe reduction in APD caused by commercial

tannins occurred on the 8% tannic acid diet (APD = 49%). Yet APD of animals on white and red oak acorns were 42% and 10%, respectively. Two explanations are offered for the greater reduction in APD by acorn tannins. First, plant-herbivore coevolution between oak trees and squirrels may have resulted in acorns containing tannins more specific for and more biologically active within the consumer than commercially produced tannins. Second, the greater effect of acorn tannins on APD may be due to their low protein content. Red and white oak acorns contained approximately 7% protein. The commercial tannin diets contained approximately 18-20% protein. Higher levels of protein in the diet have been shown to overcome the effects of tannins. The addition of 10% soybean protein to high tannin sorghum diets overcame growth suppression in rats and chicks (Rogler et al. 1985). Tannin diets with 20% protein overcame the reduced growth seen in prairie voles on tannin diets with 8% protein (Lindroth and Batzli 1984). Additional protein in the tannin diets may have alleviated effects by binding tannins in the digestive tract, thus preventing further detrimental activities (Rogler et al. 1985). The much higher protein content of the commercial tannin diets in this study may have prevented tannins from exerting their full negative effect. The low protein levels in acorns may have permitted tannins to exert greater effects on the squirrels.

A basal diet with protein levels similar to acorns was desired for the Tannin experiment. Because high levels of protein in the tannin diets were likely to obscure the effects of tannins (as they probably did), comparisons between the two experiments would be less informative. Agway PROLAB RMH 1000 was chosen for the basal diet because it was one of the lowest protein rodent chows commercially available (guaranteed protein = 14.0% min.). However, actual protein content was determined to be 19.55%. Thus, the attempt to reduce protein content was not very successful. Although a semipurified diet was one possible method of achieving a very low protein diet, squirrels would not consume any of the semipurified test diets offered during preliminary feeding trials. Despite the addition of sugar and/or peanut

flavoring, squirrels may have found the somewhat chalky textured diets foreign and unpalatable. In the future, studies intending to use commercial tannin-containing diets to elucidate the effects of natural tannins on squirrels should formulate basal diets with protein levels similar to that of the natural diet if at all possible.

Detoxification Activity

For both experiments, it was hypothesized that condensed tannins would not cause increases in glucuronidation activity because condensed tannins were unlikely to be hydrolyzed in the digestive tract (Hagerman and Butler 1989, Salunkhe et al. 1990, Hagerman et al. 1992) and thus would not be absorbed. However, hydrolyzable tannins were expected to increase glucuronidation activity because these tannins may be easily degraded in the gut and are thus likely to be absorbed (Lindroth and Batzli 1984, Hagerman and Butler 1989, Salunkhe et al. 1990). The presence of gallic acid metabolites in the urine of rats provided evidence that tannic acid was assimilated (Booth et al. 1959). Recall that higher MW gallotannins may be protected from hydrolysis by the formation of protein complexes (Hagerman et al. 1992). Thus, only simple phenols and components of low MW gallotannins ($\leq$4 galloyl groups) would be absorbed internally. Researchers have also hypothesized or shown that simple phenols may be absorbed and undergo detoxification (Booth et al. 1959, Baudinette et al. 1980, Robbins et al. 1987b). Thus nontannin phenols in the diets also were expected to increase detoxification activity.

Acorn Experiment

Acorns used in this study contained condensed tannins, hydrolyzable tannins, and nontannin phenols. However, the proportions of each of these phenolic components in the acorns were unknown. It was hypothesized that glucuronidation activity in squirrels on white and red oak acorns would be higher than the control diet because of tannin/phenolic content. In addition, it was hypothesized that the higher

levels of tannins in red oak acorns would lead to higher glucuronidation activities than white oak acorns. However, only glucuronide output (GO) of squirrels on red oak acorns was significantly higher than that of the control diet. Red oak acorns contained higher total phenols, higher levels of hydrolyzable tannins, and a higher proportion (32%) of low MW gallotannins than the white oak or control diets. Thus, levels of absorbable phenolics (tannin and/or nontannin) in red oak acorns were high enough to stimulate glucuronidation activity above that of squirrels on white oak or control diets.

Mean GO of squirrels on white oak acorns tended to be higher than the control group ($P = 0.096$), but was not significantly higher at $\alpha = 0.05$. Chemical analyses indicated that total phenol levels in white oak acorns were higher than the control diet but lower than the red oak acorns. Because total phenol values represent tannin and nontannin phenols, hydrolyzable tannins only represented a portion of the total phenols (1.37%) in white oak acorns. In addition, because low MW gallotannins represented only 22% of the hydrolyzable tannins in white oak acorns, the level of absorbable phenolics in white oak acorns may have been too low to significantly stimulate glucuronidation activity. At low doses of phenols, conjugation with sulfates rather than glucuronic acid may dominate. Only with increasing levels of phenols did the proportion of glucuronide to sulfate conjugates increase (Baudinette et al. 1980). Most of the phenolics absorbed from the white oak acorns may have been detoxified by the low capacity, high affinity conjugation pathways. Glucuronidation activity may have been only slightly stimulated. Thus, the comparison between the white oak diet and control diets resulted in $P = 0.096$.

Protein deficiency may also have contributed to the elevated glucuronide output of squirrels on red oak acorns. In tannin-fed rats, protein deficiencies induced by protein-free diets resulted in increased glucuronide outputs (Woodcock and Wood 1971). However, the level at which squirrels become protein deficient is unknown. Squirrels on red oak acorns consumed only 0.2 g digestible protein/d/kg$BW^{.75}$. This

low digestible protein intake and the fact that several animals excreted more nitrogen than they consumed indicated that animals may have been protein deficient. In addition, the higher tannin levels in red oak acorns may have caused a tannin-induced protein deficiency, thus contributing to the elevated GO levels of animals on red oak acorns.

Tannin Experiment

As a hydrolyzable tannin, tannic acid was expected to increase glucuronide output. Mean glucuronide outputs did tend to increase with tannic acid content. However, because the variability in glucuronide response increased with tannic acid content of the diet, no significant differences could be detected between tannic acid and control diets at $\alpha = 0.05$. A marginal P-value of 0.087 for the 8% tannic acid-control diet comparison draws attention to the very high glucuronide output of squirrels on the 8% tannic acid diet. This marginal P-value indicates that high levels of tannic acid may stimulate glucuronidation activity. However, such an effect may not have been detectable give the conditions of the experiment (i.e. sample sizes too small) and the degree of variation between individuals. The variation in glucuronidation response of squirrels fed 8% tannic acid may have resulted from different levels of prior exposure to tannins amongst the individuals as well as individual differences in the amount of toxins absorbed and pathways of detoxification. Thus, I believe that the higher mean glucuronide output of squirrels on 8% tannic acid is quite noteworthy.

As predicted, quebracho-containing diets did not increase glucuronidation activity relative to the control diets. Despite the presence of 0.54% and 1.02% nontannin phenolics in the 3% and 6% quebracho diets, respectively, conjugated glucuronide output of squirrels consuming these diets did not increase. The nontannin phenols in quebracho may have been present in low enough quantities that other conjugation pathways did not reach their capacities. Thus glucuronidation activity

was not stimulated.

Clausen et al. (1990) proposed that condensed tannins of blackbrush were depolymerized in the gut and absorbed, thus causing toxicity and intake depression in snowshoe hares. However, no evidence supporting this hypothesis was provided. In the present study, the capacities of sulfate and/or amino acid conjugation pathways of animals consuming quebracho diets may have been partially or fully occupied with nontannin phenols. Thus, if any depolymerization of condensed tannins had occurred, glucuronidation activity probably would have been stimulated. However, no such activity was seen. Results of this study suggest that quebracho, a condensed tannin, was not depolymerized and absorbed in the gut and thus does not significantly affect detoxification activity of gray squirrels.

Glucuronidation response to commercial tannin-containing diets may depend on individual qualities and characteristics of the tannin. In addition, the ability of the consumer (gray squirrel) to tolerate tannins through physiological adaptations must be considered. Such abilities (i.e. secretion of proline-rich salivary proteins, changes in pH of the digestive tract) may have developed as a response to the high consumption of acorn tannins during fall and winter seasons each year. A detailed examination of detoxification mechanisms may reveal that squirrels process internally absorbed tannins more efficiently than nontannin consuming species. In the future, determinations of sulfate and amino acid conjugates in the urine, in addition to glucuronides, may help determine which conjugation pathways initially respond to absorbed phenolics, the capacities of each pathway, and the point at which additional conjugation pathways take over. In addition, gray squirrels may have other defenses to prevent absorption of tannins and subsequent detrimental effects. Future studies should examine tannin intake and excretion in the feces to determine the proportion of tannins absorbed and the proportion of tannins remaining in the gut. This information would help explain the degree of internal effects (by absorbed tannins) and external effects (by unabsorbed tannins) observed in feeding trials.

Comparisons of detoxification activities between the Acorn and Tannin experiments were not made because glucuronide assays were conducted separately for each of these experiments. Because a large degree of variation occured between glucuronide assays, glucuronide values were only compared with other values within the same assay and not between assays. In addition, because dietary protein level may affect detoxification activity, the difference in protein content between diets used in the Tannin and Acorn experiments would complicate interpretations.

CONCLUSIONS

Acorn Experiment

A diet of 100% red oak acorns caused greater effects on digestion and detoxification in gray squirrels than a diet of 100% white oak acorns. Relative to white oak acorns, red oak acorns were more effective at reducing dry matter intake, apparent digestibilites, and digestible intakes and increasing detoxification activity. It is highly likely that the higher condensed and hydrolyzable tannin and total phenol content of red oak acorns were responsible for these effects. The fact that similar effects were seen in squirrels fed commercial tannin diets supports the conclusion that tannins in the red oak acorns, and not some other variable, were responsible for the reductions in digestive efficiency and the stimulation of glucuronidation activity. White oak acorns contained sufficient levels of tannins to temporarily reduce dry matter intake and to reduce apparent protein digestibility and digestible protein intake, but not enough to stimulate significant detoxification activity.

Tannin Experiment

Results of the Tannin experiment indicate that tannin composition analysis, especially for hydrolyzable tannins, may be essential to the interpretation of observed effects. Because hydrolyzable tannins were thought to be degraded in the digestive tract, thus rendering them incapable of binding protein, the reduction of apparent protein and energy digestibilities by tannic acid was unexpected. It is likely that higher molecular weight gallotannins in tannic acid formed strong complexes with protein, thus preventing hydrolysis of these tannins and allowing them to exert their digestive effects. The 8% tannic acid diet tended to increase glucuronidation activity ($P = 0.087$), supporting the hypothesis that hydrolyzable tannins may be degraded in the digestive tract and absorbed. Had the tannic acid contained a higher proportion of low MW gallotannins, which are more susceptible to degradation and absorption,

glucuronidation activity may have been stimulated more significantly.

Quebracho acted in a manner consistent with the predicted properties of condensed tannins in the digestive tract. Apparent protein and energy digestibilities of squirrels fed quebracho were reduced, indicating the formation of tannin-protein complexes. However, no effect was observed on glucuronide excretion, indicating that quebracho was not degraded or absorbed in significant quantities.

Implications in Gray Squirrel Foraging Strategy and Nutrition

Some researchers have argued that squirrels prefer white oak acorns over red. Smallwood and Peters (1986) hypothesized that squirrels preferred white oak acorns during the fall because these 'perishable' foods had to be consumed before they germinated and produced a tough radicle. Red oak acorns, which germinate in the spring, would be buried and could be consumed at any later period. It was hypothesized that gray squirrels based food preferences on the perishability of different acorns and not on the 'marginal nutritional differences' between acorns and their tannin contents. In addition, Smallwood and Peters (1986) suggested that digestive efficiencies of squirrels were not affected by tannins and that the higher tannin content of red oak acorns only served to indicate the delayed germination schedule of seed. Counter to these hypotheses, the present study found that nutritional differences between red and white oak acorns are not marginal, but significant and that digestive efficiencies are significantly reduced in squirrels fed different acorns. If tannins were leached from red oak acorns during the overwintering period, the foraging strategy proposed by Smallwood and Peters (1986) would allow squirrels to attain greater levels of nutrients from red oak acorns. However, recent experiments have found that although tannin levels were reduced in overwintered white and chestnut (Q. prinus) oak acorns, they were not reduced in overwintered red oak acorns (Appendix E, Smallwood, pers. comm.). Thus, the observation that squirrels bury more red oak acorns than white (Smallwood, pers.

comm.) may not be explained by overwinter reductions in the high tannin levels of red oak acorns. Nonetheless, the hypothesis of forage selection based on acorn perishability remains unrefuted. Additional research is necessary to clarify the relationship between food preferences, burying behavior, and how tannins influence these decisions in gray squirrels.

The significant reduction in digestible protein and energy intake and the high level of glucuronidation activity of squirrels fed red oak acorns indicate that gray squirrels may not be able to subsist on red oak acorns alone. It is likely that gray squirrels may consume large quantities of red oak acorns only when other foods are available to dilute tannin intake and provide additional energy and protein. Accordingly, in most experiments that found squirrels to 'prefer' red oak acorns, alternative foods were available. Thus, despite the apparent 'preference' of squirrels for red oak acorns, optimum squirrel habitat may also include white oaks and other species that provide low tannin, high protein mast. Thus, forest stand composition may affect condition and nutritional state of gray squirrels and ultimately, trends in population size, reproductive effort, and fecundity.

The provision of low tannin, high protein foods may be important to other acorn-consuming wildlife as well. Similar experiments with other wildlife would help determine which oak species or what combination of oaks would provide mast of the greatest overall benefit to wildlife. Experiments involving other animal species to which acorns are of varying importance in the diet also may contribute to the understanding of plant-herbivore coevolution and interaction and the effectiveness of tannins as a plant defense.

Implications in Tannin Research

Results of the Tannin experiment provide insight into the possible effects of each type of tannin. However, tannic acid preparations of varying gallotannin composition and condensed tannins other than quebracho should also be investigated

before generalizations are made about the effects of each tannin type. Differences in tannin composition must be considered before results of commercial tannin experiments are used to interpret observed effects in natural tannin experiments. The large reduction in protein digestibility and digestible protein intake caused by relatively low levels of tannins in red oak acorns (relative to moderate effects on these variables by high levels of commercial tannins) may have been due to a more biologically active composite of tannins in red oak acorns. Alternatively the low protein levels in acorns may have exacerbated the effects of tannins. In the future, this complication in interpretation may be avoided by the use of basal diets of nutrient compositions as similar to acorns as possible. Tannins also may be extracted from acorns for addition to the basal diet. Furthermore, diets containing both condensed and hydrolyzable tannins may be fed to squirrels to determine whether mixtures of tannins (as in acorns) interact to cause effects.

Much of the tannin literature to date attempts to determine what the major effects of tannins are. Intake reduction, digestive effects, and postdigestive effects (reduced efficiency of ingested energy conversion) have all been cited as major effects. It seems likely that tannins may not act by one mechanism, but by several, and that the combination of effects may depend on the source of the tannin, the nutritional composition of the diet, and the degree to which the herbivore is customarily exposed to tannins.

The lack of a prolonged reduction in dry matter intake of diets containing reasonably high levels of commercial tannins may reflect the high tolerance of acorn-consuming squirrels to astringent-tasting foods. Furthermore, the lack of significant reductions in digestible energy intake and weight loss relative to animals on the control diet indicate that the major effect of commercial tannins was not on intake and mass balance. However, had protein content of the tannin diets been lower, greater effects on these variables may have been observed. Tannins and phenolics in red oak acorns were likely responsible for the observed reductions in dry matter intake.

Thus, acorn tannins (and phenolics) may have been more unpalatable than commercial tannins. Nonetheless, because many other variables in the red oak acorns differed from white oak acorns and the commercial tannin diets, firm conclusions cannot be made.

The lower apparent protein digestibilities of red oak acorns and the tannin-containing diets indicate that tannins complexed with protein in the digestive tract. Whether the source of the additional fecal nitrogen was dietary or endogenous is unknown. Three of the ten squirrels fed red oak acorns excreted greater quantities of nitrogen than they consumed, indicating that some of the additional fecal nitrogen was of endogenous origin. Although commercial tannins had significant effects on digestive efficiency in the gray squirrel, only 8% tannic acid reduced digestible protein intake, and none of the diets affected digestible energy intake. Thus, it may be questioned as to whether commercial tannins truly caused significant negative digestive impacts in the gray squirrels.

Absorbed tannins have been shown to damage the liver and kidneys, increase metabolic requirements due to increased detoxification activities, and inhibit postdigestive metabolism of absorbed nutrients. Because squirrels were not sacrificed, it could not be determined if any of these effects occurred and to what extent. Nonetheless, elevated levels of glucuronidation activity in squirrels fed red oak acorns or 8% tannic acid indicated that tannins had been absorbed and that the potential to cause internal effects existed.

The present study has shown that natural and commercial tannins have the potential to exert effects at the intake, digestive, and internal levels in gray squirrels. However, specific effects are likely determined by tannin composition (condensed and hydrolyzable), source (natural or commercial), and dietary protein level. Future experiments that strictly control these variables may help determine the main contribution of each factor to the overall observed effects of tannins in gray squirrels.

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APPENDIX A DETERMINATION OF CONJUGATED GLUCURONIDES IN URINE

Based on the method of Mazzuchin et al. (1971) and protocol developed in the Department of Human Nutrition and Foods at Virginia Tech.

1. Sample preparation

All urine samples from a trial (15 animals) were filtered in a Buchner funnel with Whatman #54 to remove food and fecal particles. The pH of the filtrate was brought up to 6.0 to 6.5 with 5 N NaOH. Volumes of all samples were standardized at either 100 or 150 ml. Five milliliters of each urine sample were boiled, cooled, and centrifuged to precipitate any interfering proteins and salts.

2. Glucose and free glucuronic acid decomposition

Free glucose and glucuronic acid in the urine interfere with conjugated glucuronic acid determination because they react with the colorimetric reagent. To remove free glucose, glucose oxidase was added to the urine sample to convert glucose to gluconic acid, a nonreactive compound. To decompose free glucuronic acids to an unreactive form, 2 N NaOH was added to the urine sample and the mixture was boiled for 20 min.

3. Determination of conjugated glucuronides

After decomposition of free glucose and glucuronic acids, only conjugated glucuronides remained in the sample. By adding concentrated HCl and boiling the sample, glucuronide conjugates were cleaved, thus releasing free glucuronic acids. Free glucuronic acids then reacted with the modified napthoresorcinol reagent to form a purplish colored product. The colored product was then extracted from the aqueous mixture into a layer of ethyl acetate, and absorbance of the ethyl acetate layer was

determined at 564 nm on a spectrophotometer.

4. Standard curve

Glucuronic acid concentrations in each sample were determined by comparing absorbance readings to a standard curve of known glucuronic acid concentrations. Concentrations of phenolphthalein mono-β-glucuronic acid (PGIDE [Sigma Chemical, St. Louis, MO]) from 0 to 350 μg resulted in a linear progression of absorbances from 0.0 to 1.9. Standard curves were used for glucuronide determinations because the method of standard addition was not sufficiently described in Mazzuchin et al. (1971). Because PGIDE standards in urine yielded the same slopes as PGIDE in water, PGIDE standards in water were used for simplicity.

APPENDIX B PROBABILITIES OF TYPE I ERROR[1] FOR THE ACORN EXPERIMENT

Variable	P-value for comparison[2,3]		
	C vs. WO	C vs. RO	WO vs. RO
Initial weight	0.212	0.257	0.991
Weight loss	0.560	0.337	0.126
Pretrial dry matter intakes	0.015*	0.000*	0.007*
Trial dry matter intakes	0.157	0.000*	0.000*
Energy intake	0.173	0.002*	0.003*
Apparent energy digestibility	0.000*	0.000*	0.083#
Digestible energy intake	0.785	0.008*	0.002*
Protein intake	0.000*	0.000*	0.000*
Apparent protein digestibility	0.000*	0.000*	0.000*
Digestible protein intake	0.000*	0.000*	0.000*
mmol glucuronide excretion	0.096#	0.000*	0.000*

[1] Probability that differences between designated groups could have occurred by chance.

[2] C = control. WO = white oak diet. RO = red oak diet. Comparisons made by the Smith-Satterthwaite procedure.

[3] '*' indicates significance at $P \leq 0.05$. '#' highlights P-values ≤ 0.10.

APPENDIX C PROBABILITIES OF TYPE I ERROR[1] FOR THE TANNIN EXPERIMENT

	P-value for comparison[2,3]					
Variable	C - 4%TA	C - 8%TA	4% - 8%TA	C - 3%Q	C - 6%Q	3% - 6%Q
Initial weight	0.328	0.963	0.065#	0.920	0.558	0.263
Weight loss	0.151	0.110	0.811	0.958	0.526	0.385
Pretrial dry matter intakes	0.023*	0.046*	0.182	0.487	0.225	0.081#
Trial dry matter intakes	0.541	0.981	0.415	0.629	0.760	0.244
Energy intake	0.499	0.856	0.486	0.652	0.723	0.233
Apparent energy digestibility	0.001*	0.032*	0.085#	0.004*	0.011*	0.471
Digestible energy intake	0.368	0.423	0.932	0.696	0.276	0.219
Protein intake	0.401	0.572	0.694	0.879	0.307	0.157
Apparent protein digestibility	0.000*	0.006*	0.035*	0.000*	0.017*	0.368
Digestible protein intake	0.112	0.033*	0.196	0.216	0.069#	0.062#
mmol glucuronide excretion	0.190	0.087#	0.128	0.501	0.950	0.592

[1] Probability that differences between designated groups could have occurred by chance.

[2] C = control. TA = tannic acid. Q = quebracho. Comparisons by Smith-Satterthwaite procedure.

[3] '*' indicates significance at $P \leq 0.05$. '#' highlights P-values ≤ 0.10.

APPENDIX D EFFECTS OF TANNIC ACID ON WHITE-FOOTED MICE

A preliminary experiment was conducted with Fisheries and Wildlife undergraduate Sharon Gorman to determine the effects of tannic acid on dry matter intake, weight change, and apparent protein digestibility in white-footed mice (Peromyscus leucopus).

Materials and Methods

Thirty-two white-footed mice (16 male, 16 female) were selected from the colony kept by the Department of Fisheries and Wildlife in Cheatham Hall. Mice were housed individually in mesh bottom, stainless steel cages.

Tannic acid (Sigma, St. Louis, MO) was added to ground rodent chow (Agway PROLAB Rat/Mouse/Hamster 3000) at the levels of 0%, 2%, 4%, and 6%. Males were assigned randomly to one of the four diets, and females were assigned randomly to one of the four diets.

Mice were given food and water ad libitum in the feeding trial, which lasted 10 days. Four grams of food were given to mice each day. In addition, 1 gram was weighed out daily to be oven dried for dry matter determination. Mice were weighed at the beginning and end of the trial. Pans placed under each cage collected uneaten food and feces. Feces were separated from spilled food, dried, weighed, and ground for analysis. Remaining food and spilled food were dried and weighed to determine intake. Food and feces were analyzed for protein content, and apparent protein digestibility was determined as described in the Materials and Methods section for the Acorn experiment.

Because one mouse was removed from the experiment, the experiment was unbalanced. Thus, SAS General Linear Models Procedure (GLM) was used to determine if differences existed between the groups in dry matter intake, apparent protein digestibility, and weight change. Multiple comparisons were made using

the Tukey procedure and an experimentwise error of $\alpha = 0.15$.

Results and Discussion

Dry matter intake was higher for mice consuming 2% tannic acid diets. However, intake for mice consuming 4% and 6% tannic acid diets were not significantly different from the control.

Despite the higher dry matter intake for mice on the 2% tannic acid diet, no significant differences existed between the average weight changes of mice in the four groups (Table 14). Unlike the gray squirrels, not all mice lost weight. Because mice were raised in captivity, they probably did not experience cage stress and stress-associated weight loss. However, one mouse on the 6% tannic acid diet was removed from the trial because its weight loss was >4 grams in the first 4 days.

Apparent protein digestibilities (APD) were significantly reduced in mice consuming 4% and 6% tannic acid relative to the control (Table 14). No other significant differences existed.

Conclusion

The addition of 2% tannic acid increased dry matter intake. However, this effect did not continue in a dose dependent manner at levels above 2%. The addition of tannic acid at all levels had no effect on the changes in weight of mice throughout the trial. A minimum of 4% tannic acid was required to reduce APD in white-footed mice. However, the lack of significant differences between APD's of the 4% and 6% diets indicate that APD reduction did not continue in a dose dependent manner above 4%.

Table 14. Average dry matter intake, weight change, and apparent protein digestibility (APD) for white-footed mice fed tannic acid-containing diets, Blacksburg, Virginia, March 1992.

		Dry matter intake[1] (g/day)		Weight change[1] (g)		APD[1] (%)	
Diet	n	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Control	8	2.6^{A}	0.3	-0.3^{A}	0.9	58.5^{A}	8.2
2% Tannic Acid	8	3.3^{B}	0.6	0.4^{A}	0.8	48.7^{A}	15.9
4% Tannic Acid	8	2.9^{A}	0.6	1.0^{A}	1.6	46.2^{B}	9.5
6% Tannic Acid	7	3.0^{A}	0.5	0.4^{A}	1.6	45.6^{B}	7.4

[1] Means in the same column with different letters are significantly different. Experimentwise $\alpha = 0.15$.

APPENDIX E OVERWINTER CHANGES IN TANNIN LEVELS IN 3 SPECIES OF ACORNS

In the fall, squirrels have been observed to bury a disproportionate number of red oak acorns relative to their availability and relative to white oak and chestnut oak acorns (Lewis 1982, Smallwood, pers. comm.). The higher levels of tannins in red oaks have been proposed as the reason for this preferential burying of red oak acorns. It has been hypothesized that the exposure of red oak acorns to ambient winter conditions reduces the tannin content of these acorns, perhaps by leaching or internal biochemical changes. Thus, squirrels may consume low tannin, white oak acorns immediately and bury red oak acorns to allow tannin levels to decline before consumption. The following experiment was conducted with Dave Hewitt of the Department of Fisheries and Wildlife to test the hypothesis that tannins are reduced in acorns left to overwinter.

Materials and Methods

Northern red (Quercus rubra), white (Q. alba), and chestnut (Q. prinus) oak acorns were collected from several sites on the Virginia Tech campus from mid-September to early October 1991. Acorns were randomly assigned to one of 3 protective wire mesh cages and were placed in groups of 10-20 acorns (by species) on a bed of dirt and leaves under an oak-hickory canopy at the Center Woods Facility. One group of acorns of each species was collected from each cage at approximately 45-day intervals from midSeptember 1991 - March 1992. Thus, 3 replicates existed for each species at each time period, and each replicate consisted of 10-20 acorns.

After collection, acorns were shelled, frozen, lyophilized, and ground in a Wiley mill. Worm-infested acorns were discarded. Acorn powder was extracted with 70% acetone. Tannin levels in the extract were determined by the radial diffusion assay, which measures the protein-precipitating activity (PPA) and uses purified tannic acid

as a standard (Hagerman 1987). PPA is reported as mg tannic acid equivalents/g dry weight acorn. The values obtained reflect tannin content, but do not distinguish between tannin types (condensed or hydrolyzable).

A two-way ANOVA was used to determine if time and/or species were significant factors determining tannin content. One-way ANOVA's were also performed to determine if time was a significant factor affecting tannin content for each species. Multiple comparisons were made with Tukey's studentized range test (experimentwise $\alpha = 0.05$).

Results and Discussion

Both acorn species and time significantly affected the tannin content ($P = 0.0001$). Tannins were highest in the red oak acorns and lowest in the white oak acorns (Table 15). A notable interaction occurred between time and acorn species ($P = 0.082$), indicating that tannin levels may not have declined in all acorn species or may not have declined at the same rate. One-way ANOVA's determined that time had a significant effect on tannin levels in chestnut and white oak acorns ($P = 0.0001$), but not red oak acorns ($P = 0.452$) (Table 15). The lack of a time effect on tannin levels in red oak acorns explains the interaction observed in the two-way ANOVA and is apparent in Figure 7.

Conclusion

Results of this experiment indicate that tannin levels were reduced in overwintered white oak and chestnut oak acorns, but not in red oak acorns. Thus, the hypothesis that tannins are leached from acorns exposed to ambient winter conditions is supported for the 2 of the 3 species. However, because tannin levels were not reduced in red oak acorns, the hypothesis does not explain the preferential burying of red oak acorns.

Table 15. Tannin levels (mg tannic acid/g dry wt acorn) in 3 species of acorn overwintered at Blacksburg, Virginia, September 1991 - March 1992.

Acorn		Day of experiment acorns were collected (actual date)				
		Day 1 (15 Sept 91)	Day 30 (15 Oct 91)	Day 77 (1 Dec 91)	Day 120 (13 Jan 92)	Day 168 (2 Mar 92)
White[1]	$\bar{x}$	41.0^{A}	34.6^{A}	24.6^{B}	19.2^{BC}	14.7^{C}
	SE	2.1	1.0	2.1	1.2	1.0
Chestnut[1]	$\bar{x}$	74.6^{AB}	79.7^{A}	71.6^{AB}	66.8^{B}	56.7^{C}
	SE	5.8	3.2	3.2	2.9	0.8
Red[1]	$\bar{x}$	98.9^{A}	101.2^{A}	104.1^{A}	91.9^{A}	94.0^{A}
	SE	5.2	2.5	7.4	4.3	4.4

[1]Means in the same row with different letters are significantly different. Experimentwise $\alpha = 0.05$.

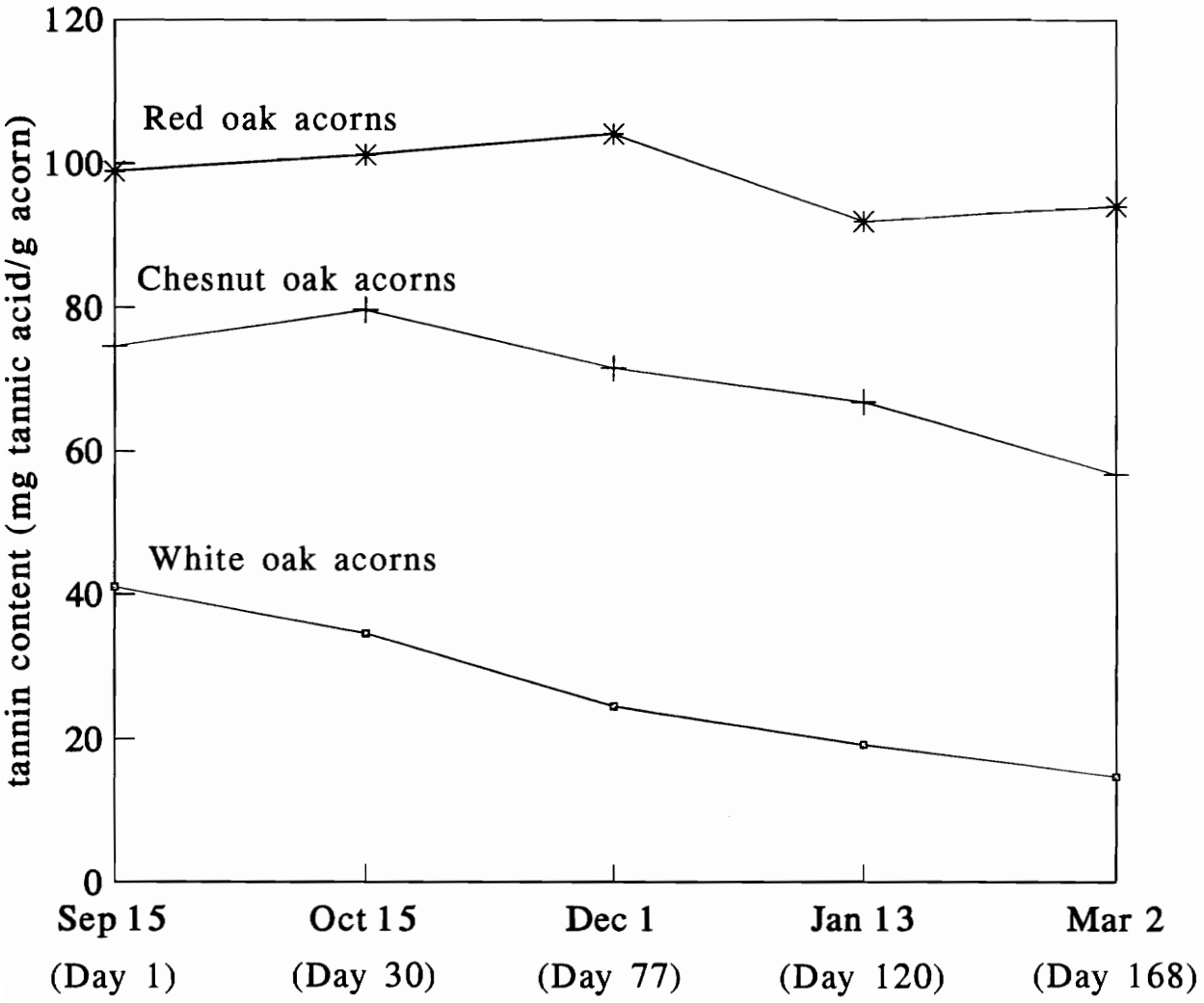

Figure 7. Overwinter changes in tannin levels in 3 species of acorns, Blacksburg, Virginia, Sept. 1991 - March 1992.

VITA

Alice L. Chung-MacCoubrey was born the daughter of Eric and Nancy Chung on September 14, 1966 in Philadelphia, Pennsylvania. She was raised in Bryn Mawr, Pennsylvania and graduated from Haverford Senior High School in Havertown, Pennsylvania in 1984. She received her B.S. in Biochemistry with highest honors from Rutgers University, Cook College, in June 1988. In 1989, because of her changing interests, she decided to pursue post-baccalaureate studies in Wildlife Science at Oregon State University. In 1991, she was accepted into the Master of Science program in Fisheries and Wildlife Sciences (Wildlife Science) at Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Alice L. Chung-MacCoubrey