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Phenolic profile of shrub live oak and its relation to goat diets in central Arizona

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PHENOLIC PROFILE OF SHRUB LIVE OAK AND ITS RELATION TO GOAT DIETS IN CENTRAL ARIZONA

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by HILTON DE SOUZA GOMES entitled PHENOLIC PROFILE OF SHRUB LIVE OAK AND ITS RELATION TO GOAT DIETS IN CENTRAL ARIZONA,

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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George B Ruyle — 2/20/90

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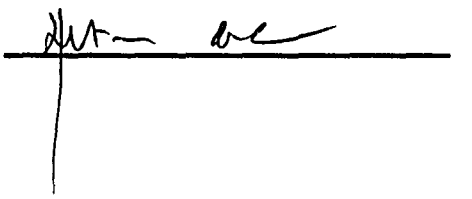
SIGNED: 

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ABSTRACT

The occurrence and concentration of substances like glycosides, alkaloids, certain amino acids, and, especially, phenolics in vegetation has been seen as a defense against environmental perturbations. Whether this phenomenon is associated with the disruptive effect of man, herbivore predation or the invasion of arthropods and microorganisms remains to be ascertained by ecologists. However, contemporary research shows that most so-called secondary metabolites in plants render them less sensitive to adverse factors in the natural environment. Likewise, herbivores that adapted to chemical defense in plants were rewarded with wider food source and might have been able to more efficiently exploit rapidly changing foraging environments.

This study focuses on relationships between goats, their ruminal microbes and tannin-rich plants in a central Arizona chaparral vegetation type dominated by shrub live oak (Quercus turbinella). The study tested hypotheses involving goat dietary selection, estimated through microhistological analysis of feces; phenolic profile of shrub live oak, expressed as condensed and hydrolyzable tannins, protein binding capacity of oak

leaves extract, expressed as bovine serum albumin (BSA) precipitation;and apparent dry matter digestibility of mature and immature oak leaves by goat ruminal microbes, measured at 6, 12, 24 and 48 hours.

Findings of this research indicated that goats prefer leaf and shoot ends of shrubs, mainly shrub live oak, despite the constant high concentration of tannins in that plant. Hydrolyzable tannins in oak are synthesized during summer/fall and late spring, and the synthesis of condensed tannins is mostly restricted to winter and early spring.

Precipitation of BSA by extract of oak leaves, apparently associated with hydrolyzable tannins, did not discourage oak consumption by goats. The digestibility of younger and older oak leaves was more influenced by time of incubation than level of phenolics in plant tissue.

This research emphasizes the adaptability of goats to ecological zones where presence of toxins and digestion reducing compounds in plants adversely affects survival and fitness of non-adapted herbivores.

INTRODUCTION

It has been hypothesized that as plants moved from aquatic to terrestrial environments they passed through genetic changes that rendered them more adapted to many adverse factors. In addition to physical factors such as ultra violet radiation and gradients of temperature and humidity there was a sequence of biological factors beginning with arthropods, then dinosaurs and reptiles, and ultimately, mammalian herbivores and man (Myers 1979).

The plant defense theory points out that the concentration of substances like glycosides, fatty acids, amino acids, alkaloids, phenolic compounds and many others in most terrestrial plants is much greater than the basic needs of the plant for physical and chemical processes involving growth and species perpetuation. Most of these substances, are collectively called "secondary compounds" due to the inability of modern science to ascertain their absolute necessity for plant life. For example, the phenolics are, after carbohydrates, the most abundant group of substances in plants but the knowledge of their function still remains fragmentary.

Most of these secondary compounds are believed to play, or to have played in the past, a defensive role in the plants in which they occur by mediating interactions with biotic and abiotic elements of the environment.

Since the dawn of civilization, man has used products from plant origin for varied purposes such as raw materials like wood and fiber, in processes such as leather, beer and wine making, as food like fruits, and beverages, medicinal drugs, not to mention the inexorable attractiveness of people in ancient and modern civilizations to cocaine from (Erythroxylon coca) and cannabinoids from (Canabis sativa) (Luckner 1984).

A distinct feature of plants is the variability in abundance, in time and space, of most of their primary or secondary compounds. This tri-dimensional variability of food and poison in the environment makes diet selection a dynamic process in which the value of feeding locations, plant species and plant parts is dictated by the herbivore's nutritional requirements and its foraging ability, metabolic capability and knowledge of the changing forage conditions.

Herbivores have counteracted to the armory of allelochemicals evolved by plants by morphological,

physiological and behavioral adaptations. One of the most fascinating examples of this is the development of the rumen and the increasing level of voluntary intake of phenolic-rich plants observed in the continuum of grazers to intermediate browsers to browsers.

Goats (Capra hircus), are one of the most valuable group of animals for studies on plant-herbivore interactions because they have a short generation interval, exert a great impact on the environment by eating a multi-species diet, and accept foodstuff not eaten by other ruminants. Perhaps for these reasons, and others, approximately 96 percent of the 476 million goats of the world are in Mediterranean and drier tropical environments of less developed countries (FAO 1983).

The management of deserts, savannas, caatinga and chaparral biomes where patterns of soil fertility, aridity, herbivory and occupation by man contributes to or is affected by the presence of high levels of chemical defense in the existing vegetation requires the understanding of resource allocation and the counteraction to physical and biotic environmental factors by plants and animals.

This research attempts to provide insight into the coevolution of goats, one of the earliest domesticated ruminants (Zeuner 1963), and shrub live oak - the dominant species of the Arizona chaparral (Kearney and Peebles 1960). Shrub live oak is grouped into a genus whose presence on earth has been traced back to the Miocene epoch, Tertiary Period, between 12 and 26 million years ago (Plumb and McDonald 1981).

The study of the phenolic profile of shrub live oak as mediator of general chemical defenses in that plant in addition to studies on dietary selection by goats on oak-dominated chaparral and efficiency of ruminal digestion of oak leaves contributes to the understanding of the value of oak-dominated ranges for goats, the pattern of phenolic biosynthesis in shrub live oak, and its biological implications for herbivory.

LITERATURE REVIEW

Growth and self-perpetuation of living systems requires the capture of free energy in the environment for use as chemical energy necessary to convert simple substances into complex cytoplasmic structures. Although chemical activity or increases in size and reproducibility, when considered separately, may not identify life in a system, it can be said that growth, reproduction, response to the environment and another process, metabolism, are true features of living things (Salisbury and Ross 1978). Metabolism, as defined by several authors, is a life process common to all organisms, where a series of enzyme-mediated reactions make it possible for some chemical compounds to be synthesized (anabolized) and others to be degraded (catabolized) *in vivo* (Martin et al. 1985).

Plants, as autotrophic organisms, couple their metabolism with exergonic reactions as the anabolic process of photosynthesis and rely also on catabolic processes like respiration to obtain the energy they need. The metabolism of heterotrophic organisms, like animals, stands in sharp contrast to plant metabolism in that the first obtain free energy by coupling their

metabolism to the reorganization of complex molecules in their environment (food), making possible the maintenance of the cellular machinery and processes like muscular activity and homothermy.

Primary and Secondary Metabolism

The formation and breakdown of compounds essential for survival and well-being of an organism is defined as primary metabolism. All living things possess similar, if not identical, metabolic pathways to synthesize and utilize primary metabolites such as most carbohydrates, common fatty acids, amino acids, nucleotides, and macromolecules such as polysaccharides, lipids, proteins, DNA and RNA. All of these are important chemical entities for cells and tissues.

The analysis of chemical make-up of cells, made possible by improvements in microscopy and analytical procedures at the end of last century, brought a teleological approach to the field of biology and started some debate and speculation about the structure, function and chemical and ecological role of secondary metabolites.

Sachs (1882) defined tannins, polyterpenes, alkaloids, and even lignin in plants as "by products of

metabolism." Kossel (1891) proposed the term "secondary components" to denote protoplasmic constituents that did not occur in every cell and were neither incidental nor absolutely necessary for life of the plant as primary metabolites.

The need to investigate the relationship of certain cellular components to plant life lead Pfeffer (1897) to precipitate tannins in Spirovira, a blue green algae, by applying a thiazine dye. He observed a continuous growth of the algae following precipitation of its tannins and accepted the hypothesis of no physiological function of the tannins in Spirogyra.

Many other scientists offered terminology for secondary metabolism and its major end-products. Bonner and Galston (1952) favored the terms "highways and byways of metabolism" despite earlier terminology, "processes of secondary character", to name basically the same thing (Czapek 1921). Czapek (1921) additionally classified basic nitrogenous metabolites (alkaloids) and other compounds that seemed to perform no physiological function in plants as excretory products or end products of metabolism. Zenk (1967) suggested the term "natural products" arguing that elements of isotopically labeled

secondary metabolites are reintroduced into biological cycles. He stated that "secondary compounds" was not appropriate terminology nor could these metabolites be seen as wastes of metabolism as Czapek (1921) had proposed.

Other designations for secondary compounds were based on functions they appeared to perform in the systems where they occurred. Brattsten (1979) proposed the term allelochemicals because of the involvement of secondary products in many biochemical interactions among organisms. This terminology also is not precise enough and can be misleading because the term allelopathy, as coined by Molisch (1937), refers to inhibitory and stimulatory biochemical interactions between plants and may include microorganisms in the process. The presence of secondary compounds in microorganisms during specific developmental stages prompted Demain (1976) to name them idiolytes.

Stahl (1888) drove the argument from terminology to a more pragmatic ecological approach, suggesting a possible advantage for plants in possessing secondary compounds; the concept of deterrence of herbivores. It was several decades until Fraenkel (1959) discussed in

more detail the value of secondary metabolites for plants and provoked more work on the biosynthesis of several secondary compounds and on plant-herbivore relationships. Although, some workers kept calling secondary compounds plant wastes (Muller 1969, 1970; Whittaker 1970), they admitted that those substances acquired functions later in the development of their possessors.

A growing number of ecologists now believe that most secondary compounds are substances that function as mediators of ecological interactions involving microorganisms, plants and animals. Contrary to earlier ideas of secondary compounds as an exclusive feature of plants (Sachs 1882; Czapek 1921) the occurrence and importance of these metabolites in lower and higher classes of living organisms is now widely accepted (Mann 1987).

Microorganisms do not possess pathways for synthesis of certain secondary compounds such as alkaloids which are very common in plants. They did evolve metabolic routes for production of antibiotics, however. The pharmacological literature is full of examples of secondary metabolites produced by bacteria and fungi. Most of these compounds depress growth and

destroy microorganisms and, due to a potent effect against many higher animals, have been widely used in medicine. Common examples include tetracyclines such as terramycin, produced by soil actinomycetes and chloramphenicol, a shikimate metabolite produced by Streptomyces. This last one is a large spectrum antibiotic applied in the cure of typhoid (Herbert 1989). Other antibiotics have a high toxicity that precludes their clinical use (Hutchinson 1981).

Secondary compounds in animals act as intraorganistic signals, pheromones, defensive compounds and detoxicants. Examples of internal signals include epinephrine and nonepinephrine, also known as adrenaline and nonadrenaline. These substances, chemically classified as cathecolamines, are tyrosine and tryptophan derivatives synthesized and stored in the sympathetic nervous neurotransmitters of animals and participate in several processes including metabolism of fatty acids and glycolysis (Hagen and Hagen 1964).

Secondary compounds in animals are also known to confer defense against predators. For example,

Cantharidin in the blood of the blister beetle (Epicauta pestifera) is a strong feeding deterrent to insects and a systemic poison to higher vertebrates (Carrel and Eisner 1974). This substance, only produced by male blister beetle, is supposedly an aphrodisiac and is transferred to females during mating (Mann 1987). Other uses of secondary metabolites by animals include territorial markers, trail identification and mate attractants. The participation of secondary metabolites in detoxication systems of animals has also been well documented (Luckner 1984).

Animals surpass microrganisms and plants in the ability to eliminate excess or waste of metabolism. While microorganisms release unwanted materials in their surroundings, higher animals dispose "unnecessary" products via kidneys, liver and other excretory organs. High concentration of secondary products in animal cells or tissues is found in very few species (e.g., defense glands in beetles, wings of butterflies). The low excretory efficiency of unwanted metabolites and the ability to polymerize chemicals and sequester toxic substances out of the protoplasm milieu afforded plants a widespread and efficient use of secondary metabolites.

The abundance of secondary metabolites in plants is accepted by most ecologists not only as a consequence of poor excretory system but also as a mechanism evolved to cope with adverse biotic and abiotic conditions encountered when they moved from an aquatic to a terrestrial environment (Mann 1987). Of course, not all secondary compounds found in plants have a known defensive function or confer competitive advantage to their possessors. Some of them may even be waste, mutation, or end products of teratologic pathways as suggested by Muller (1969) and Whittaker and Feeny (1971). However, it should also be mentioned that plants do actively respond to stimuli from their environment.

There is growing evidence that plants react to mechanical perturbations like wind and trampling by animals and machinery through mediation of endogeneous ethylene that produce changes in their growth pattern and render them hardier and better adapted (Jaffe and Telewski 1984). The action of ethylene has also been documented as a pheromone transferring information from damaged plants to undamaged ones (Rhoades 1985). Works such as these, revealing the electrical and chemical mechanisms involved in nastic movements in plants and the presence of compounds like gentisic acid glycosides

mediating response to touch in plants (Pickard 1973) have contributed to cast out the view of plants as static resources for herbivores and provided support for the proclaimed ecological role of most plant secondary products.

The ecological significance of secondary compounds in the plant kingdom embraces the role of these substances in attraction of pollinators, feed deterrency, avoidance of pathogens and competitors and the maintenance of biochemical and physiological functions in plants. Rhoades (1979) defends the ecological function of these metabolites in plants by arguing that they are, in most cases, end-products of energy-demanding pathways and as such, there must have been great selective pressure for this diversion of metabolic resources that otherwise could be used in growth and reproduction. Current ecological theory suggests that features of living organisms must either confer advantage or be innocuous for their possessors, implying that "lack of function" must be a limitation of our understanding of processes.

Besides their function as attractants and repellents, plant pigments and other secondary compounds are involved in regulatory processes of the plant.

According to del Moral (1972) phenolics like chlorogenic and isochlorogenic acid have a role in adaptation of sunflower plants (Helianthus annuus) to stress situations. He mentions that absorption of UV by plant phenolics helps preserve cellular organization. The protection of nucleic acids, NADH and other coenzymes from high intensity ultra violet light by phenolics was also observed by Robbercht and Caldwell (1978).

Perhaps the more obvious function of secondary metabolites in plants is the role of lignin in tensile strength, elasticity and resistance to microbial degradation of cell wall, in addition to anti-gravity support. Lignin, as defined by North Cote (1972), is a insoluble, hydrophobic, aromatic compound derivative of high molecular weight, synthesized by dehydrogenation and polymerization of plant alcohols.

Just as lignin, was once seen as a by-product of plant metabolism (Sachs 1882), the function of many other secondary metabolites has only recently been elucidated. Most of these functions are so related to survivability of the plant that it has become difficult to make a distinction between primary and secondary metabolism. Seigler and Price (1976) and Seigler (1977) visualized

this fact and advocated "primary functions" for secondary compounds by mentioning that in certain plants nitrogen and other elements from secondary metabolites are recycled for synthesis of carbohydrates, amino acids and other primary metabolites (e.g., nicotine recycling in tobacco plants).

It appears that secondary metabolites in certain plants may function as storage of carbon and nitrogen for periods of great need, as is observed in the sharp decline of these substances in legumes, after seed germination and by the fluctuation of levels of many secondary compounds throughout phenological stages of plants (Janzen 1969; Ellis 1974).

The interconnection between primary and secondary metabolism is more evident yet by the function of certain primary compounds in both areas of metabolism. Acetic acid for example, which occurs in living organisms as coenzyme A ester, constitutes the starting material in the synthesis of many long chain fatty acids and also serves as a precursor of secondary compounds like polyacetylenes, terpenes, prostaglandins, steroids, isoterpenes, macrocyclic antibiotics and some polyphenols (Mann 1987; Hebert 1989).

Another example are the amino acids lysine, ornitine, phenylalanine and triptophan which supply carbon skeletons for alkaloids and peptide antibiotics like penicillins and cephalospirin (Herbert 1989).

Finally, condensation of phosphoenolpyruvate from glycolysis with erythrose-4-phosphate from the pentose phosphate pathway, leads to the synthesis of shikimic acid that is the precursor of aromatic amino acids and a variety of secondary metabolites of aromatic character (Fig. 1).

Contemporary definition (Mann 1987; Herbert 1989; Luckner 1984) for secondary metabolites excludes intermediate and end-products of primary metabolism as well as photosynthetic and oxygen-carrying pigments. Features that best characterize plant secondary compounds are:

a) Occurrence restricted to certain genera and species.

b) Bizarre and heterogeneous chemical structure.

c) Formation by enzymes encoded by unusual genes or combinations of genetic material.

d) Expression of cell specialization and biogenesis coupled with amount and activity of required enzymatic material.

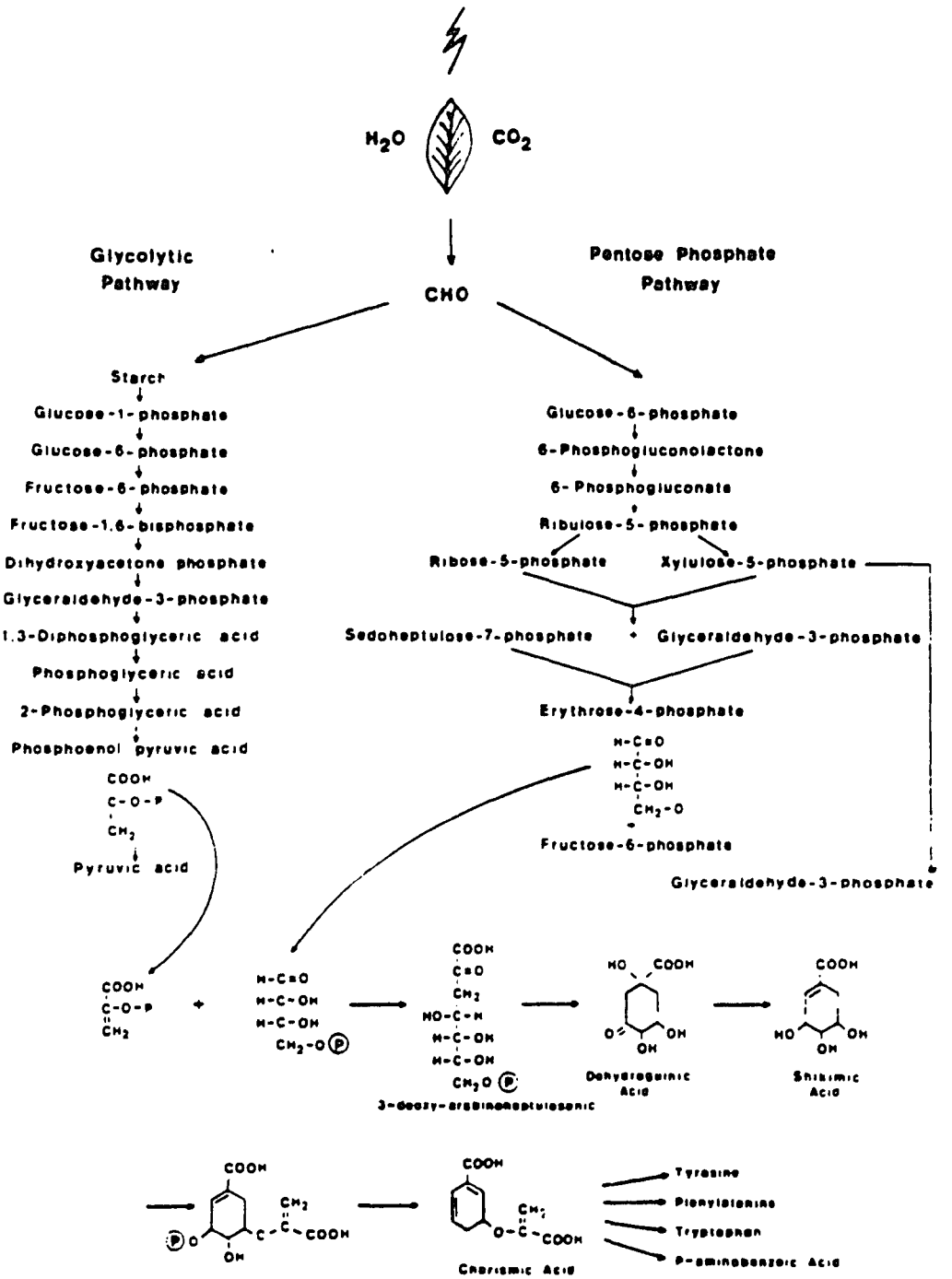


Fig. 1. Shikimic acid pathway as related to glycolysis and pentose phosphate pathway (Martin et al. 1985; Floss 1986).

e) Synthesis through pathways activated at particular stages of growth and development of the producing organism and during stress caused by nutritional limitation and microbial attack.

f) Compartmentalization of enzymes, precursors, intermediates and products participating in their metabolic pathways, storage and breakdown.

g) Apparent lack of explicit function to the synthesizing cell but present, or past, importance to the economy of the producing organism as a whole.

h) The function of a given secondary compound in one organism may be disparate or even absent in another.

Phenolics

An interesting group of secondary products, the phenolics, has attracted much attention recently for their occurrence in animals, plants and in the environment.

Phenolics are a group of hydrophylic, aromatic substances bearing one or more hydroxyl groups in the aromatic ring which gives them an acidic character. The designation "aromatic" does not necessarily have anything to do with aroma. Although the term was first applied to fragrant oils and resins found in leaves, flowers, buds

and exudates of certain plants. It in fact refers to compounds containing a cyclic structure of carbon atoms with benzene as a parent substance. Plant phenols embrace not only compounds of recognizable color and odor but also the odorless tannins.

Phenolics are often perceived as environmental pollutants discharged by activities such as coal coking, oil refineries, gas works, agricultural pesticides and dye manufacturing (Buikema et al. 1979). The presence of these compounds in soil, lakes, rivers and other water bodies represents a serious threat for elements of the flora and fauna.

Naturally occurring phenolics in animals are synthesized in specialized tissues, stored inactive, and later released by stimulus to act in regulation of metabolic processes (epinephrine and nonepinephrine) and maturation and sexual activity (estradiol). Other phenolics in animal metabolism include serotonin, a heart stimulant in crustaceans and mollusks and found in intestinal cells of humans, functioning in vasoconstriction and activity of smooth muscles; and dopamine, found in the adrenal gland (Ramwell and Sherra 1964).

Regarding the synthesis of phenolic compounds in animals, it must be mentioned that animals do not carry the shikimic acid, prephenic and anthranilic pathways for aromatic biosynthesis and rely on plants for acquisition of the starting materials (Baillie et al. 1972). This fact illustrates not only how animals depend on plants but also the interactions that might prevail between them. Research has shown that a great number of phenolics which occur in animal systems (cathecolamines, indolacetic acid, dopamines, bufotenine) also occur in plants, some of them with functions not completely understood (Finkle 1967).

The thousands of phenolics produced by plants are classified according to the number of carbon atoms in their skeleton. As seen in Table 1, phenolics vary from simple, monocycle, six-carbon compounds such as cathecol and hydroquinone to large polymerized forms like melanins, lignins and tannins. Except for the flavonoids, all plant phenolics are derivatives of the shikimic acid pathway (Fig.1). Of the aromatic rings in flavonoids, one is originated from phenylalanine and the others come from Acetyl-CoA via polyketide pathway (Goodwin and Mercer 1983). Flavonoids, the largest groups of phenolics, are

Table 1. Classification of phenolics found in higher plants.

Basic skeleton	Class
C_6	phenol
C_6-C_1	phenolic acid
C_6-C_2	hydroxycinnamic acid
C_6-C_3	hydroxycinnamic acid coumarins isocumarins phenylpropenes chromones
C_6-C_4	naphthoquinones
C_6-C_1-C_6	xantones
C_6-C_2-C_6	stilbenes anthraquinones
C_6-C_3-C_6	flavonoides
$(C_6$-$C_3)_2$	lignans neolignans
$(C_6$-C_3-$C_6)_2$	biflavonoids
$(C_6$-$C_3)_n$	lignins
$(C_6)_n$	melanins
$(C_6$-C_3-$C_6)_n$	tannins

Source: Finkle 1967; Goodwin and Mercer 1983.

responsible for flower pigments (anthocyanin) in many angiosperms and most of the flavour of food and drink of plant origin.

Striking characteristics of plant phenolics include their abundance, chemical stability, low phytotoxicity, anti-microbial activity and easy identification. Phenolic compounds are produced in large amounts by all higher plants, through inheritable biosynthetic pathways. According to Levin (1971), phenolics are the second most abundant group of substances in plants, being edged out only by carbohydrates. Their great chemical stability in plants results from a frequent association with sugars such as glycosides and a low reactivity. Even though plant phenolics are highly soluble in water, they offer low phytotoxicity because of their location in the vacuole and a their frequent state of polymerization. However, these substances provide mechanical (lignins) and chemical (lignins,tannins) defense against invading microorganisms. The strong UV absorption by phenolics eases their identification by routine laboratory techniques (Levin 1971; Harborne 1973).

Although Crawley (1983) mentions that any carbon fixed by plants is destined to be eaten either by a herbivore or some kind of microbial life, it can also be argued that plants are not subservient hosts for herbivores because most species have avoided extinction by developing ways to escape or minimize injuries from the environment. Common counteractions by plants to hrebivory consist of alterations in photosyntetic rate, morphology and palatability that reduce their digestibility, metabolizability and nutritional value to herbivores conferring escape, resistance and defense.

Escape and resistance to herbivores, phytophagous insects and microorganisms is possible by mimicry, above ground regrowth, long-lived seedbeds, growth of ungrazeable reserve organs, rootstock survival and migration made possible by seed dispersal (Dahl and Hyder 1977; Caldwell et al. 1981).

Defense from herbivores can be biological, physical and chemical. Biological defense has been observed in very few cases (e.g., ants conferring predator avoidance to plants) but seems to be an efficient mechanism.

Physical barriers include several means of thickening the surface cuticle in plants which prevents penetration of pathogens and arthropods or their access to enclosed nutrients. Other physical barriers are the presence of stings, thorns, silica, cork, resins, waxes and lignins (Crawley 1983; Cooper and Owen-smith 1986).

Chemical defenses can be classified in two sub-systems. First, the induced, also called facultative or defensive response and, second, the preformed or constitutive defenses. The first group is mostly seen defending plants against pathogens and arthropods. These responses can follow herbivore attack very rapidly, as hypersensitive cell death, in the long term as in cell wall modification or in an intermediate time span, as in the synthesis of phytoalexins (Ayers et al. 1985).

Preformed or constitutive plant defenses are used against feeding herbivores and also pathogen and insects. According to Feeny (1976) and Rhoades and Cates (1976) preformed defenses can be qualitative and quantitative. They maintain that qualitative defenses are possessed by "unapparent plants", that are ephemeral, early successional, rare and deciduous plants, while quantitative defenses are predominantly found in

evergreen plants. Although this concept was criticized by Crawley (1983) as meaningless, and unhelpful due to the hassle of measuring plant apparency, the definitions for quantitative and qualitative defenses remain accepted.

Qualitative defenses are metabolic toxins effective against generalistic herbivores and less harmful for specialist phytophagous organisms. Examples of toxic compounds in plants include alkaloids, cyanogenic substances, cardenolides, and glucosinolates. They occur in small concentrations in plants, have been mostly identified in short lived species and seem to cost less to plants than quantitative defenses.

Quantitative defenses are digestion reducing compounds such as calcium oxalate crystals, resins, lignins and tannins. These substances are more abundant in the plant kingdom than qualitative defenses and occur in very high concentrations in most woody species. Digestion reducing substances (e.g.,tannins) are one of the most ancient and successful group of plant defensive compounds. Due to a bitter taste to all mammals and some birds, action by quantitative effect and a low likelihood

of being neutralized, tannins are considered effective against both generalist and specialist herbivores (Swain 1979).

Herbivores have shown some ability to sequester toxins in their body, become adapted to them and, in rare cases, use dietary toxins for their own defensive systems. A classic example of this is the monarch butterfly (Danaus plexippus) that by accumulation of ingested plant toxins are avoided by predatory birds (Brower 1969). Another example of detoxication is elimination of volatile plant toxins as terpenoids by body heat and movement of ingesta in ruminants (Cluff et al. 1982). However, it seems that ruminants had to counteract in other ways because the rumen has a limited ability to handle phenolic-based plant defenses, the most abundant feeding deterrent present in their multi-species diet.

One explanation for the multi-species dieting of herbivores is offered by the "Optimal Defense Theory"(Rhoades and Cates 1976; Freeland and Janzen 1974; White 1974). They contend that herbivores, mostly browsers, feed from a great variety of plants in order to avoid ingesting an overdose of toxic secondary

metabolites. This view is in agreement with the pioneer observation of Fraenkel (1959) that quality and amount of deterrent substances in plants, rather than their nutritional value, is what determines the likelihood of plants to be attacked by herbivores. Furthermore, a great number of studies have failed to prove that a multi-species diet serves the purpose of maximizing intake of energy and nutrients (Bryant et al. 1983; Owen-Smith and Novellie 1982).

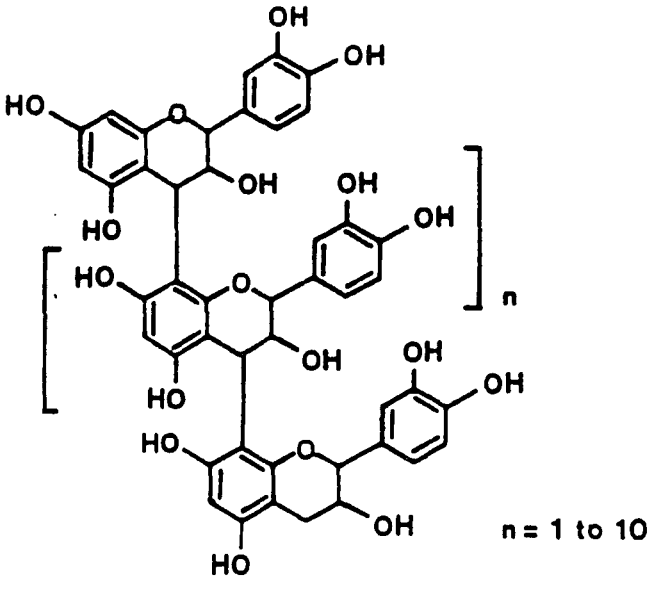
Studies on feeding behavior of herbivores seem to indicate that plants have a much greater impact on herbivores than the impact herbivores impinge upon them (Crawley 1983). Quality and concentration of defensive plant substances vary with organ and age of tissue in a plant and also with kind of plant, time of the day, season, phenological stage, plant health and nutritional status, edaphic and climatic conditions (Scalbert and Haslam 1987; Harborne 1972; Mckey 1979). The study of Feeny (1970) for example, demonstrated that young oak leaves (Quercus rubur) are vulnerable to herbivory attack due to their higher level of nitrogen and lack of chemical defense (tannins) which is found in the older leaves.

Vegetable Tannins

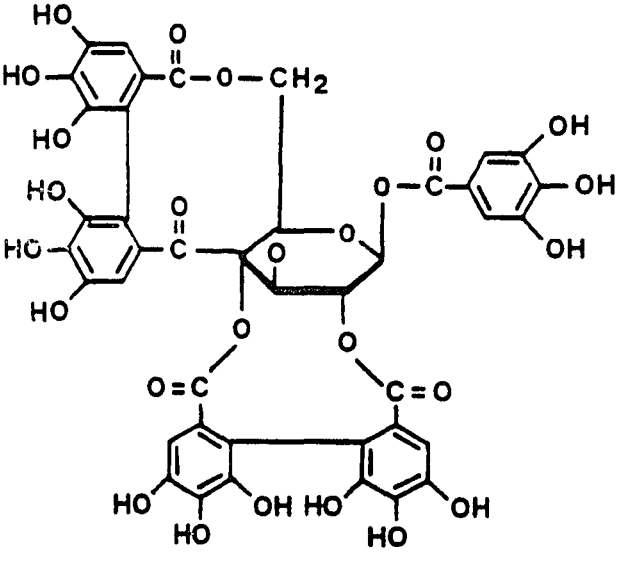
Of thousands of naturally occurring phenolics, one group of polymers, the tannins, appears as the most important plant secondary metabolite for their role in interactions between plants and biotic or abiotic environmental factors. The term "tannin" was coined in 1796 by A. Seguin (Haslam 1981) to define material present in oak galls which had the ability to form leather when animal hides were exposed to an aqueous infusion of oak galls. These polyphenols are notorious for their ability to precipitate alkaloids, gelatin and other proteins (Bate Smith and Swain 1962; Swain 1979).

Tannins are defined as water soluble substances bearing one or more hydroxyl groups and having molecular weight between 500 and 3,000, although unusual molecular weights as high as 28,000 have been recorded for sainfoin (Onobrychis viciifolia) (Jones et al. 1976). They are a cosmopolitan group of substances, found in late successional plants from tropical to temperate zones (Bate Smith 1954; Haslam 1981).

There are two classes of tannins: condensed (proanthocyanidins), and hydrolyzable tannins (Fig 2). Condensed tannins, which include delphinidins, are



Condensed Tannin



Hydrolyzable Tannin

Fig. 2. Structural formula of condensed and hydrolyzable tannin.

polymers of flavan-3-ols, flavan-3,4-diols, catechin and epicatechin linked by carbon-carbon bonds between C-4 and the C-6 or C-8 monomers (Haslam 1981). Proanthocyanidins are mostly found in woody species but can also occur in grasses and herbs (Haslam 1977).

Hydrolyzable tannins, also called gallotannins and ellagitannins, consist of gallic acid or hexahydroxydiphenic acid esters of carbohydrates or polyols. Unlike condensed tannins, gallotannins occur only in dicotyledons (Swain 1979).

Condensed and hydrolyzable tannins can also be distinguished by the nature of phenolic nuclei they have, and by the kind of linkage between them and by the end-product of their hydrolysis. Condensed tannins do not yield carbohydrate and are degraded by heat to form anthocyanidins. When treated with acid, these tannins yield condensation, hydrolysis and anthocyanidin formation. Hydrolyzable tannins, on the other hand, when cleaved by acid, alkali or enzymes, yield carbohydrates and one or more of its acid constituents. As their name implies, these tannins are easily hydrolyzable by acid, bases and enzymatically by tannase.

The biosynthesis of vegetable tannins has its origin in the shikimic acid pathway (Fig. 3). Formation

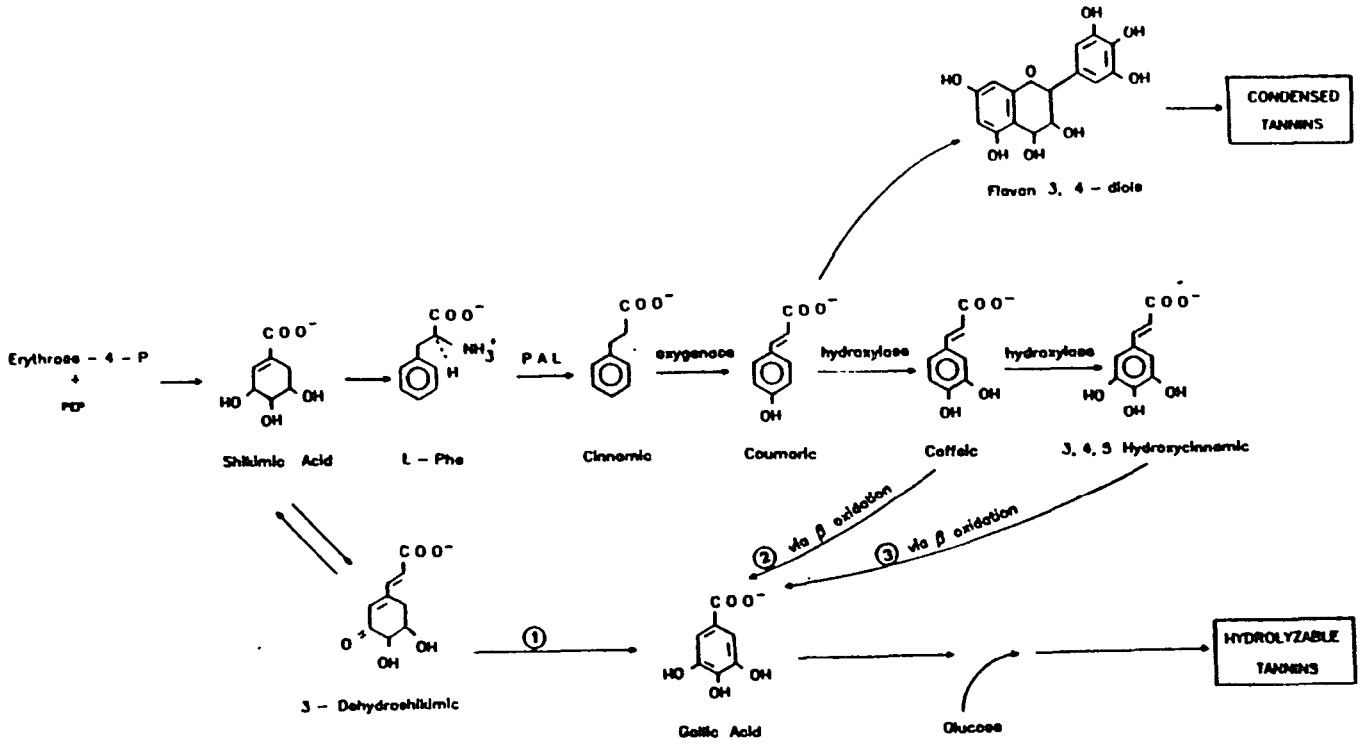


Fig. 3. Postulated biosynthetic pathways for condensed and hydrolyzable tannins (Haslam 1981; Swain 1979).

of condensed tannins starts with conversion of phenylalanine to cinnamic acid under enzymatic activity of phenylalanine-ammonia-lyase (PAL). A subsequent incorporation of oxygen into cinnamic acid leads to formation of coumaric acid and flavan-3-ols, flavan-3, 4-diols that by self-condensation form proanthocyanidin (Jones 1984).

Hydrolyzable tannins are formed from gallic acid by glucose dimerization of hexahydroxydiphenic acid. Gallic acid is found in free state in the bark of some trees and in the woody tissue of herbaceous plants. Three metabolic routes to gallic acid have been demonstrated and it is possible that, as isotopic tracer techniques improve, other biosynthetic pathways will be known. One pathway, identified in 1964 by Zenk (Haslam 1981), shows L-phenylalanine forming 3,4,5-trihydroxycinnamic acid that by Beta-oxidation yields gallic acid. Other pathway proposed by Chen et al. (1964) shows that Beta-oxidation of caffeic acid yields protocatehuic acid that hydroxylated gives gallic acid. The third metabolic route devised by Haslam (1981) indicates direct dehydrogenation of 3-dehydroshikimic acid yielding gallic acid (Fig. 3).

Tannins in Plant-herbivore Relationships

The ecological significance of tannins and their importance for plants, microorganisms, herbivores and man is due to the striking feature of these polyphenols: their ability to interact with proteins to form insoluble complexes (Swain 1979).

Tannin-protein complexes are formed mostly by hydrogen bonding between hydroxyls in the polyphenol and carbonyl groups in the peptide but ionic and hydrophobic bonds may also occur (Van Buren and Robinson 1969; Hagerman and Klucher 1986). According to Oh et al. (1980), hydrophobic interactions are just as important as hydrogen bonds in the formation of tannin-protein complexes.

Crucial points in the complexation with proteins are molecular weight of the tannin and conformational mobility and flexibility in the tannin substrate. Beart et al. (1985) mention that increase in molecular size when associated with abundance of phenolic groups on the periphery of the tannin enhances complexation with proteins. Endres and Herman (1963) also pointed out molecular weight and number of hydroxyl groups as prerequisites for protein complexation by a tannin. The

occurrence of reduced conformational mobility and flexibility in the tannin substrate lowers the protein binding capacity of the tannin.

Other factors such as temperature of the medium, pH, presence of electrolytes and nature of the protein are known to affect the formation of tannin-protein complexes. Oh and Hoff (1987) found that the best pH for protein precipitation is near the isoelectric point of the protein which suggests that electrostatic charges interfere with bond formation. Calderon et al. (1968) demonstrated that as far as complexation is concerned, the ability of condensed and hydrolyzable tannins to precipitate gelatin was affected by pH and nature of the medium. It has been recently demonstrated that tannins are very specific for proteins, that is, not all proteins are alike for these polyphenols. Hagerman and Butler (1980, 1981) demonstrated that sorghum tannins have higher affinity for proteins with hydrophobic amino acids such as glycine and glutamic acid, open loose compounds, substances of high molecular weight and, most importantly, proline-rich proteins. These proteins have a random coil structure with a great number of carbonyl groups available for bond formation.

The most obvious effect of vegetable tannins on animals seems to be their bitter taste and the reduction of lubrication in the mouth of herbivores due to a complex formation with glycoprotein and mucoprotein (Bate Smith 1954). Provenza and Malechek (1984) studied goat diets in relation to primary and secondary metabolites in blackbrush (Coleogyne ramosissima) and found that the animals preferred eating old twigs rather than current seasons growth. However, in blackbrush, high tannin levels were associated with higher protein levels and greater digestibility values. This was evident in the better performance shown by goats that were forced to eat current season twigs. The authors concluded that in the case of blackbush, goat nutrition was more affected by the effect of tannins on palatability than by the effect of tannins on digestibility. Goat selection against tannin-containing forage could represent a nutritional mistake to goats. Besides being an astringent taste that lowers feed palatability, tannins cause griping, diarrhea and constipation that also lead herbivores to avoid or eat smaller amounts of tannin-rich plants. Ingestion of tannin-containing plants can have a detrimental effect on animal growth (Reddy et al. 1985).

The growth-depressing effect of tannins in monogastric and ruminants is mostly the result of the high affinity of these phenolics for proteins, which adversely affects digestibility of protein, decreases nitrogen retention in the animal and lowers feed efficiency ratios (Price and Butler 1980; Laurena 1984).

The effect of tannins on rumen microbes was studied by Nastis and Malechek (1981) in a feeding trial with goats consuming diets made up of pure alfalfa hay, alfalfa plus 40 percent mature Gambel oak (Quercus gambelii); alfalfa plus 80 percent mature oak and alfalfa plus 80 percent immature oak. The study showed that intake was adversely affected by the presence of oak, mostly in the immature form, in the diet. Tannin-containing diets were digested to a less extent than alfalfa and resulted in higher levels of fecal nitrogen, indicating the formation of tannin-protein complexes. Other workers also observed a drop in voluntary intake of ruminants feeding on tannin-rich diets (Kumar and Singh 1984).

Although Krumholz et al. (1986) suggested that rumen microbes have a certain ability to degrade plant phenolics, the deleterious effect of tannins on the

ability of microbes to breakdown leaf protein, and also the occurrence of other tannin-inhibited microbial systems, are well documented in a number of studies (Watson 1975; Tagari et al. 1965). They all show inhibition of microbial activity as the result of a complex formation between the tannin and microbial enzymes. Smart et al. (1945) and Lohan and Negi (1979) noticed inhibition of rumen cellulase activity by extracts of sericea (Lespedeza cuneata) and oak (Quercus icana) respectively. According to Kumar and Singh (1984) enzymatic innactivation by tannins is due to either the formation of insoluble enzyme-tannin complexes or the formation of soluble but inactive complexes.

The level of dietary tannin necessary to elicit food rejection by grazing animals is around 20 mg/g of dry matter (Donnelly and Anthony 1969). However, the effect of tannins on ruminants goes beyond causation of food avoidance and detrimental interactions with rumen biota. In the omasum and abomasum, dietary tannins interact with digestive enzymes (Kumar and Singh 1984; Griffiths 1982) and react with the outer layer of the gut, decreasing the absorptive capacity of the animal (Mitjavila et al. 1977) and ultimately causing a

depression in food intake , poorer feed efficiency ratio and the appearance of signs of toxicity in herbivores.

Due to the huge molecular size of the tannin-protein complex, it cannot easily cross the intestinal membrane of herbivores. However, its accumulation at the site causes gastritis and intestinal edema. Condensed tannins are more easily attached to proteins than hydrolyzable tannins. On the other hand, hydrolyzable tannins are easily broken down and can be absorbed causing deleterious effects. Studies with herbivores ingesting hydrolyzable tannins have shown that these phenolics, and sometimes their metabolites, cause several toxic effects including accumulation of fat in the liver and kidney toxicity (Price and Butler 1980).

There are also reports of tannin toxicity in rabbits (Dollahite et al. 1962), sheep (Fowler et al. 1965) and cattle (Fowler 1965; Sandusky et al. 1977; Dollahite 1966; Panicera 1978; McLeod 1974). The way tannins and other phenolics cause toxicosis is still a matter of investigation but it is known that common symptoms in ruminants and monogastrics include elevated phenolic content in the blood, liver damage and, cellular sodium

imbalance (Camp 1967; Reddy et al. 1970; Freeland et al. 1985).

Research by Kingsbury (1965) and James et al. (1980) indicated that intake of oak leaves beyond 75 percent of the diet by browsing mammals could cause death. Cooper and Owen-Smith (1985) mentioned a threshold of 5 percent condensed tannins for herbivores, before which animals consuming tannin-rich plants must shift their forage preference to avoid death. Harper et al. (1988) discuss the high susceptibility of cattle to oak poisoning and the economic threat it represents for the Intermountain region of the United States.

The work of Korpassy et al. (1951), Dollahite et al. (1962, 1966) and Camp et al. (1967) provide support for the idea that hydrolyzable tannins may deserve more attention than condensed tannins as toxic substances because they can be easily broken down and being absorbed cause toxicity. On the other hand, condensed tannins require close attention in a nutritional sense because the non-hydrolyzed state indicates greater potential for biding dietary, microbial and endogeneous protein in herbivores which affect nitrogen economy of the animal.

There have been some reports in the literature concerning desirable effects of tannins on ruminant diets, mostly regarding a role on ruminal by-pass of dietary proteins and bloat prevention in cattle. In order to efficiently by-pass ruminal degradation, proteins must have low solubility to avoid their conversion to ammonia in the rumen in addition to being highly degradable to make possible cleavage at the abomasum. Price and Butler(1980) mention that tannin-protein complexes that do not dissociate post-ruminally serve no purpose to the animal. They also advert that most reports on desirability of tannis in ruminant diets are conflicting in regard to tannin effect on protein ruminal by-pass but seem to agree on the value of tannins on bloat prevention. At the moment, reports on deleterious effects of tannins on herbivores outnumber references on their desirability in animal diets.

Mammals counteraction against tannins in plant has been identified through exploratory behavior (cautious trial of novel foods), aversion to high-tannin plants and also the prevention of complexation of tannins with dietary protein and digestive enzymes. Robbins et al. (1987a) have found that production of proline-rich-

proteins (PRP) by parotid glands of browser mammals give them advantage over mixed-feeders and grazers in relation to utilization of tannin-rich plants. PRP's form soluble complexes with tannins and the dietary protein and enzymatic systems of the browser remain available to the animal. This naturally occurring system of tannin inactivation is by far more efficient than most others pursued.

Because tannins are genetically-based defenses (Luckner 1984) that work not only against ungulates but protect the plant against other environmental constraints (Zucker 1983; Swain 1979; Mann 1987) plant breeding for low-tannin forages may also result in lower primary productivity. On the other hand, the removal of tannins via chemical methods, as in treatment with NH4OH, NaOH and Ca(OH)2 or adsorption by polyvynilpirrolidone and polyethyleneglycol (Kumar and Singh 1984) have problems associated with the cost of these chemicals and loss of dry matter in treated feedstuffs.

The problematical aspects of tannin removal imposes the use of adapted animals as the best solution to the utilization of many tannin-containing agricultural wastes and agroindustrial by-products, in addition to

forage in savannahs, chaparral and other arid zones where the short supply of nitrogen and abundance of photosyntates favour the presence of plants with high content of tannin and other phenolics.

Range Management Considerations

Limitations to land use practices imposed by degree of aridity and other physical and even cultural factors in vast areas of the world determine a pattern of land occupation where a co-adaptive resource-use among plants, animal populations and humans remains to be optimized. For example, about 96% of the 460 million goats of the world live in the so-called Less Developed Countries (LDC) where they contribute to health, nutrition and economy of the people (Winrock International 1983). Most of the herding systems in the LDC are examples of negative feedback, control mechanisms and adaptive structures produced by plant, animal populations and humans in response to selective pressures. Examples are plant anti-herbivore defenses, herbivore counteraction and intervention by man (grazing, brush control, fire, etc.)

Ecologically-sound management of biomes like chaparral, savannas, caatinga and other arid type

environments throughout the world is dependent on the understanding of plant-animal interactions as affected by plant chemicals such as phenolic compounds whose abundance in the plant kingdom makes them the class of allelochemicals that herbivores are most likely to encounter (Rhoades and Cates 1976, Swain 1979).

Goats are one of the most valuable animals for studies on plant/herbivore relationships because they exert significant impact on the environment and tend to eat a multi-species diet (Knipe 1982, Sidahmed 1981).

Due to a need to overcome problems of having a small gut capacity and a higher nutrient requirement per unit of body weight than larger ruminants (Demment and Van Soest 1983) goats evolved anatomical structures and behavioral adaptations like prehensile lips and bipedal stance that help them to exert a better selection of plant parts, presumably the more nutritious. Furthermore, they possess physiological adaptations such as a faster rate of passage which enable them to obtain a greater nutritional reward per unit of feeding bout than larger herbivores provided the right decision on what to eat has been made (Van Soest 1982).

Several studies have reported goats as shrub or forb preferring, intermediate feeders with a high perception for changing forage conditions (Devendra and Burns 1983) in addition to being able to perform well on vegetation considered toxic to other herbivores (Cooper and Owen-Smith 1985, Harrington 1980). According to Nastis and Malechek (1981) and Davis et al. (1975), goats can utilize Quercus sp. in a productive manner, without suffering nutritional constraints or toxicity imposed by the presence of tannins in those plants. The toxic level of tannins for goats is an intake between 8-10 percent of the diet while the tannin-toxicity for cattle is around 3-5 percent of the diet (Kumar and Singh 1984).

The interior chaparral, also called Arizona Chaparral, a discontinuous vegetation association covering approximately 1.2 million hectares across the central part of the state, from north to southwest, along the slopes of the Mogollon Rim (Pond and Schmutz 1984) is an example of a vegetation type dominated by tannin-rich plants. Arizona chaparral has six species of oak (Quercus sp.). One of them, shrub live oak (Quercus turbinella), comprises more than 50% of vegetation composition by weight (Knipe et al. 1979). Ruyle et al.

(1986) observed that Quercus turbinella is very abundant in Arizona Chaparral, being the most widely distributed of 12 native oaks and making up to 40 percent of cattle diets on this vegetation type during the dormant season. The abundance of tannins in oak has been documented in a number of studies (Nastis and Malechek 1981; Harper et al. 1988).

This research focuses on the diet selection by goats on Arizona chaparral, identification of the phenolic profile and *in vitro* digestibility of shrub live oak, and the impact of condensed and hydrolyzable tannins on goats.

MATERIAL AND METHODS

Working Hypotheses and Crucial Experiments

This study was undertaken to investigate the following aspects of plant and herbivore interactions through a shrub/goat model: (1) diet selection by goats grazing on a shrub live oak community in the interior chaparral of Arizona; (2) phenolic profile of shrub live oak (Quercus turbinella) in Arizona Chaparral;(3) digestibility of shrub live oak (Quercus turbinella) by goat rumen biota throughout various phenologic stages; and (4) protein precipitating capacity of extract from shrub-live oak.

Following a methodology proposed by Platt (1964), the research pursued the hypotheses below through the respective experiments:

Hypotheses

1) The diet of goats in Arizona chaparral includes species that have been regarded as poisonous to other herbivores or inhibit ruminal digestibility.

2) Spatial and temporal allocation of phenolic compounds in highly preferred plants affects the pattern of goat herbivory by forcing animals to reduce consumption of these plants when phenolic compounds are abundant and/or to adopt multi-species dieting to dilute the toxic effect of the phenolic compounds.

3) The protein precipitating capacity of extracts from the most abundant plants in the diet of goats on Arizona chaparral influences goat herbivory.

4) Phenolic-containing plants are valuable constituents of the diet of goats on Arizona chaparral due to the ability of the goat rumen biota to utilize herbage from such plants.

Experiments

1) Estimate botanical composition of diets of Spanish goats on Arizona chaparral.

2) Estimate amounts of condensed and hydrolyzable tannins in shrub live oak selected by goats grazing on Arizona chaparral.

3) Estimate protein precipitating capacity of shrub live oak herbage.

4) Estimate apparent <u>in vitro</u> dry matter

digestibility of mature and immature oak leaves by goat rumen microbes.

Study Area

The study site was located on the Conway Ranch in Greenback Valley, 26 km SE of Punkin Center, Tonto Basin, in the central part of the Arizona Chaparral, within Sections 4,5,8, and 9; Township 6N, Range 12E (Fig. 4). The terrain is rugged and rocky and the topography is rolling to hilly with slopes derived from diabase parent material.

Precipitation records from a neighboring station show an average of 508 mm per year of which 60 percent falls from October to May and 40 percent from June to September. Previous studies in the area (Pond and Schmutz 1984) indicated that part of the winter precipitation is stored in the soil and used by the vegetation in the spring.

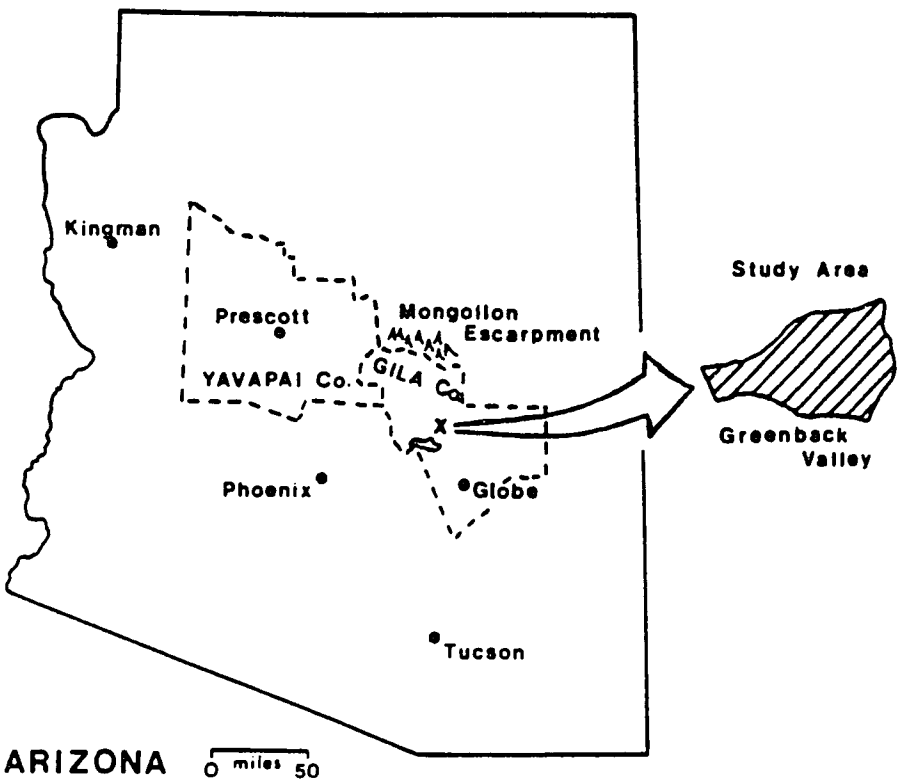


Fig. 4. Location of Conway Ranch in Greenback Valley, Tonto Basin, Globe, Arizona where the field studies were conducted.

Vegetation

The vegetation of the area is characterized by a dominance of shrub live oak-mixed shrub community, formed by Quercus turbinella and a mix of other shrubs (Knipe et al. 1979). This vegetation type has been described by Brown and Lowe (1980) as interior chaparral. Besides shrub live oak, which accounts for more than half of the species composition by weight in this vegetation, desert ceanothus (Ceanothus greggii), and manzanita (Actostaphylos pringlei and A. pungens) rank second in abundance. Other shrubs include Palmer oak (Q. chrysolepis), mountain mahogany (Cercocarpus betuloides and C. montanus), catclaw (Acacia greggii), wait-a-bit (Mimosa biuncifera), skunkbush sumac (Rhus trilobata), pricklypear (Opuntia engelmannii), hollyleaf buckthorn (Rhamnus crocea), and false mesquite (Calliandra eriophylla).

Other perennial plants in the area include the half-shrub buckwheat (Eriogonum wrightii), broom snakeweed (Xanthocephalum sarothrae), and rough menodora (Mendora scabra). Spring and summer moisture make possible the appearance of forbs and grasslike plants like jimson-weed (Datura stramonium), encelia (Encelia

sp.), Fremont goosefoot (Chenopodium fremontii), James bundleflower (Desmanthus cooleyi), filaree (Eriodium cicutarium), and scurf-pea wild alfalfa (Psoralea lanceolata). Trees include one-seed juniper (Juniperus monosperma, pinyon (Pinus edulis), Emory oak (Quercus emory), and velvet mesquite (Prosopis velutina). In the open spaces of the understory and along the washes there are grasses such as red three-awn (Aristida longiseta), gramas (Bouteloua gracilis and B. curtipendula), red brome (Bromus rubens), curly mesquite (Hilaria belangeri), bottlebrush squirreltail (Sitanion hystrix), and fluff grass (Erioneuron pulchellum).

Diet Study (Experiment 1)

A group of 10 goats out of a herd of approximately 500 were monitored for diet selection from May 1988 to April 1989. The goat herd was kept in a short duration high stocking density grazing system (Savory 1978) in operation since February 1984. The total area was 435 ha, divided into 17 paddocks of varying sizes, enclosed with a 6-wire perimeter electric fence and cross fenced with 4-wire electric fence.

In order to avoid neonatal deaths due to coyote (Canis latrans) predation, improve postpartum nutritional

condition of the does and minimize kidding interval the herd was kept in a 40-acre, predator-free, pasture where it was fed alfalfa hay Ad libitum plus "scratch feed", a mixture of corn, sorghum and wheat having 9% crude protein, 25% crude fat, 4% crude fiber and 3% ash (Southwest Co-operative Wholesale, Phoenix, Az.).

The diet was determined by microhistological analysis of fecal material collected once a month. At each sampling time the animals were followed throughout the area and the feces were collected immediately after defecation.

Fecal samples were preserved in alcohol and analyzed at the Range Laboratory of the School of Renewable Natural Resources, University of Arizona, following procedures outlined by Sparks and Malechek (1968). Each sample was ground in a blender for 3 minutes with hot water, then removed and rinsed through a .1 mm sieve. From the remaining material, two microscope slides were prepared. The slides were examined under a 125 power microscope using 20 random fields to determine frequency of identifiable plant epidermial fragments. Plant species were identified in the slides using reference slides prepared from all

plants in the study area. The frequency percentages were converted to particle density and relative densities were considered as an estimate of plant percent composition by weight for each species observed (Curtis and McIntosh 1950, Sparks and Malechek 1968).

Analysis of data on percent of oak in the diet of goats was performed by using the Statistical Analysis System (SAS 1985), following a two way mixed ANOVA according to model $y_{ij} = \bar{x} + a_i + m_j + e_{ij}$, as indicated in Table A2 and A3.

Phenolic Profile of Shrub Live Oak (Experiment 2)

The most important component of the diet of goats, shrub live oak, was analyzed to detect the presence of low-molecular-weight phenolics, condensed tannins and hydrolyzable tannins. The Acid-Butanol method (Porter 1986) was applied to separate low-molecular-weight phenolics and to determine condensed tannins (proanthocyanidins). Hydrolyzable tannins were measured by the rhodanine-gallic acid assay (Inoue and Hagerman 1988).

Collection of plant material

Mature and immature leaves of shrub live oak were collected once a month, between May 1988 and April 1989. The sampling was done by following the goats in the pasture during their major morning and afternoon feeding bouts and removing mature and immature leaves from oak plants visited by them. The area of the pasture was divided into three locations for leaf collection and the samples were collected from a total of at least 30 shrubs from each location. Both mature and immature leaves were hand-removed at the nodes from various points within the canopy.

Once removed from the plant, the leaves were placed in a plastic bag, identified, frozen overnight and lyophilized as soon thereafter as possible in a freeze-dryer with maximum temperature of 18^{o} C during sublimation. Lyophilized leaves were ground in a Wiley mill fitted with a 1mm screen. The finely ground material was placed in plastic bags stored at room temperature away from oxygen, moisture and light as suggested by Rey (1977) until used for subsequent analysis. These leaf samples were utilized in all analysis of plants involved in this research.

Condensed Tannins (Acid-butanol assay)

All chemicals were reagent grade or the best available.

Preparation of leaf extracts

Duplicate samples of 500 mg of lyophilized leaf powder were extracted in 5 ml of 70% aqueous acetone for 30 minutes at room temperature by rotation in a Labquaque Shaker (Labindustries Inc., Berkeley, California 94710). After extraction, the material was centrifuged at 200 rpm for 1 minute at room temperature and the supernatant was discarded. The average volume of crude extract obtainable from mature (3.75 ml) and immature (3.73 ml) oak leaves was determined from a group of ten samples of each kind of leaves.

Assay

An aliquot of 0.2 ml of crude extract was transferred to a culture tube with screw cap and diluted with 0.8 ml of 70% acetone. To this sample, 6.0 ml of Butanol-HCl (95:5 v/v) and 0.2 ml of 2% (w/v) Ferric ammonium sulfate were added. The mixture was vortexed and absorbance at 550 nm (A 550) was read and compared to a control tube having all chemicals but the extract. This background absorbance was considered an

effect of the low molecular phenolics present in the sample. Then, the tubes were heated with lose screws in a shaking water bath at 90^{o} C for 50 minutes. After cooling in ice for 10 minutes the samples were read for absorbance at 550 nm. The background absorbance was subtracted from the final absorbance (Porter et al. 1986).

The assay was standardized with purified sorghum tannin (Hagerman and Butler 1980) by applying the same procedures used for leaf extract to solutions of .074, .0147, .245, .30, .60 and 1.0 mg of sorghum tannin in 70% acetone. The equation of the regression line was:

$$Y = 5.04 \text{ (mg tannin)} + 0.019$$

with a R squared of 0.988, a standard error of 0.140 for the slope and 0.045 for the intercept.

Absorbance values were converted to mg of tannin by the formulas below where b = slope and a = intercept of regression line.

$$Abs = b \text{ (mg tannin)} + a$$

and $$\frac{\text{mg tannin}}{0.2 \text{ ml}} * \frac{\text{ml extract}}{0.5 \text{ g plant}} = \text{mg tannin/ gram of plant}$$

The experimental design was a randomized block design repeated over time. Data on mg tannin / gram dry matter of mature and immature leaves and monthly variation of condensed tannins in oak were analyzed by a two way analysis of variance by using the Statistical Analysis System (SAS 1985) according to the ANOVA table shown in Table A6.

Hydrolyzable Tannins (gallic acid assay)

The determination of hydrolyzable tannins in oak leaves was made on the basis of the reaction between rhodanine (2-thio-4-Ketothiazolidine) and gallic acid as described by Thies and Fischer (1973) following the procedures outlined by Inoue and Hagerman (1988).

Reagent grade chemicals were used throughout the study. Rhodanine was purchased from Sigma Chemical Company (St.Louis, MO) and gallic acid was obtained from MCB (Norwood, OH). Rhodanine was kept below 0^{o} C and gallic acid was stored at room temperature. The assay was standardized with solutions of 10 mg of gallic acid dissolved in 100 ml of 0.2 N H2 SO4. Aliquots of 0.2, 0.4, 0.7 and 1.0 of the gallic acid solution along with a blank were taken. These were assayed with rhodanine by adding 1.0, 0.8 , 0.6, 0.3 and 0 ml of 0.2 N H2 SO4

aqueous solution respectively and treating all samples with the addition of 1.5 ml solution 0.667% rhodanine / methanol. After waiting 5 minutes, 1.0 ml 0.5 M KOH aqueous solution was added; after 2.5 minutes the samples were diluted with 25.0 ml of distilled water. Between five and ten minutes later the absorbance at 520 mm was read. Least squares linear regression on 4 replicate analyses generated the following equation:

$$Y = 5.08 \text{ (mg gallic acid) } + 0.018$$

with a R squared of 0.959 and standard errors of 0.039 and 0.247 for the intercept and slope respectively.

Assay

A sample of 500 mg of lyophilized leaves was placed in a test tube and extracted with 5 ml of 70% acetone for 18 hours at 4^{o} C with continuous shaking in a Labquaque Shaker (Labindustries Inc., Berkeley, Ca., 94710). After extraction the samples were centrifuged at 200 rpm for 1.5 minute and the supernatant was removed. A 1.0 ml aliquot of each sample was transferred to one ampule and 5.0 ml of 2 N H2 SO4 was added. The ampule was vacuum sealed and heated for 26 hours at 100^{o} C. After this acid hydrolysis, one aliquot (120 micro liters) of the hydrolyzate was transferred to a graduated tube, 880

micro liters of distilled water was added and that 1.0 ml was assayed with rhodanine.

The assay was performed by adding 1.5 ml of .667% methanolic rhodanine solution to each sample. After 5 minutes, 1.0 ml 0.5 N aqueous KOH solution was added. Then, after 2.5 minutes, distilled water was added to the mixtures to bring the volume to 25.0 ml. Between 5 to 10 minutes later, the absorbance at 520 nm was read. After converting absorbance to mg of gallic acid by the formula generated by a standard curve the amount of gallic acid per gram of plant (X) was calculated by taking into account all dilutions and the mass of the samples by the formula:

$$\frac{\text{mg gallic acid}}{\text{1 ml hydrolyzate}} * \frac{\text{20 ml}}{\text{1.0 ml extract}} * \frac{\text{3.0 ml extract}}{\text{0.5 g plant}} = X$$

For both condensed and hydrolyzable tannins the experimental design was a randomized block design repeated over time and statistical analyses of variance were performed to test for differences between mature and immature leaves and differences between monthly levels of tannins in leaves of oak as seen in Table A6 and A8.

All data were analyzed using the Statistical Analysis System (SAS 1985).

Protein Precipitating Capacity of Extract of Oak Leaves (Experiment 3)

The assessment of protein binding capacity of phenolics present in the staple food of goats on Arizona Chaparral was done through the radial diffusion method developed by Hagerman (1987). The procedure is similar to radial immunodiffusion assay described by Vaerman (1981) in which the phenolic is diffused through a protein-containing gel resulting in the formation of a visible disk-shaped precipitate that can be measured.

Chemicals used were Agarose (type I) and Bovine Serum Albumin (BSA) fatty acid free, fraction V, purchased from Sigma Chemical Company (St. Louis Missouri).

A buffer was prepared from 50 mM Acetic Acid and 60 mM ascorbic acid adjusted pH 5.0.

Preparation of the medium

A 1 % w/v solution of agarose was prepared in the buffer by heating it to boil and adding agarose. The solution was cooled in a water bath at 45^{o} C. When the

temperature reached 48° C the protein (0.1 % w/v) BSA was added while gently stirring the solution. Then aliquots of 9.5 ml were pipeted in plastic Petri dishes of 8.5 cm diameter and allowed to cool on a level surface in order to form slabs of uniform thickness. After preparation the Petri dishes were sealed with parafilm and stored at 4° C to avoid bacterial growth.

Plant Extraction

Extract from mature and immature oak leaves were prepared by identical procedures followed in the tannin analysis.

Assay

Four wells of 4.0 mm diameter spaced of 1.5 cm were perforated in the Petri dishes with a punch (Biorad Co., Richmond, California). Three aliquots of 8 ul of centrifuged leaf extract were added to the wells with a Hamilton microsyringe. Then, the Petri dishes were identified and covered with parafilm and incubated for 120 hours in a oven at 30° C. The tannins diffused through the medium and formed a visible disc precipitate that was measured as the square of the average of two diameters. Since the extract contained both kinds of

tannins, and their protein precipitating capacity are additive in this medium (Hagerman 1987) they were analyzed as the amount of protein precipitated per ml of leaf extract. The experimental design was a randomized block design repeated over time. Data on diameter squared of protein precipitated were analyzed using SAS (1985) and following a two way analysis of variance according to Table A10.

Apparent Dry Matter Digestibility of Oak Leaves (Experiment 4)

A two-stage in vitro digestion was performed according to procedures of Tilley and Terry (1963) and Goering and Van Soest (1970) to measure the effect of leaf age and phenolic content on apparent dry matter digestibility for goats and on rumen microbes and their enzymes.

Two female goats selected from the herd to serve as inoculum donors were taken to the Animal Care Unit at the University of Arizona to receive permanent ruminal fistulae. The canulas used were the plastisol type, 3.5 cm in diameter with 5.0 cm of wall thickness, (5C made by Bar Diamond, Inc. Parma, Idaho 83660). The surgery was

performed at the University of Arizona Hospital by Veterinarians of the Vet-Diagnostic Lab of the U of A. The procedure consisted of a two-stage operation starting with a laparotomy for dissection of abdominal muscles followed by suture of the rumen to the skin. Then, a incision was made through the exposed rumen wall to create a fistula through which the cannula was inserted according to procedures described by Hecker (1969).

After surgery, the goats were taken back to the study area for recovery and readaptation of host animal and rumen biota to the browse diet for five weeks.

Incubation

All chemicals utilized were reagent grade or the best available. Before the incubation, rumen fluid and ingesta were taken through the cannula with a large spoon, placed in a preheated thermal chest, flushed with CO2 from a portable tank and transported to a lab facility set up nearby. Rumen fluid were pipeted into test tubes within half an hour from removal from the goats. In the lab, rumen contents were flushed again with carbon dioxide and blended for 3 minutes and strained through two layers of muslin.

The microbial digestion was conducted by fermenting duplicate samples of 300 mg of lyophilized, mature and immature leaves in plastic 50 ml X 25 mm X 1300 mm nalgene tubes containing 24 ml medium and 1.2 ml reducing solution (Goering and Van Soest 1970, Milchunas and Baker 1982). Both the medium and the reducing solutions were gassed with CO2 to ensure anaerobic conditions throughout the incubation.

A 6.0 ml aliquot of rumen inoculum was added to each tube, then a flush of CO2 was applied and the tubes were sealed with a rubber cork fitted with a 1 mm bunsen gas valve to release fermentation gases and were put in water bath at 40° C. The incubation times for both kinds of leaves were 6, 12, 24 and 48 hours. Tubes containing inoculum and medium but no substrate (blank) and others containing a sample (300 mg) of alfalfa hay (standard) were run simultaneously. All tubes were shaken every two hours during incubation.

Upon removal from the water bath, 1.0 ml toluene was added to each tube as a preservative and they were stored in freezer until a pepsin digestion was done to eliminate insoluble portion formed by unchanged leaf protein plus microbial protein.

Pepsin digestion

Rumen biota activity was checked with 2.0 ml 6 N HCl added to each tube prior to the addition of 300 mg of Pepsin N. P-7125 from Sigma Chemical Company (St. Louis, MO 63178) and 1.0 ml of toluene. The tubes were then incubated at 40° C for 6, 12, 24 and 48 hours. After incubation, tubes were washed with water and acetone in filter paper (Whatman N.2), dried at 100° C and weighted.

Calculations for apparent dry matter digestibility (X) were performed by the formula:

$$[\ 100-[\ (R - F) - \text{Blank} / \text{oven dry residue}\ ]\ ] * 100 = X$$

where, R = weight of residue and filter paper, F = weight of filter paper and , R - F = weight of the blank (Goering and Van Soest 1970).

The data on apparent dry matter digestibility were analyzed using SAS (1985) in a three-way analyses of variance to test for differences between digestibility at each month, digestibility of mature and immature leaves and differences among incubation times in a randomized block design repeated over time (Table A12).

RESULTS AND DISCUSSION

Dietary Selection

Shrub live oak (Quercus turbinella) was the staple browse species for the entire period of this study. During 10 out of 12 months goat diets included between 50 and 72 percent of this species. In November and March, oak still comprised 36 and 39 percent of the diet, respectively (Fig. 5 and Table A1). Tables A2, A3, and A4 show the difference ($P = .0001$) between months in terms of percent oak in the diet.

Focal animals seemed to prefer new leaves and shoot ends at all times devoting little attention to more mature tissues of oak.

The average number of plant species in the diet for the whole period of the study varied between 5 and 10 (Table A14). The goats showed a high response to changes in foraging conditions as observed by the fluctuation of relative and absolute values of oak throughout the study. The results do not support the multi-species concept as suggested by Bryant et al. (1985) that browsing mammals which ingest plants with high levels of defense compounds during the winter need to expand their range of species

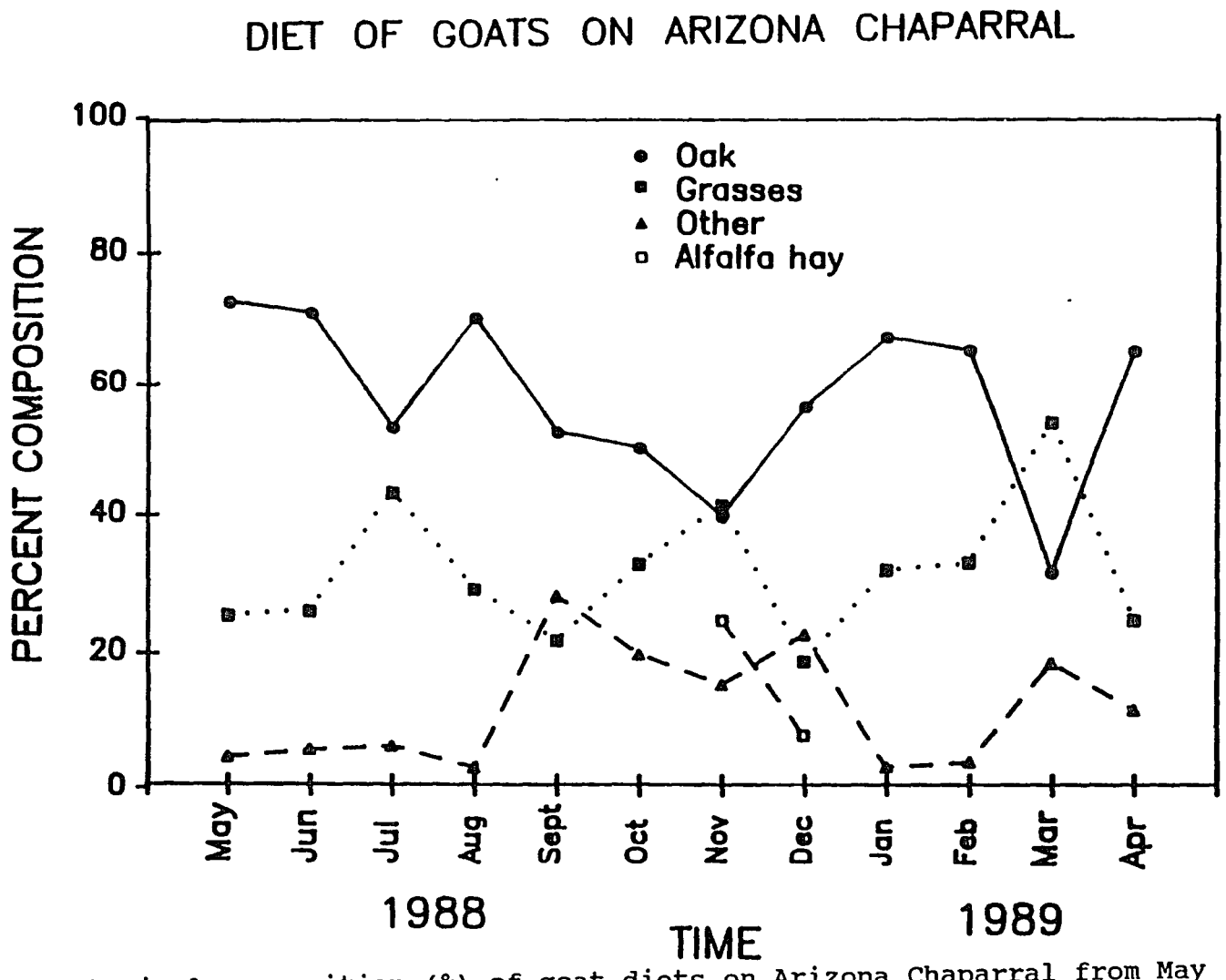


Fig. 5. Botanical composition (%) of goat diets on Arizona Chaparral from May 1988 to April 1989.

acceptability in order to dilute the effects of toxic compounds. In this study, when oak increased in the diet of goats fewer species were recorded in the overall diet.

These data seem to better fit the model of Owen-Smith and Novellie (1982) which predicts that as forage resources deteriorate animals tend to expand the range of plant acceptability by consuming less palatable species. For example, by late summer (August) goats were eating high amounts of oak and feeding on an average of 5 different plant species (Table A14). During the fall, when immature leaves became less available, goats fed on an average of 9 different species including other shrubs and perennial grasses. During the winter they restricted the number of species in the diet feeding on 6 to 8 different species between December and February (Table A14). By March the goats increased the number of species in their diets, presumably in response to good spring growing conditions. They ate large amounts of forbs and shrubs other than oak. Annual grasses were almost absent from the diets. In April they returned to eat large amounts of oak and decreased to about half the number of species in their diets.

This sharp shift in oak utilization from March to April could be interpreted as a response to an ephemerally high level of protein, common to shrub live oak at this time of the year (Pond and Schmutz 1984). Assessment of nutritional value of shrub live oak in the same area, for two consecutive years by these authors showed that crude protein peaked at 22 and 16% by mid April of 1962 and 1963, respectively, and dropped in May to values near 8% staying at that level for the rest of the year. Selection for plants with higher levels of leaf protein may not only increase the proximate nutritional quality of the diet for goats but may also allow more room for dietary components with elevated levels of protein precipitating substances.

Overall, the results confirm several general observations. First of all, goats are primarily browse eaters and exhibit a high level of selectivity for plant parts. A strong preference for shrubs was demonstrated during November and December when they were fed alfalfa hay ad libitum and still included considerable amounts of oak, grasses and other shrubs and forbs in their diet. They also take advantage of changes in foraging conditions by including grasses in their diets and shifting from their most preferred browse species to

sample new growth of shrubs and forbs whenever it is available, for example, they ate considerable amounts of Rhus trilobata, Ceanothus greggii, Opuntia engelmannii, Prosopis velutina, Erodium cicutarium, Mimosa biuncifera, and Encelia farinosa.

Phenolic Profile of Oak Leaves

Condensed tannins in oak

Acetone/water extracts of oak leaves contained both condensed and hydrolyzable tannins in addition to low-molecular-weight phenolics that received no further attention in this study due to their inability to precipitate protein and little impact on feeding behavior and physiology of herbivores (Haslam 1981). Table A5 and Fig. 6 show the amounts of condensed tannins in mature and immature oak leaves throughout the year , measured as mg of condensed tannin per gram of leaves on a dry matter basis. Table A6 shows a significant difference ($P = .0001$) between montly values of condensed tannins in oak and also between tannin amounts in young and older leaves.

The pattern of accumulation of tannins in plants is not completely understood. Baldwin et al. (1987) mention that the amount of tannins can be influenced by

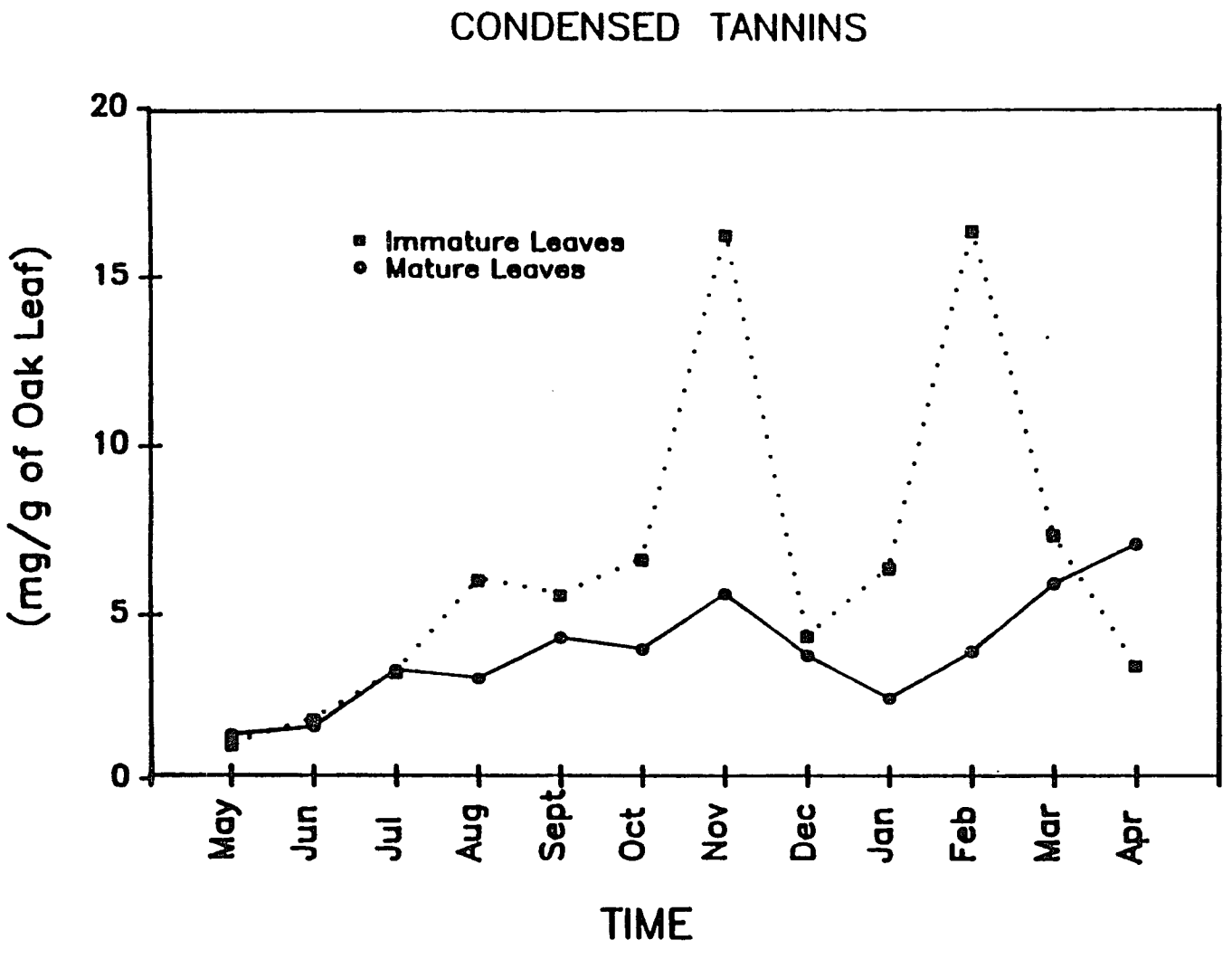


Fig. 6. Variation in condensed tannins (Proanthocyanidins) in mature and immature oak leaves in Arizona chaparral.

season, age of the plant, genotypic differences, physical stress and presence of pathogens. This study showed that condensed tannins in shrub live oak are mostly synthesized during winter/early spring (Fig. 6).

Haslam (1977) maintained that a striking feature of procyanidin-producing plants is the conspicuous biogenesis of these polyphenols at a late stage of growth as seen in the autumnal coloration of leaves in many plants. The nature of this phenolic content has been observed to vary tremendously within a species (Coley et al. 1985). In this study (Fig. 6), the concentration of condensed tannins tended to be higher in immature leaves. During late spring (Mar-Apr) and early summer (May-Jul), levels of proanthocyanidins in young leaves remained close to the amounts found in older foliage. But by August, the abundance of condensed tannins in newly initiated leaves began to increase progressively until it reached a value 19 times higher than mature leaves in November. Following the November boost, the amount of condensed tannins dropped during December and January, peaked again on February but decreased in April. Abrupt increases in phenolic content were also observed by Feeny (1970) in Quercus robur when levels of tannin showed a tenfold increase from April to September.

The level of condensed tannins in older leaves varied little from May to December but increased continuously after that to a twofold value in March and dropped again in April. The more intense synthesis of condensed tannins in oak younger leaves during the fall and winter is presumably a means to protect tissue that will initiate carbon fixation in the following growing season. Feeny (1970) showed that the pattern of predator protection in immature oak leaves is either by a decrease in protein availability via phenolic precipitation or by an increase in the repellent astringent taste that lowers the hedonic value (palatability) of these leaves to non-adapted herbivores. On the other hand, the protection of older leaves is exerted by a decrease in protein content that becomes more prominent with maturation. Robbins et al. (1987) mentions that the decrease in nitrogen availability in leaves can not only be due to precipitation of proteins by polyphenols but also by an increase of non-digestible, fiber-bound protein as deposition of lignin takes place in shrubs.

The synthesis of increased concentrations of condensed tannins in November 1988 and February 1989 showed in this study (Fig. 6) may be a response to water stress during these months. Precipitation recorded in the study area (Table A13) shows that November 1988 and February 1989 were drier than the same months in the last three years. Moreover, the total precipitation in 1988 was lower than that in the previous year.

Water stress and its associated nutrient shortage decreases the rate of protein synthesis, reduces growth and affects many other biochemical processes in plants. Tan (1980) showed that nutrient stress, mainly nitrogen and potassium, increases the activity of phenylalanine ammonia-lyase. Under this condition, deaminated phenylalanine and unused photosynthates are diverted to the synthesis of lignin precursors and tannins (del Moral 1972). It has been documented in a number of studies that unfavorable conditioins for protein synthesis result in an accumulation of large amounts of phenolic compounds in plants (del Moral 1972; Tan 1980; Gershenzon 1983). Dement and Mooney (1974) observed that photosynthates produced by Heteromeles arbutifolia in California chaparral during the winter, before favorable conditions for leaf initiation appear, are utilized by the plant

for synthesis of both protective and structural compounds.

Increased production of condensed tannins during periods of water and nutrient shortage may have an adaptive value for oak because it can help reduce herbivory at a time when the cost to replace foliage taken by herbivores and pathogens is higher than it would be during periods of adequate conditions of water and nutrient supply.

Hydrolyzable tannins in oak

Hydrolyzable tannins selectively determined in the crude extract of oak leaves in the presence of condensed tannins and other phenolics is shown in Table A7 and Fig. 7. The findings in this study indicate that, as in the case with condensed tannins, phenological stage of the plant determined a significant variation in the amount of gallotannins in oak. The synthesis of gallotannins in oak seems to be restricted to periods of more intense physiological activity: in late spring and summer. These polyphenols may reduce herbivory on oak foliage in a juvenile growth-stage since the mature foliage had significantly lower values of hydrolyzable tannins during most of the year. Table A8 shows

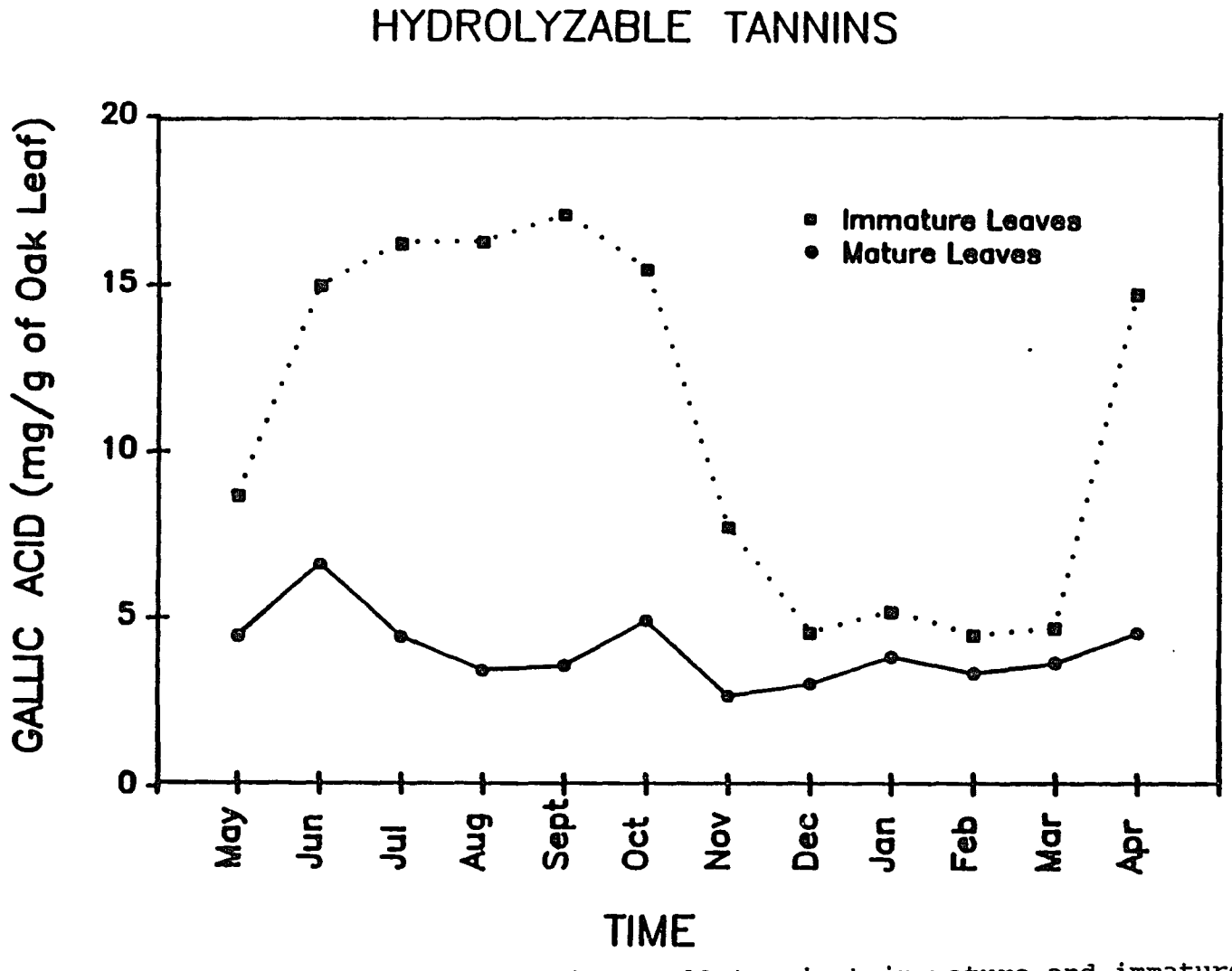


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Fig. 7. Variation in hydrolyzable tannins (gallotannins) in mature and immature oak leavess in Arizona chaparral.

significant differences (P = 0.0001) between levels of hydrolyzable tannins in oak during different months and between young and older oak foliage.

A plausible explanation for this pattern of defense is the role of polyphenols in the economy of higher plants as reported in several investigations. Beart et al. (1985) pointed out that biosynthesis of high concentrations of tannins involves a substantial input of energy and interferes with photosynthetic efficiency of the plant. Furthermore, the cost of production of secondary compounds plus the cost of sequestering them away from cytoplasmic processes draws energy that could be used for growth. As indicated in Fig. 3, hydrolyzable tannins may have a cheaper metabolic cost to the plant. The production of condensed tannins by oak during periods of shorter daylength and the production of hydrolyzable tannins at times of more intense vegetative growth represent a use of photosynthates to produce more "expensive" tannins only at times when conditions for growth were not prevalent.

Our data indicate that hydrolyzable tannins in newly initiated oak leaves increased from 8.52 mg/g dry matter at the onset of the growing season in May to values between 15 and 16 mg /gram dry matter throughout

summer and fall (July-October). Between November and March the level of these phenolics in younger leaves had a dramatic decline to values close to that in older foliage. At the beginning of the spring growth conditions in April hydrolyzable tannins that were at 4.55 mg/g dry matter the month before increased to 14.57 mg/g dry matter, a 3-fold increase.

Leaf chemistry and food selection

The pattern of tannin allocation in oak in this study (Fig. 6 and 7) covers all periods of physiological activity of the plant. A comparison between presence of tannins in shrub live oak documented here and the abundance of tannins in gambel oak (Quercus gambelii) as reported by Nastis and Malechek (1981) indicates a rather small concentration of tannins in shrub live oak. For example, plant species differ in the magnitude of their chemical defenses but the variation between the size of phenolic pool in shrub live oak as reported in this study and the findings reported by Nastis and Malechek (1981) is more likely attributable to the methods utilized. The

use of Folin-Denis method by Nastis and Malechek (1981) lead them to overestimate tannin content in gambel oak since that technique is a redox assay which measures total phenolics and includes non-tannin phenolics, protein, catechin - a monomeric unit of condensed tannins, and other oxidizable compounds that do not precipitate protein.

By isolating low molecular phenolics, breaking down phenolic content into condensed and hydrolyzable tannins and determining the protein binding capacity of extract of oak leaves, the values presented here more likely represent the biological significance of tannins in oak (Hagerman and Robbins 1987). A look at levels of both condensed and hydrolyzable tannins throughout the year (Figs. 6 and 7) reveals that, except for December and January, and to a less extent in March, there were always high levels of tannins in oak. The levels of hydrolyzable tannins in oak were approximately constant throughout the summer while condensed tannins did not appear until late June. These results corroborate the findings of Feeny and Bostock (1968) who studied changes in tannin content of Quercus robur. Bryant and Kuropat (1980) also observed that the phenolic compounds in

various plants in a subarctic habitat tended to complement one another and forced herbivores to eat a varied rather than a single species diet.

The goats in this study included a large number of species in their diets (Fig. 5) (Tables A1 and A14) despite the limited chaparral vegetation diversity. Goats also demonstrated a great ability to handle both condensed and hydrolyzable tannins in their diets and showed a marked preference for oak, consuming considerable amounts of it even when alfalfa hay was offered ad libitum during November and December (Fig. 5). The oak consumption by goats in July, November and March, shown in Fig. 5 indicates an opportunistic feeding behavior. Goats included in their diets some of the rarer ephemeral species that appeared in those months. However it is noticeable that these dietary shifts occurred at times of boosts in hydrolyzable tannins (July) and during the first peak of condensed tannins in November and shortly after the second peak in February.

It appears that peaks of condensed and hydrolyzable tannins in oak have a lagged effect on the dietary selection since following peaks in tannin content

in oak leaves the goats reduced intake of oak for a short time and then they quickly adapt to consume higher levels of tanniferous forage (Fig. 5, 6, and 7). For example, as levels of hydrolyzable tannins increased from 8.52 mg/g dry matter in May to 14.81 mg/g dry matter in June the amounts of oak in the diet remained the same. After a further increase in the amounts of hydrolyzable tannins to 16.07 mg/g dry matter in July the goats selected a diet with significantly lower amount of oak. By August the presence of oak in the goats diets returned to values similar to the levels in May. With the maintenance of higher levels of hydrolyzable tannins throughout September and October, the goats again reduced their oak consumption to amounts similar to their intake in July when the increase of hydrolyzable tannins began to stabilize.

From November to March hydrolyzable tannins in immature leaves stayed near the lower values found in older leaves. Amounts of oak in the diet dropped considerably by November corresponding with the peak in the synthesis of condensed tannins. This peak in tannin levels was followed by an apparent period of plant dormancy in December and January when the consumption of

oak by goats increased steadily (Fig. 5). Another drop in oak consumption was recorded in February (Fig. 5) after another steep increase in the amount of condensed tannins in oak (Fig. 6). In March, with the onset of growing conditions, the plants began to produce hydrolyzable tannins and kept the values of condensed tannins in new leaves close to that of the older leaves. At this time, the percent oak in the diet of goats was increased to levels similar to those observed during the summer.

Recent related investigations have concluded that plant defense against browsers operates mostly as toxicosis created by the absorbed phenolics rather than digestion reduction since most phenolics that can reduce digestibility in browsers occur at smaller fractions in most plants (Robbins et al. 1987; Bryant et al. 1985). The observation that hydrolyzable tannins occur in greater abundance than do condensed tannins in oak made in this study (Fig. 6 and 7) tends to support these conclusions.

An explanation for high levels of oak in the diet of goats could be the presence of salivary proline-rich proteins produced by goats (Robbins et al. 1987b) or the

possibility that both condensed and hydrolyzable tannins in goats form complexes with salivary proteins and are detoxified in the liver via conjugation with glucuronate or sulfate anions and excreted in urine as glucuronide (McSweeney et al. 1988). Robbins et al. (1987b) suggested that the biological activity of phenolics in browsers can be different than that in other herbivores.

A possible reason for the variation in amounts of oak in the diet seen in all seasons in our study could be a need of the goats to balance intake with rate of detoxification and excretion as was suggested by McLeod (1974) as an explanation for intake of plants rich in hydrozylable tannins by herbivores.

Protein precipitation by oak leaf extracts

The precipitation of proteins in plants and in the digestive tract of herbivores is one of the most agreed upon functions of tannins. The biological activity of tannins in plants can be assessed by their protein precipitating capacity. The protein binding assay with bovine serum albumin (BSA), a standard soluble protein was used because it mimics the protein complexation processes that occur *in vivo* (Asquith and Butler 1985; Hagerman 1987). Protein precipitating capacity of leaf

extracts has been observed to be a reliable parameter to estimate protein digestibility in large herbivores (Robbins et al. 1987).

Results of the protein binding assay (Tables A9 and A10 and Fig. 8) show that acetone/water extract of oak leaves gave significantly different (P = 0.0001) precipitates with BSA for monthly values and for precipitation capacity of extracts of young versus older leaves. During the period between May to October and again in March and April, but only for newly developing leaves. The protein precipitation of oak leaves in this study was higher at times of greater concentration of hydrolyzable tannins in leaves. Moreover, the peaks of protein precipitation capacity occurred during vegetative stages (summer and spring) when protein levels are normally elevated. This affects the nature of protein-complexes as demonstrated by Hagerman and Robbins (1987). When protein to tannin ratios are beyond a equivalence point (excess of protein)there is not enough tannin available to form cross-links from the protein molecules resulting in formation of soluble tannin-protein complexes and increase in digestibility of the feed and

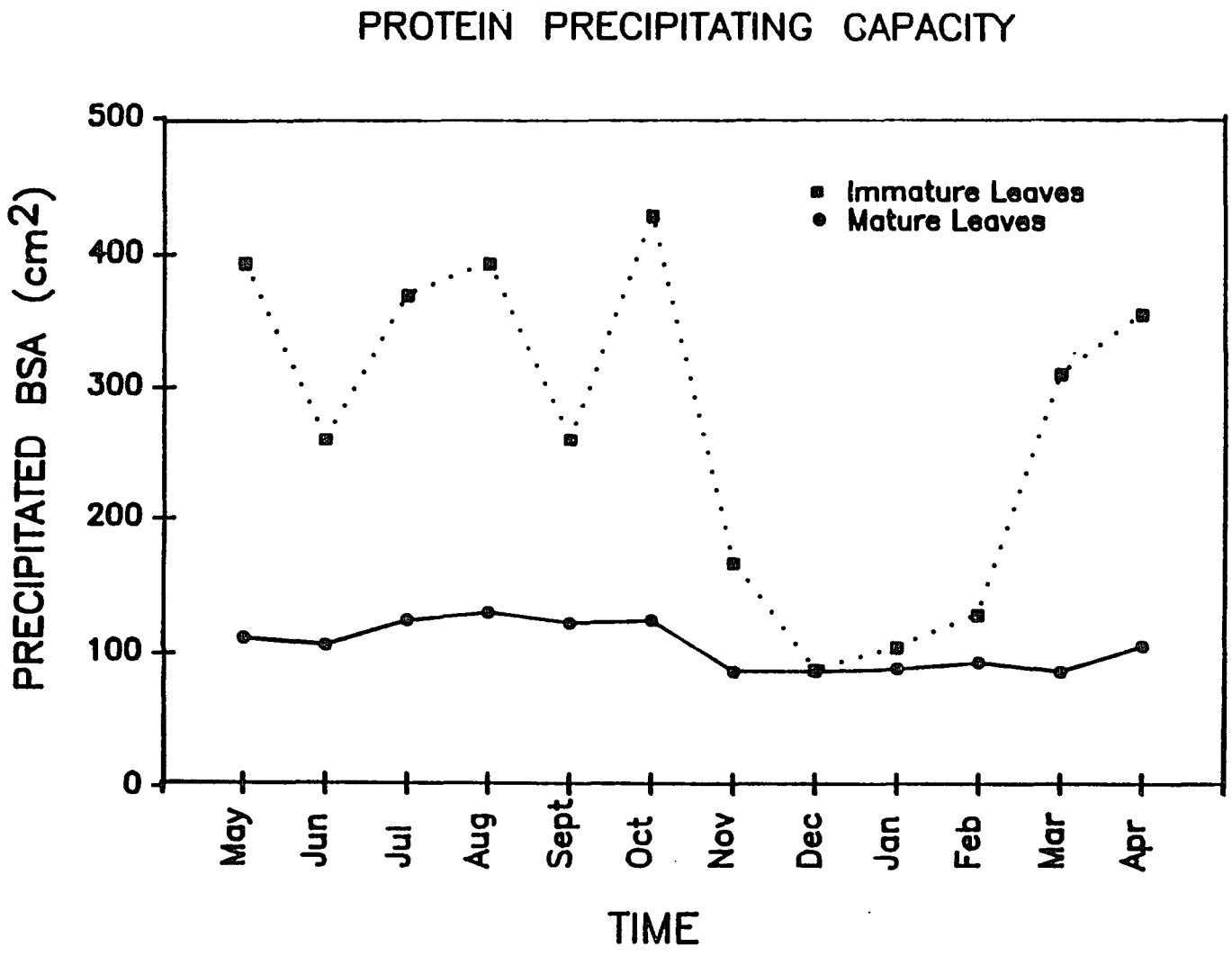

Fig. 8. Protein precipitation by extract of immature and mature oak leaves in

absorption of the tannin. Bate-Smith (1977) demonstrated that, on a weight basis, hydrolyzable tannins are more effective protein precipitants than condensed tannins. Moreover, the fact that hydrolyzable tannins are easier to break down (Haslam 1977) corroborates the finding of this study that goats can accommodate high levels of tannin uptake without serious consequences. McLeod (1974) and Jones and Mangan (1977) showed that tannin-protein complexes may undergo dissolution resulting in protein available for trypic digestion in the abomasum with good nutritional consequences if the herbivore has ability to handle the toxic tannin. McLeod (1974) and Nastis and Malechek (1981) showed that browsing ruminants such as goats are less susceptible to tannin toxicity than generalistic herbivores.

The presence of hydrolyzable tannins at times of intense vegetative growth in oak (Fig. 7) coupled with the high levels of protein precipitating capacity verified at these times (Fig. 8) suggest the function of these tannins in oak as a toxic defense instead of digestion reducing compound because hydrolyzable tannins and their complexes with protein are easily broken down and produce toxic phenolic products (Zucker 1983; Martin

and Martin 1984; Robbins et al. 1987). It is well accepted that the nutritional impact of tannins on herbivores is mostly in their nitrogen economy and as toxic compounds. In the light of our data on diet, phenolic profile and protein precipitation, we can argue that for animals that have developed pathways to detoxify and eliminate phenolic compounds hydrolyzable tannins in forage will have no nutritional significance. The recent discovery (Robbins et al. 1987) of complexes between hydrolyzable tannins and salivary protein in browsers is an example of this counteraction.

The protein binding capacity of oak leaves as related to amounts of oak in the diet may indicate that the adaptation to dietary tannins by goats was very rapid. The selection of only new leaves and growing tips of oak and the high voluntary intake of oak all times in this study suggests an adaptation of goats to phenolic compounds. In a similar case of adaptation to tanniferous browse, Spalinger et al. (1986) showed that when elk faced reduction in protein availability in their diets they tended to select more succulent plant parts with higher nutritional value that promote faster rate of passage.

Adaptive benefit from increase in tannin content in plants is only possible if the higher phenolic level confers to the plant a lower critical threshold of available energy, protein or other nutrient as related to other fodder species in the area, and imposes limitations to the herbivore's handling time and metabolic capabilities (Robbins et al. 1978b). Spalinger et al. (1986) noticed that deposition of tannins in plants being consumed by deer because of its relative high proximate value and digestible cell wall structure caused an increase in voluntary intake as the cervids continued to eat to meet nutritional requirements. Our data on diet and protein precipitation from summer and spring (Fig. 5 and 8) showed a similar trend since the increase in production of protein-precipitating tannins was followed by the goats increasing their consumption of oak.

Apparent Dry Matter Digestibility of oak leaves

The low nutrient content , slow rate of breakdown and high concentration of lignin-cutin and other secondary metabolites in most shrubs impose limits on the amount of energy and nutrients that can be extracted by herbivores. Evolutionary counteraction to those impediments by herbivores includes differences in

buffering ability and volatile fatty acids absorption, nature and efficiency of rumen biota, patterns of food selection, rumination, rate of passage and metabolic and fecal output (Van Soest 1988).

Apparent digestibility of oak leaves incubated at 6, 12, 24, and 48 hours in goat rumen fluid and further digested in pepsin was significantly ($P = 0.0001$) affected by age of the leaf, phenological stage of the plant and length of incubation time (Fig. 9, Tables A11 and A12). In general, the dry matter digestibility tended to be low at the onset of the growing season from May to July, increased considerably during the summer/fall and dropped continuously thereafter until February, increasing again towards the months of March and April. Except for the 6-hour fermentation, lower values of digestibility in relation to the standard alfalfa were recorded for both mature and immature leaves sampled during winter/early spring.

Most of the purported differences in digestibility of goat diets as compared to other herbivores result because of a greater selectivity toward less lignified plant tissue (Devendra and Burns 1983). According to Van Soest (1988), conventional digestion trials may underestimate digestibility when selectivity and rate of

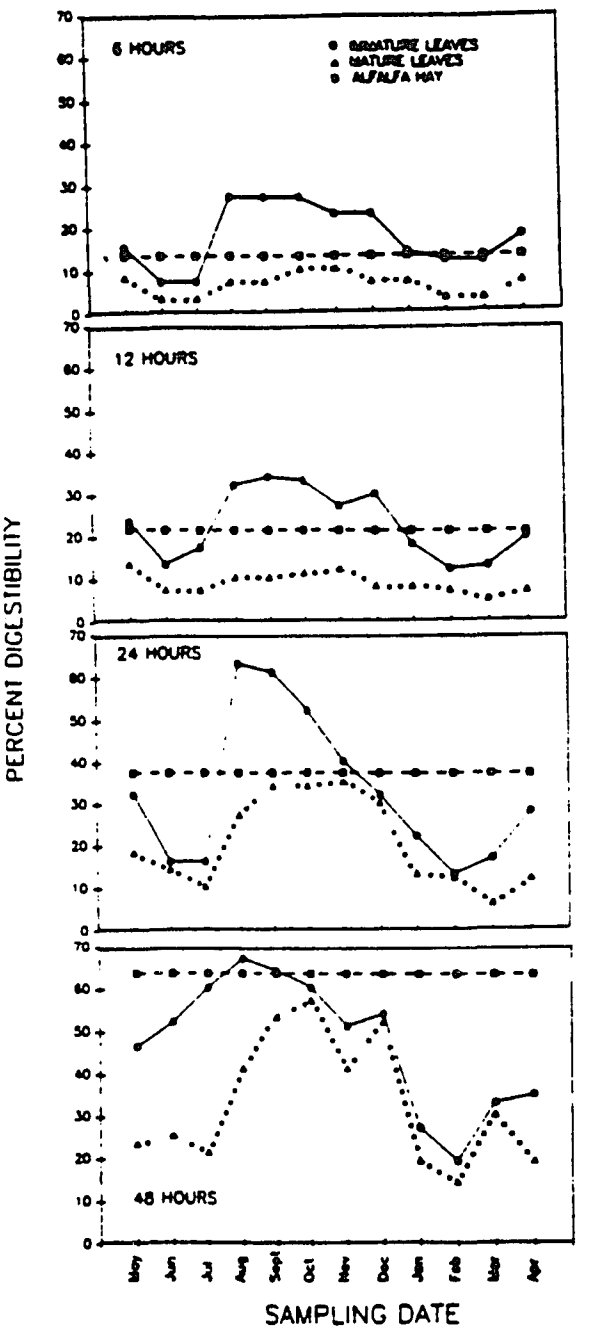

Fig. 9. Apparent digestibility (IVDDM) of mature and immature oak leaves measured at 6, 12, 24, and 48 hours incubation in goat rumen fluid (alfalfa hay used as a standard).

passage of food through the alimentary tract of the animal are not considered.

Analysis of variance of digested dry matter of mature and immature oak leaves at 6,12,24 and 48 hours of incubation in inoculum from goats revealed that difference in digestibility between immature and mature oak leaves is significant for all the months observed, there is a significant month x age of leaf interaction, and the effect of time of incubation is the same regardless the age of the leaf (Table A12). These results indicate a remarkable rumen microbial adaptation in goats to tanniferous substrate. In a related experiment Nastis and Malechek (1988) compared the _in vitro_ digestibility of mature and immature oak leaves (_Quercus gambelii_) by goat rumen microbes and found that the effect of tannin on digestibility was mostly due to a reduction in pepsin activity. In their experiment the older foliage had greater enzyme reduction power than immature foliage which resulted in non-significant differences in total reduction of pepsin activity by material differing in tannin content as the older leaves also had less tannin than the young leaves.

Because incubation time increased digestibility regardless of leaf age this study (Fig. 9) indicated that rumen microbes adapt to tanniferous substrates and suggests a reduction in enzyme activity in older leaves as their tannin content was significantly lower than in young leaves in all months.

Increases in content of hydrolyzable tannins in oak leaves from May to August did not affect the dry matter digestibility in this study (Fig. 9). A similar lack of effect of hydrolyzable tannins on digestibility of cell wall contents by sheep was observed by McSweeney et al. (1988).

The gradual decrease in disappearance of dry matter observed from October to February at all times of incubation is associated with both the reduction of nutritional proximate value and the boosts of condensed tannins during that time. Our results for digestibility of young leaves during the winter, especially January, are in agreement with a 28% digestibility found for Gambel oak for the same time of the year in Colorado (Kufeld et al. 1981) and are also consistent with low digestibility recorded for shrubs in most studies (Urness et al. 1975, Nastis and Malechek 1981). This low

digestibility could be attributable either to the presence of tannins or another polyphenol, lignin, in the leaves that time of the year.

Regarding the effect of leaf age on digestibility it should be stressed that shrubs generally have low proximate nutritional value in spite of phenolic content. This is due to the time limitation imposed by the slowly fermenting cellulose-lignin fraction. Animals with short forage retention time (Castle 1956) such as goats are most susceptible to this limitation. Interpretation of the results from the ruminal digestion reported here should be tempered by the limitations of *in vitro* technique. Due to the difficulty to correct for the effects of dilution of phenolics entering the rumen pool, the continuous addition of saliva prior and during *in vivo* trials, the absorption and differential flow of food particles from the rumen and the shortcoming between *in vitro* solubility and in *vivo* absorbability, the effect of tannins on microbes is only approximated (Robbins et al. 1987a).

Although shorter exposures to rumen microbes gave the expected lower digestibility estimates, the values obtained for digestion of younger leaves collected during

summer/fall, were at all incubation times, higher than the digestion of alfalfa hay used as standard in this study. If goats could sense digestibility it would be a nutritional mistake for them to eat alfalfa hay in lieu of oak leaves at that time. In fact, the diet of goats in Table A1 and Fig. 5 shows that, though alfalfa hay was supplied from November-December goats included oak in their November (24%) and December (7%) diets. Oak digestibilities were 39.5 and 32%, respectively, for the 24 hour incubations during November and December (Fig. 9).

CONCLUSIONS

Shrub live oak (Quercus turbinella) in the interior chaparral of Arizona produces high levels of tannins throughout the year. The partitioning of tannin allocation in oak plants shows a higher concentration in newly developed leaves than in older foliage.

The biosynthesis of hydrolyzable tannins in shrub live oak is mostly restricted to summer/fall, and the production of condensed tannins occurs during periods of less intensive metabolic activity.

Due to an alternation of tannins being produced, shrub live oak possesses a omnipresent high level of these allelochemicals.

It appears that anti-herbivore defense via precipitation of proteinaceous substances is a mechanism present mostly in younger oak leaves.

Water/acetone extracts of immature leaves of shrub live oak collected during periods of fast growth formed copious precipitates with a standard and soluble protein indicating an ecological role of hydrolyzable tannins as toxic compounds and as a potential hazard for the nitrogen economy of non-adapted browsers.

The phenolic profile of oak as seen in this study, in the light of the biological and ecological role of phenolic compounds, indicates that nutritional success and increased fitness in biomes such as Arizona chaparral, where shrub live oak comprises more than 50 percent of the vegetation, require herbivores to possess evolutionary adaptations to detoxify and eliminate tannins and to reduce the waste of nitrogen.

Goats (Capra hircus) consumed shoots and younger leaves of shrub live oak throughout the year regardless of the high content of condensed and hydrolyzable tannins and the protein binding capacity of this forage. Although neither forage or tannin intake were measured in this study the amounts of tannin observed in oak and the presence of oak in the diet of goats suggest that goat diet selection was influenced by factors other than tannin content. Older oak foliage avoided by goats contains deterrency factors such as leaf toughness, less moisture content and attachment of protein to the lignified cell wall.

Goat rumen biota showed increases in efficiency of fermentation at greater incubation times in spite of

leaf age and amount and concentration of condensed and hydrolyzable tannins.

Phenolic profile of shrub live oak, the protein binding capacity and the apparent dry matter digestibility of oak leaves indicate that tannins confer anti-herbivore defense in oak mostly as a toxic compound rather than as a digestion reducing substance.

Shrub live oak provided the bulk of the diet of goats in 10 out of 12 months of the year in Arizona chaparral with a level of dry matter digestibility beyond 50% during November and December, a critical period for many browse fodders in that vegetation type.

APPENDIX A:

TABLES

Table A1. Botanical composition (%) of goat diets on Arizona chaparral from May 1988 to April 1989.

	May 1988	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1989	Feb	Mar	Apr
Oak	72.2	70.5	52.5	69.8	51.8	49.4	39.2	55.8	66.7	64.7	31.2	64.6
Grass	3.6	4.7	5.3	2.1	27.4	18.9	14.5	21.9	2.3	3.1	17.8	10.9
Other	24.2	24.9	42.2	28.1	20.7	31.9	40.4	17.7	31.2	32.2	53.1	24.0
Alfalfa							23.6	6.7				

Table A2. Analysis of variance for percent of oak in the diet of goats on Arizona chaparral from May 1988 to April 1989.

Source of variation	df	Mean square	F value
Months	11	1700.516	6.27
Animals	9	85.912	.32
Error	99		
Total	119		

Due to the significance of the F found (rejection of Ho) a conservative LSD test was run using 9 degrees of freedom to compare populations of means in the diet study. It considered t - 12, = 10 animals and t -1 0 as the numerator df = 11 (t - 1) r - 1) 0 as the denominator df = 99 and we took 0 = $\underline{1}$ = $\underline{1}$ according to Steel and Torrie (1980).

Table A3. Analysis of variance for percent of oak in the diet of goats in Arizona chaparral from May 1988 to April 1989.

Source of variation	df	Mean square	F
Model	20	973.945	3.59
Error	99	271.019	
Corrected total	119		

Table A4. Fisher's (protected) LSD values for dietary levels (%) of oak leaves for goats on Arizona chaparral from May 1988 to April 1989.

Mar	Nov	Oct	Sep	Jul	Dec	Apr	Feb	Jan	Aug	Jun	May
31.2	39.2	49.4	51.8	52.5	55.8	64.6	64.7	66.7	69.8	70.5	72.2
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)

LSD

$$t_{\alpha/2} \quad .025 \quad 9 \text{ df} = 2.262 \sqrt{2\left(\frac{85.913}{10}\right)} = 9.376$$

Table A5. Variation in condensed tannins (Proanthocyanidins) in mature and immature oak leaves (mg/g dry matter) in Arizona chaparral.

	May 1988	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1989	Feb	Mar	Apr
Immature leaves	0.83	1.67	3.21	6.28	5.42	6.53	15.88	4.14	5.98	16.22	7.16	3.26
	0.87	1.40	3.25	5.62	5.44	6.56	16.27	3.97	6.61	16.41	7.10	3.22
	0.84	1.79	2.77	5.71	5.38	6.31	16.31	4.32	5.99	16.15	7.16	3.23
Mean	0.85	1.62	3.08	5.87	5.41	6.46	16.15	4.14	6.19	16.26	7.14	3.24
Mature leaves	1.21	1.45	3.27	2.90	3.75	3.81	5.29	2.47	3.75	5.60	7.00	2.52
	1.18	1.44	3.14	3.07	4.16	3.84	5.59	2.21	3.82	5.90	6.90	2.06
	1.18	1.45	3.18	2.83	4.58	3.81	5.60	2.27	3.66	5.84	6.95	2.22
Mean	1.19	1.45	3.20	2.93	4.16	3.82	5.49	2.32	3.74	5.78	6.95	2.27

Table A6. Analysis of variance for levels of condensed tannins in mature and immature oak leaves in Arizona chaparral from May 1988 to April 1989.

	ANOVA		
Sources of Variation	d.f.	Mean Square	F
Months	11	62.347	2156.88
Blocks/months (error A)	24	.040	1.39
Mature vx. immature leaves	1	137.175	4745.59
Month x mature vs. immature leaves	11	239.601	753.59
Blocks x mature (error B) vs. immature leaves/month	24		
Total	71		

Table A7. Variation in hydrolyzable tannins (gallotannins) in mature and immature oak leaves (mg/g of dry matter) in Arizona chaparral.

	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
	1988								1989			
Immature leaves	8.83	15.20	16.31	16.07	16.98	15.52	7.58	4.41	5.10	4.43	4.70	14.77
	8.16	14.57	15.94	16.03	16.80	15.44	7.82	4.41	5.13	4.41	4.41	14.77
	8.57	14.67	15.98	16.25	16.97	14.93	7.40	4.44	4.90	4.18	4.55	14.53
Mean	8.52	14.81	16.07	16.12	16.92	15.30	7.60	4.42	5.04	4.34	4.55	14.57
Mature leaves	4.24	6.86	4.34	3.46	3.31	4.68	2.56	2.92	3.75	3.30	3.60	3.35
	4.57	6.39	4.38	3.21	3.61	4.81	2.50	3.05	3.66	3.22	3.68	5.15
	4.13	6.18	4.23	3.34	3.53	4.93	2.72	2.92	3.84	3.28	3.44	5.15
Mean	4.32	6.48	4.32	3.33	3.48	4.81	2.59	2.96	3.75	3.27	3.57	4.55

Table A8. Analysis of variance for levels of hydrolyzable tannins in mature and immature oak leaves in Arizona chaparral from May 1988 to April 1989.

Source of variation	df	Mean square	F
Month	11	52.546	520.09
Blocks/months (error A)	24	.063	8081.87
Mature vs. immature leaves	1	816.870	359.31
Month x mature vs. immature leaves	11		
Blocks x mature (error B) vs. immature leaves/month	24		
Total	71		

Table A9. Protein precipitation (cm^2 of BSA slab) by extract of mature and immature oak leaves in Arizona chaparral from May 1988 to April 1989.

	May 1988	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1989	Feb	Mar	Apr
Immature leaves	380	256	400	400	256	420	169	81	100	132	306	361
	400	256	380	400	225	420	156	81	100	132	306	361
	400	256	324	380	240	420	156	81	100	100	306	342
	380	256	361	380	225	441	169	90	100	132	306	342
Mature leaves	110	100	121	121	110	121	81	90	81	81	81	100
	100	100	121	132	100	121	81	81	81	90	81	100
	100	110	121	132	144	121	90	81	90	100	81	110
	121	100	121	121	121	121	81	81	90	90	90	100

Table A10. Analysis of variance for protein precipitation by extract of mature and immature oak leaves in Arizona chaparral from May 1988 to April 1989.

Source of variation	df	Mean square	F
Month	11	37358.889	275.87
Blocks/months (error A)	36	97.184	.72
Mature vs. immature leaves	1	644028.844	4755.78
Month x mature vs. immature leaves	11	24813.502	183.23
Blocks x mature (error B) vs. immature leaves/month	36		
Total	95		

Table A11. Apparent digestibility (IVDDM) of mature and immature oak leaves from Arizona chaparral measured at 6, 12, 24, and 48 hours incubation in goat rumen fluid.

		May 1988	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1989	Feb	Mar	Apr
Immature leaves													
Time	6 hr	13	7	7	27	27	27	22	21	14	12.5	12	19
of	12 hr	23	13	16.5	32	34	32	26.5	29.5	18	11	13	20
incubation	24 hr	32.5	16	16	61.5	60.5	52.5	39.5	32	21.5	18.5	12.6	15
	48 hr	47	51.5	59	67.5	64	59	50.5	54	27.5	18.5	12.5	15
Mature leaves													
Time	6 hr	7	3	3	7	7	10	11	7	7	3	3	7
of	12 hr	13	6	6	10	10	11.5	12	9	8.5	7	5.5	5
incubation	24 hr	17.5	14.	10	27	34	34	35.5	30	11	12	6.5	12.5
	48 hr	21.5	24.5	21	40.5	54	58	40.5	52	19	18	29	19.5

Table A12. Analysis of variance for apparent dry matter digestibility (IVDMD) of mature and immature oak leaves from Arizona chaparral measured at 6, 12, 24, and 48 hours of incubation in goat rumen fluid.

A) Source of variation	df	Mean square	F
Month (M)	11	633.241	18.60
Age of leaf (A) (mature vx. immature)	1	3888.760	114.20
M x A	11	69.999	2.06
Time of incubation (T)	3	3867.281	113.57
A x T	3	10.100	.30
M x T	33	74.656	2.19
M x A x T (error B)	33		
Total	95		

B) Source of variation	df	Mean square	F
Month	11	633.241	9.05
Age	1	3888.760	55.55
Month x age (error)	11	69.999	

Table A13. Precipitation in Punkin Center station at East-Central Arizona (NOAA 1986-1989).

	1986	1987	1988	1989
Jan			3.96	3.17
Feb	1.37	3.29	1.10	.80
Mar			.28	1.70
Apr			2.60	0
May			0	
Jun			.18	
Jul			1.73	
Aug			1.54	
Sep			1.03	
Oct			1.22	
Nov	1.70	3.35	.95	
Dec			.34	
Total	19.58		14.93	

Table A14. Multispecies dieting by goats grazing on oak-dominated Arizona chaparral.

Month	Average number of species in the diet of 10 goats
May 1988	9.5
Jun	6.6
Jul	9.0
Aug	4.9
Sep	9.2
Oct	9.4
Nov	9.7
Dec	8.4
Jan 1990	6.5
Feb	7.9
Mar	9.7
Apr	6.4

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