AGE AND BODY CONDITION OF GOATS INFLUENCES CONSUMPTION OF REDBERRY JUNIPER (*Juniperus coahuilensis*) AND DISPOSITION OF FOUR MONOTERPENES

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ABSTRACT

Age and Body Condition of Goats Influences Consumption of Redberry Juniper (*Juniperus coahuilensis*) and Disposition of Four Monoterpenes

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Redberry juniper (*Juniperus coahuilensis*), is an invasive, evergreen tree that is rapidly expanding throughout western and central Texas. Goats will consume some juniper on rangelands, however, intake is limited. The objective of our research was to determine how the age and body condition of goats influences their consumption of juniper and the disposition of 4 monoterpenes found in redberry juniper. Four main trials were conducted from Summer of 2003 through Summer of 2005 at the Texas Agricultural Experiment Station (TAMU), in Sonora, TX and the University of Idaho Sheep Research and Teaching Center in Moscow, ID.

In the Summer of 2003 a series of pen studies examined whether age and body condition of goats influenced the formation of a conditioned flavor aversion and the consumption of live redberry juniper foliage. An aversion was formed in half of the goats with no indication that age or body condition influenced the formation of an aversion. Goats in low body condition consumed more juniper foliage than those in high body condition. Young animals consumed more juniper foliage than mature animals within the body conditions. Conclusively, age and body condition both influence intake of juniper although these attributes do not seem to contribute to the formation of conditioned flavor aversions.

In a follow-up experiment in the Winter of 2004, goats were offered a synthetic ration treated with twice the amount of monoterpenes that occur in juniper foliage. Again, goats in low body condition consumed more of the terpene treated feed than animals in high body condition. Age was not a contributing factor for intake of the treated feed.

The next experiment examined the influence of age and body condition of goats on the elimination and concentration of monoterpenes in the bloodstream. Goats were dosed intra-ruminally with 4 monoterpenes found in redberry juniper and a series of blood samples were taken in a pharmacokinetic study. Body condition significantly impacted elimination and concentration of monoterpenes in the bloodstream. Age did not appear to influence the elimination and concentration of monoterpenes in the bloodstream of goats.

The final set of experiments conducted in the Winter of 2004 examined the influence of age and body condition on the propensity of goats to sample novel foods following dosing with LiCl paired with a novel food. Body condition did not affect acceptance of novel foods, but mature animals more readily accepted novel foods than young animals.

Collectively this research adds to the existing knowledge base of how animals differing in age and body condition are affected by toxic plants. Clearly, these two animal attributes influence intake and dietary preferences. However, further research is needed to clarify exactly how these attributes influence diet selection to enhance animal production and to expand the capacity for animals to be used in vegetation management.

Key words: Age, Body Condition, Monoterpenes, *Juniperus coahuilensis*, Aversion, Intake and Goats

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CHAPTER ONE: Research Overview

Introduction

Prescription grazing is the application of livestock grazing at a specified season, duration and intensity to accomplish specific vegetation management goals. Controlled grazing of this type is being employed throughout North America on public and private land and is proving to be a promising tool in the battle against weeds. Awareness of invasive exotic weeds has raised concern over the potential role of livestock in spreading these weeds, however, other areas are welcoming livestock in an effort to heal the very lands they were held partially responsible for degrading.

With careful management, selective grazing can be used to alter the community composition in favor of native species. Competition is a two-way street, and healthy perennial bunchgrasses can successfully compete with invaders and inhibit their spread (Ferrell et al. 1998). Grazing animals can influence weeds directly by eating or damaging the plants, or indirectly by "conditioning" the pasture and making the desirable vegetation more competitive and better able to resist subsequent weed invasion (Popay and Field 1996). The goal of using livestock to control weeds is to manipulate patterns of defoliation to place a target plant at a competitive disadvantage relative to other plants in the community. There are two approaches to placing an invasive plant at a competitive disadvantage in the community: 1) use grazing management that harms the target weed by grazing at the time and frequency when the weed is most vulnerable, and 2) modify the grazing behavior of animals to cause them to concentrate their grazing efforts on the target weed instead of the desirable forage (Walker et al. 1994). These two approaches form the basic framework of prescription grazing.

It is common practice for vegetation managers to separate their grazing animals by age, body condition, sex, or physiological state depending on their specific management objectives. However, they admit that there is no real basis for this practice, only observed differences in the effectiveness of their animals in achieving the vegetation goal. Digestive capabilities and nutrient requirements vary throughout an animal's life. While animals can be encouraged to select specific foods, they will never habitually consume them if they do not receive a nutritional benefit from the plant. Therefore, species, breed, age, body condition, sex, and physiological state could have a profound effect on diet selection and preferences. A better understanding of how these attributes affect diet selection will be immediately applicable to the vegetation management industry, as well as aiding livestock managers everywhere in improving grazing management.

Animal Attributes that Influence Diet Selection

Species

The most obvious place to start in selecting the right animal for the job is to select the right species.

Grazers vs. Browsers. All herbivores are not created equal when it comes to digestive abilities and foraging skills. Ultimately, animals consume foods that they are physiologically adapted to digest and those that meet their nutritional requirements. Herbivores are born with digestive constraints that determine what types of plants can be included in their diet (Hoffman 1989). Herbivores are often classified into 3 major groups; grazers, browsers, and intermediate feeders. Grazers, including cattle and horses, primarily consume grass and have the necessary digestive capabilities to handle large quantities of this relatively low quality

forage resource. Browsers, such as goats and deer, consume the leaves and stems of shrubs and forbs which have a thinner cell wall and a greater proportion of highly soluble cell contents. Intermediate feeders, like sheep, select both grasses and browse. The difference in diet selection is governed by several physiological characteristics of the animals. Grazers tend to have relatively wide mouths made for harvesting large quantities of uniform forage while browsers have narrower mouths, and longer more dexterous tongues that allow for selecting individual leaves among thorns and branches of browse. Furthermore, because browse often contains toxins such as tannins or terpenes, browsers are equipped with salivary glands that bind tannins, have a larger liver in relation to body mass for transformation and excretion of toxins and may possess specialized rumen microbes that allow for detoxification of alkaloids and other toxins (Shipley 1999).

Breed

Diet composition varies between breeds and even among individual animals (Pritz et al. 1997, Winder et al. 1995, and Launchbaugh et al. 1997). Studies with Rambouillet, Karakul, and Barbado sheep and Spanish and Angora goats, found that Barbado differed from Rambouillet and Karakul sheep and that Spanish and Angora goats differed from all the sheep breeds in diet composition (Warren et al. 1984). Breeds of animals also differ in their dry matter intake, digestibility and energy conversion (Langlands 1968, Ramsey et al. 1998, Osuro et al. 1999, (Ramirez-Perez et al. 2000). However, other studies have found no difference in diet composition between breeds of cattle (Herbel and Nelson 1966), goats (Dziba et al. 2003) or found with sheep that differences occurred only occasionally on some pastures (Langlands 1968). Reasons for the variation in findings could be accounted for by

the wide variety of breeds, animal and forage species studied, and the different data collection methods used to determine diet composition in these studies.

However, several research projects with the toxic plant juniper have yielded conclusive results regarding the differences in consumption between goat breeds. Riddle et al. (1996) concluded that Spanish goats consume juniper more readily than Angora. Similarly, Pritz et al. (1997) found that naive Spanish goats consumed more juniper than naive Angora goats, while Launchbaugh et al. (1997), found that Ibex goats consumed more than both Spanish and Angora goats. These differences may be explained by the degree of human selection each breed has experienced in the past. Ibex goats are largely feral and have received virtually no human selection within their breed. Angoras have been highly selected for hair production, while Spanish goats are raised primarily for meat production and have experienced much less selection pressure by managers. Therefore, while selecting for these performance traits, managers could have been inadvertently selecting for or against physiological traits that influence diet selection, primarily the ability to handle various plant toxins.

Where a breed was developed by humans also influences its habitat selection which may, in turn, influence diet selection. For example, breeds of cattle developed in mountainous terrain may graze rugged rangeland more uniformly than breeds that were developed in gentler terrain (Bailey et al. 2001). This is important considering one of the major advantages to using livestock for weed control is that they can traverse rough terrain that is unaccessible to more conventional methods of weed control.

Individual variation and heritability

Even with the species and breed guidelines there is one powerful force that influences intake. That is individual animal variation. Within almost every study, there are individuals that consume much greater amounts of poisonous plants than the average. Riddle et al. (1996) found that there were significant differences among individual animals within breeds (Spanish and Angora). Nearly 25% of the Angora goats consumed as much or more juniper as the average Spanish goat (Pritz et al. 1997). Many times there is not a definitive characteristic that sets these animals apart, it is something physiological that allows them to tolerate or metabolize the toxins better than the average animal. There is some interest in trying to identify these individuals and to determine if their dietary abilities can be perpetuated through breeding. While not an easily recognizable trait like conformation or weaning weight, there is evidence that diet selection is somewhat heritable. A study conducted in Idaho found that genetic factors significantly influence dietary preferences of sheep grazing mountain big sagebrush (*Artemisia tridentata*; Snowder et al. 2001). Conversely, Frost et al. (2003), determined that the ability of lambs to consume bitterweed was not strongly influenced by the sire's genetic characteristics and Ellis et al. (2005), confirmed that the ability to consume redberry juniper (*Juniperus coahuilensis*) is only mildly heritable (11%) in goats.

Age

The age class of animals can also have a profound affect on diet selection and secondary compound tolerance. It is difficult to separate age and experience as experience generally increases with age. Experiences, especially those early in life, can affect more that just food preferences, they have the potential to change the structure and physiology of the

body. For example, exposure to blackbrush early in life resulted in increased reticulorumen sizes in goats (Distel and Provenza 1991). Goats and sheep raised on poor quality forages have larger rumens, recycle urea nitrogen more efficiently and eat more poor-quality forages later in life than those raised on high-quality diets (Distel et al. 1996). However, exposing young animals to toxins early in life has variable results. In some cases, early exposure to toxins may increase the liver's ability to metabolize those toxins, but in other cases, it may cause a decrease in liver function depending on the toxin and its dose. Pritz et al. (1997) found that exposure to monoterpenes early in life resulted in decreased consumption of juniper later in life, presumably due to liver damage. Exposure to some alkaloids can result in accumulation of the toxin in the liver that leads to a decrease in function and upon repeated exposure can result in death (Stegelmeier et al. 1996). Therefore, care should be taken when exposing young animals to high levels of toxic plants.

Young animals face unique foraging challenges because of their higher nutritional requirements and lack of foraging experience (Provenza and Malecheck 1986). Metabolic rate declines with age, so older animals do not need as much food and consequently graze less (Henry 2000), while most young animals spend more time foraging and less time ruminating. This combined with their higher nutritional requirements necessitates the selection of a higher quality, more digestible diet overall (Hodgson and Jameson 1981). Diets of younger animals are usually higher in crude protein concentrations and energy density, while lower in fiber than diets of adults (Cazcarra and Petit 1995; Grings et al. 2001). However, this search for a more nutritious diet often leads to higher energy output and increased weight loss. This combined with a lack of foraging knowledge can make younger

animals more willing to try novel foods and to retry foods that made them sick in the past (Ralphs and Provenza 1999).

Generally as herbivores age, their incisor teeth suffer wear and they exhibit a decreased ability to graze and achieve maintenance requirements, particularly on short forage (Coop and Abrahamson 1972). Some compounds, like the essential oils of redberry juniper are quite volatile and may be released pre-ingestion through mastication (Cluff et al. 1982). Therefore, the efficiency with which an animal chews and the length of time spent chewing may affect the amount of toxin that actually reaches the rumen. Furthermore, incisor wear influences which forages animals decide to graze. Goats with worn teeth avoided grasses and chose a higher proportion of tender-leaved shrubs than goats with unworn incisors (Mellado et al. 2005). Lack of teeth is more of a handicap when forage is short (Newton and Jackson 1983). The body's primary detoxification organ, the liver, also undergoes changes as animals age. Fernandez et al. (1988), found that ageing in rats is accompanied by a diminution of the total oxidative activity of the liver tissues, notably that of the cytochrome P-450 function. Additionally, the activity of some hepatic enzymes related to glucuronic acid conjugation (a phase II reaction active in monoterpene metabolism) decreased with increasing age (Santa Maria and Machado 1988). While reduction in functioning capacity of enzymes appears to be the major change in animals influencing drug metabolism, a reduction in hepatocyte mass and total liver blood flow appears to be the main agent influencing drug metabolism in humans (Jansen 2002).

Body condition

The nutritional status of an animal has long been known to play an important role in animal reproduction and maintaining animals in good body condition for breeding has been

the subject of much research. However, nutritional stress (consuming a diet that does not meet maintenance requirements) can also influence diet selection, particularly the inclusion of plants containing allelochemicals. This occurs primarily because of the nutritional "cost" of metabolizing a toxic compound (Foley et al. 1995, Illius and Jessop 1995). For a chemically defended plant to be contained in the diet, it must be quite nutritious itself, or there must be sufficient other nutritious forage available to help meet energy requirements. However, when forage availability becomes limited, as is usually the case on rangeland for at least a portion of the year, the animal must decide if the chemically defended plant is worth consuming. To do this, the herbivore needs to determine which results in greater weight loss, the increased metabolic costs of detoxifying the food, or the weight loss resulting from decreased intake rate.

The metabolism of toxins like phenolics and terpenes results in an increase in systemic acid loads that can negatively affect protein and nitrogen metabolism. The goal of allelochemical metabolism is to convert the product to a water soluble compound that can be easily excreted via the urinary system. Biotransformation of monoterpenes occurs in 2 phases: Phase 1 reactions involve oxidation, reduction, or hydrolysis to make the compound more polar; Phase 2 reactions are conjugations that produce a water-soluble and highly ionized metabolite, usually a strong organic acid. Metabolic acidosis can lead to losses of bone calcium and more important to this study, the breakdown of skeletal muscle to provide glutamine necessary to maintain acid-base homeostasis (Foley et al. 1995). Therefore, consumption of toxic foods by animals that are already catabolizing muscle tissue for maintenance is more harmful than beneficial despite needed nutrients the plants may contain. This was demonstrated by the model created by Illius and Jessop (1995) where the nutritive

value of food is diminished due to dilution and detoxification costs. Once the allelochemical:nutrient ratio crosses a threshold, the food then becomes unacceptable to the herbivore.

The activity of visceral organs, including the liver, accounts for 40-50% of total energy requirements in domestic animals (Noziere et al. 1999). The microsomal mixed function oxidase (MFO) system is involved in detoxification of many secondary compounds and activity of this system is higher in the liver than in other organs (Wattenburg et al. 1976). Two of the monoterpenoids found in redberry juniper, (α-pinene, and myrcene) are potent inducers of MFO activity immediately following consumption suggesting the importance of this system in detoxification of monoterpenes (Brattsten and Wilkinson 1977). Prolonged periods of nutritional stress can result in a loss of liver mass (Noziere et al. 1999), and low protein diets decrease the amount of activity of the cytochrome P450 enzyme system of the liver further hampering the animal's detoxification abilities (McLean and McLean 1969). This was demonstrated by sheep dosed with silvery lupine in that animals in low body condition had higher levels of alkaloids in their system for longer time periods than those in average condition (Lopez-Ortiz et al. 2004). Consequently, sheep in low body condition were exposed to the toxins for a longer period of time and suffered a greater toxic insult than those in average body condition.

Further conflicting the problem is the need for malnourished herbivores to alleviate deficiencies. This leads to an increase in dry matter intake of all types of foods. Sheep in low body condition consumed more forage than sheep in average body condition by maintaining a higher bite rate and by increasing time spent grazing (Arnold and Birrell 1977, Sibbald and Kerr 1994). However, nutritional stress on rangelands generally occurs during periods of low

forage availability, which can cause animals to turn to toxic foods. Cattle in low body condition begin including lupine in their diet before cattle in high body condition and consumed greater amounts overall (Lopez-Ortiz 2002). Intake of ponderosa pine by cattle in low body condition was 5 times greater than for cattle in average body condition (Pfister et al. unpublished data). Therefore, while animals in low body condition are not as able to metabolize the toxins found in plants, they are more likely to consume poisonous plants in an effort to alleviate nutritional deficiencies.

Sex of animal

Along with other more obvious difference, males and females also experience differences in diet selection. This is due in part to differences in size, feed conversion efficiency and overall nutrient requirements during reproduction activities. Differences in diet composition between males and females have been documented in Rocky Mountain bighorn sheep (Shank 1982) and in muskoxen (Forchhammer 1995). Most dietary differences related to animal gender have been tied with various seasons of the year when either the males segregated to other parts of the range (Rocky Mountain bighorn sheep), or they increased their energy intake to prepare for the upcoming breeding season (muskoxen). Dietary differences between genders may be confounded by other morphological and physiological attributes. Males are generally larger in both stature and muzzle size than females and may have greater energy needs (Grings et al. 2001). Grings et al. (2001) and Mohammad et al. (1996), both found that cattle diets of different genders were relatively similar except during periods of low forage quality when steer diets were less digestible than diets of heifers. Mellado et al. (2005) found similar results with goats where bucks generally chose a lower quality diet, especially during periods of drought, when they evidently traded quality for

quantity of forage. However, Aregheore (1995), found that sex of the animal had no effect on voluntary dry matter intake of goats, but significant effects on growth rate and feed efficiency.

Production cycle

Research has clearly demonstrated that an animal's dietary choice are greatly influenced by its nutritional state and requirements. However, these change dramatically throughout the stages of life. A herd of lactating females has a much higher nutrient demand than a herd of wethers and therefore may be more or less suited for different types of vegetation management jobs. While there is little evidence that pregnant females consume less toxic food than non-pregnant females, certain toxins are particularly harmful to females during gestation and should be avoided, like Ponderosa pine, lupine, and veratrum (Binns et al. 1963; Shupe et al. 1967; Gardner et al. 1994). Furthermore, the high nutrient content of some invasive plants can be utilized to meet the nutritional requirements of lactating females and growing lambs or kids. Studies indicate that sheep grazing leafy spurge wean heavier lambs than their counterparts grazing uninfested rangeland (Walker et al. 1994).

However, not all invasive plants are highly nutritious and it is important to make sure that animals have adequate alternative forage to maintain body condition prior to breeding and to meet nutrition requirements throughout gestation and lactation.

Redberry Juniper

Redberry juniper (*Juniperus coahuilensis*), was the plant selected for study in this project. It is an invasive, evergreen shrub that has been rapidly expanding throughout western and central Texas, southwestern Oklahoma, southeastern New Mexico, and northeastern

Mexico since the early 1900's (Adams and Zanoni 1979). A native plant in the southwest, juniper was historically confined to rocky outcroppings, and areas of shallow soil or poor productivity. However, heavy season-long grazing and the reduction of frequency and intensity of fire in the ecosystem has contributed to its rapid expansion into more productive areas (Ueckert et al. 1994). Currently, the shrub is posing serious problems for livestock producers and land managers due to the plant's profound affect on rangeland hydrology (Thurow and Hester 2001). The tree has a dense lateral root system that enables it to be a staunch competitor for moisture and soil nutrients. Not only do the trees alter the amount and distribution of water reaching the soil, they are also highly competitive plants that reduce the productivity of grasses and forbs and increase the amount of bare soil, which further perpetuates the establishment of juniper. This results in a dramatic decline in the herbaceous vegetation and reduces carrying capacity for livestock and wildlife (Dye et al. 1995).

Because the shrub is a basal resprouter, redberry juniper is very difficult to control and manage. This combined with the economic cost of implementing brush control (Johnson et al. 1999) has led managers to look toward goats in the battle against juniper. Goats will consume some juniper on rangelands, however, intake is generally limited to less than 30% of the diet by the presence of monoterpenes (Pritz et al. 1997). These essential oils inhibit consumption of juniper by herbivores in several ways. First, they have antimicrobial properties that are detrimental to the microbial populations of ruminants (Schwartz et al. 1980, Cluff et al. 1982). Secondly, they produce conditioned taste aversions by causing the herbivore to experience illness following consumption of juniper (Launchbaugh et al. 2001).

However, juniper does contain adequate nutrients to meet requirements during important periods of a goat's reproductive cycle (Riddle et al. 1999). Compared to

herbaceous plants during the growing season, juniper is only moderately nutritious with crude protein values of 6-9% and digestible organic matter of 57-66%. However, as the season progresses to fall and the herbaceous vegetation becomes dormant, juniper becomes a relatively good forage (Launchbaugh et al. 2001) (Straka 2001).

Previous studies have demonstrated that goats prefer Ashe juniper (*Juniperus ashii*) over Redberry juniper (Riddle et al. 1996). While the nutritional value of the plants is quite similar, they contain a different mixture, of both kind and concentration, of monoterpenes. Redberry juniper contains significantly higher levels of α-pinene, β-pinene & sabinene, myrcene, and terpineol (Owens et al. 1998). Pritz et al. (1997), determined that each of these monoterpenes was negatively correlated with preference for the shrubs by goats. Therefore, several of these compounds were chosen for more detailed study in Chapters 2 and 4.

Conclusion

This body of research describes a series of trials conducted between 2002 and 2005 to examine the influence of age and body condition of goats on the consumption of redberry juniper foliage, the long term consumption of feed treated with 4 monoterpenes found in redberry juniper foliage, the response of animals of different ages and body conditions to novel foods and aversions, and a pharmacokinetic study of the disposition of these 4 monoterpenes in goats. The results are presented in 3 chapters. Chapter 2 details the procedures and results of the intake trials, while Chapter 4 highlights the findings of two separate trials to assess animal willingness to accept novel foods despite potentially adverse consequences. Chapter 3 focuses on the pharmacokinetic parameters of 4 monoterpenes administered directly into the rumen and how age and body condition affect absorption and

elimination of these compounds. While this work is not comprehensive in how age and body condition influence consumption of plants containing monoterpenes, it does add to the sparsely existing knowledge of how these animal attributes influence diet selection. It is hoped that this knowledge will contribute to the efficacy of using animals in vegetation management projects and to the overall knowledge of how they contribute to livestock responses to poisonous plants. The need for sustainable, non-polluting methods for managing vegetation is increasing throughout the world as invasive plants continue to threaten the ecological integrity or rangelands and the economic viability of agriculture. While livestock grazing is proving to be a promising tool, new technologies must be developed to increase its effectiveness and to educate land managers in its proper application. It is essential that we continue to gather and share information so we can constantly sharpen this “new” tool in range management.

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CHAPTER TWO: Intake of Redberry Juniper (*Juniperus coahuilensis*) and 4 Monoterpenes by Goats Differing in Age and Body Condition.

Abstract

Redberry juniper (*Juniperus coahuilensis*), is an invasive, evergreen tree that is rapidly expanding throughout western and central Texas. Goats will consume some juniper on rangelands, however, intake is limited. The objective of our research was to determine how the age and body condition of goats influences their consumption of juniper and a feed made with 4 monoterpenes. Thirty-nine goats either young (< 2 yrs) or mature (> 6 years) were fed appropriate basal rations to reach either a high (HBC) or low (LBC) body condition. Experiment 1 tested whether a dose of 0.25 ml/kg BW of 4 monoterpenes found in redberry juniper was sufficient to produce a conditioned flavor aversion (CFA) to a novel food (wheat) and if age or body condition affected the magnitude of this aversion. An aversion (intake of wheat was less than 25% of the total offered) was formed in half of the goats with no indication that age or body condition influenced the formation of an aversion. Experiment 2 examined the intake of redberry juniper foliage. Goats in LBC consumed more juniper than those in HBC ($P < 0.05$). Young animals consumed more than mature animals within the body conditions ($P = 0.04$). In a final experiment, goats were offered a synthetic ration treated with 20.8 g/kg of 4 monoterpenes found in redberry juniper. Animals in LBC consumed more (24.5 g/kg BW ± 1.2 SE vs.16.9 g/kg ± 0.8 SE) than animals in HBC ($P < 0.01$). Age did not influence intake of the treated food ($P = 0.13$). A better understanding of how these attributes affect diet selection will be applicable to the vegetation management industry and aid livestock producers in improving grazing management.

Key words: Age, Body Condition, Monoterpenes, Redberry Juniper, Aversion, and Goats.

Introduction

Redberry juniper (*Juniperus coahuilensis*), is an invasive, evergreen tree that has been rapidly expanding throughout western and central Texas since the early 1900's (McPherson and Wright 1990). The small tree has a dense lateral root system that makes it a competitor for moisture and soil nutrients. Not only do trees alter the amount and distribution of water reaching the soil, their competitive nature reduces the productivity of grasses and forbs increasing the amount of bare soil (Dye et al. 1995). This results in a dramatic decline in the herbaceous vegetation and reduces carrying capacity for livestock and wildlife while creating conditions that further perpetuate the establishment of juniper (Thurow and Hester 2001).

Redberry juniper is a basal resprouter, making it very difficult to control and manage. This combined with the economic cost of implementing brush control has led managers to consider goats in the battle against juniper. Goats will consume some juniper on rangelands, however, intake is generally limited to less than 30% of their diet (Taylor et al. 2001). There is much interest in increasing livestock consumption of juniper either as a control measure or simply to use the plant as a source of forage. Juniper is only moderately nutritious compared to herbaceous plants during the growing season, with crude protein values of 6-9% and digestible organic matter of 57-66% (Houston et al. 1981). However, as the season progresses to fall and the herbaceous vegetation becomes dormant, juniper becomes a comparatively good forage.

Juniper contains monoterpenes which are aromatic compounds believed to inhibit consumption by herbivores in several ways. First, monoterpenes in juniper have antimicrobial properties that are detrimental to rumen microbial populations (Schwartz et al. 1980). Secondly, they produce conditioned flavor aversions by apparently causing digestive malaise

or illness following consumption of juniper (Launchbaugh et al. 1997a). Monoterpenes are lipophilic compounds that must be converted to hydrophilic substances to be excreted from the body. This conversion process involves both phase I and phase II biotransformations and occurs in the liver (Foley et al. 1995).

Riddle et al. (1996), determined that some of the monoterpenes found in redberry juniper were negatively correlated with preference by goats. Schwartz et al. (1980) found that preferences for juniper species by mule deer were inversely related to their concentrations of volatile oils and that the deer avoided foliage with higher concentrations of oxygenated monoterpenes. Sheep decreased intake of alfalfa pellets treated with α-pinene, but other terpenes found in juniper including limonene, camphor, sabinene and myrcene did not appear to influence consumption (Estell et al. 1998, 2000, 2002). However, most of these studies were conducted in a fairly short time frame (5 days) and maintenance requirements were met with a basal ration, so inferences to natural foraging situations are unclear.

Rangeland animals commonly experience noticeable weight loss and nutritional stress at times of low forage quality or availability or periods of high demand such as during lactation. These conditions potentially limit the amount of energy available for detoxification and biotransformation of the monoterpenes found in plants like redberry juniper. As body condition declines, animals lose functioning liver mass and may begin catabolizing muscle tissue to meet maintenance requirements. Animals in low body condition typically increase dry matter intake which can result in the inclusion of less palatable plants in the diet (Mellado et al. 2004). However, if the increased intake includes chemically defended plants, the animal must pay the metabolic “cost” of detoxifying the compounds. Foley and colleagues (1995) contend that animals under nutritional stress may need to adjust their foraging strategy by

decreasing consumption of toxic plants to maintain homeostasis, particularly the systemic acid load of the body. They basically argue that concentrations of secondary plant compounds set intake limits and animals with compromised detoxification capacity should exhibit reduced intake of plants containing secondary compounds. But, recent research has demonstrated that herbivores may behave otherwise. Cattle in low body condition began consuming lupine sooner and ingested greater quantities overall than those in average body condition (Lopez-Ortiz 2002). Therefore, animals in low body condition may not alter their foraging strategy to limit intake of toxic plants as some have hypothesized.

Age also influences diet selection in herbivores. Young animals face unique foraging challenges because of their greater nutritional requirements and lack of foraging experience (Provenza and Malecheck 1986). A mammal's metabolic rate declines with age, so older animals do not need as much food and consequently graze less, while young animals generally spend more time foraging and less time ruminating (Henry 2000). These conditions necessitate the selection of a more digestible diet by younger animals (Hodgson and Jameson 1981). Diets of younger animals are usually higher in crude protein concentrations and energy density, while lower in fiber than diets of adults (Grings et al. 2001; Cazcarra and Petit 1995). However, the search for a more nutritious diet can require higher energy output and leads to increased weight loss. This greater nutritional demand and lack of foraging knowledge in younger animals can make them more willing to try novel foods and to retry foods that previously made them ill (Ralphs and Provenza 1999).

As animals age they accumulate foraging experiences that alter their harvesting and digestive capabilities. Experiences, especially those early in life, can affect food preferences, and have the potential to change the morphology and physiology of the body. For example,

exposure to blackbrush (*Coleogyne ramosissima*) early in life resulted in increased reticulorumen size in goats (Distel and Provenza 1991). Goats and sheep raised on poor quality forages have larger rumens, recycle urea nitrogen more efficiently and eat more poor-quality forages later in life than those raised on high-quality diets (Distel et al. 1996). In some cases, early exposure to toxins may result in physiological changes such as increased liver capacity to metabolize specific secondary compounds (Smith 1992). But in other cases, exposure to plant secondary metabolites may impair liver function and detoxification processes depending on the toxin and its dose. Pritz and colleagues (1997) found that exposure to monoterpenes early in life resulted in decreased consumption of juniper later in life, presumably because of apparent liver damage. We do not know if mature animals respond differently to monoterpenes than young animals.

Clearly, the age and body condition of an animal can have direct effects on diet selection with implications for rangeland management. Furthermore, both of these animal attributes are easily recognized or manipulated by livestock managers. Many invasive plants on rangelands are chemically defended, often decreasing their value as forage. Increasing consumption of these chemically defended plants could increase the effectiveness of prescribed grazing practices for vegetation management. Therefore, it is important to understand how these attributes influence consumption of chemically defended plants so they can either be utilized as tools or managed to prevent undesirable range degradation. The purpose of this research was to determine: 1) if conditioned flavor aversion based on feedback from monoterpenes is influenced by age and body condition, 2) if age and body condition influenced the consumption of live redberry juniper foliage, and 3) if 4 monoterpenes found in redberry juniper influence intake by goats differing in age and body

condition over an extended time period. We hypothesized that mature animals in high body condition may consume more juniper and monoterpenes as they are better equipped physiologically to process the monoterpenes found in juniper.

Materials and Methods[1]

Experiment 1 - Formation of Conditioned Flavor Aversions

Aversions are formed when an animal experiences negative post-ingestive feedback following consumption of a food that results in decreased preference for that food at the next encounter. The interactions between taste and feedback are influenced by an animal's physiological condition with preferences formed for foods that alleviate deficiencies or cause satiation and aversions formed for foods that result in malaise from excesses of nutrients of toxins (Provenza 1996). Previous research has established that a dose of 0.22 ml/kg body weight (BW) of the monoterpenes found in redberry juniper foliage was sufficient to produce an aversion (Pritz et al. 1997). The objective of this trial was to determine if four juniper monoterpenes (alpha pinene, limonene, myrcene, and alpha terpineol), cause sufficient negative feedback in goats to form an aversion and if the magnitude of this aversion varied by age and body condition.

Conditioning Period. Research was conducted at the Texas A&M University (TAMU)- Agricultural Experiment Station, near Sonora, TX. Thirty-nine female Boer X Spanish crossbred goats from the same herd were selected from two age groups: young were 2 yrs old (n=20) and mature goats were greater than 6 yrs of age (n=19). Half of each age

[1]All methods involving animals were approved by the University of Idaho, Animal Care and Use Committee (Protocol 2003-46; Appendix D).

group was assigned to either a high body condition (HBC) or low body condition (LBC) treatment, resulting in 4 treatment combinations of 2 factors: age and body condition. The goats were maintained in individual pens with free-choice access to water and trace mineralized salt. For a 10-week conditioning period, those animals in high body condition treatment groups were offered *ad libitum* access to an above-maintenance complete ration, while those assigned to the low body condition treatment received 1.5% BW of a below-maintenance ration in two allotments offered at 0700 and 1700 each day (Table 2.2). Body condition was evaluated by weight and by visual and tactical evaluation (scored on a 1 to 5 scale) once a week by two trained technicians, one which was blind to the treatments. At the end of the conditioning period those animals in the HBC group had an average body condition score of 3.7 ± 0.08 SE, while those in the LBC group had an average score of 1.4 ± 0.12 SE. Weights of the BC groups were 46.55 kg ± 2.3 SE and 29.35 kg ± 1.2 SE for the HBC and LBC groups respectively.

The trial was initiated with a 6-day conditioning period during which the goats were offered 100 g of a novel food for 30 minutes each morning before receiving an allocation of alfalfa pellets (2.0% BW). The novel foods consisted of rolled barley offered on days 1 and 2, white rice offered on days 3 and 4, and rolled barley flavored with 2.0% garlic powder offered on days 5 and 6. A variety of novel foods were offered to increase the likelihood that animals would accept a novel food in subsequent phases of this research (Launchbaugh et al. 1997b).

On day 7, all goats were offered 100 g of the novel food, wheat. Following consumption of wheat and the morning ration of alfalfa pellets, each goat was ruminally infused with a mixture of 4 monoterpenes found in redberry juniper. The mixture was

composed of alpha pinene, limonene, myrcene, and alpha terpineol (Table 2.1), designed to reflect the concentrations found in redberry juniper (Owens et al. 1998). The mixture was administered directly into the rumen by inserting a 10 cm stylus on the animal's left side behind the ribs. The terpenes were administered at a concentration of 0.25 ml/kg BW.

Forty-eight hours after dosing with the monoterpene mixture, the goats were offered a choice between 100 g of wheat, the novel food offered immediately before dosing, and 100 g of whole oats, also a novel food. After 30 minutes, or when one of the foods was completely consumed so that the goat no longer had a food choice, all orts were collected and weighed to determine intake.

Experimental Design and Analysis. This experiment was a 2 x 2 factorial in a completely randomized design nearly balanced with 10 animals per treatment (except young-LBC with nine animals). The model included body condition, age, and wheat consumption expressed as a percent of the total consumed (oats + wheat) and arcsine transformed to achieve normality. Analysis of variance was performed to examine % of wheat eaten with means compared using least squared means when a significant F-test ($P < 0.05$) was observed. All statistical analyses were performed using the statistical software SAS (SAS 2004).

Experiment 2 - Live Juniper Intake

The goats in this experiment were the same goats used in experiment 1 and this research was also conducted at the TAMU- Agricultural Experiment Station, near Sonora, TX.

Experimental Period. Five days before the experiment, all animals were converted to a daily ration of 2.0 % BW (as fed basis) of alfalfa pellets divided into two allotments fed at

0700 and 1700. The goats were maintained in individual pens with continuous access to water and shade. This feeding protocol was continued throughout a 5-day juniper consumption trial during which goats were also offered fresh juniper foliage from 0900 until 1300 each day. Redberry juniper branches were harvested daily from male trees within a 300 m^2 area. The leaves were stripped from the branches by hand and amounts in excess of intake were offered to each goat from 0900 until 1300 each day for the 5 day trial. Orts were collected and adjusted for moisture loss by placing two feed troughs with 250 g fresh juniper outside the pens each day during the trial. The juniper was re-weighed at the end of the 4 hours and the percent of moisture loss was calculated.

Experimental Design and Analysis. This experiment was a 2 x 2 factorial in a completely randomized design nearly balanced with 10 animals per treatment (except young-LBC with nine animals). The model included body condition and age with repeated measurements over sampling time (5 days). An analyses of variance procedure was performed to examine intake. Effects included in the model were body condition, age, body condition * age, animal nested within body condition * age, and all possible interactions of these factors and time. Means for main effects were compared using least squared means when a significant F-test ($P < 0.05$) was observed. All statistical analyses were performed using the statistical software SAS (SAS 2004).

Experiment 3 - Consumption of Terpene Treated Feed

Conditioning Period. The trial utilized 36 goats and was conducted at the University of Idaho Sheep Research and Teaching Center near Moscow, Idaho. The female Boer X Spanish crossbred goats were selected from two age groups: young were 2 yrs old (n=18) and mature goats greater than 6 yrs of age (n=18). Half of each age group was assigned to

either a high body condition (HBC) or low body condition (LBC) treatment, resulting in the same 4 treatment combinations as Experiment 1 and 2. Goats were maintained in a large pen with free choice water and trace mineralized salt, except during the feeding period when the goats were individually penned in stalls measuring 1.16 m^2. Animal conditioning was accomplished in an 18-week feeding period during which LBC does were offered an average of 1.88 % BW[2] of an alfalfa-straw pellet (60% alfalfa, 40% straw), while those assigned to the HBC treatment were offered an average of 2.68% BW of a diet comprised of about 92 % alfalfa pellets and 8 % rolled barley (Table 2.3). Beginning in week 3 of the conditioning period, all animals were offered 100 g of chopped bluegrass hay before their conditioning ration to provide fiber for proper rumen function.

Goats were weighed weekly and amounts of feed were adjusted by treatment and individual to ensure timely progress toward desired body conditions. Beginning in week 15, body condition was scored weekly on a 1-5 scale by tactile and visual estimation of body fat depositions by three trained technicians who were blind to the treatments (Spahr 2004). The mean body condition at the end of the conditioning period was 1.98 $\pm$ 0.09 SE for LBC and 3.06 $\pm$ 0.09 SE for HBC animals. Weights of the BC groups were 47.6 kg ± 1.7 SE and 37.2 kg ± 1.6 SE for the HBC and LBC groups respectively. Seven days before the trial began, all animals were converted to a diet consisting of 100 g chopped bluegrass hay and 1.5% BW of alfalfa pellets.

Experimental procedure. During a 6-day pre-trial adjustment period and 23-day intake trial, goats were offered a feed treated with a mixture of alpha-pinene, limonene, myrcene,

[2]All weights and amount of feed were determined on an as fed basis.

and alpha terpineol (Table 2.1). The terpenes were applied at a concentration of 20.8 g of monoterepenes per kg of feed (as fed basis), which is 2 times the total concentration of monoterpenes in redberry juniper foliage (Owens et al. 1998). The feed was a bluegrass pellet that resembled redberry juniper in nutritional quality (Table 2.3; Houston et al. 1981). The terpenes were mixed according to weight the day before feeding, stored overnight in a glass bottle and applied via a 3.7 L garden sprayer (Chapin Manufacturing, Inc. Batavia, NY). The sprayer tank was metal coated with polypropylene, and the spray nozel was set at a course mist to minimize volatilization, yet allow even coverage of feed pellets. The sprayer was calibrated using the amount of monoterpenes required to treat 27 kg of feed (563 g). This amount was put into the sprayer which was pressurized using 40 pumps and then sprayed back into a flask. The weight of the retrieved monoterpenes determined that about 10 g of monoterpenes were lost each time to sprayer residue and volatilization, therefore 10 additional grams of monoterpenes were added to the sprayer each time a batch of feed was mixed.

About 5 kg of bluegrass pellets were put into the polyethylene drum of an electric concrete mixer (model # 350, Kushlan Products, Inc. Goldendale, WA). The mixer was rotated with pellets while the monoterpenes were applied at a coarse mist for about 10 seconds. Then an additional 5 kg of feed was added followed by another 10 seconds of spraying monoterpenes. This process was repeated until 27 kg of feed was in the mixer and the sprayer was empty. Thereafter, the mixer was turned off and the feed was stirred by hand using a large metal spoon to ensure even mixing of the batch. The initial offering of 750 g of the treated feed was weighed into a 3.78 L (1 gallon) polypropylene feed bucket. About 20

minutes elapsed from the final mixing until the feed was offered to goats. Subsequent batches of feed were mixed with amount depending on demand.

On days 1-6 of the trial, animals were offered one-third of their basal ration (0.05% BW) at 0900 followed by a 4-hour exposure to a feed treated with monoterpenes. These 6 days were used as a training period to familiarize the goats with the monoterpene feed and the testing schedule. The amount of monoterpene treated food offered was gradually increased from 500 g to 1000 g and then reduced to 750 g which was determined to be the optimum amount of feed to offer.

Beginning on day 6, the goats were offered access to the treated feed from 1100 to 1500. After 4 hours all orts were collected and weighed and the goats were returned to a larger holding pen and allowed to drink for 30 minutes. Animals were then returned to their individual pens and offered 1% BW of alfalfa pellets as the remainder of their basal ration.

Orts were collected and intake recorded daily for 23 days. Difficulty in obtaining monoterpenes led to 1 missed day during the experiment. On day 9, there were only enough monoterpenes to offer each animal 250 g of treated feed. On day 18, monoterpene supply was only sufficient to offer the goats 750 g of treated feed and on day 19 the monoterpenes were not offered until 0300. Day 9 was dropped from the analysis because none of the goats were able to achieve desired intake levels, however, there was enough monoterpene treated feed to satisfy most animals on day 18, so it remained in the analysis. Dropping day 9 from the analysis did not influence the overall results of the trial.

Feed Analysis. Samples of the monoterpene treated feed were taken on 2 separate days that were representative of air temperature throughout the trial. The samples were taken immediately following mixing, upon offering the feed, 1 hr, 2 hr, 3 hr and 4 hr after offering

the feed to quantify volatilization of the monoterpenes. Analysis of the monoterpene concentration of the feeds was accomplished by liquid extraction and gas-chromatography-mass-spectrometry (GC-MS) analysis. About 1 g of feed was weighed into a 20 ml scintillation vial containing 10 ml of methanol/diethyl ether (1:4). The vial was shaken for 45 min and extract transferred to a 2 ml vial for GC-MS analysis. The GC-MS analysis was done on a Polaris Q Mass Spectrum Trace GC 2000 coupled to an AS2000 autosampler, both manufactured by Thermo Finnigan, San Jose, CA. The gas chromatograph was equipped with a ZB-1 column (15m X 0.25 mm X 0.25 μm) wall-coated with 100% dimethyl-polysiloxane, (manufactured by Phenomeaux, Torrance, CA). The oven temperature was initiated at 40°C and held for 1 min, then was ramped to 200°C at 5°C per minute and held for 10 minutes. One μl of sample was injected at 210°C with an inlet termperature of 205°C and a constant flow of 1.2 ml/min with vacuum compensation. Ultra high purity helium was used as the carrier gas.

Terpene concentration was quantified by measuring the base peak of the individual monoterpenes. A retention index was calculated based on the retention times derived from a set of standards of the monoterpene mixture diluted to 3 concentrations, (0.2 , 0.02, and 0.002 mg/ml) designed to encompass the range of monoterpenes found in the feed. Peak identification and measurement was performed with Xcalibur 1.3 software.

Neutral detergent fiber (NDF) of feeds was determined by the Van Soest method (Van Soest et al. 1991) and crude protein (CP) analysis of feeds were determined by carbon/nitrogen analysis based on the Dumas method of combustion (Sweeney 1989).

Results

Experiment 1 - Aversion Trial

It appears that only 11 of 22 goats (4 mature-HBC; 3 young-HBC; 1 mature-LBC; and 3 young-LBC) formed an aversion to wheat as indicated by wheat constituting < 25% of total consumption when offered as a choice with oats. Physical reactions to the dosing were observed in most of the animals in the HBC treatment. Some animals laid down, while others assumed postures indicative of abdominal pain. Two animals vocally expressed discomfort. Five goats (1mature-HBC, 1 mature LBC and 3 young-HBC) refused any feed the following day, including alfalfa pellets, and were subsequently dropped from the choice test. Three goats (2 mature-HBC and 1 young-HBC) were dropped from the test because they failed to consume measurable amounts of either oats or wheat. Nine animals were dropped from the test because of experimental error (4 young-LBC and 3 mature-LBC, and 2 mature-HBC).

Neither body condition ($P = 0.77$) nor age ($P = 0.83$) explained the formation of aversion in goats based on monoterpenes (Figure 2.1). An interaction between age and body condition was marginally significant ($P = 0.09$). The only group that expressed an avoidance of wheat was the young-LBC group. These goats ate 23.6% ± 8.9 SE wheat which was lower ($P = 0.04$; $t = 2.96$) than 50% of their total intake indicating a preference for oats over wheat.

Experiment 2 - Live Juniper Intake

While all goats consumed 100% of their maintenance ration of alfalfa pellets, there was considerable variation in intake of juniper foliage among animals within treatments. Intake was adjusted for moisture loss and is expressed as g/kg BW. Goats in LBC ate more ($P < 0.01$) juniper than those in HBC (8.6 g/kg ± 0.7 SE compared to 2.3 g/kg ± 0.3 SE, respectively) and young animals consumed more ($P < 0.05$) juniper than mature goats across

body condition treatments (7.2 g/kg ± 0.7 SE compared to 3.9 g/kg ± 0.5 SE, respectively). Intake increased for all treatment groups throughout the 5 day trial ($P < 0.01$). At the end of the trial period, all animals were consuming nearly 4 times the amount of juniper they had initially.

Goats in the young-LBC treatment decreased intake on day 3 resulting in a Day * BC ($P < 0.01$) and Day * Age interaction ($P < 0.05$; Figure 2.2). No three way interaction (Day*Age*BC) was observed. The young-LBC animals more than doubled their consumption of juniper from day 1 to day 2 and may have experienced sufficient negative post-ingestive feedback to deter their consumption of juniper on day 3. The young-LBC group was the only treatment group that noticeably decreased intake during the trial, however, no other treatment group approached the 13 g/kg BW intake by the young-LBC animals on day 2. The mature-LBC group consumed 11 g/kg BW on the last day of the trial. Had the experiment continued another day, the mature-LBC may have experienced a marked decrease in intake as well.

Experiment 3 - Consumption of Terpene Treated Feed

Goats increased intake during the 6 day pre-trial period and were readily accepting the feed by the first day of the trial, suggesting the odor and flavor of the monoterpene mixture was not overly offensive to the goats. While intake varied by day ($P < 0.01$), there was no overall upward or downward trend in intake for any treatment group throughout the 23 days of the trial (Figure 2.3). Age did not affect intake of the treated feed ($P = 0.13$), so data are presented as values averaged across age. Animals in LBC consumed more ($P < 0.01$) of the terpene treated feed than did those in HBC (24.5 g/kg BW ± 1.2 SE compared to 16.9 g/kg ± 0.8 SE, respectively). Additionally, total daily intake of monoterpenes by the LBC group

during this trial was roughly double the dose administered in the aversion trial (0.49 g/kg vs. 0.21 g/kg BW) or shown to create aversions by Pritz et al. (1997). The HBC goats were also able to maintained intake levels above the effective dose of monoterpenes (0.34 g/kg BW). Total intake in g of the treated feed and the basal ration was the same for both LBC and HBC goats (Figure 2.4), however, when total intake was examined as a proportion of body weight, the LBC goats consumed 4.0 % BW while the HBC goats consumed 3.2 % BW (Figure 2.5).

Cyclical intake was evident for all treatment groups throughout the trial (Figure 2.3), however it did not differ from normal cyclical intake with non-toxic foods when compared to ad libitum alfalfa pellet intake by the HBC goats for 25 days of the conditioning period. There was a significant day * BC interaction ($P < 0.01$) for the second day following the shortage. No other interactions (BC * Age, Day * Age, or BC * Day * Age) were observed. Both LBC and HBC animals gained weight throughout the 23 day trial, however, as a group the LBC gained more ($P < 0.01$) than the HBC (2.1 kg ± 0.5 SE vs. 0.1 kg ± 0.5 SE, respectively).

Monoterpene concentration of feed. There was no decline in the concentration of monoterpenes on the feed even after 4 hrs (($P = 0.68$; $R^2 < 0.01$; Figure 2.6). The feed was treated with 20.8 g/kg and the average monoterpene concentration detected was 18.97 g/kg ± 0.45 SE. Therefore, volatilization of the monoterpenes should not have influenced intake.

Discussion

We examined conditioned flavor aversions formed to digestive feedback induced by infusions of juniper monoterpenes because we believed it would indicate an animal's ability to process and negate the deleterious effects of these plant secondary metabolites. We

assumed that animals readily able to detoxify the administered dose of monoterpenes would express weaker aversion to wheat than goats with compromised detoxification abilities. Half of the goats in our experiment expressed aversion based on monoterpene feedback, however, age and body condition did not explain patterns of aversion.

Young goats in the low body condition treatment expressed an aversion to wheat suggesting that young-LBC goats experienced negative post-ingestive feedback from the dose. The severe reactions to dosing observed in the high body condition goats may have been caused by the large amount of monoterpenes administered. Three high body condition goats were removed from the experiment because they consumed less than 20 grams of feed on the test day and 7 of the 10 remaining goats expressed aversions (i.e., ate less than 25% wheat of the total wheat and oats consumed). Some of the animals received nearly 3 times the amount of monoterpenes administered to the low body condition animals. It is unlikely that all the additional weight of these animals was active in metabolizing the monoterpenes, so they may have suffered a more intense toxic insult to their rumen and digestive system than goats in the LBC groups. Specifically, intra-abdominal fat deposits in obese ewes have been shown to decrease filling capacity of the gastrointestinal tract, especially the rumen (McCann et al., 1992). Therefore, the large dose combined with a smaller rumen in relation to body size may have initiated the severe reaction to the dosing event.

We do not know of any aversion trials that were able to detect differences in detoxification or processing abilities of secondary plant metabolites. Because not all animals in our trial expressed aversions, the dose used in this experiment was apparently sensitive to animal differences and should have revealed a difference in the level of aversive feedback if it was clearly related to age or body condition. It is likely that potential differences in intake

of juniper based on body condition or age may not be simply related to digestive feedback based on monoterpenes. It is also possible that an experimental protocol based on conditioned flavor aversions is not sensitive enough to reveal small differences in digestive malaise experienced by animals.

Goats in low body condition ate more live juniper than those in high body condition. The need for malnourished herbivores to alleviate deficiencies can lead to increased dry matter intake of many types of foods. Animals can accomplish this by increasing their biting rate (Arnold and Birrell 1977; Sprinkle et al. 2000) or time spent grazing (Sibbald and Kerr 1994; Sibbald 1997). Alternatively, as we suggest here, animals may reach above normal intake by increasing diet breadth and consuming chemically defended foods that would normally be rejected. For example, cattle in low body condition began including lupine in their diet before cattle in high body condition and consumed greater amounts overall (Lopez-Ortiz 2002). Therefore, while animals in low body condition may not be as able to metabolize the toxins found in plants, they are more likely to consume toxic plants.

Supplementation, particularly of protein, has been demonstrated to increase consumption of redberry juniper (Taylor et al. 2001). Animals in low body condition had been receiving a below-maintenance ration until 2 weeks before the trial when they began consuming alfalfa pellets. The alfalfa pellets provided a greater protein source than their conditioning ration, so, in a way, they were being supplemented with protein while the alfalfa provided only a portion of the total nutrients the high body condition animals had been receiving. Research with sheep conducted by Koong et al. (1985) and Ferrell et al. (1986) demonstrated that nutritionally deprived animals have lower basal metabolism that consequently decreases maintenance requirements. If the low body condition goats in our trial

had lower basal metabolic rates, then more energy may have been available for detoxification allowing for greater juniper consumption.

Young animals consumed more juniper than mature goats within body conditions. Young animals have higher nutritional requirements and consequently select diets that are higher in nutrients than mature animals, when possible (Cazcarra and Petit 1995; Grings et al. 2001). But, a lack of foraging experience can result in young animals sampling more novel foods or returning to foods that made them ill in the past, especially when forage choice is limited, as is often the case on rangelands for at least a portion of the year (Ralphs and Provenza 1999).

At the end of the 5 day trial, all animals were consuming nearly 4 times the amount of juniper they had initially. Gradual increase in the acceptance of novel foods is a well known ruminant behavior, however, because these goats were all raised in an area where juniper is a common plant, it is unlikely that it was a novel food to them. This suggests that animals may have been acclimating to the trial protocol and that the juniper may have been providing some nutritional benefit to the animals. However, Pritz and colleagues (1997) found that juniper consumption results in a negative nitrogen balance in goats so this does not seem likely. Mellado et al. (2004) found that goats in low body condition included a higher proportion of low quality and chemically defended shrubs in their diet, presumably in an attempt to maintain high intake rates. Therefore, it is more likely that low body condition animals were trying to increase intake rates and juniper was the only available food to accomplish intake.

When we examined intake of terpene treated feed, there was no upward or downward trend in intake for any treatment group throughout the 23 day trial. Therefore, none of the

animals appeared to be experiencing any cumulative toxic effect that would have resulted in a decline in intake, despite the fact that the feed was treated with twice the amount of total monoterpenes that would be found in redberry juniper (Owens et al. 1998). However, redberry juniper contains a much wider array of monoterpenes than the 4 used in this study (Owens et al. 1998). Studies by Estel et al. (1998, 2000, 2002) examined the influence of 15 individual monoterpenes on the consumption of alfalfa pellets treated with up to 10 times the amount of the compounds naturally occurring on tarbrush (*Flourensia cernua*). Only 4 of the 15 monoterpenes influenced consumption of alfalfa pellets. Furthermore, previous studies determined that limonene, the terpene that made up the largest proportion of the mixture, was not strongly correlated with intake of redberry juniper by goats (Riddle et al. 1996). This suggests that the suite of monoterpenes in plants has a potentially synergistic effect, not accomplished by individual monoterpenes. However, total intake of monoterpenes during the treated feed trial was also greater than the dose observed to create aversions in this and related research (about 2 times greater for LBC and 1.5 times greater for HBC goats). Again, this could be related to the swift introduction of monoterpenes administered in pure form directly into the rumen in aversion trials versus the much slower release of monoterpenes experienced during consumption. It is also possible that the total amount of monoterpenes on the feed did not enter the rumen, perhaps from volatilization during the chewing or rumination process (Cluff et al. 1982).

A decrease in body condition is accompanied by changes in the hormonal balance of animals that can influence intake patterns. A decrease in body condition is generally associated with a decrease in plasma leptin concentrations. Leptin is a peptide synthesized and secreted by adipocytes. It has the potential to regulate intake by acting on the

hypothalamus to decrease appetite and increase energy expenditure (Vernon et al. 2001). However, leptin is now believed to function more as a signal for too little adipose tissue rather than too much. Fasting results in a rapid decline in plasma leptin levels. This fall in plasma leptin initializes adaptations such as increased metabolic efficiency and appetite. In the absence of leptin, appetite control is lost, therefore, decreased plasma leptin concentrations signal the animal to increase intake of nutrients in an attempt to achieve a more stable nutritional state (Vernon et al 2001). This increase in appetite was demonstrated by the low body condition goats as they consumed the same amount of feed on a daily basis as the high body condition animals. This resulted in the low body condition animals consuming a greater proportion of their body weight in feed than the high body condition animals.

The fact that the low body condition goats were capable of gaining weight during the trial suggests that the feed offered sufficient nutrients to facilitate detoxification and result in weight gain. This indicates that metabolism of the 4 monoterpenes did not leave the animals in an energy deficit, despite the relatively low quality of the bluegrass pellets and the limited amount of alfalfa pellets (1.5% BW). It also suggests that the higher intake expressed by the low body condition group was due solely to compensatory intake often observed in animals in low body condition (Gunn et al. 1991; Sibbald 1997; Sibbald and Rhind 1997). However, Foley et al. (1995) and Illius and Jessop (1995) would argue that for animals to consume poisonous plants there must either be enough nutrients in the toxic plant itself to fuel detoxification or in the rest of the diet. The goats were receiving less than maintenance requirements from the basal ration and it constituted only about 25% of the low body

condition group's total intake, so about 75% of the low body condition group's total intake was comprised of the terpene treated feed.

This raises speculation that monoterpenes may not be as influential as previously thought on intake as goats in low body condition continued to maintain a higher overall level of intake throughout the 3 week trial despite the monoterpene content of the feed. This is supported by the fact that several studies suggesting monterpenes inhibit consumption were conducted with animals in good nutritional status that were not on a restricted basal diet (Schwartz et al. 1980; Vourch et al. 2002). This has also been observed with redberry juniper as goats will consume larger quantities in pen situations vs range situations where goats have access to other forage species. So monoterpenes may be inhibiting enough that, when given a choice, animals will avoid them, but not so aversive that herbivores are not willing to include substantial quantities in their diet to maintain intake. For example, mule deer are capable of maintaining body condition on a diet containing as much as 69% sagebrush when other forage is limited (Pederson and Welch 1982).

The toxic effects of monoterpenes post ingestion in ruminants are not well understood. While it is widely believed that monoterpenes are anti-microbial, studies with deer (Pederson and Welch 1982) and goats (Straka 2001) determined that monoterpenes do not inhibit digestive processes. Most research investigating the toxic effects of monoterpenes has involved the forced dosing of animals directly into the rumen with either pure monoterpenes or foliage from monoterpene containing plants (Johnson et al. 1976; Pritz et al. 1997; Straka 2001). However, research by Cluff et al. (1982), determined that monoterpene concentration in the rumen of mule deer was about 80% lower than expected given the concentration of monoterpene containing plants in the diet. This suggests that profound

changes in concentration, and thus toxic effects, can occur from the natural processes of ingesting juniper (i.e. mastication and a slower intake rate) vs animals dosed directly into the rumen.

All animals increased intake dramatically the day immediately following the shortage of monoterpenes then decreased intake the next day, presumably in response to negative post-ingestive feedback. However, the low body condition animals decreased intake much more than the high body condition animals suggesting they experienced stronger negative feedback. Whether this was a function of LBC, regular cycles in intake, or total concentration of monoterpenes consumed is unclear.

Management Implications

Animals in low body condition will clearly consume more redberry juniper in a pen situation, however, it is unclear if this indicates an increased willingness to include chemically defended plants in their diet, or whether it is simply a result of increased intake patterns to alleviate nutritional deficiencies. Furthermore, animals in low body condition were able to maintain consistently higher consumption of monoterpenes than the high body condition animals for at least 3 weeks as indicated by our research.

Animals grazing rangelands undergo natural fluctuations in body condition related to forage availability and reproductive cycle. It is important to recognize that the diet of animals in low body condition may vary not only in total amount consumed, but in the species richness and proportions that they occur in the diet from that of animals in high body condition. For this reason, it is important to consider the state of the animal when

implementing range management strategies, such as calculating stocking rate based on intake as a percent of body weight.

These findings also have important application for the selection and care of animals in prescribed grazing plans to control invasive plants. Because most animals, particularly those involved in redberry juniper control programs are generally used for production and vegetation management, it is not recommended that animals be maintained in low body condition. Livestock production will generally take priority over vegetation management until researchers and managers can increase juniper consumption to a point that economic returns from brush control can compare to sales of meat and offspring. However, the economic demands of ranching are encouraging producers to diversify and juniper control may constitute a way to add value to an enterprise without seriously decreasing production. Therefore, goat producers may use this knowledge to take advantage of periods where animals are naturally in low body condition to achieve greater utilization of juniper. Further research into how age and body condition influences consumption of other plant secondary metabolites has the potential to expand the usefulness of these animal attributes in vegetation management.

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Table 2.1. Simulated redberry juniper (*Juniperus coahuilensis*) monoterpene mixture used in an aversion trial in goats (Experiment 1) and on a terpene treated feed trial (Experiment 3).

Monoterpene	Purity (%)	Percent in mixture[1]	Manufacturer
1R (+) Limonene	99	58	Acros Organics
Myrcene	88	24	Acros Organics & Sigma Aldrich
Alpha Pinene	98	13	Acros Organics
1R (+) Alpha Terpineol	99	5	Acros Organics

[1] = Percent on weight basis

Table 2.2. Nutrient composition of mixed rations fed to goats high body condition (HBC) or low body condition (LBC) treatments during conditioning period. Animals in the HBC treatment had *ad libitum* access to the feed for a 10-week conditioning period, while LBC animals were offered 1.5% BW daily (as fed basis) for the 10 weeks.

Nutrient	HBC Ration	LBC Ration
	---------- % Dry Matter ----------	
Crude Protein	16.24	9.02
Total Digestible Nutrients	71.08	56.08
Crude Fat	2.60	2.35
Acid Detergent Fiber	24.45	43.75
	----------Mcal/kg----------	
Net Energy - maintenance	0.75	0.53

Table 2.3. Neutral detergent fiber (NDF) and crude protein(CP) content of composite samples of feeds used in the terpene-treated feed conditioning period, basal ration, and experimental ration.

Feed	NDF	CP
	---------- % Dry Matter ----------	
Alfalfa/straw pellets	57.9	11.3
Rolled barley	49.3	14.4
Alfalfa pellets	47.1	16.5
Bluegrass hay	63.9	5.3
Bluegrass pellets	54.6	10.4

Figure 2.1. Wheat consumption by young or mature goats in low or high body condition (BC) when wheat was offered in a choice test with oats. The preference test was conducted to examine evidence of an aversion formed after its initial consumption was paired two days before this test with intra-ruminal infusions of monoterpenes found in juniper.

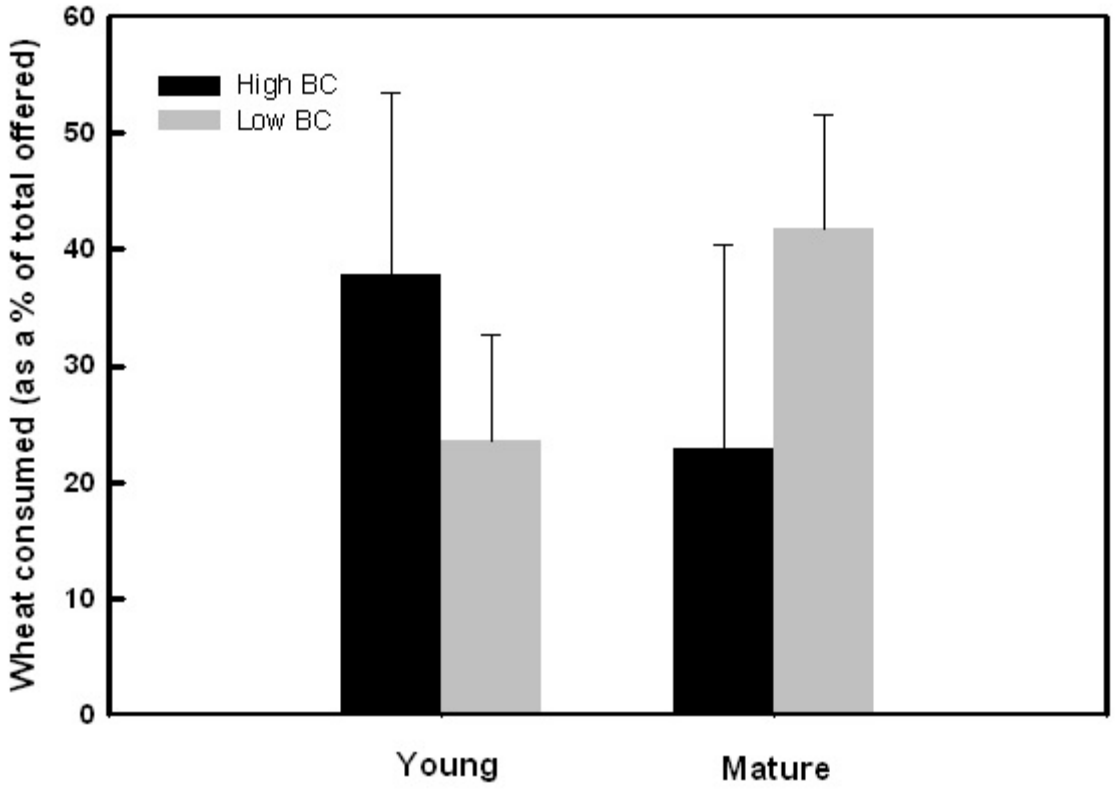


Figure 2.2. Mean intake of live juniper foliage by domestic goats for 5 days. Intake was adjusted for moisture loss and expressed as g^{-}/kg BW. Treatments were of goats in low or high body condition (LBC or HBC) either young (2 yrs old) or mature (> 6 yrs of age).

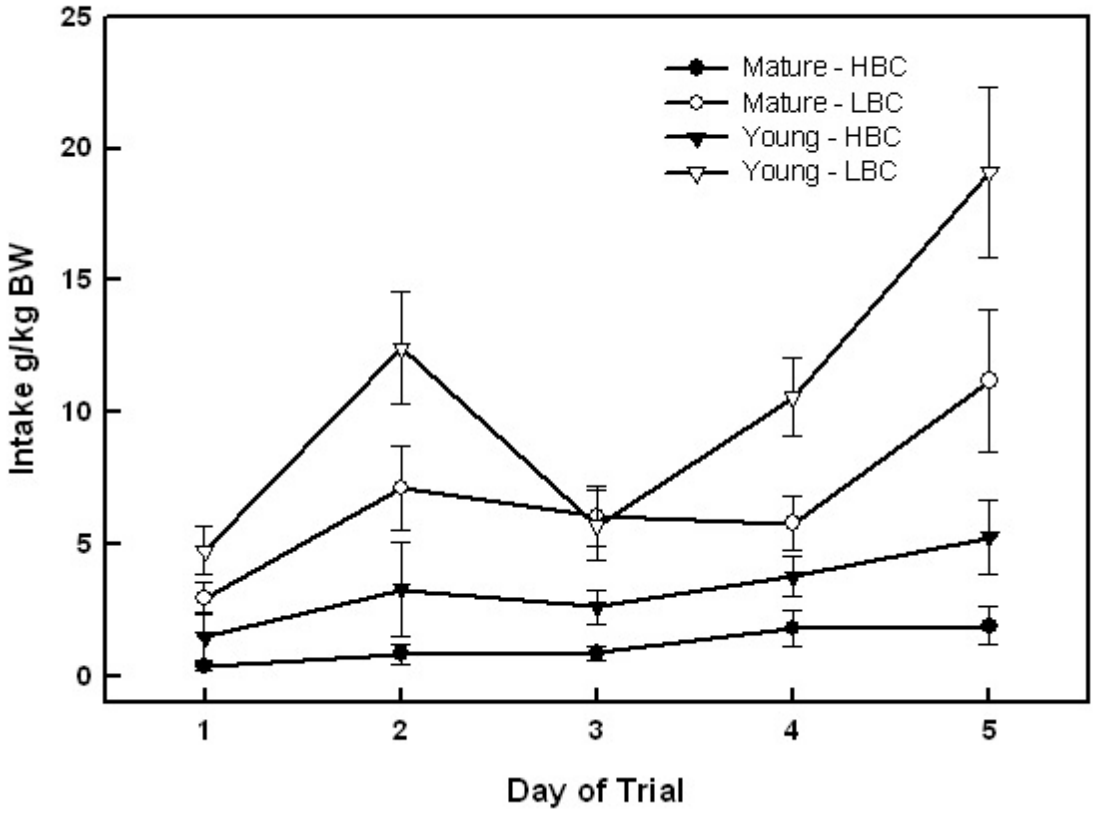


Figure 2.3. Mean intake of bluegrass pellets treated with 20.8 g/kg of a monoterpene mixture. The treated pellets were offered in excess for 4 hours for 23 consecutive days. Treatments were low body condition (LBC) and high body condition (HBC). Day 9 (gray) was not included in analysis because of limited terpene availability.

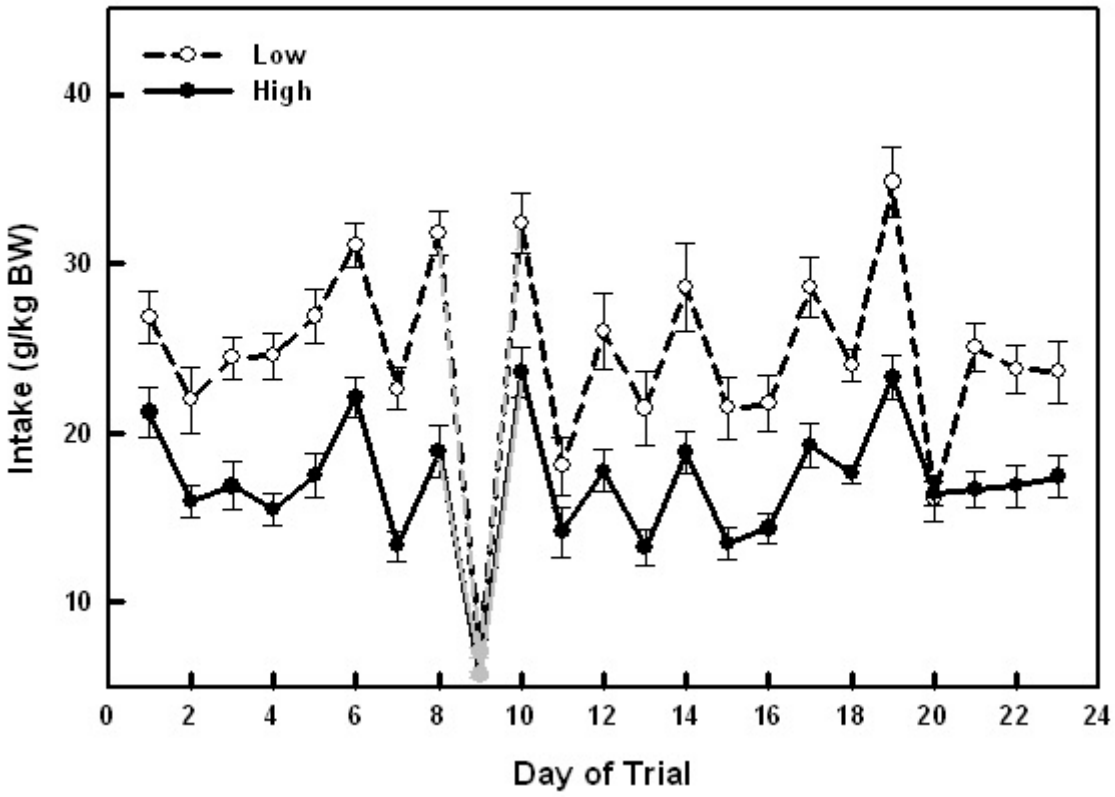


Figure 2.4. Mean total intake per day of bluegrass pellets treated with 20.8 g/kg of a monoterpene mixture and basal ration alfalfa pellets. Treatments were low body condition (LBC) and high body condition (HBC).

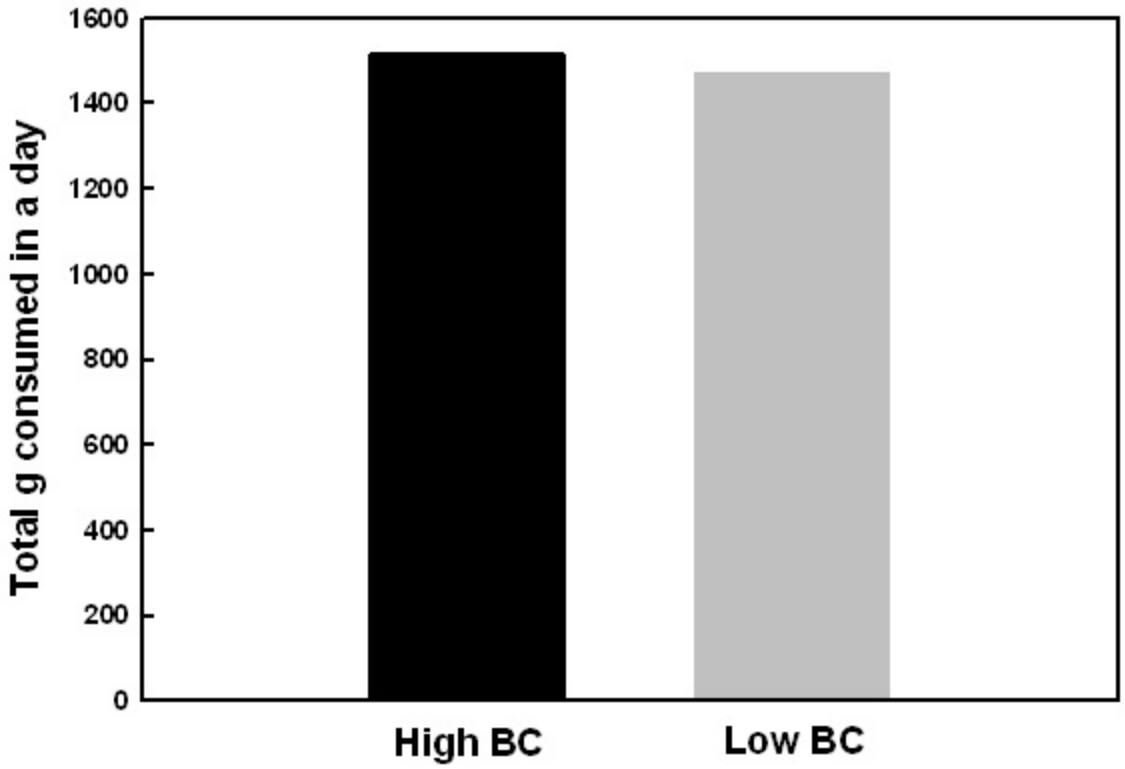


Figure 2.5. Mean total intake per day as a percent of body weight of bluegrass pellets treated with 20.8 g/kg of a monoterpene mixture and basal ration alfalfa pellets. Treatments were low body condition (LBC) and high body condition (HBC).

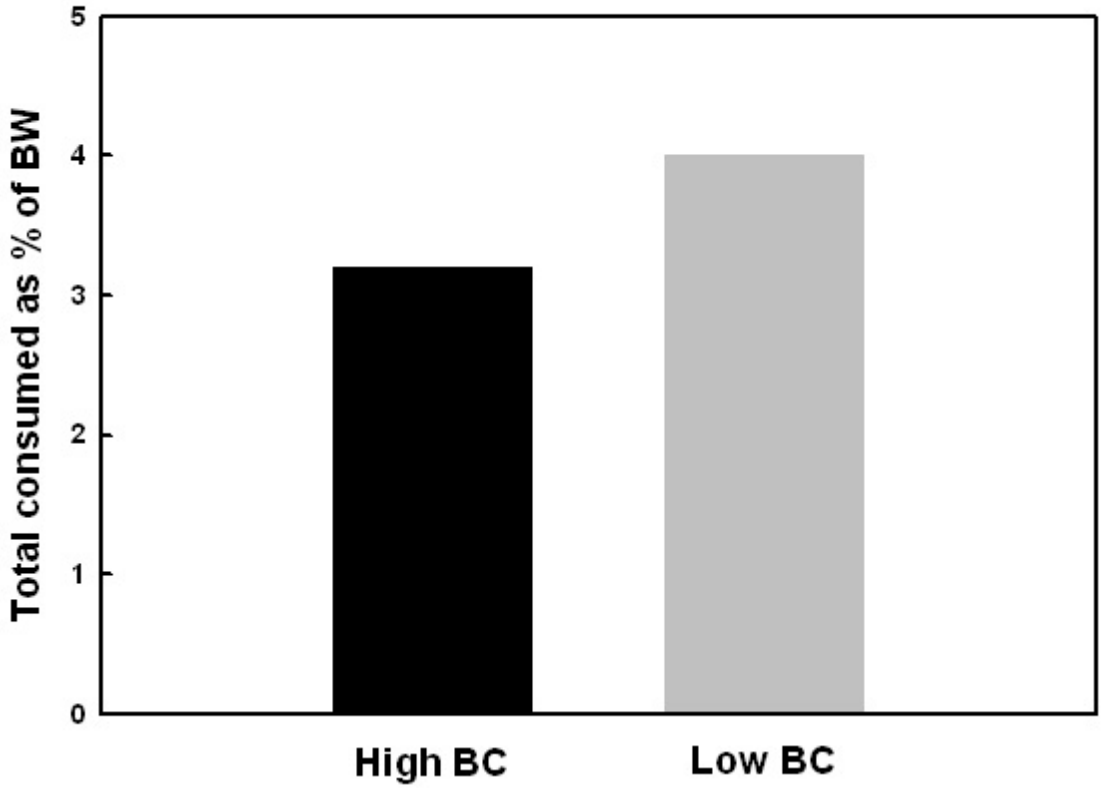
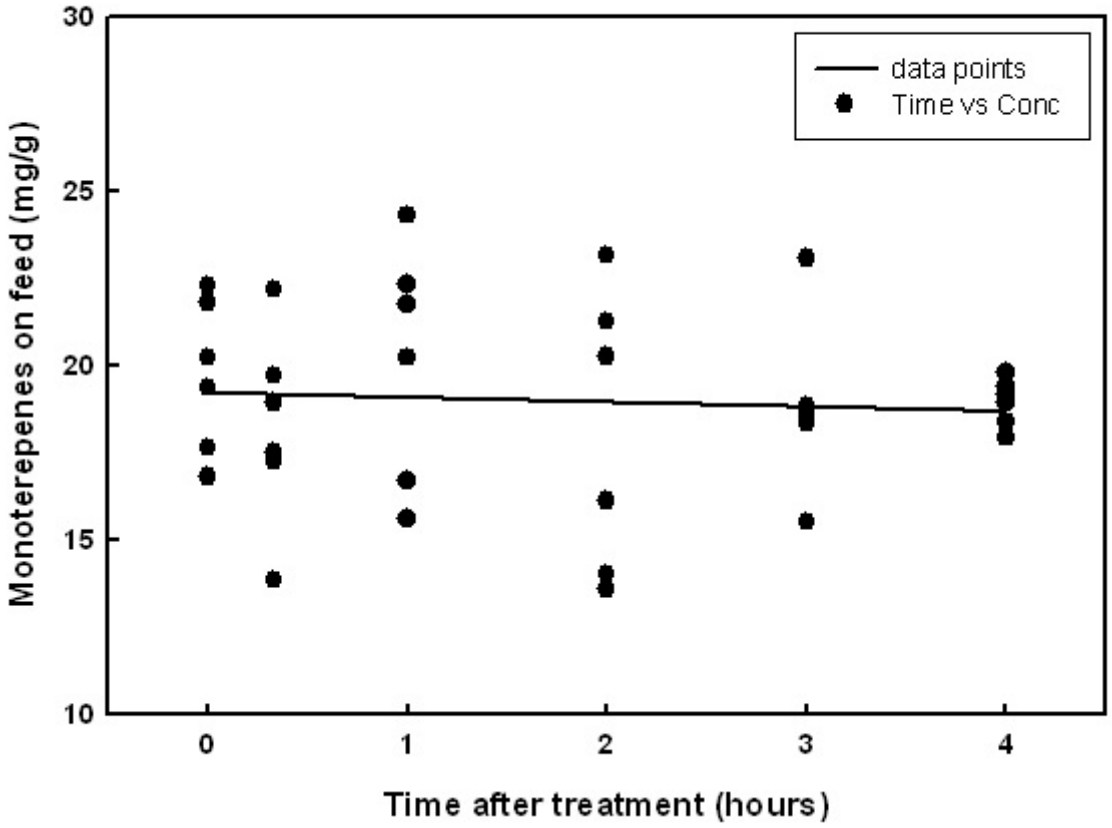


Figure 2.6. Regression of monoterpene concentration on bluegrass pellets treated with 20.8 g/kg of a monoterpene mixture for the four hour period it was offered in excess.



CHAPTER THREE: Effects of Age and Body Condition on Disposition of 4 Monoterpenes found in Redberry Juniper (*Juniperus coahuilensis*)

Abstract

Animals in low body condition, or suffering from nutritional stress, may not be as well equipped to consume plant secondary metabolites (PSMs) as their counterparts in good nutritional status because of limited capacity to process ingested PSMs. The age and experience of animals may also profoundly affect diet selection and tolerance of secondary compounds. The objective of our study was to determine if there is a difference in the way that animals of different ages and body conditions eliminate monoterpenes from the bloodstream. The trial used 32 domestic female goats that were selected by age groups: young were 2 yrs old (n=16) or mature being greater than 6 yrs of age (n=16). Half of each age group was assigned to either a high or low body condition (HBC or LBC) treatment, resulting in 4 treatment combinations of 2 factors: age and body condition. Goats received an intra-ruminal dose (0.35 ml/kg BW) of a mixture of 4 monoterpenes and samples of whole blood were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 18 hrs post dosing. Blood samples were analyzed for monoterpene concentrations using gas chromatography and were analyzed for actual concentration of monoterpenes in whole blood and concentrations of monoterpenes adjusted by dose. Mature animals in high body condition had a greater area under the curve (AUC) for whole blood concentrations of monoterpenes plotted against time than LBC ($P = 0.03$; 1256.72 ng/ml ± 291.71 SE vs. 688.89 ng/ml ± 53.11 SE, respectively). However, when blood terpene concentration was examined as a proportion of the dose, LBC animals expressed greater levels of total monoterpenes in their bloodstream than the HBC

animals ($P < 0.01$). Neither AUC, ($P = 0.30$), nor half life ($P = 0.17$) differed between LBC and HBC. While HBC had greater total blood concentration of monoterpenes, they did not experience slower elimination rates. Furthermore, because LBC animals had greater monoterpene concentrations in the blood, despite a lower dose, they appear to be less efficient at eliminating the monoterpenes from the bloodstream. Therefore, body condition influences blood levels of monoterpenes which may influence the feeding strategy of herbivores, particularly browsers.

Key words: Age, Body Condition, Monoterpenes, *Juniperus coahuilensis*, Pharmacokinetics, and Goats.

Introduction

The capacity of an herbivore to detoxify and eliminate plant secondary metabolites (PSMs) directly influences the amount of chemically defended plants the animal can ingest (Freeland and Janzen, 1974). Herbivores have developed a variety of mechanisms to counteract the negative digestive and metabolic effects of PSMs (Brattsten, 1979). However, the efficiency of these detoxification systems can be influenced by both the internal and external environment of the herbivore. Herbivores can experience toxic effects from PSMs when detoxification pathways become saturated, or when there are insufficient quantities of necessary nutrients or metabolic products to complete the biotransformation process. Foley et al., (1995) describes how saturation of detoxification pathways can threaten acid-base homeostasis, causing browsing mammals to break down muscle tissues to provide substrates for buffering or energy for biotransformation. This "cost" of detoxification has been demonstrated to influence diet selection in several species of herbivores (Illius and Jessop, 1995; Guglielmo et al., 1996; Wang and Provenza, 1996). Therefore, the nutritional status of an animal may be an important factor determining diet selection of free ranging herbivores.

Animals in low body condition, or suffering from nutritional stress, may not be as well equipped to consume PSMs as their counterparts in good nutritional status for several reasons. The biotransformation of toxins requires nutrients in excess of those for maintenance (Illius and Jessop 1995; Guglielmo et al., 1996). The activity of visceral organs involved in detoxification, including the liver, accounts for 40-50% of total energy requirements in domestic animals (Noziere et al., 1999). The microsomal mixed function oxidase (MFO) system is involved in detoxification of many secondary compounds and activity of this system is higher in the liver than in other organs (Wattenburg et al., 1976). Prolonged periods

of nutritional stress can result in a loss of liver mass (Noziere et al., 1999). Low protein diets decrease the activity of the cytochrome P450 enzyme system of the liver further hampering the animal's detoxification abilities (McLean and McLean, 1969).

Low body condition may reflect several internal conditions that could limit an animal's ability to ameliorate and process ingested PSMs. This was demonstrated when domestic sheep of low and average body condition were dosed with lupine (*Lupinus spp.*) and blood alkaloid levels were monitored (Lopez-Ortiz et al., 2004). Animals in low body condition had higher levels of alkaloids in their system for longer time periods than those in average condition. Consequently, sheep in low body condition were exposed to the toxins for a longer period of time and suffered a greater toxic insult than those in average body condition.

The age and experience of animals may also profoundly affect diet selection and tolerance of secondary compounds. Experiences, especially those early in life, can affect food preferences, potentially changing the morphology and physiology of the body. For example, exposure to blackbrush (*Coleogyne ramosissima*) early in life resulted in increased reticulorumen sizes in goats and increased consumption of this plant later in life (Distel and Provenza, 1991). However, exposing animals to phytochemicals early in life has variable results. In some cases, early exposure to toxins may increase the liver's capacity for detoxification (Smith, 1992). In other cases, it may impair liver function depending on the toxin and its dose. Pritz et al., (1997) found that exposure to monoterpenes early in life resulted in decreased consumption of juniper later in life, presumably due to apparent liver damage. Exposure to some alkaloids can result in accumulations of the toxin in the liver that decrease function and with repeated exposure can result in death (Stegelmeier et al., 1996).

Thus, older animals may express enhanced or compromised detoxification efficiency depending on dietary experience. Young animals may also face unique foraging challenges that arise from their higher nutritional requirements and lack of foraging knowledge that can make them more apt to try novel (potentially toxic) foods and to re-try foods that initiated illness in the past (Ralphs and Provenza, 1999).

Browsing herbivores face unique foraging challenges because of the wide array of PSMs found in browse that make it almost impossible to avoid consuming some quantity of allelochemicals. Widespread toxins, including tannins and terpenes, are found in many browse plants throughout the western United States. Monoterpenes occur in wide-spread plant genera such as junipers (*Juniperus spp*.), sagebrush (*Artemisia* spp.), and pines (*Pinus* spp.). This class of aromatic chemicals is believed to inhibit herbivory by grazing and browsing ruminants by exerting negative effects on rumen microbial populations (Ngugi et al., 1995), by producing conditioned flavor aversions as a result of the herbivore experiencing illness following consumption (Launchbaugh et al., 1997), and by influencing feeding patterns and intake (Dziba et al., 2004; Chapter 2). Monoterpenes can therefore have serious implications for foraging behavior and the ecology of plant communities dominated by monoterpene-producing plants.

Redberry juniper (*Juniperus coahuilensis*) is an invasive, evergreen tree that has been rapidly expanding throughout western and central Texas, southwestern Oklahoma, southeastern New Mexico, and northeastern Mexico since the early 1900's (Adams and Zanoni, 1979). Juniper is native to the southwest, but was historically confined to rocky outcroppings and areas of shallow soil or poor productivity (Ueckert et al., 1994). However, heavy season-long grazing and the reduction of frequency and intensity of natural fires has

contributed to its rapid expansion into more productive areas. Currently, juniper is posing serious problems for livestock producers and land managers because it can profoundly affect rangeland production and hydrology (Thurow and Hester, 2001). The tree has a dense lateral root system that enables it to be a staunch competitor for moisture and soil nutrients. Juniper trees also alter the amount and distribution of water reaching the soil (Thurow and Hester, 2001). This results in a dramatic decline in the herbaceous vegetation and reduces carrying capacity for livestock and wildlife while creating conditions that further perpetuate the establishment of juniper (Dye et al., 1995). There has been increasing interest in enhancing livestock consumption of juniper, particularly goats, in an attempt to restore the balance between woody and herbaceous plants and to gain some value from juniper in the form of livestock forage. However, before this can be accomplished, it is imperative that researchers gain a better understanding of how animals respond to monoterpenes, particularly during different production cycles on rangelands.

The objective of our study was to determine if there is a difference in the way that animals of different ages and body conditions eliminate monoterpenes from the bloodstream. We hypothesized that mature animals in high body condition would be more efficient at clearing monoterpenes from the blood and thereby have a lower total blood concentration of monoterpenes. We accomplished this research by examining 4 monoterpenes found in redberry juniper.

Materials and Methods[3]

Conditioning Period

Research was conducted at the University of Idaho, Sheep Research and Teaching Center near Moscow, ID. The trial utilized 32 domestic female goats that were selected by age groups: young were 2 yrs old (n=16) or mature being greater than 6 yrs of age (n=16). Half of each age group was assigned to either a high body condition (HBC) or low body condition (LBC) treatment, resulting in 4 treatment combinations of 2 factors: age and body condition. Goats were maintained in a large pen with free choice water and trace mineralized salt except during the feeding period when the goats were penned individually in feeding stalls measuring 1.16 m^2. Animal conditioning was accomplished in an 18-week feeding period during which LBC does were offered an average of 1.88% of body weight (BW)[4] of an alfalfa-straw pellet (60% alfalfa, 40% wheat straw), while those assigned to the HBC treatment were offered an average of 2.68% BW of a ration comprised of 92% alfalfa pellets and 8% rolled barley (Table 3.1). Beginning in week 3 of the conditioning period, all animals were offered 100 g of chopped bluegrass hay before their conditioning ration to maintain good rumen function. Goats were weighed weekly and amounts of feed were adjusted by treatment and individual to ensure timely progress into desired body conditions. Beginning in week 15, body condition was scored weekly on a 1-5 scale by tactile and visual estimation of body fat depositions (Spahr, 2004) by three trained technicians who were blind to the treatments. The mean body condition scores at the end of the conditioning period were 1.98 $\pm$

[3]All methods involving animals were approved by the University of Idaho, Animal Care and Use Committee (Protocol 2003-46, Appendix D).

[4]All weights and amount of feed were determined on an as fed basis.

0.09 for LBC and 3.06 ± 0.09 (mean ± SE) for HBC animals. The mean weights at the end of the conditioning period were 36.30 kg ± 1.32 SE for the LBC goats compared to 46.58 kg ± 1.44 SE for the HBC goats.

Blood Clearance Trial

Preparation. The 32 goats in this experiment were randomly assigned to one of two groups with each group having equal numbers of goats from each treatment. Group 1 underwent the experiment on 09 and 10-Oct-2004, while group 2 underwent the experiment the following week on 16 and 17-Oct-2004. The experiment was conducted in two time phases because the dosing and blood collection required time sensitive application and we were not able to accommodate all animals in one trial. There was no difference between the 2 groups except the day on which the trial was conducted. Seven days before each trial period, all animals were offered a ration of 2.0% BW alfalfa pellets and 100 g of chopped bluegrass hay for 30 minutes to equalize rumen fill and microbial environment among animals and treatments.

On day 1 of the trial, goats were fitted with an 18-gauge 5 cm indwelling catheter placed in the jugular vein on the left side of the neck. The catheters were fitted with a 76 cm extension and secured in place with vet wrap and Elasticon®. The catheters were all flushed with 10 ml of heparin at a concentration of 10 units/1000 mLS .

Dosing Procedure. A mixture composed of 13 % α-pinene, 58% limonene, 24% myrcene, and 25% alpha terpineol was prepared the day before ruminal dosing (Table 3.2). This terpene mixture was designed to reflect the concentrations found in redberry juniper (Owens et al., 1998). The mixture was administered directly into the rumen by inserting a 10 cm stylus on the animal's left side behind the ribs. A syringe was attached to the needle and

back-filled to determine if the needle had penetrated the rumen. Once rumen penetration was confirmed, the terpene mixture was injected and the needle flushed with 5 mls of additional water. The monoterpenes were administered at 0.35 ml/kg BW and mixed with 20 ml of water to mediate initial reactions from the dosing. Previous research established this dose as non-lethal, yet enough to cause noticeable discomfort in animals. Animals were observed for 15 minutes after dosing and reactions to the dosing were recorded. The exact time of dosing was recorded for each animal.

Blood collection. A sample of whole blood was collected 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 18 hrs post dosing. For each blood collection, 10 mls of blood were first drawn via the catheter and discarded to ensure that all heparin was removed from the catheter and extension and that a clean blood sample was obtained. Then, 10 mls of blood were collected in a clean polypropylene syringe and immediately transferred to a vacutainer™ tube containing freeze dried sodium heparin to prevent clots. After thoroughly mixing the blood with the sodium heparin, the blood was transferred to a 7 ml polypropylene storage vial. The labeled vials were transferred to a freezer and stored at -17° C until analysis. Whole blood was used in this study because we determined that there was no difference in the monoterpene recovery between whole blood and plasma using the extraction method employed in this experiment (Appendix A).

Following each blood draw, the catheters and extensions were flushed with 15 mls of heparin to prevent clots. For the first 6 blood draws the catheters were flushed with heparin at a concentration of 1 unit/1000 mls and subsequently increased to 100 units/1000 mls because of the increased time between blood collections. Animals were maintained within their feeding stalls with free access to water during the 18-hr collection period. After the final

blood sample was drawn, the catheters were removed, goats were administered 1 ml of Penicillin G Procaine (Pen G; manufactured by GC Hanford, Syracuse, NY) to prevent infection, and moved to a large common holding pen.

Sample preparation and analysis. In preparation for analysis, samples were thawed and were mixed for 15 seconds on a Vortex mixer at high speed. All blood samples were prepared in duplicate. Two ml of blood were transferred into a 10 ml glass test tube using a micropipette. Two ml of ethyl ether containing 0.02% naphthalene was added to the blood. Ethyl ether was compared to n-Hexane and to a 1:1 ethyl ether:n-hexane mix as a solvent and determined to be the most consistent with regard to monoterpene recovery for this experiment (Appendix B). The blood and ether were mixed for 30 seconds by a Vortex mixer on high speed. The samples were then centrifuged at 5000 rpms for 15 minutes, after which about 1 ml of clear liquid was pulled off the top with a glass Pasteur pipette and transferred to a 2 ml glass vial for a robotic autosampler for GC analysis.

A Clarus 500 GC coupled to an autosampler, (both manufactured by Perkin Elmer, Wellesley, MA), was employed for the analysis. The gas chromatograph was equipped with a Restek Rtx-5 column (30m x 0.25 mm x 0.25 µm) wall-coated with 5% diphenyl polysiloxane and 95% dimethyl-polysiloxane (Bellefonte, PA). One µL of sample was administered via splitless injections (split time of 0.5 min) under the following conditions: injection temperature was 200° C and the detector temperature was 325° C. The oven temperature was initiated at 40° C and held for 0.5 min. The first oven ramp took the oven temperature to 110° C at a rate of 5° / min with a 0 min hold time. A final oven ramp took the oven to 300° C at 20° C per min1 (0 min hold time). The time elapsed for 1 run was 24

minutes. Helium was the carrier gas delivered at a constant 39 cm/s and the detector gases were hydrogen (30 ml/min), nitrogen (30 ml/min), and air (400 ml/min; Kimball et al., 2004).

Terpenes were quantified by measuring the base peak of the individual monoterpenes. Detector responses were calculated based on the retention times derived from a set of standards of the monoterpene mixture at 3 concentrations that represented the ranges of 0.01, 0.001, and 0.0001 mg/ml, calculated from previous samples to represent the expected values of monoterpenes found in the bloodstream. Peak identification and measurement was performed with TotalChrom Workstation Version 6.2.1 software.

Toxicokinetic Evaluation and Statistical Analysis. Only six of the original thirteen times were analyzed for monoterpenes in the full set of experimental animals. Four goats, (one from each treatment), were randomly selected for GC analysis of all thirteen time points. Samples from these animals were analyzed in triplicate and a coefficient of variation was calculated for each triplicate set to test for consistency of the method. The data from these animals were used to determine the shape of elimination curve and to select sample times necessary to characterize that curve. Six times were chosen at intervals along the curve (0.5, 1, 3, 4, 8, and 18 hours) to ensure good fit and attempted to capture peak concentration of monoterpenes for all treatments.

The data were fit using an exponential model given by: $y = a * \exp^{(-b * hour)} + c + e$. Where y = concentration of monoterpenes, a is an intercept term, b is a rate, c is the final concentration, *hour* is time and e is an error term assumed to be normal with mean 0 and Σ constant variance. Each treatment was fit separately using a "dummy" variable regression (DVR) technique. Parameters for DVR estimation were calculated with a simple exponential model. The estimated values were then calculated using weighted non-linear regression

(NLMIXED) in SAS (SAS 2004). The following pharmacokinetic parameters were estimated for total and individual monoterpenes in all treatment groups; area under the curve (AUC) and biological half life ($t_{½}$). The AUC was determined using a trapezoidal method of concentration vs. time graph. This same analysis was run on both the predicted values of the absolute blood concentrations recovered from GC analysis and on the predicted values of the concentration as a proportion of the initial dose administered to each goat. Individual monoterpenes were also analyzed based on absolute concentration and values as a proportion of dose. All calculations were performed in SAS (SAS 2004).

Results

There was no difference between the two trial periods. The dose of 0.35 ml/kg BW produced no visible physical reaction, such as noticeable discomfort, in any of the goats.

Total monoterpenes - absolute concentration. There was a significant interaction between age and body condition for the concentration of monoterpenes in the blood of goats post dosing (P = 0.04; Figure 3.2). The age groups differed when compared only among goats in the HBC treatment (P < 0.01). Mature goats tended to have higher AUC than young goats for blood terpene levels (P = 0.06; 1256.72 ng/ml ± 291.71 SE vs. 688.89 ± 53.11 SE, respectively). Body condition also effected the kinetics of monoterpenes within the young goats (P <0.01) however, neither AUC or $t_{½}$ were sufficient to describe these differences.

The main effect body condition affected the concentrations and elimination of monoterpenes from the blood (P = 0.05) though age did not (P= 0.1; Table 3.3). The effect of body condition was most clearly observed as a difference in the AUC between the HBC and LBC animals for total monoterpenes (P = 0.03; 1945.62 ng/ml ± 296.50 SE vs. 1214.23 ±

139.01 SE, respectively). The greater values of the HBC goats for AUC suggests that the HBC goats were exposed to higher blood monoterpene levels overall following an oral dose (Figure 3.2). There were no differences in AUC, or $t_{½}$ for the main effect of age.

The model fit the data well with all the parameters significant for total monoterpenes and most significant for individual monoterpenes (Table 3.4). The disposition of the monoterpenes followed an exponential first-order rate of elimination, and the response was similar for all treatments (Figure 3.1). Concentration of total monoterpenes peaked in the blood by 0.5 hr post dosing with only residual values found at 18 hr (Figure 3.2). Chromatograph peaks with the same retention time of limonene and alpha terpineol were observed in the pre-dose blood draw (0 hr) and monoterpene concentrations post-dosing were adjusted for these values (Figure 3.3).

Total monoterpenes - adjusted for dose. The interaction between age and body condition was still significant when the data were expressed as a proportion of the original dose ($P = 0.02$; Figure 3.5). Within the HBC groups, mature goats differed from young goats ($P < 0.01$), with mature goats appearing to have a greater concentration of monoterpenes throughout the entire sampling period. Within the LBC groups, the young goats differed from the mature goats ($P < 0.01$) with the young goats appearing to have a greater concentration of monoterepenes throughout the entire sampling period. The metrics AUC and $t_{½}$ were not sufficient to describe any differences between the age groups within the body conditions.

The main effect of body condition also affected the concentration and elimination of monoterpenes ($P < 0.01$), however, in exactly what way is not as clear because neither AUC, ($P = 0.30$; Table 3.5), or $t_{½}$, ($P = 0.17$) were different for BC. The greatest differences

between body conditions appeared to occur at the initial sample of 0.5 hr where it appeared the LBC had higher concentrations in their blood (Figure 3.5). The main effect of age did not contribute to monoterpene kinetics (P = 0.1).

The model fit the data well (Figure 3.4) with all parameters contributing to significance (Table 3.6) when the data was analyzed adjusted for the dose of monoterpenes administered to each animal. Total monoterpenes in the blood expressed as a proportion of original dose peaked at 0.5 hr post dosing and only residual values remained at 18 hr post dosing (Figure 3.5).

Individual monoterpenes - absolute concentration. There was a significant interaction between age and body condition for alpha pinene (P < 0.01; Figure 3.6). Within the low body condition groups, the young animals appeared to have greater concentrations than the mature animals (P < 0.01). The mature animals differed by body conditions in concentrations with the HBC group having a higher concentration of monoterpenes than the LBC group (P = 0.03). However, within the young groups, the LBC appeared to have a higher concentration of monoterpenes than the HBC group (P < 0.01).

The main effect of body condition also influenced blood concentration and clearance of two of the individual monoterpenes; alpha pinene (P = 0.03), and limonene (P = 0.03). The influence of BC was most evident for limonene where AUC was marginally significant (P = 0.06) with the HBC goats having greater total area under the curve than the LBC (586.66 ng/ml ± 148.48 SE vs. 293.53 ng/ml ± 56.71 SE, respectively). The overall effect of age was not significant for any of the individual terpenes, but age did influence monoterpene disposition within the HBC treatment for myrcene (P = <0.01), limonene (P = 0.02) and alpha terpineol (P = 0.04). For each of these monoterpenes, the mature-HBC group appears to have

greater concentrations of monoterpenes, through the 8 hr blood sample (Figure 3.6). There were no differences between treatments for AUC or biological half-life ($t_{½}$) for any of the 4 individual monoterpenes used in this trial (Table 3.3).

The model fit each individual monoterpene well (Appendix C) with most of the parameters significant (Table 3.4). There were still residual concentrations of each monoterepene in the blood at 18 hr; except for myrcene which was not found in any treatment past the 8 hr sample (Figure 3.6). This suggests that myrcene clears the blood of goats more rapidly than the other 3 monoterpenes used in the trial.

Individual monoterpenes - adjusted for dose. There was a significant interaction between age and body condition for alpha pinene ($P < 0.01$; Figure 3.7). Within the low body condition groups, the young animals appeared to have greater concentrations than the mature animals ($P < 0.01$). However, within the young groups, the LBC appeared to have a higher concentration of monoterpenes than the HBC group ($P < 0.01$).

The main effect of body condition was also significant for myrcene ($P = 0.02$), limonene ($P = 0.03$), and alpha pinene ($P < 0.01$) when these monoterpenes were adjusted for original dose. Alpha pinene was the only terpene for which the overall influence of age was significant ($P = < 0.01$) However, age differed within the HBC for myrcene ($P < 0.01$), limonene ($P = 0.02$), and alpha pinene ($P = 0.05$). For each monoterpene it appears that mature-HBC goats had greater total proportions of the dose than young-HBC goats (Figure 3.7). No differences were found between treatments for AUC or $t_{½}$ for any of the 4 individual monoterpenes used in this trial (Table 3.4). The model fit the data well (Appendix C), with most of the parameters contributing significantly when the individual monoterpenes were expressed as a proportion of the dose administered to the animal (Table 3.6).

Discussion

Body condition influenced the concentration and elimination of monoterpenes from the blood for both total and individual monoterpenes analyzed as absolute concentration and adjusted for dose. While we were unable to isolate the source of the contribution with either AUC or $t_{½}$, this does not diminish the importance of body condition on the cumulative process of detoxification of monoterpenes. It is well documented that conversion of toxic compounds in the body to inert, excretable substances requires energy over and above that required for normal body maintenance (Foley et al., 1995; Illius and Jessop, 1995). Monoterpenes are lipophilic compounds that require extensive biotransformation to eliminate from the body (Brattsten and Wilkinson, 1977). This metabolism requires energy that may need to be provided by the breakdown of skeletal muscle or adipose tissue if it is not available from dietary resources. This has been substantiated for monoterpenes in grouse and woodrats that were not able to maintain their body weight on diets with high concentrations of monoterpenes (Guglielmo et al., 1996; Dearing et al., 2000). Therefore, it is believed that animals in low body condition are less able to metabolize toxic compounds than animals in higher body condition (Foley et al., 1995). This led to our hypothesis that animals in low body condition would experience greater total blood concentrations of monoterpenes and slower elimination rates than high body condition animals because of compromised detoxification processes.

The significant interaction of age and body condition confirm that these two factors do not work independently to influence blood kinetics of monoterpenes. Animals within the low body condition treatments did not appear to differ in the concentrations of monoterpenes in the blood, however, age appears to be very important in the high body condition animals.

We currently do not have an explanation for this as weight differences were similar between ages within body conditions. It is possible that the mature high body condition animals experienced higher concentrations of monoterpenes in their bloodstream due to a reduction in metabolic rate compared to young animals (Henry 2000).

When the main effects were analyzed as absolute concentration in the blood, our research demonstrated the opposite of our hypothesis in that animals in high body condition had greater total blood concentration of monoterpenes, although they did not experience slower elimination rates. This was unexpected because prolonged periods of nutritional stress are known to result in a loss of liver mass and activity, and monoterpenes are subject to detoxification systems in the liver (Noziere et al., 1999). We did not conduct any tests to determine if liver mass and efficiency differed among treatments, so we cannot assume liver function was responsible for the observed differences.

The greater AUC expressed by the high body condition goats was likely a result of them receiving a greater dose of the monoterpenes than the goats in low body condition because the dose was administered on a BW basis. This is of interest because the mature-HBC animals weighed the most as a group (49.52 kg ± 2.28 SE), and consequently received the largest dose by volume. It is unlikely that rumen size, blood volume and liver function increase linearly with weight, and a portion of the HBC animal's weight was composed of adipose tissue which is metabolically inactive, so the HBC animals could have actually been receiving a more concentrated dose for their functioning metabolic system than the LBC animals. The concentration of a toxin found in the blood is dependant on its volume of distribution (Rozman and Klassen, 1995). Body water volume decreases as fat increases, so HBC animals likely had less total blood volume per unit of body weight, potentially resulting

in the higher blood concentrations observed in HBC goats (Scholz et al., 1990; Zahn et al.,1991).

The interaction remained significant when we examined the total monoterpenes as a proportion of dose, but the influence of the main effect body condition changed. The young-LBC animals appear to have a greater proportion of the dose of monoterpenes in their blood, as well as a greater maximum observed proportion of the dose remaining in the blood at 0.5 hr. If this could be substantiated, it would suggest that although LBC animals received a smaller dose of monoterpenes, they experienced higher concentrations in their blood that could have resulted in physiological damage or the stimulation of negative post-ingestive feedback that could alter foraging patterns (Dziba et al. 2004).

Total recovery of monoterpenes was lower (only 8%) of that reported for recovery of 1-8 cineole in lambs dosed intra-ruminally (Dziba et al., 2006). While differences in the method of terpene extraction and evaluation could have been somewhat responsible for the decreased levels, it is also probable that different classes of monoterpenes behave differently when introduced to the body. More likely it was the fact that animals were offered feed about 2 hours before dosing, so they received the toxic insult after they had begun active digestion. Most pharmacokinetic studies administer the toxic dose following a fast or simultaneously with feed. However, the peak in concentration of total monoterpenes occurred between 0.5 hours and 1 hour, which coincides with time of 1-8 cineole peak concentration observed in sheep (Dziba et al., 2006), that of limonene observed in rats (Chen et al., 1997), and that of perillic acid, a major metabolite of d-limonene, in human plasma (Chow et al., 2002) suggesting the presence of food in the rumen did not noticeably alter rate of absorption. The lack of difference in the distribution or elimination of any of the 4 individual monoterpenes

suggest that they all respond similarly in the body and that age and body condition are not influential in their individual metabolism.

The only study we know that examines toxin clearance in ruminants differing in body condition was conducted with sheep exposed to the quinolizidine alkaloids found in lupine (*Lupinus argenteus*). Lopez et al., (2004) found that when dosed with alkaloids, sheep in low body condition suffered from increased concentration of total serum alkaloids and experienced slower elimination rates of alkaloids than animals in average body condition. If their results had been adjusted for dose, which was administered as a proportion of BW, the difference between the high body condition and low body condition blood concentrations would have increased dramatically. However, the quinolizidine alkaloids used to dose the ewes do not undergo the same detoxification process in the body as monoterpenes (Gardner and Panter, 1994). The decrease in body condition may have been sufficient to impair the alkaloid metabolism, but not the metabolism of monoterpenes in this study.

Conclusions

This research suggests that goats in low body condition do not suffer a greater toxic insult than their counterparts in high body condition from an intra-rumen dose of 4 monoterpenes found in redberry juniper. Animals in low body condition typically increase dry matter intake, so low body condition goats may be more likely to consume juniper on rangelands, especially if alternative forage is limiting. This potential increase in juniper consumption could be utilized for vegetation management or simply to take advantage of juniper as an alternative forage.

While it is unknown if the levels of monoterpenes experienced by the high body

condition group were sufficient to result in any physiological damage to the liver or other organs, it could manifest itself in other ways, like an adjustment to feeding behavior. Specifically, animals in high body condition may be less likely to include substantial amounts of browse containing monoterpenes in their diet. Further research is needed to determine if there are any differences in physiological effects to animals in low or high body conditions consuming monoterpenes.

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Table 3.1. Neutral detergent fiber (NDF) and crude protein content (CP) of composite samples of feeds used in the conditioning period, basal ration, and experimental ration.

Feed	NDF	CP
	----------% Dry Matter----------	
Alfalfa/straw pellets	57.9	11.3
Rolled barley	49.3	14.4
Alfalfa pellets	47.1	16.5
Bluegrass hay	63.9	5.3

Table 3.2. Simulated redberry juniper (*Juniperus coahuilensis*) monoterpene mixture used to dose goats intra-rumenally in a trial designed to measure blood clearance rates of monoterpenes by goats differing in age and body condition. Mixture was delivered at a concentration of 0.35 ml/kg on a volume basis.

Monoterpene	Purity (%)	Percent in mixture[1]	Manufacturer
1 R (+) Limonene	99	58	Acros Organics
Myrcene	88	24	Acros Organics & Sigma Aldrich
Alpha Pinene	98	13	Acros Organics
1 R (+) Alpha Terpineol	99	5	Acros Organics

[1] = Percent on weight basis

Table 3.3. Significant treatment contrasts for absolute blood concentration of total monoterpenes of goats ruminally dosed with a mixture of 4 monoterpenes. Treatments are young or mature within high body conditions (HBC) or low body conditions (LBC).

Treatment Contrast	F value	P value*
Total Monoterpenes (Absolute concentration)		
Main Line Contrasts ----------		
Mature vs Young	2.11	0.10
HBC vs LBC	2.72	0.05
Interaction	2.79	0.04
Mature-HBC vs Mature-LBC	2.42	0.07
Mature-HBC vs Young-HBC	9.94	< 0.01
Mature-LBC vs Young-LBC	0.02	0.99
Young-LBC vs Young-HBC	6.70	< 0.01
Area Under the Curve (AUC) Contrasts** ----------		
HBC vs LBC	4.99	0.03
Mature vs Young	2.78	0.10
Interaction	3.24	0.07
Young-LBC vs Young-HBC	0.52	0.47
Mature-HBC vs Young-HBC	0.06	0.06
Mature-HBC vs Mature-LBC	0.02	0.04
Biological halflife ($t_{1/2}$) * *----------		
HBC vs LBC	2.22	0.14
Mature vs Young	1.66	0.20
Interaction	2.31	0.13
Young-LBC vs Young-HBC	0.00	0.97
Mature-HBC vs Young-HBC	2.62	0.11
Mature-HBC vs Mature-LBC	2.63	0.11

* Contrast considered significant if $P \leq 0.05$.

** AUC and $t_{1/2}$ only run when main line contrast was significant**.**

Table 3.4. Parameter estimates for total terpenes used in the exponential model given by: $y = a * \exp^{(-b * hour)} + c + e$. Where *y* = concentration of monoterpenes, *a* in an intercept term, *b* is a rate, *c* is the final concentration, *hour* is time and *e* is an error term assumed to be normal with mean 0 and Σ constant variance. Additional parameters are biological half-life (t½) and area under the curve (AUC).

Treatment			Rate of elimination	Final concentration		
Age	BC	y-intercept (a)	(b)	(c)	tT ½ (h)	AUC
Total Terpenes						
Mature	High	78.48 ± 12.18*	0.19 ± 0.07*	19.65 ± 6.76*	3.56 ± 0.92*	1256.72 ±
Mature	Low	215.00 ± 33.02*	0.36 ± 0.08*	71.86 ± 6.44*	1.92 ± 0.41*	596.22 ±
Young	High	235.00 ± 16.66*	0.34 ± 0.03*	49.96 ± 2.92*	2.03 ± 0.21*	688.89 ±
Young	Low	209.10 ± 27.03*	0.34 ± 0.06*	71.45 ± 5.37*	2.05 ± 0.35*	618.01 ±
Myrcene						
Mature	High	25.18 ± 4.05*	0.32 ± 0.08*	0.40 ± 0.35	2.14 ± 0.52*	77.59 ±
Mature	Low	28.27 ± 3.95*	0.39 ± 0.07*	0.29 ± 0.28	1.77 ± 0.33*	73.04. ±
Young	High	29.03 ± 2.82*	0.53 ± 0.08*	0 ± 0.28	1.32 ± 0.21*	55.17 ±
Young	Low	27.53 ± 2.72*	0.26 ± 0.11*	0 ± 3.14	2.63 ± 1.08*	104.29 ±
Alpha Terpineol						
Mature	High	67.81 ± 18.78*	0.22 ± 0.11*	21.67 ± 6.93	3.10 ±1.54	303.72 ±
Mature	Low	109.90 ± 73.82*	1.43 ± 0.79*	23.86 ± 1.36	0.48 ± 0.27	76.87. ±
Young	High	39.12 ± 9.74*	0.49 ± 0.22*	19.74 ± 2.00	1.40 ± 0.61*	79.22 ±
Young	Low	35.51 ± 9.66*	0.46 ± 0.24*	24.83 ± 3.38	1.50 ± 0.79	76.76 ±
Alpha Pinene						
Mature	High	81.70 ± 8.67*	0.17 ± 0.05*	4.30 ± 4.49	4.05 ± 1.17*	477.95 ±
Mature	Low	63.10 ± 6.92*	0.23 ± 0.05*	8.39 ± 3.22*	2.96 ± 0.59*	269.30 ±
Young	High	96.70 ± 8.33*	0.24 ± 0.03*	2.50 ± 2.09	2.88 ± 0.41*	401.18 ±
Young	Low	78.30 ± 8.59*	0.20 ± 0.05*	9.77 ± 2.46	3.39 ± 0.76*	383.10 ±
Limonene						
Mature	High	78.48 ± 12.18*	0.19 ± 0.07*	19.65 ± 6.76	3.62 ± 1.40*	409.85 ±
Mature	Low	67.34 ± 13.69*	0.40 ± 0.13*	35.65 ± 3.93*	1.73 ± 0.56*	167.70 ±
Young	High	66.67 ± 7.47*	0.38 ± 0.06*	24.92 ± 1.75	1.83 ± 0.32*	176.81 ±
Young	Low	53.75 ± 9.36*	0.43 ± 0.10*	33.09 ± 2.35	1.62 ± 0.40*	125.83 ±

* Asterisk indicates that the parameter made a significant ($P < 0.05$) contribution to the model

Figure 3.1. Concentration of total monoterpenes over time in whole blood of goats ruminally dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment. Lines represent data fit to an exponential first-order rate of elimination.

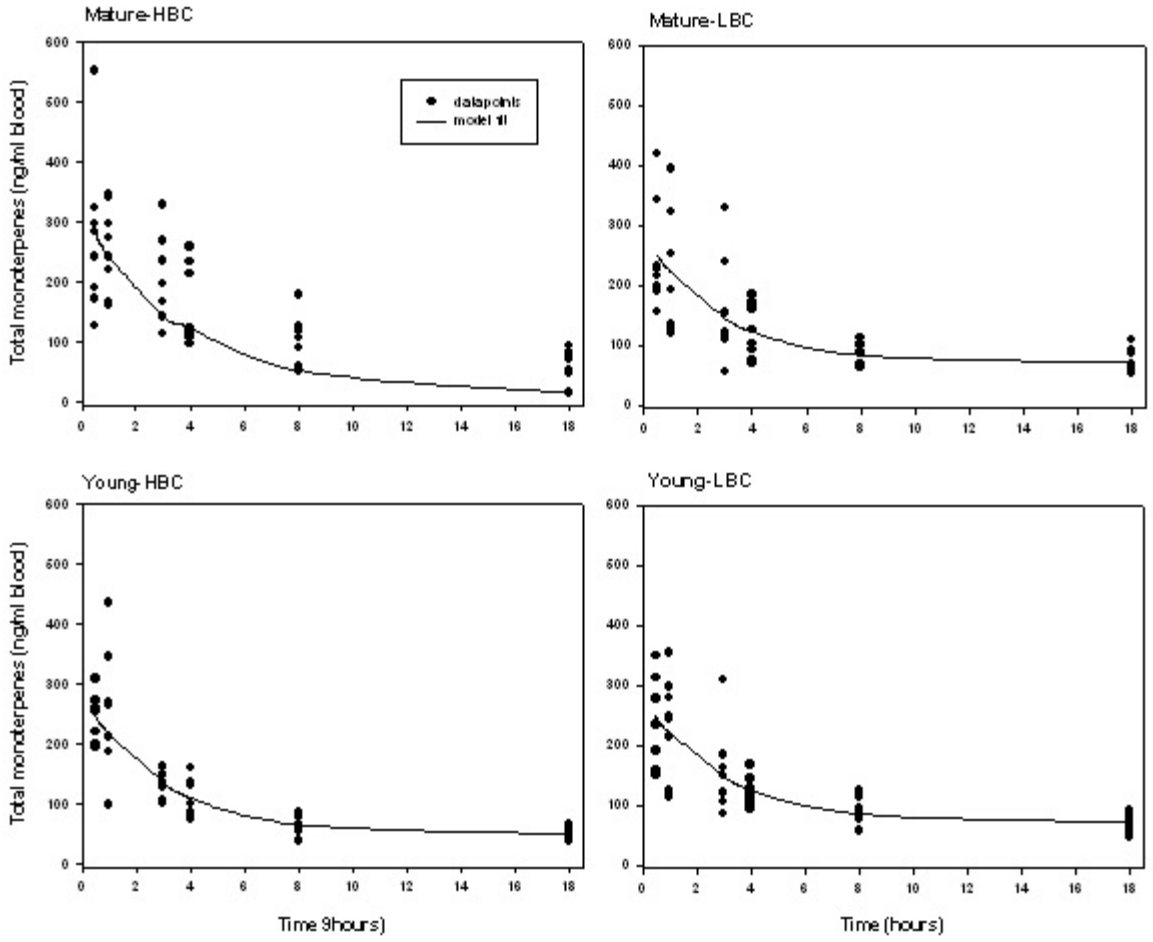


Figure 3.2. Plot for predicted value-time curve of total monoterpene concentrations in whole blood from goats ruminally dosed with a mixture of 4 monoterpenes at 0.35 ml/kg body weight. Treatments were young and mature goats in either high (HBC) or low (LBC) body condition.

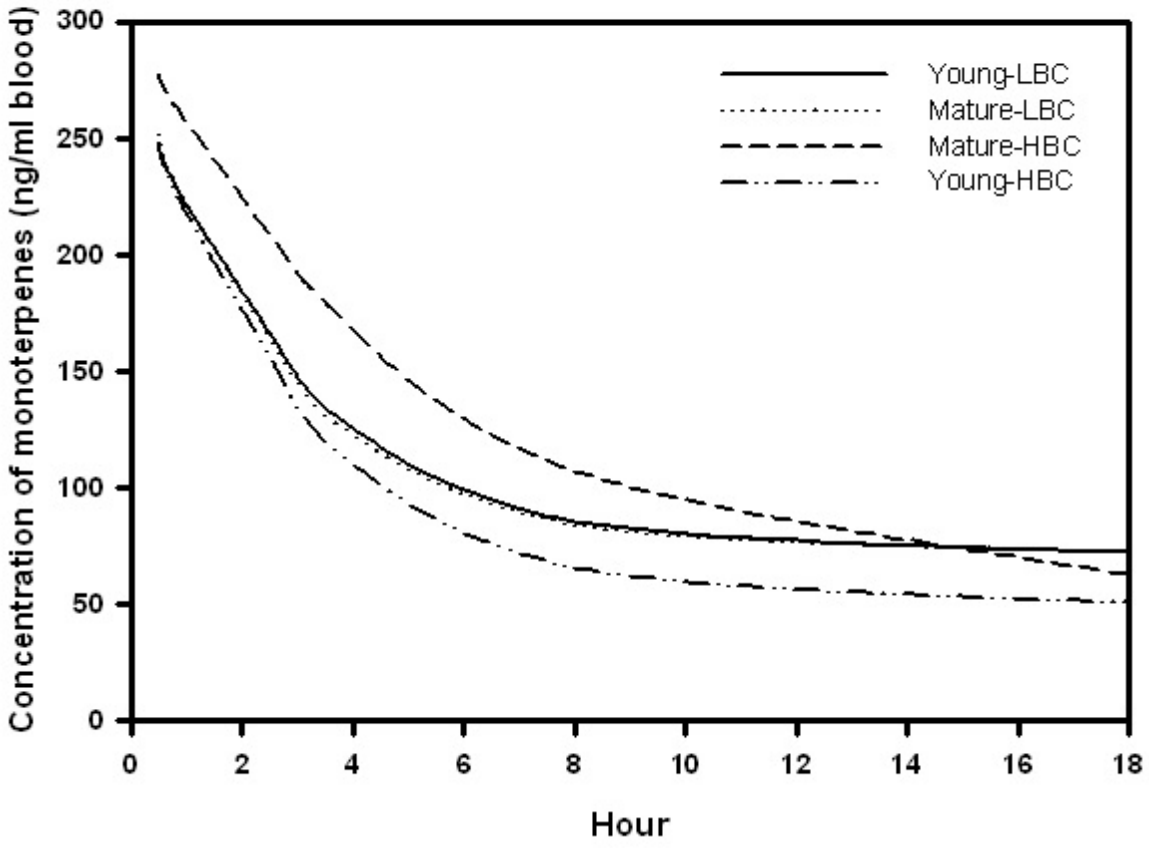


Figure 3.3 Chromatograms from gas chromatography of (a.) monoterpenes recovered from synthetic standards, (b.) the whole blood of goats before dosing, (c.) monoterpenes recovered at 0.5 hr after being dosed with 0.35 ml/kg BW of a mixture of 4 monoterpenes.

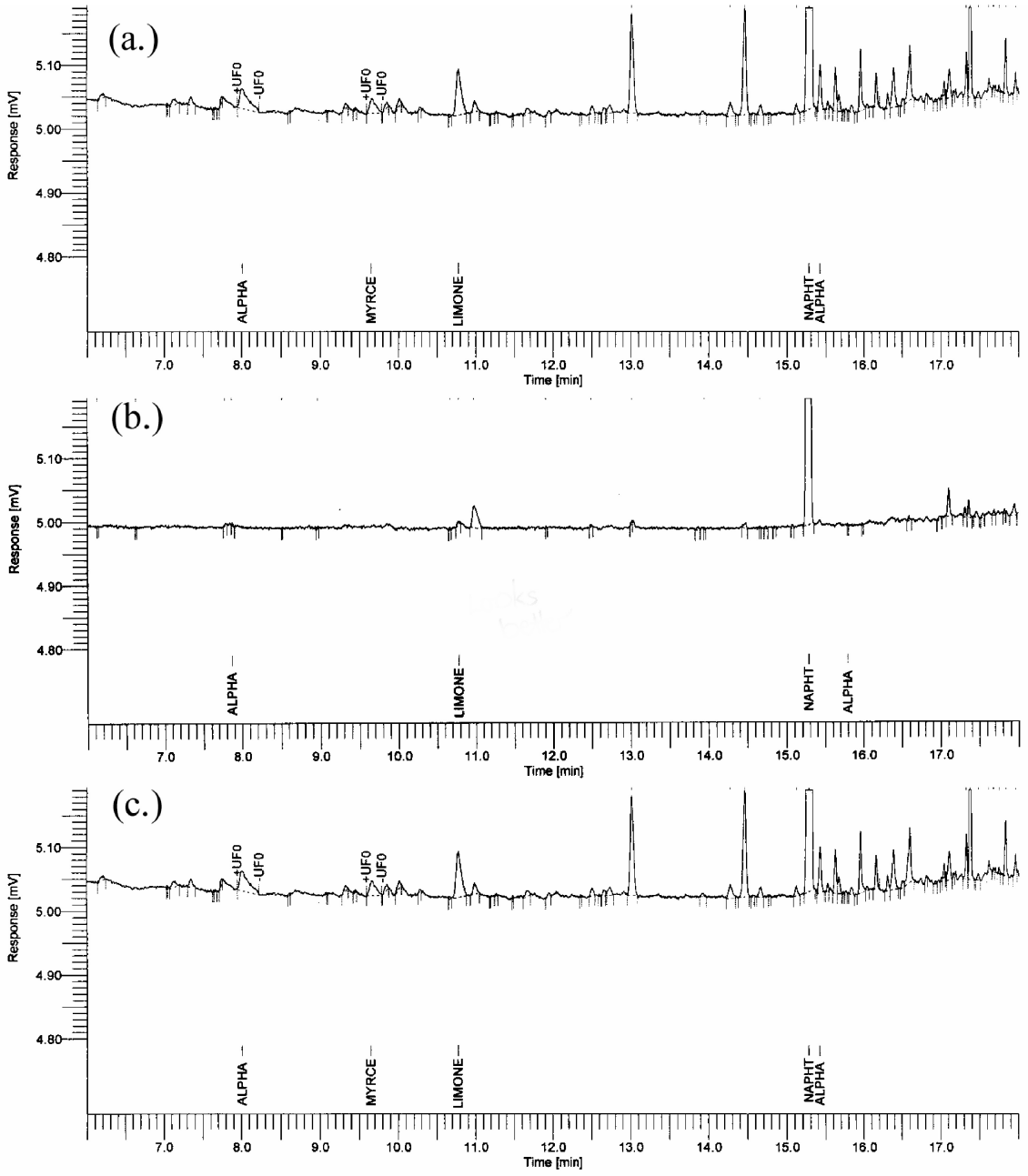


Table 3.5. Significant treatment contrasts for concentration of total monoterpenes adjusted as a proportion of the original dose of 4 monoterpenes administered ruminally to goats. Treatments are young or mature within high body conditions (HBC) or low body conditions (LBC).

Treatment Contrast	F value	P value*
Total Monoterpenes (As proportion of dose)		
Main Line Contrasts ----------		
Mature vs Young	0.65	0.59
HBC vs LBC	9.93	< 0.01
Interaction	3.21	0.02
Mature-HBC vs Mature-LBC	1.53	0.21
Mature-HBC vs Young-HBC	5.80	< 0.01
Mature-LBC vs Young-LBC	0.98	0.40
Young-LBC vs Young-HBC	21.33	< 0.01
Area Under the Curve (AUC) Contrasts** ----------		
HBC vs LBC	1.10	0.30
Mature vs Young	1.12	0.29
Interaction	2.08	0.15
Young-LBC vs Young-HBC	0.35	0.56
Mature-HBC vs Young-HBC	2.05	0.15
Biological halflife ($t_{1/2)}$ * * ----------		
HBC vs LBC	1.10	0.30
Mature vs Young	1.12	0.29
Interaction	1.86	0.17
Young-LBC vs Young-HBC	0.00	0.97
Mature-HBC vs Young-HBC	2.43	0.12

* Contrast considered significant if $P \leq 0.05$.

** AUC and $t_{1/2}$ only run when main line contrast was significant.

Table 3.6. Parameter estimates for total terpenes adjusted for dose used in the exponential model given by: $y = a * \exp^{(-b * hour)} + c + e$. Where *y* = concentration of monoterpenes, *a* in an intercept term, *b* is a rate, *c* is the final concentration, *hour* is time and *e* is an error term assumed to be normal with mean 0 and Σ constant variance. Additional parameters are Biological half-life (t½) and area under the curve (AUC).

Treatment			Rate of elimination	Final concentration		
Age	BC	y-intercept (a)	(b)	(c)	T ½ (h)	AUC
Total Terpenes						
Mature	High	17.39 ± 1.85*	0.19 ± 0.06*	4.13 ± 1.15*	3.73 ± 1.11*	93.77 ±
Mature	Low	19.33 ± 3.12*	0.35 ± 0.09*	6.94 ± 0.79*	1.96 ± 0.50*	54.73 ±
Young	High	19.23 ± 1.49*	0.35 ± 0.04*	4.08 ± 0.28*	1.97 ± 0.22*	54.62 ±
Young	Low	21.58 ± 2.59*	0.36 ± 0.06*	7.75 ± 0.72*	1.95 ± .035*	67.78 ±
Myrcene						
Mature	High	7.5 ± 1.23*	0.3 ± 0.08*	0.13 ± .013	2.27 ± 0.6*	24.62 ±
Mature	Low	11.30 ± 1.5*	0.41 ± 0.07*	0.09 ± 0.12	1.69 ± 0.28*	27.56 ±
Young	High	9.85 ± 0.98*	0.53 ± .08*	0 ± 0.09	1.32 ± 0.21*	18.70 ±
Young	Low	12.22 ± 1.26*	0.29 ± 0.11*	0 ± 1.13	2.43 ± 0.93*	42.89 ±
Alpha Terpineol						
Mature	High	96.59 ± 25.30*	0.22 ± 0.11*	32.46 ± 10.62*	3.37 ± 1.75*	444.92 ±
Mature	Low	188.5 ± 129.79	1.31 ± 0.81	47.33 ± 3.33*	0.81 ± 0.44	120.09 ±
Young	High	64.93 ± 18.41*	0.53 ± 0.25*	32.58 ± 3.68*	1.21 ± 0.60*	120.83 ±
Young	Low	71.86 ± 16.87*	0.44 ± 0.22*	51.11 ± 6.67*	1.57 ± 0.78*	165.52 ±
Alpha Pinene						
Mature	High	44.39 ± 4.27*	0.16 ± 0.05*	2.21 ± 3.18	4.38 ± 1.38*	280.78 ±
Mature	Low	46.84 ± 5.67*	0.24 ± 0.05*	6.37 ± 2.52*	2.95 ± 0.64*	199.14 ±
Young	High	61.04 ± 5.10*	0.24 ± 0.03*	1.49 ± 1.18	2.84 ± 0.38*	249.88 ±
Young	Low	62.80 ± 6.39*	0.20 ± 0.04*	8.08 ± 2.31*	3.44 ± 0.75*	311.61 ±
Limonene						
Mature	High	9.60 ± 1.43*	0.18 ± 0.08*	2.46 ± 1.03*	3.90 ± 1.69*	54.05 ±
Mature	Low	11.10 ± 2.17*	0.40 ± 0.14*	5.80 ± 0.74*	1.75 ± 0.60*	28.05 ±
Young	High	9.32 ± 1.06*	0.38 ± 0.07*	3.46 ± .0.26*	1.84 ± 0.33*	24.69 ±
Young	Low	9.09 ± 1.32*	0.40 ± 0.11*	5.94 ± 0.59*	1.75 ± 0.48*	22.97 ±

* Asterisk indicates that the parameter made a significant ($P < 0.05$) contribution to the model

Figure 3.4 Concentration of total monoterpenes adjusted for dose detected in the whole blood of goats. Dots represent concentrations over time for each treatment. Lines represent data fit to an exponential first-order rate of elimination.

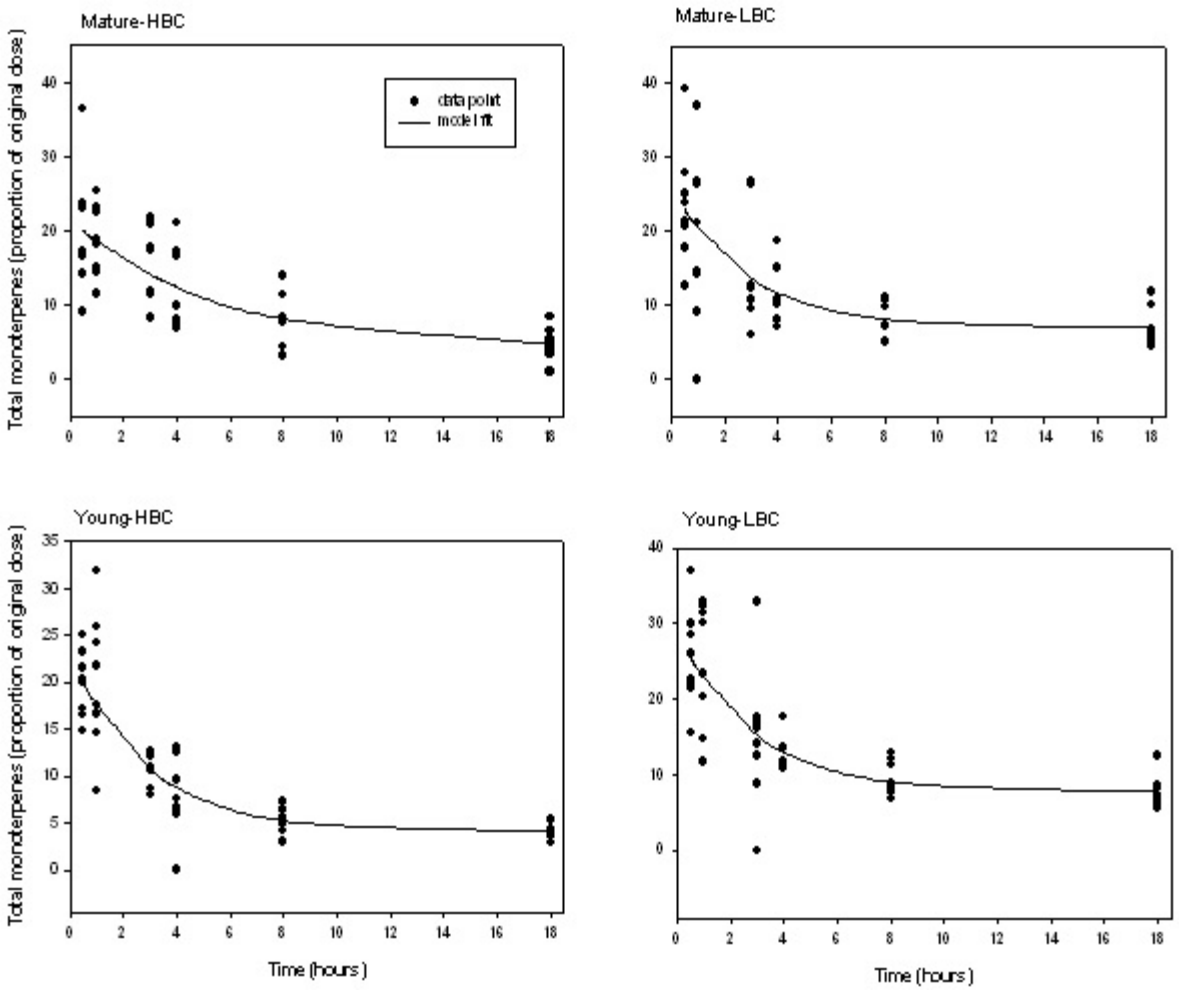


Figure 3.5. Plot for predicted value-time curve of total blood monoterpene levels in goats adjusted for original dose of 4 monoterpenes. Treatments were mature or young-high (HBC) or low (LBC) body condition.

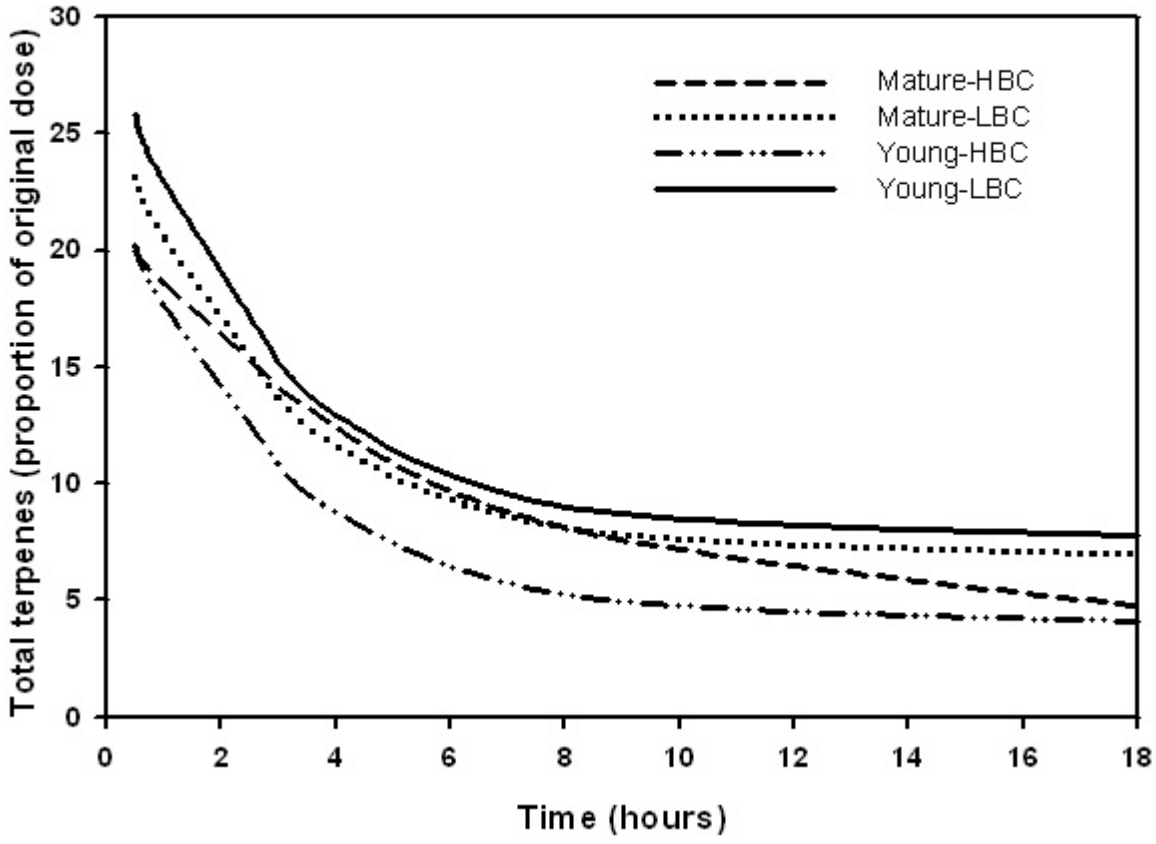


Figure 3.6. Plot for predicted value-time curve of individual monoterpene levels in whole blood from goats dosed with a mixture of 4 monoterpenes at 0.35 ml/ kg BW. Terpenes were a. myrcene, b. alpha terpineol, c. limonene, and d. alpha pinene. Treatments were mature or young goats in high body condition (HBC) or low body condition (LBC).

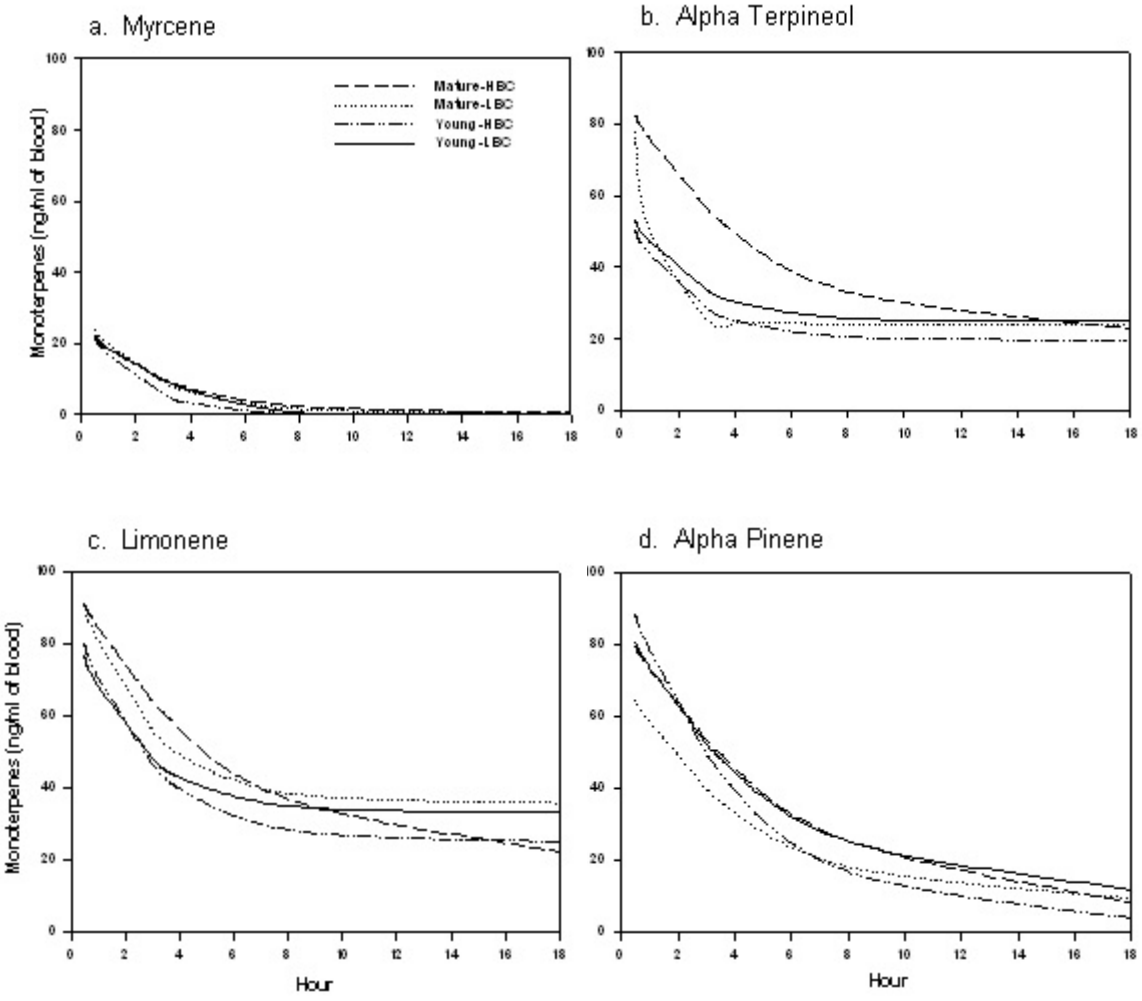
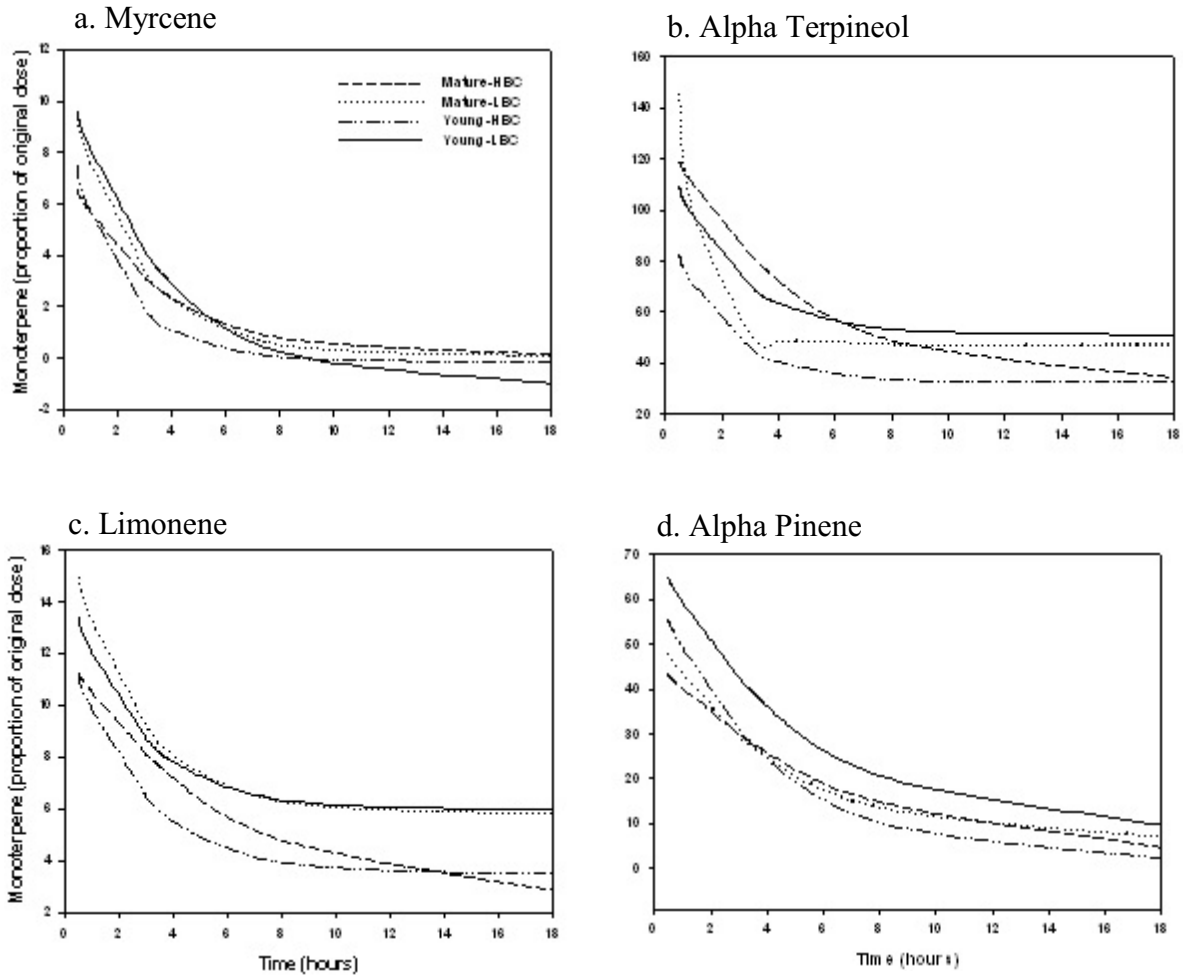


Figure 3.7. Plot for predicted value-time curve of concentration of individual monoterpene levels adjusted for dose in whole blood from goats ruminally dosed with a mixture of 4 monoterpenes at 0.35 ml/ kg BW. Terpenes were a. myrcene, b. alpha terpineol, c. limonene, and d. alpha pinene. Treatments were mature or young goats in high body condition (HBC) or low body condition (LBC).



CHAPTER FOUR: Novel Food Acceptance by Goats Differing in Age and Body Condition

Abstract

Ruminants are reluctant to include novel foods in their diet. Two experiments were performed to assess whether age or body condition of animals influenced their propensity to try novel foods and their reaction to aversions. Goats were assigned to 1 of 4 treatments: young, low body condition (young-LBC); young, high body condition (young-HBC); mature, low body condition (mature-LBC); or mature, high body condition, (mature-HBC). For Experiment 1, animals were offered 3 novel foods on consecutive days; whole flax seed, rye berries, and rye berries flavored with 2.0% garlic powder. Animals were then offered 200 g cinnamon-flavored oats and were dosed with lithium chloride (LiCl) at a concentration of 150 mg/kg of their current body weight. Three days later, a choice between cinnamon-flavored oats and ginger-flavored wheat was offered to all animals. For Experiment 2, all animals were offered 200 g of grape-flavored soybean meal (SBM) and then dosed with 150 mg/kg LiCl of their pre-treatment body weight. Mature animals accepted all three novel foods presented in the conditioning period more readily than young animals (74 % $\pm$ 4.2 SE vs. 58 % $\pm$ 5.2 SE; $P < 0.01$). For both days of the choice test in Experiment 1, the LBC animals consumed more of the cinnamon flavored oats than did the HBC animals (46 % $\pm$ 7.1 SE vs. 12 % $\pm$ 4.6 SE ; $P < 0.05$;). However, body condition had no influence on choice of foods in Experiment 2 ($P = 0.97$), while age was a significant factor as mature animals consumed more of the grape flavored SBM than young (58 % ± 5.7 SE vs. 36 % ± 5.7 SE;

$P < 0.05$) animals. While these experiments do not indicate that body condition influences how animals sample and accept novel foods, it does support previous research that animals in LBC are more likely to consume plants and foods, even if they receive negative feedback. Age does appear to influence acceptance of novel foods, however, these results may have been confounded with previous dietary experiences.

Key words: Age, Body Condition, Novel Foods, Aversion, Diet Selection, and Goats.

Introduction

It is well known that ruminant herbivores are slow to accept novel foods, presumably as a survival strategy to avoid toxicosis when foraging in natural environments (Chapple and Lynch 1986; Chapple et al. 1987; Thorhallsdottir et al. 1987; Provenza et al.1995). However, ruminants in a low nutritional state need to alleviate deficiencies and or maladies and may broaden their diet in search of the necessary nutrients to accomplish this nutritional goal (Provenza 1995). Another theory is that only animals on a high plane of nutrition will be more likely to broaden their diet because they are more physiologically equipped to handle a possible encounter with a toxic food (Murden and Risenhoover 1993). Plant secondary compounds cost an animal valuable energy and other nutrients to metabolize and eliminate them from the body. Illius and Jessop (1995) contend that when food supply becomes limiting, animals must decrease their intake of toxic plants and low quality forage to meet maintenance requirements. This narrowing of the diet implies that animals are better off to stick with known, valuable food sources, no matter how scarce, than to risk the additional loss of energy and nutrients associated with most detoxification processes. Very little research has been conducted to determine if animals in a deprived nutrient state are more willing to accept novel foods than those in a high nutritional state.

Age can also influence diet selection. Mature animals are believed to be more reluctant to try novel foods than younger animals; though this is not well tested (Burritt, personal communication). Furthermore, the diets of younger animals are usually higher in crude protein concentrations and energy density, while lower in fiber than diets of adults (Cazcarra and Petit 1995; Grings et al. 2001). The search for a more nutritious diet requires higher energy output for travel and time spent grazing and often leads to weight loss. This

combination of higher energy requirements and decreased foraging knowledge may make younger animals more willing to try novel foods and to retry foods that made them ill in the past (Ralphs and Provenza 1999). To examine animal attributes that affect acceptance of novel foods we conducted two experiments to: 1) determine if there is a difference in the way that animals of different ages and body conditions accept novel foods and if this changes after they have been exposed to a toxic novel food, and 2) determine if the total dose of lithium chloride, determined by the weight of the animal, was more important than the body condition of the animal in determining its response to toxic novel foods.

Methods[5]

Research was conducted on the University of Idaho, Sheep Research and Teaching Center in Moscow, ID. Thirty-seven, open, non-lactating Boer cross female goats were selected by age groups with young goats (n=19) of 2 years age and mature goats (n=18) of 6 years or more. Then 9 goats in each age group were assigned to either a high (HBC) or low body condition (LBC) treatment. Does were stratified according to weight and randomly assigned to treatments. This resulted in 4 treatment combinations of 2 factors: age and body condition. Goats were housed as a group to minimize stress and penned daily in feeding stalls measuring 1.16 m^2 for a 2 hr feeding period.

Conditioning period. Doe conditioning to create assigned body conditions was accomplished in an 18-week feeding period. Does assigned to the LBC treatment were

[5] All methods involving animals were approved by the University of Idaho, Animal Care and Use Committee (Protocol 2003-46, Appendix D).

offered an average of 1.88% BW[6] of an alfalfa-straw pellet (60% alfalfa, 40% straw), while those assigned to the HBC treatment were offered an average of 2.68% BW comprised of 92% alfalfa pellets and 8% rolled barley (Table 4.1). Beginning in week 3 of the conditioning period, all animals were offered 100 g of chopped bluegrass hay preceding their conditioning ration to maintain sufficient dietary fiber for good rumen function. Goats were weighed weekly to assess progress into assigned body conditions with amounts of feed adjusted by treatment and individual. Beginning in week 15, body condition was scored on a weekly basis on a 1-5 scale by tactile and visual estimation of body fat depositions (Spahr, 2004) by three trained technicians who were blind to the treatments. The mean body condition at the end of the conditioning period was 1.97 $\pm$ 0.09 SE for LBC and 3.06 $\pm$ 0.09 SE for HBC animals. Seven days before beginning the trial, all animals were converted to a diet consisting of 100 g chopped bluegrass hay and 2.0% BW of alfalfa pellets. The does remained on this basal diet throughout the trial.

Experiment 1- Novel food acceptance

A series of novel foods was offered over 4 days to ascertain the animal's willingness to try novel foods and to prepare them for accepting the novel foods that were to be paired with an aversion in later trials. Animals received all their basal ration at 09:00 h each morning and a novel food at 11:00 h each morning. On days 1 and 2 of the trial, all animals were offered 200 g of whole flax seed, a novel food, for 10 minutes. On day 3 all animals were offered 200 g of whole rye berries (grain) and on day 4 they were offered 200 g rye berries flavored with 2% garlic powder. Orts were collected at 12:00 h and weighed to determine intake.

[6]All weights and amount of feed were determined on an as fed basis.

Experiment 2 - Aversion based on actual weight

At 05:00 hr on day 4 of the novel food acceptance trial (Experiment 1) all animals were offered about 25 g of whole oats flavored with 2% ground cinnamon to encourage consumption of 100 g on the following day. On day 5, animals were offered 200 g of cinnamon-flavored oats. Orts were collected and weighed after 10 minutes and then returned to the animals for an additional 20 minutes to encourage consumption of at least 100 g. Those animals consuming more than 100 g of the cinnamon flavored oats were dosed with a gelatin capsule containing 150 mg/kg BW (Table 4.2) of lithium chloride (LiCl). This dose was determined to be effective in producing an aversion in sheep (duToit et al. 1991). Dosing began about 6 hours following consumption of the novel food and was accomplished by administering a size OO gelatin capsule filled with the appropriate dose of lithium chloride using a pair of pill forceps. If there was doubt about whether the pills were swallowed, a 30 ml syringe filled with water was used to flush the mouth and encourage the goat to swallow the pill. Two goats were not dosed with lithium chloride as they only consumed 8 and 36 g of the novel food, respectively. Three consumed only 66 g, 62 g, and 85 g respectively, however, they were dosed despite eating < 100 g of the novel food. Animals were only offered their basal ration on day 6 and 7 to allow recovery from illness induced by the lithium chloride. On day 8, all the goats were offered a choice between 200 g of cinnamon-flavored oats and a fourth novel food (whole white wheat flavored with 2% ginger powder) presented simultaneously in 2 identical feed buckets set side by side. Orts were removed and weighed after 10 minutes or as soon as 1 of the foods was completely eaten thus eliminating the choice between foods.

Experiment 3- Aversion based on actual body weight

Three days after completing experiment 1, goats were again offered 200 g of a food both novel in flavor and texture, soybean meal with 2% grape flavoring. The grape flavoring was accomplished by adding unsweetened grape flavored KoolAid® powder mixed thoroughly with the soybean meal. Those animals consuming at least 100 g of the novel food were dosed with 150 mg/kg BW of lithium chloride according to their pre-conditioning weights (Table 4.2). The preconditioning weights were used to separate the influence of weight and body condition. Dosing followed the same schedule and procedure as described above. All goats consumed at least 100 g of the novel food and were dosed with the lithium chloride. Again, the goats were only offered their basal ration for the 2 days following the dosing. On the third day after dosing, all the goats were offered a choice between 200 g of the grape flavored soybean meal and another novel food (canola meal flavored with 2% lemon lime flavoring) presented simultaneously in 2 identical feed buckets set side by side. The lemon-lime flavoring was accomplished by adding unsweetened lemon-lime flavored KoolAid ® powder mixed thoroughly with the canola meal. Orts were removed and weighed after 10 minutes or as soon as 1 of the foods was completely eaten thus eliminating the choice between foods.

Statistical analysis

This experiment was a 2 x 2 factorial in a completely randomized design nearly balanced with 9 animals per treatment (except young-HBC, n = 10 animals). The model included body condition and age with day as the repeated measure. Intake of the averted food was analyzed as a percentage and arcsine square root transformed to achieve normality. Analyses of variance were performed to examine intake daily. Effects included in the model

were body condition, age and all possible interactions of these factors and time. Means for main effects were compared using least squared means when a significant F-test ($P < 0.05$) was observed. All statistical analyses were performed using the statistical software SAS (SAS, 2004).

Results

Experiment 1 - Novel food acceptance

Mature animals accepted all three novel foods presented in the conditioning period more readily than young animals (74% $\pm$ 4.2 SE vs. 58% $\pm$ 5.2 SE; $P < 0.01$). There was a difference ($P < 0.01$) in the acceptance of each of the three feeds with rye being the most readily accepted (97.69 % $\pm$ 1.6 SE) followed by whole flax seed (65.81 % $\pm$ 5.8 SE), then garlic flavored rye (34.05 % $\pm$ 3.8 SE).

Experiment 2- Aversion based on actual weight

In this experiment, an attempt was made to avert all animals to cinnamon-flavored oats using lithium chloride administered as a percentage of actual body weight. Malaise was apparently accomplished in all animals as evidenced by a reduced consumption of their basal diet following administration of LiCl. However, for both days of the choice test, the LBC animals consumed more of the cinnamon flavored oats (expressed as a % of the total eaten) than did the HBC animals (46 % $\pm$ 7.1 SE vs. 12 % $\pm$ 4.6 SE; $P < 0.05$;). There was no day effect ($P = 0.31$) and no interactions were significant.

Experiment 3 - Aversion based on pre-conditioning weight

In this experiment, an attempt was made to avert all animals to grape-flavored SBM using lithium chloride administered as a percentage of pre-conditioning body weight. The

treatments differed in their acceptance of SBM, the novel food presented following the LiCl dosing associated with cinnamon oats. There was a trend for the LBC goats to consume more of the flavored soybean meal during the first 10 minutes of the trial, however it was not significant. At the end of the 4 hour period, there was no difference in amount of SBM consumed by any of the treatment groups.

Results were quite different from experiment 1 in that body condition had no influence on choice of foods (P=0.97; Figure 4.2). Furthermore, age was a significant factor as mature animals consumed more of the grape flavored SBM than young (58 % ± 5.7 SE vs. 36 % ± 5.7 SE; $P < 0.05$) animals. Consumption of SBM was greater than canola meal (CM) for all treatments on both days except for Y-LBC on day 2 (Figure 4.3). There were no significant interactions among treatments or days.

Discussion

Experiment 1-Novel Food Acceptance

Young animals are generally believed to be more apt to try novel foods and incorporate them into their diet as they are still developing their forage habits and diet breadth, while it is usually difficult to get mature animals to sample novel foods (Burritt, personal communication). However, we found the mature animals consumed more than young animals of all three of the novel foods offered in the acceptance phase of the trial. One possible explanation is the uncertainty of the dietary history of the animals. While all the goats in the trial were obtained from the same herd, the mature animals were purchased by the producer from a variety of sources as foundation stock. Given that producers engage in a variety of supplementation regimes depending on their location, circumstances, and the price

of feedstuffs, there is no concise knowledge of their previous dietary experiences, so it is possible that these were not novel foods to the mature animals. Also, animals more readily accept a novel food if they have had exposure to a variety of novel foods in the past (Launchbaugh et al. 1997). The young animals were all raised by the provider and so they shared virtually the same dietary experiences, and were unlikely to have had past encounters with any of the foods or flavors used in this experiment. Conversely, the mature animals may have had a variety of experiences with novel foods. Our results suggest that specific dietary experiences may be more influential than simply examining the age of an animal.

Body condition did not influence acceptance of novel foods in this trial. This substantiates the findings of Provenza et al. (1995) who determined that food restriction did not increase the rate of acceptance of a novel food by lambs.

Experiment 2- Aversion based on actual weight

Body condition of the goats affected their consumption of the cinnamon flavored oats following dosing with LiCl. Previous studies contend that animals in a deprived nutritional state should avoid toxic foods as they lack the excess energy and nutrients required to metabolize the toxins (Foley 1995; Illius and Jessop 1995; Guglielmo 1996). Wang and Provenza (1996) observed this with lambs in that as food deprivation increased, consumption of foods containing lithium chloride decreased, even if the foods provided high levels of energy. However, research by Lopez-Ortiz (2002) and the results of Chapter Two in this document provide evidence that animals in low body condition are willing to consume greater quantities of toxic plants than their counterparts in average body condition, presumably in an attempt to alleviate nutrient deficiencies. Herbivores often include poisonous plants in their diets, generally below levels that cause toxicity or in combination

with adequate amounts of high quality forage that aid in the detoxification process (Provenza 1995; Freeland and Janzen 1974). Many poisonous plants are highly nutritious even though they may contain deleterious secondary metabolites. So animals undoubtedly receive some benefit from including them in their diet. The willingness of low body condition goats to include the cinnamon oats in their diet could have been a result of mixed feedback. While all animals ultimately experienced negative feedback as evidenced by their reduced intake of alfalfa pellets the day following dosing with LiCl, they also undoubtedly experienced positive feedback from the high energy value of oats. This energy feedback may have resulted in a stronger preference in the low body condition animals as they were in a long-term energy deficient state. Therefore, the preference for the energy rich oats may have been strong enough in the low body condition animals to override the negative feedback from the LiCl.

Furthermore, the methods of the trial may have contributed to the difference in consumption of cinnamon-flavored oats. While a conditioned taste aversion can form if feedback follows consumption within 12 hours, it is more effective the closer the temporal contiguity between consumption and feedback (Burritt and Provenza 1991). We did not dose the animals until 6 hours after the novel foods were consumed because of an unexpected experimental error. Therefore, aversive feedback may have been only weakly associated with the cinnamon oats. Additionally, although all animals received a dose of 150 mg/kg BW of LiCl, the weight differences in the treatments resulted in a substantially lower total dose of LiCl for the low body condition animals. While animals in high body condition may have had a larger liver mass and more available energy for detoxification, the full mass of their body (including muscle and adipose tissue) is not active in metabolizing toxins (Koong et al. 1985; Noziere et al. 1999).

Experiment 3 - Aversion based on pre-conditioning weight

When the animals were dosed according to their pre-conditioning weights, those in the high body condition received about 118 mg/kg LiCl of current BW, while those in low body condition received about 147 mg/kg current BW of LiCl. The dose received by the high body condition group may have been insufficient to produce an aversion in at least some of the animals (duToit et al. 1991). More likely is the aforementioned difficulty in getting mature animals to sample novel foods. The canola meal was of an unfamiliar texture and the strong lemon-lime flavoring may have discouraged its acceptance. It was not until day 2 that any group consumed more of the canola meal than the soybean meal and then it was only the young-low body condition group. So the increased consumption of the soybean meal may have been due to an unwillingness to try the canola meal.

Furthermore, animals have the ability to generalize flavors to post-ingestive consequences (Villalba and Provenza 2000). Because the flavoring was unsweetened there may not have been much discernable differences between the grape flavoring and the lemon-lime flavoring. Both were mixed at a sufficiently high level to create a bitter taste. It is possible that the goats were not able to distinguish between the 2 bitter flavors, and generalized a response among them.

Implications

While these experiments do not indicate that body condition influences how animals sample and accept novel foods, it does support previous research that animals in low body condition are more likely to consume plants and foods, even if they receive negative feedback. Therefore, producers should ensure there are adequate alternative forages available

for their animals during periods of nutritional stress, caused by high energy demand or low forage availability. Avoid moving animals to new areas with novel forage or a high proportion of toxic plants. Animals, particularly those in low body condition, may over-ingest some plants resulting in illness or death before receiving sufficient negative feedback to decrease consumption. Additionally, older animals may not be as neophobic as commonly believed. Past dietary experiences may play a larger role in the acceptance of novel foods, so older, more experienced animals may be even more willing to try novel foods than young animals.

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Table 4.1. Neutral detergent fiber (NDF) and crude protein content (CP) of composite samples of feeds used in the conditioning period and basal ration.

Feed	NDF	CP
	----------% Dry Matter----------	
Alfalfa/straw pellets	57.9	11.3
Rolled barley	49.3	14.4
Alfalfa pellets	47.1	16.5
Bluegrass hay	63.9	5.3

Table 4.2. Weights used in Experiment 2 and 3 where Lithium chloride (LiCl) was administered at a dose of 150 mg/kg BW. Goats were young or mature and in low body condition (LBC) or high body condition (HBC).

Treatment	Weight in kg	Standard Error
----------Experiment 2 - Actual Weight----------		
Young- LBC	36.44	± 0.99
Mature- LBC	41.67	± 2.60
Young- HBC	45.45	± 1.16
Mature- HBC	52.58	± 2.62
----------Experiment 3 - Pre-Conditioning Weight----------		
Young- LBC	36.57	± 1.01
Mature- LBC	39.92	± 1.91
Young- HBC	36.07	± 0.94
Mature- HBC	41.04	± 2.24

Figure 4.1. Mean percent intake by treatment of each of 3 novel foods (whole flax seed, whole rye berries, and whole rye berries flavored with 2.0% garlic powder) offered during the conditioning period. Treatments are young or mature animals in low body condition (LBC) or high body condition (HBC).

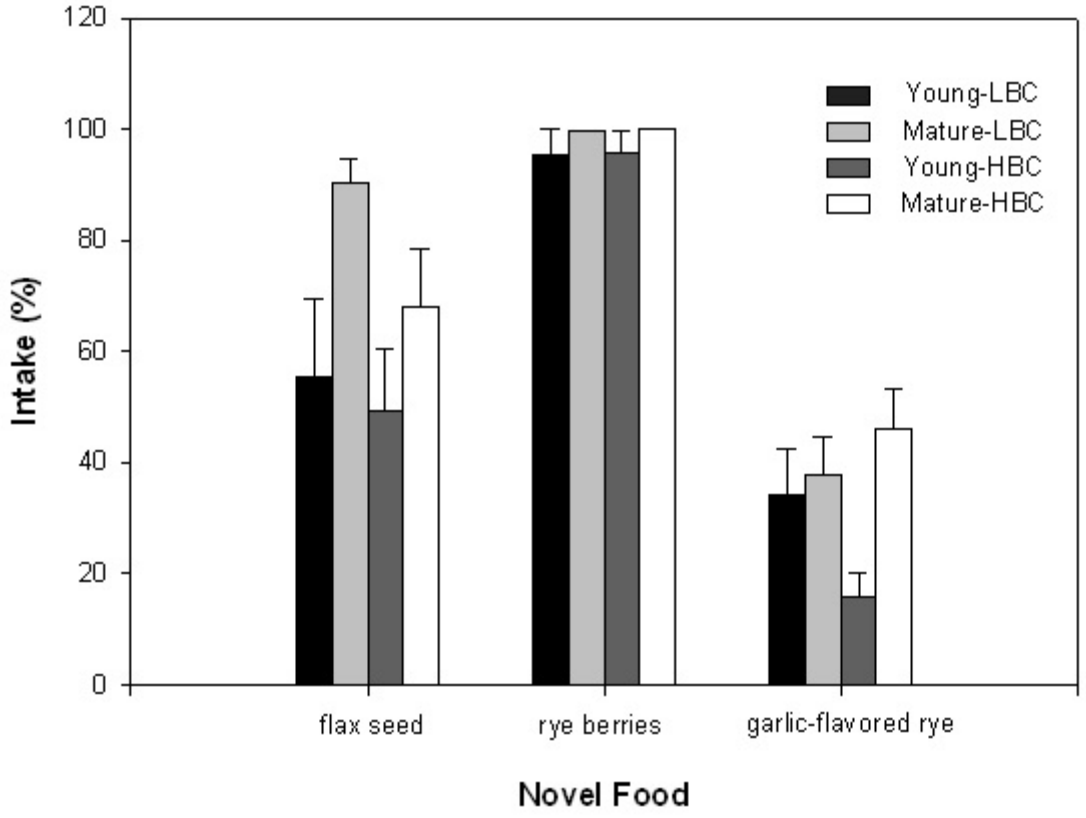


Figure 4.2. Change in consumption of averted food (%) by body condition, high (HBC) or low (LBC), from Experiment 2 to Experiment 3. Animals were dosed with 150 mg/kg BW of LiCl based on their actual weight in Experiment 1. For Experiment 2 goats were dosed with 150 mg/kg BW of LiCl based on their pre-conditioning weights.

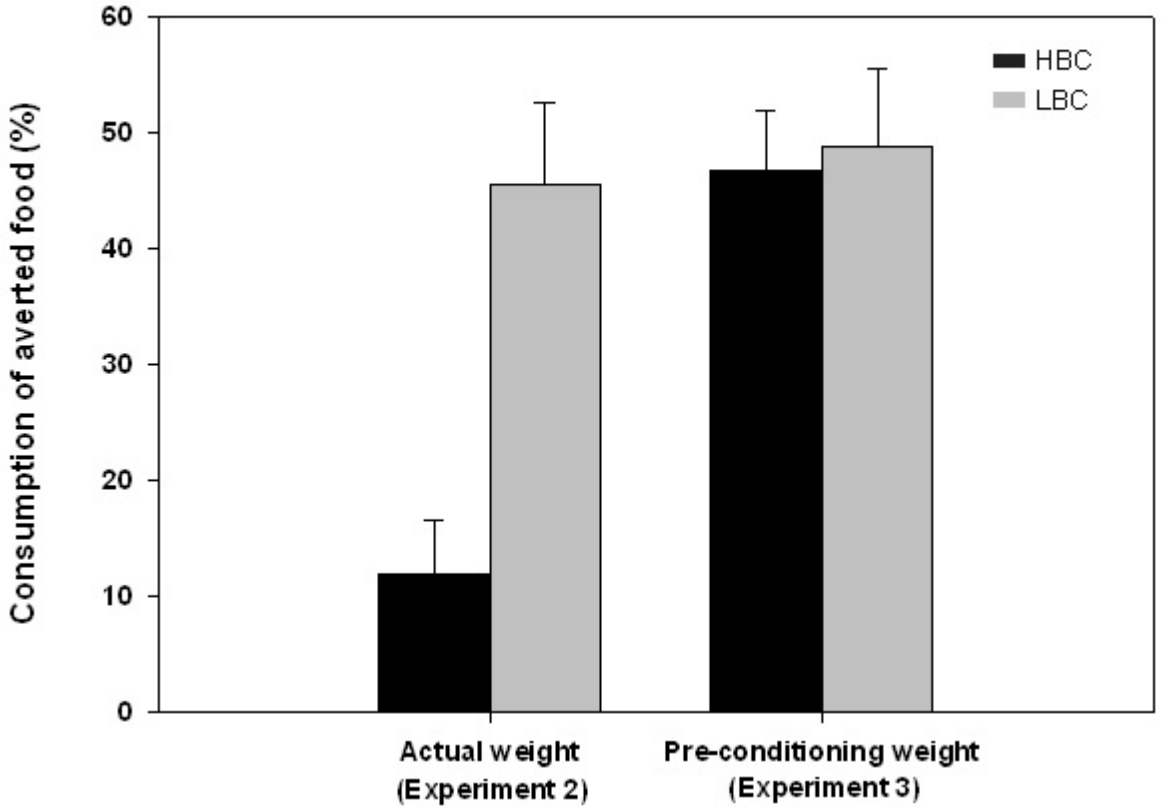
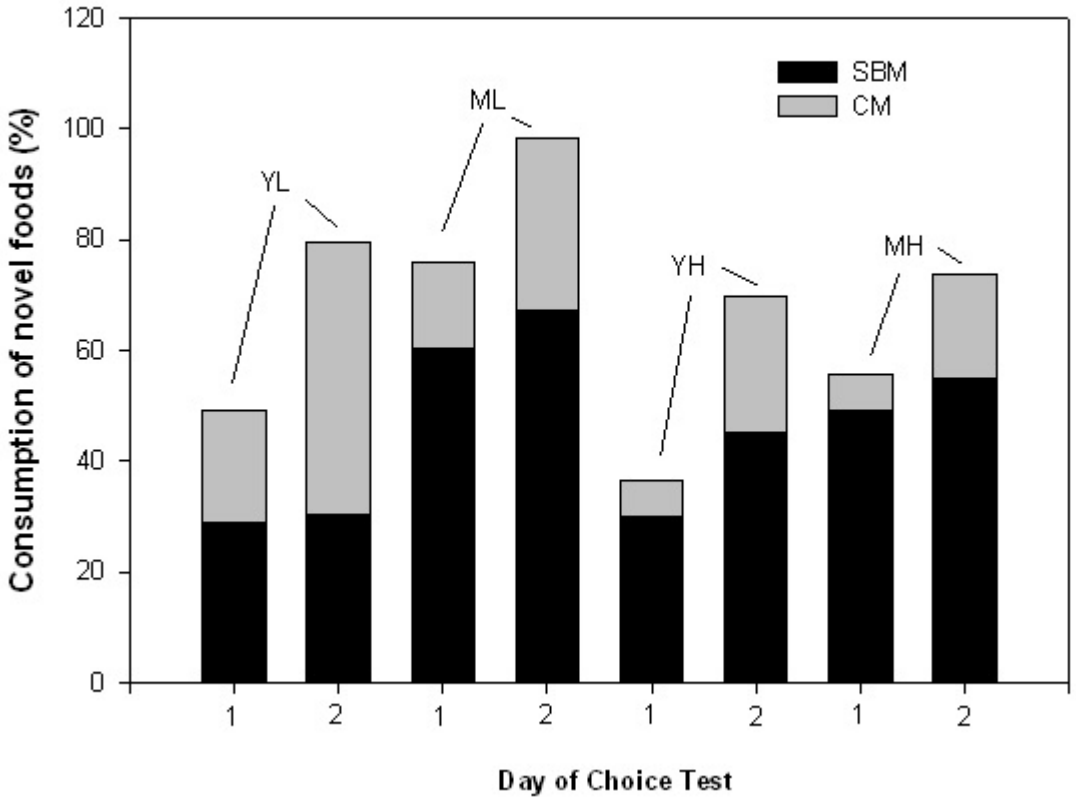


Figure 4.3. Total consumption of foods offered as a choice on day 1 and day 2 in Experiment 3. Feeds were grape-flavored soybean meal (SBM) and lemon lime-flavored canola meal (CM). Foods were offered as a choice for 10 minutes.



APPENDIX A: Whole Blood Vs. Plasma as a Medium for GC Analysis of Monoterpenes in Goats

Introduction

When blood is collected and prepared for any pharmacokinetic analysis, one primary decision is to keep samples as whole blood or spin it to plasm. Plasma is the primary medium used for pharmacokinetic studies involving monoterpenes (Egorin et al. 1996, Chow et al. 2002, Kimball et al. 2004). However, whole blood offers a distinct advantage to researchers in sample handling time, especially where many samples must be processed. Additionally, the development of a field test to determine consumption of monoterpenes is highly desirable in goats to facilitate selection of individuals that habitually consume more juniper than their counterparts. Whole blood is a more useful medium for taking and storing field samples where no immediate laboratory equipment is available. Therefore, the purpose of this trial was to determine if whole blood provided a comparable medium to plasma for analysis of monoterpene levels in the bloodstream of goats.

The cellular constituents of whole blood could potentially clog the columns resulting in poor recovery when using solid phase extraction of monoterpenes as described by Kimball et al. (2004). However, this is not a concern when employing liquid extraction. Chen et al. (1998) compared the concentrations of *d*-limonene recovered from the whole blood and plasma of rats and found higher concentration in the whole blood vs. the plasma. The efficacy of whole blood as an analysis medium has been documented for other biological studies including; therapeutic drug monitoring of the immunosuppression drug, FK 506, (Winkler et al. 1994) and HIV viral load (Mwaba et al. 2003). Both studies confirmed that whole blood

gave equal or better analysis results than plasma in the context of total analyte recovery and sample consistency. Therefore, our hypothesis was that there would be no difference in monoterpene recovery from either whole blood or plasma when using the liquid extraction method employed for analysis of monoterpenes (Chapter Three of this document).

Methods

Research was conducted at the TAMU Agricultural Experiment Station, Sonora, TX. The trial utilized four mature female Spanish goats. The goats were housed in individual pens with free-choice access to water and maintained on a diet of 2.0% BW sun cured alfalfa pellets offered at 0800 hours each morning.

Dosing Procedure

Goats were dosed with a mixture composed of 13 % α-pinene, 58% limonene, 24% myrcene tech., and 5% alpha terpineol. The mixture was administered directly into the rumen by inserting a 10 cm stylus on the animal's left side behind the ribs. A syringe was attached to the needle and back-filled to determine if the needle had penetrated the rumen. Once rumen penetration was confirmed, the terpene mixture was injected and the needle flushed with 5 mls of additional water. The monoterpenes were administered at 0.30 ml/kg BW and mixed with 20 ml of water to mediate initial reactions from the dosing. Previous research established this dose as non-lethal, yet enough to cause noticeable discomfort in animals. Animals were observed for 15 minutes after dosing and reactions to the dosing were recorded. The exact time of dosing was recorded for each animal.

Blood collection

Two blood samples were collected from each goat via jugular vein puncture at 15, 30, 45, 60, 90, 180, and 360 min post dosing. About 10 mls of blood was collected in each of two vacutainer™ tubes containing freeze dried sodium heparin to prevent clots. One sample from each goat was then centrifuged at 5000 rpms for 15 minutes, and the clear plasma removed and transferred to a 7 ml polypropylene storage vial. The other sample was transferred to a 7 ml polypropylene storage vial as whole blood. The labeled vials were then transferred to a freezer and stored at -17° C until analysis.

Blood sample preparation

In preparation for analysis, samples were thawed and mixed for 15 seconds on a Vortex mixer at high speed. All samples were prepared in triplicate. Two mls of each sample was transferred into a 10 ml glass test tube using a micropipette. Then 2 mls of ethyl ether containing 0.02% naphthalene was added to the sample. The sample and ether were mixed for 30 seconds by a Vortex mixer on high speed. The samples were then centrifuged at 5000 rpms for 15 min, after which about 1 ml of the upper layer of clear liquid was transferred with a glass Pasteur pipette to a 2 ml glass vial for a robotic autosampler for GC analysis.

GC-MS analysis

A Clarus 500 GC coupled to an autosampler, both manufactured by Perkin Elmer, Wellesley, MA, was employed for the analysis. The gas chromatograph was equipped with a Restek Rtx-5 column (30 m x 0.25 mm x 0.25 μm) wall-coated with 5% diphenyl polysiloxane and 95% dimethyl-polysiloxane, (Restek, Bellefonte, PA) . One μL of sample was administered via splitless injections (split time of 0.5 min) under the following conditions: injection temperature was 200° C and the detector temperature was 325° C. The

oven temperature was initiated at 40° C and held for 0.5 min. The first oven ramp took the oven temperature to 110° C at a rate of 5° per minute with a 0 minute hold time. A final oven ramp took the oven to 300° C at 20° C per minute (0 minute hold time). The time elapsed for 1 run was 24 minutes. Helium was the carrier gas delivered at a constant 39 cm/s and the detector gases were hydrogen delivered at 30.0 ml/min, nitrogen delivered at 30.0 ml/min, and chromatographic grade compressed air delivered at 400 ml/min (Kimball et al. 2004).

Monoterpenes were quantified by measuring the base peak of the individual monoterpenes. Detector responses were calculated based on the retention times derived from a set of standards of the monoterpene mixture at 3 concentrations that represented the ranges of 0.01, 0.001, and 0.0001 mg/ml, calculated from previous samples to represent the expected values of monoterpenes found in the bloodstream. Peak identification and measurement was performed with TotalChrom Workstation Version 6.2.1 software.

Statistical analysis

The data were fit using an exponential model given by: $y = a * \exp^{(-b * hour)} + c + e$. Where y = concentration of monoterpenes, a is an intercept term, b is a rate, c is the final concentration, *hour* is time and e is an error term assumed to be normal with mean 0 and Σ constant variance. Each treatment was fit separately using a DVR technique. All computations were done using weighted non-linear regression (NLMIXED) in SAS (SAS 2004).

Results and Discussion

The dose of 0.30 ml/kg BW produced no visible reaction in any of the 4 goats. The following pharmacokinetic parameters were estimated for total monoterpenes in the plasma and whole blood groups; y-intercept (*a*), terminal elimination rate constant (*b*), and final concentration at 18 hours (*c*). The disposition of the monoterpenes followed an exponential first-order rate of elimination (Figure A.1). Although there was some difficulty in fitting the model to the data as evidenced by lack of significance of some parameters, (Table A.1), the response was similar for both treatments (Figure A.2). No differences in monoterpene content were found between whole blood and plasma (P=0.72), or the parameters; *a, b* or *c* (Table A.2). The model was analyzed for individual terpene concentration and, again, no difference was found between whole blood and plasma.

Conclusions

For this method of liquid extraction, whole blood provided a comparable medium to plasma for GC analysis of these 4 monoterpenes (alpha-pinene, limonene, myrcene, and alpha terpineol) in the bloodstream of goats. This offers researchers more flexibility when in the field as whole blood samples can be frozen for later analysis without having to be spun down within a certain time frame. Furthermore, the analysis of whole blood results in less total blood extracted from each goat. Ten mls of blood yields approximately 4 mls of plasma, therefore, if multiple samples are required from the same animal, it is necessary to collect more than twice as much whole blood to yield an equal quantity of plasma.

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Table A.1. Parameter estimates used in the exponential model given by: $y = a * \exp^{(-b * hour)} + c + e$. Where *y* = concentration of monoterpenes, *a* in an intercept term, *b* is a rate, *c* is the final concentration, *hour* is time and *e* is an error term assumed to be normal with mean 0 and Σ constant variance.

Parameter	Estimate	Standard Error	P value
a_1	49.9	17.38	<0.01
b_1	0.4	0.35	0.32
c_1	20.5	18.95	0.29
a_2	70.4	13.26	<0.01
b_2	0.5	0.28	0.11
c_2	18.2	9.43	0.06
sig	0.8	0.17	<0.01

* P values ≤ 0.05 were considered significant.

Figure A.1. Concentration of total monoterpenes detected in the plasma and whole blood of goats (n = 4) dosed intra-ruminally with a mixture of 4 monoterpenes. Dots represent monoterpene concentrations measured in the plasma or whole blood of individual goats. Lines represent fits of the data to an exponential first-order rate of elimination.

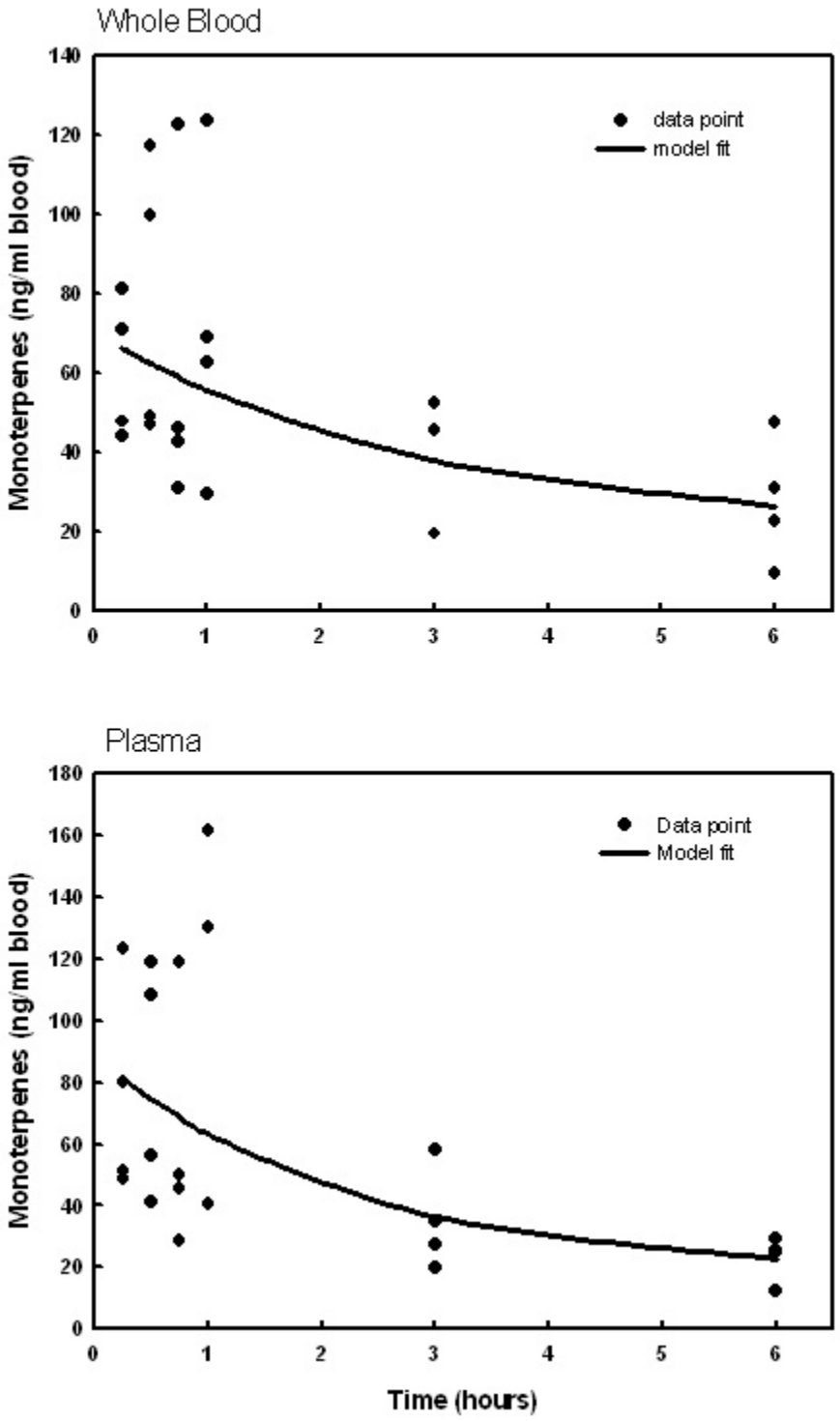


Figure A.2. Plot for predicted values-time curve of total monoterpene levels in whole blood and plasma from goats dosed with a mixture of 4 monoterpenes at 0.30 ml/kg body weight. Predicted values were obtained from an exponential model given by: $y = a * \exp^{(-b * hour)} + c + e$. Where *y* = concentration of monoterpenes, *a* is an intercept term, *b* is a rate, *c* is the final concentration, *hour* is time and *e* is an error term assumed to be normal with mean 0 and Σ constant variance. Predicted values were obtained by fitting each treatment separately using a DVR technique.

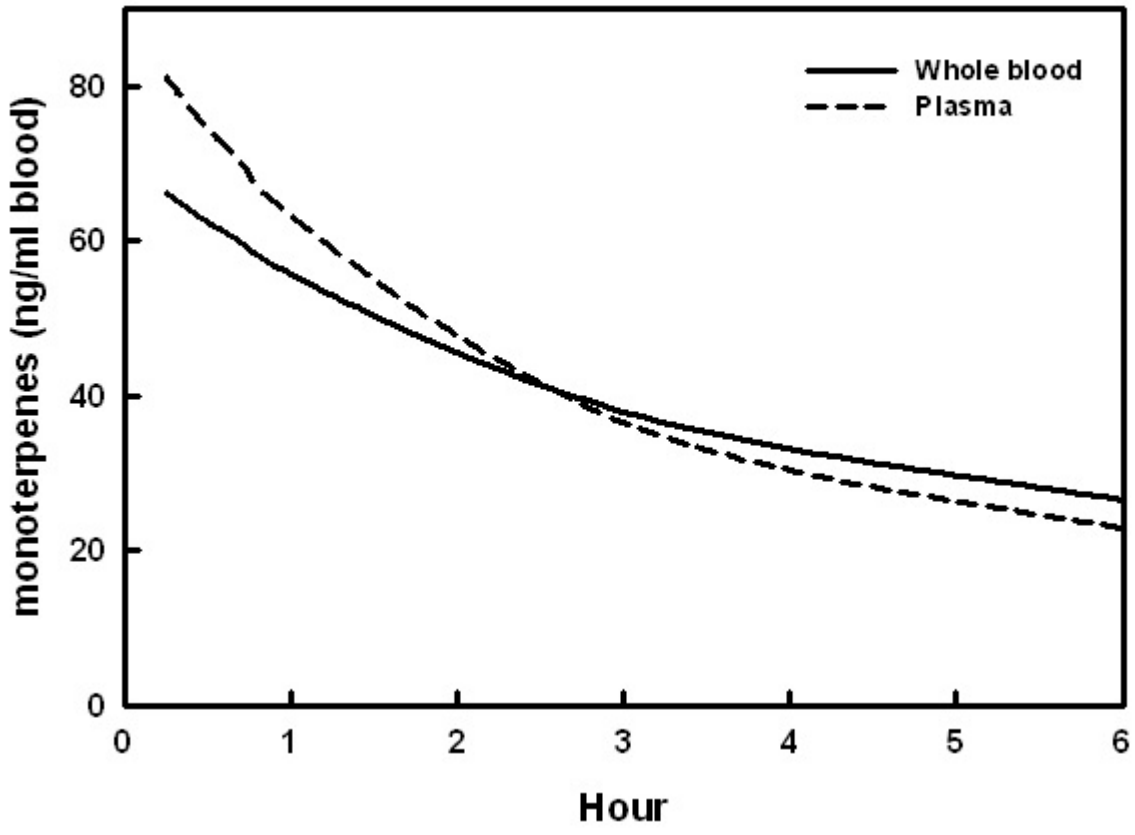


Table A.2. Statistical values for the contrast of whole blood and plasma where *a* is an intercept term, *b* is a rate of depletion, and *c* is the final concentration.

	DF	F value	P value*
Blood vs Plasma	3	0.44	0.72
a	1	0.88	0.35
b	1	0.05	0.82
c	1	0.01	0.92

*Values were considered to be significant if $P < 0.05$

APPENDIX B: Comparison of Liquid Solvents for the Extraction of Alpha Pinene, Limonene, Alpha Terpineol, and Myrcene from the Whole Blood of Goats

Introduction

Numerous chemical solvents are employed in the extraction of compounds for gas-chromatography (GC). Ethyl ether and n-Hexane are both used in the liquid extraction of monoterpenes from plasma and other substances (Mastelic 2001; El-Shazly et al. 2002; Dubey et al. 2003). N-Hexane has properties more suited for binding with monoterpenes which are lipophilic. However, the more water-soluble ether does not bind as readily with the fats and other constituents of whole blood and is more easily separated for GC analysis. Consistency across replications was of chief concern for sample preparation in this project. Therefore, the purpose of this study was to compared the solvents: ethyl ether, n-hexane and a 1:1 ethyl ether and n-hexane mix to test for consistency of extraction of a mixture of alpha-pinene, limonene, myrcene, and alpha terpineol from whole blood samples obtained from goats.

Materials and Methods

Blood sample preparation

In preparation for analysis, 3 whole blood samples obtained from goats used in Chapter 3 were thawed and mixed for 15 seconds on a Vortex mixer at high speed. Each blood sample was divided into 3 sub-samples used to test one solvent for consistency. Sub-samples from Sample 1 were prepared with ethyl ether, Sample 2 with n-hexane, and Sample 3 with a 1 to 1 ethyl ether:n-hexane mix. Two ml of each sub-sample was transferred into a

10 ml glass test tube using a micropipette and an equal amount of the assigned solvent was added. The diluted samples were mixed on a Vortex mixer and then centrifuged to separate the solvent and monoterpenes from the whole blood following the method described in Chapter Three of this document. The resulting top layer of solvent was transferred via glass pipette to a 2 ml autosampler vial for gas chromatography (GC) analysis, which was conducted following the method described in Chapter Three.

Total monoterpenes were quantified by measuring the base peak of the individual monoterpenes. Detector responses were calculated based on the retention times derived from a set of standards of the monoterpene mixture at 3 concentrations; 0.01, 0.001, and 0.0001 mg/ml, designed to represent the expected values in the blood sample. Peak identification and measurement was performed with TotalChrom Workstation Version 6.2.1 software.

Statistical analysis

Because each solvent used a different blood sample, no comparison could be made of terpene recovery across samples, only consistency among the three replications done with each solvent. The mean (M), standard deviation (SD) and a coefficient of variation (CV) were calculated for all samples within each solvent and the CV compared across solvents. The CV was obtained by the formula: $CV = (M / SD)$

Results and Discussion

The ethyl ether solvent provided the most consistency as evidenced by the lowest CV value (Table B.1). Furthermore, the ethyl ether was the easiest compound to handle during the extraction process; providing the greatest volume of clear extract and separating easily from the whole blood. The n-hexane mixture resulted in a gel-like extract that was difficult to

transfer to vials for analysis and resulted in solid clumps that were not conducive to GC analysis. The ethyl ether:n-hexane mix produced similar results as the pure n-hexane. Therefore, ethyl ether was chosen as the solvent of choice for extraction of monoterpenes from whole blood based on consistency and ease of sample preparation. While ethyl ether is not commonly used as a solvent for extraction of monoterpenes from biological fluids, it is widely employed in the extraction of monoterpenes in the wood industry. However, care should be taken when handling and storing the ether:napthalene standard mixture because of its volatility. Ethyl ether based standards should not be stored for long time periods between analysis as some volatilization of the ether could potentially alter the concentration of the standard mix.

Literature Cited

Dubey, V. S., R. Bhalla, and R. Luthra. 2003. Sucrose mobilization in relation to essential oil biogenesis during palmarosa (*Cymbopogon martinii* Roxb. Wats. var. *motia*) inflorescence development. Journal of Bioscience. 28:479-487.

El-Shazly, A., G. Dorai, and M. Wink. 2002. Composition and antimicrobial activity of essential oil and hexane-ether extract of *Tanacetum santolinoides* (DC.) Fenbr. and Fertig. Z. Naturforschung. 57: 620-623.

Mastelic. J. 2001. The essential oil co-distillation by suerheated vapour of organic solvents from aromatic plants. Flavour and Fragrance Journal. 16:370-373.

Table B.1. Mean, standard error and coefficient of variation for recovery of monoterpenes from three replicate whole blood samples. The monoterpenes were extracted using ethyl ether, n-hexane, or a 1:1 mix of ethyl ether and n-hexane as liquid solvents.

Solvent	Mean	Standard Deviation	Coefficient of Variation
Ethyl Ether	1110.8	± 29.2	0.03
n-Hexane	840.5	± 124.4	0.15
Ethyl Ether & n-Hexane*	959.7	± 93.9	0.10

* 1:1 mixture of ethyl ether and n-hexane on a volume basis

APPENDIX C: Model Fit for Individual Monoterpenes Expressed as Both an Absolute Value and an Amount Adjusted by Proportion of the Original Dose

Figure C.1. Concentration of alpha terpineol over time in whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.

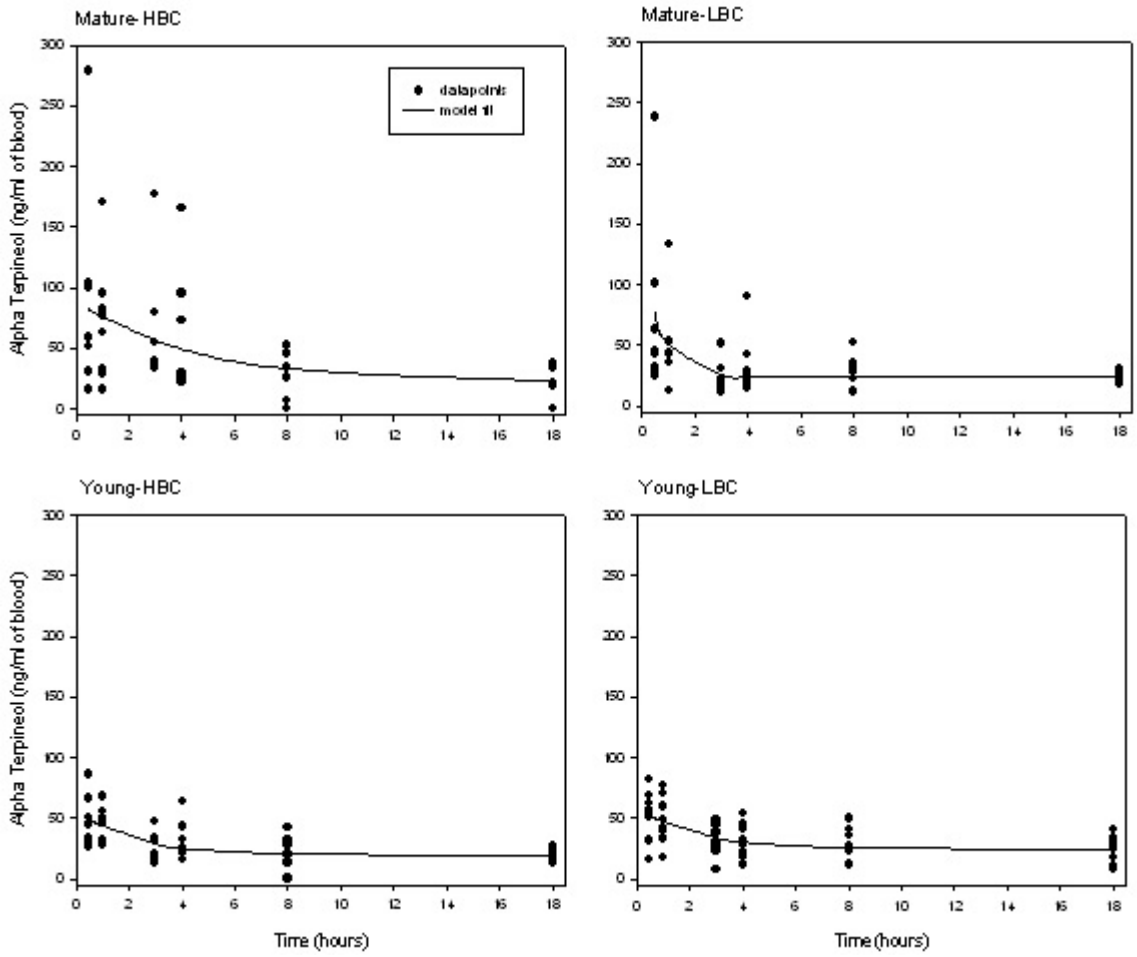


Figure C.2. Concentration of myrcene over time in whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.

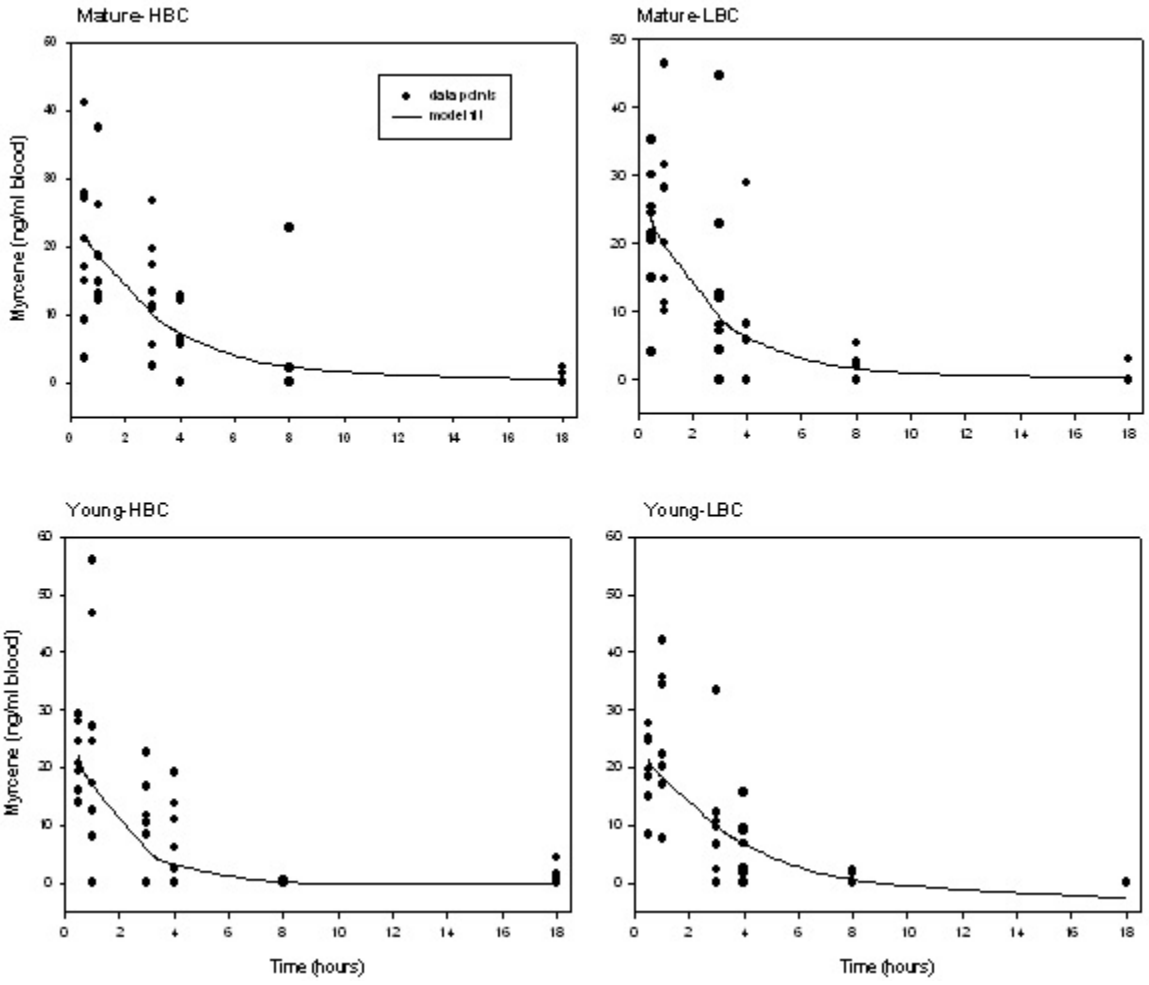


Figure C.3. Concentration of alpha pinene over time in whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.

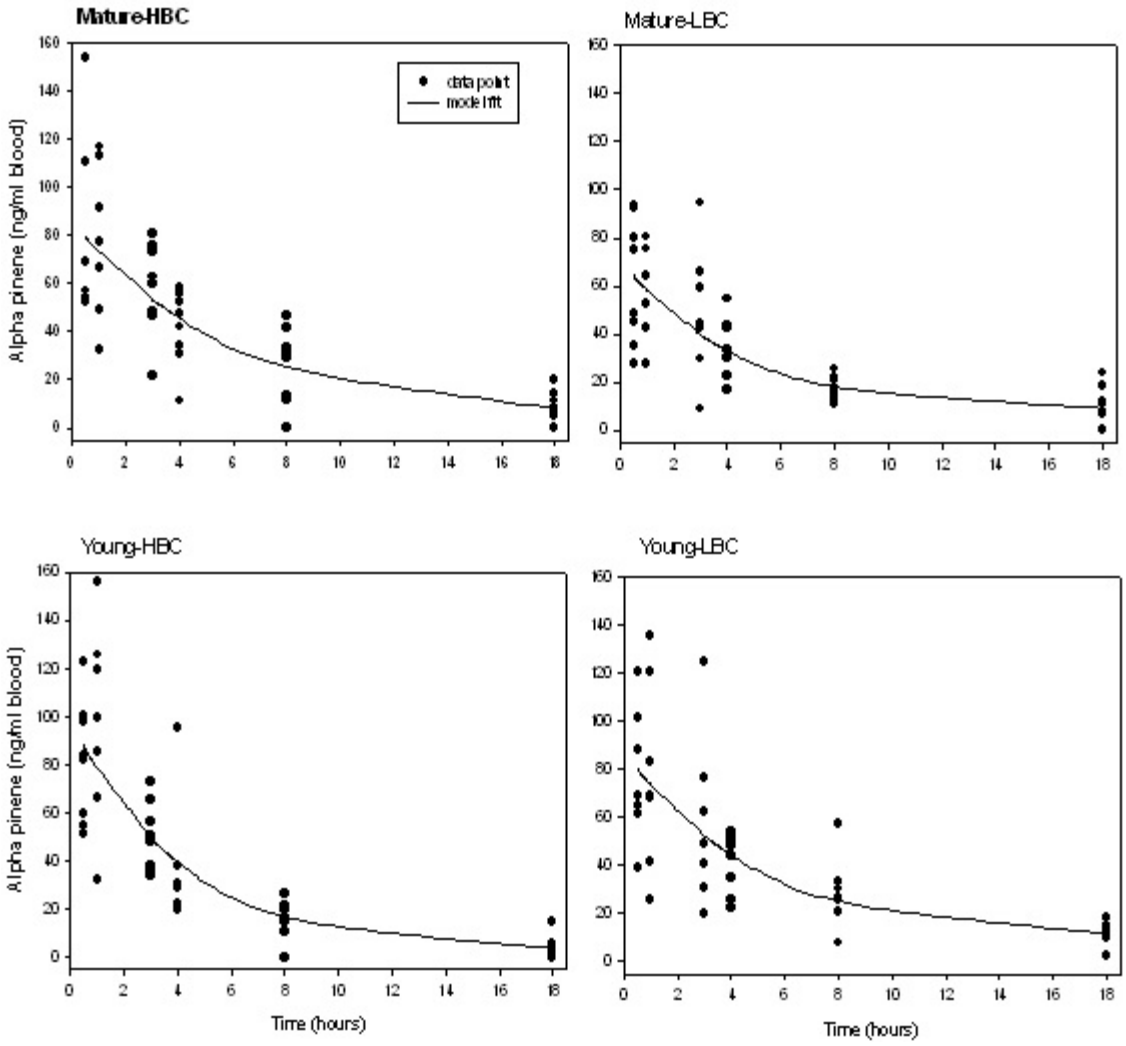


Figure C.4. Concentration of limonene over time in whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.

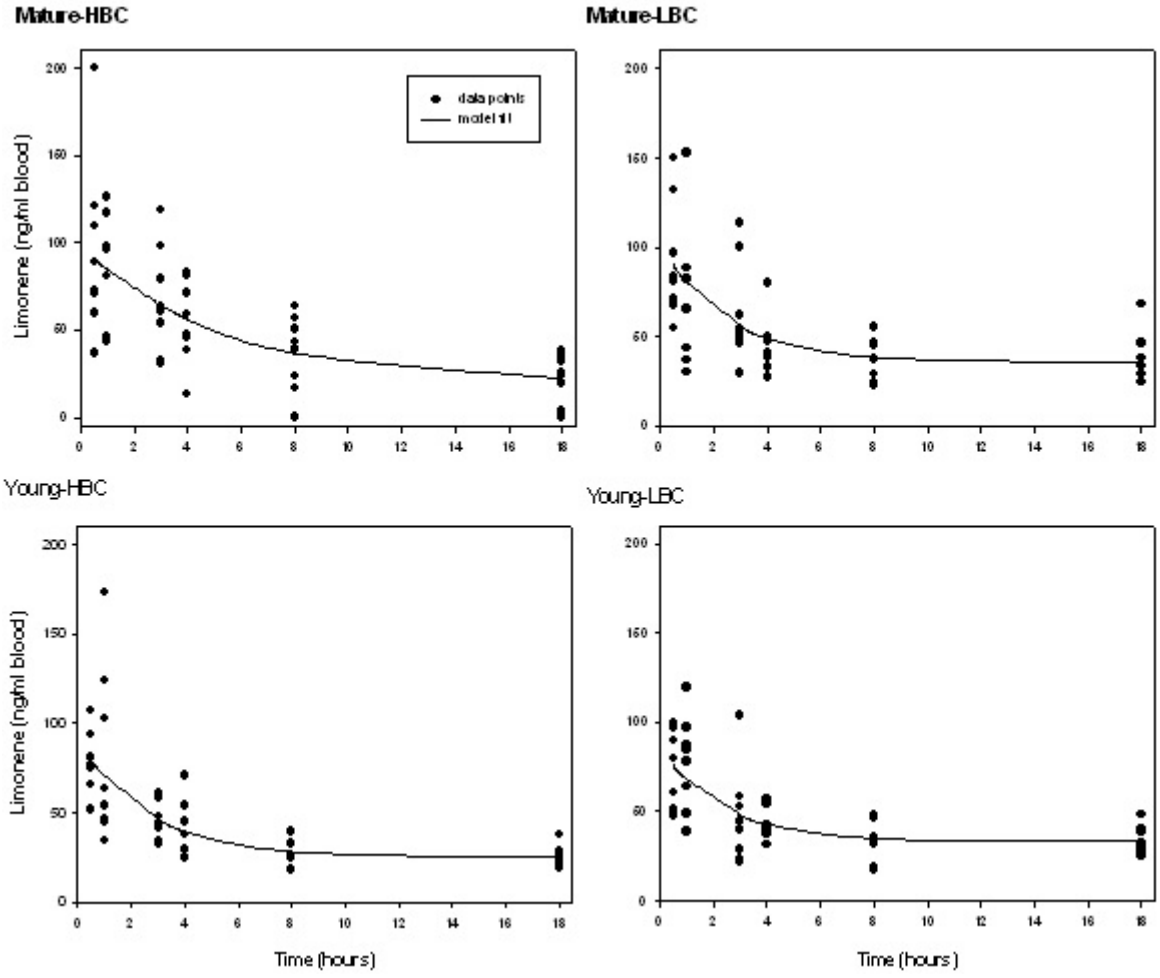
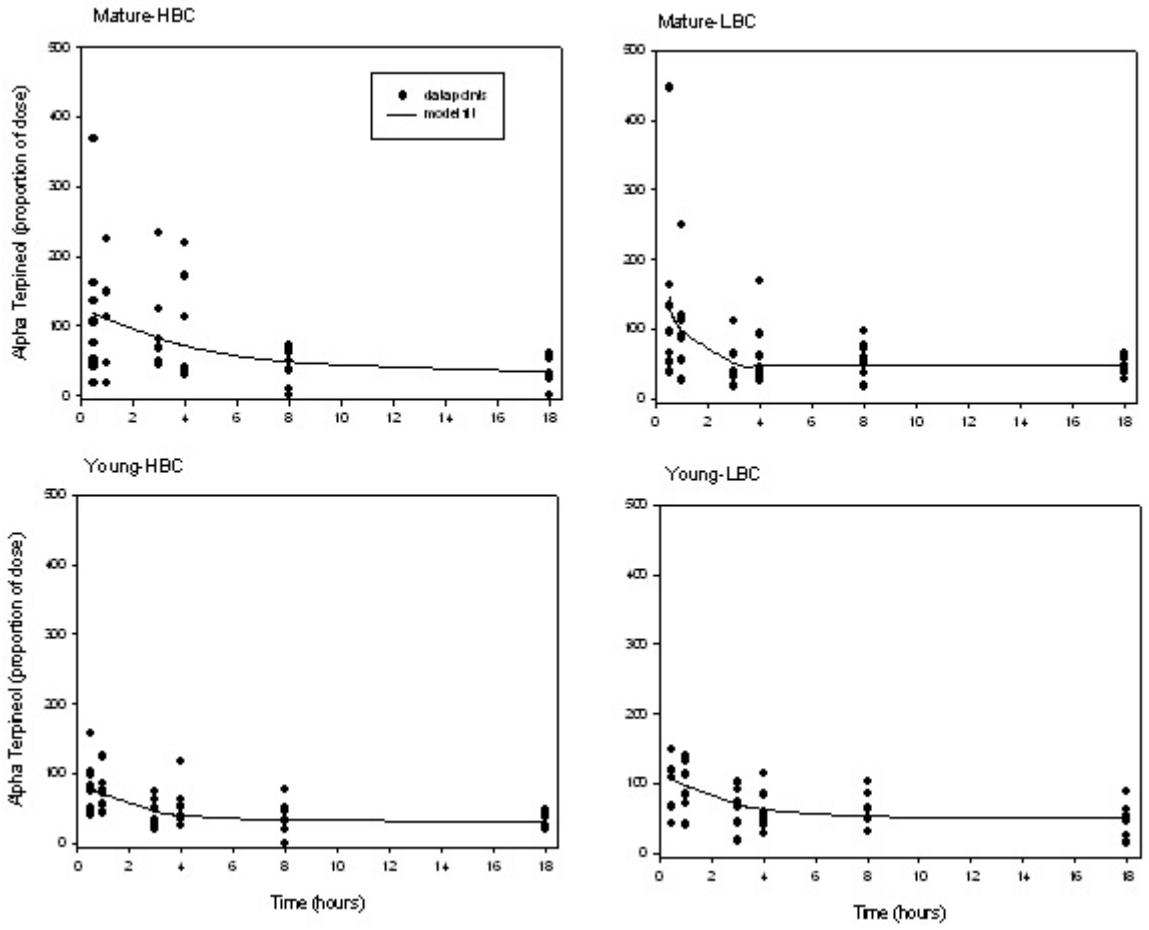


Figure C.5. Concentration of alpha terpineol adjusted for dose detected in the whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.



.**Figure C.6**. Concentration of myrcene adjusted for dose detected in the whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.

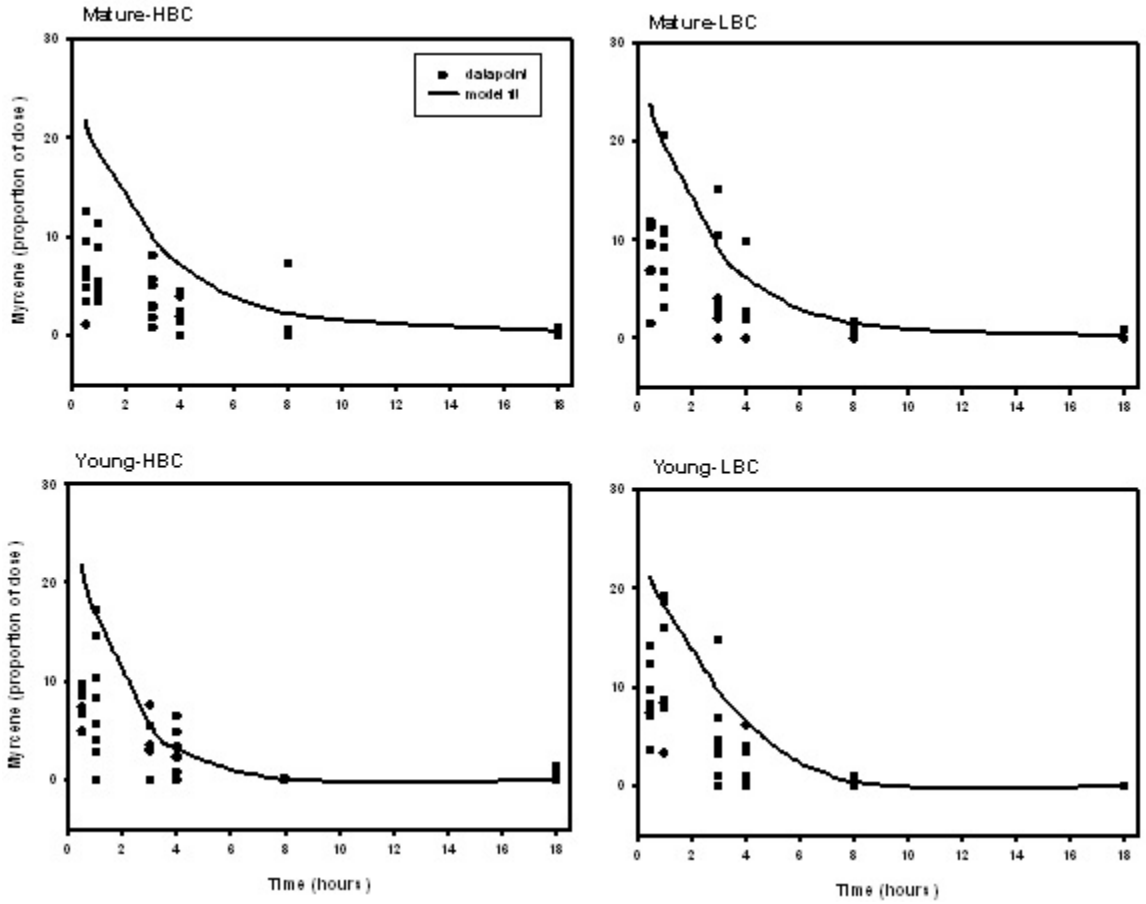


Figure C.7. Concentration of alpha pinene adjusted for dose detected in the whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.

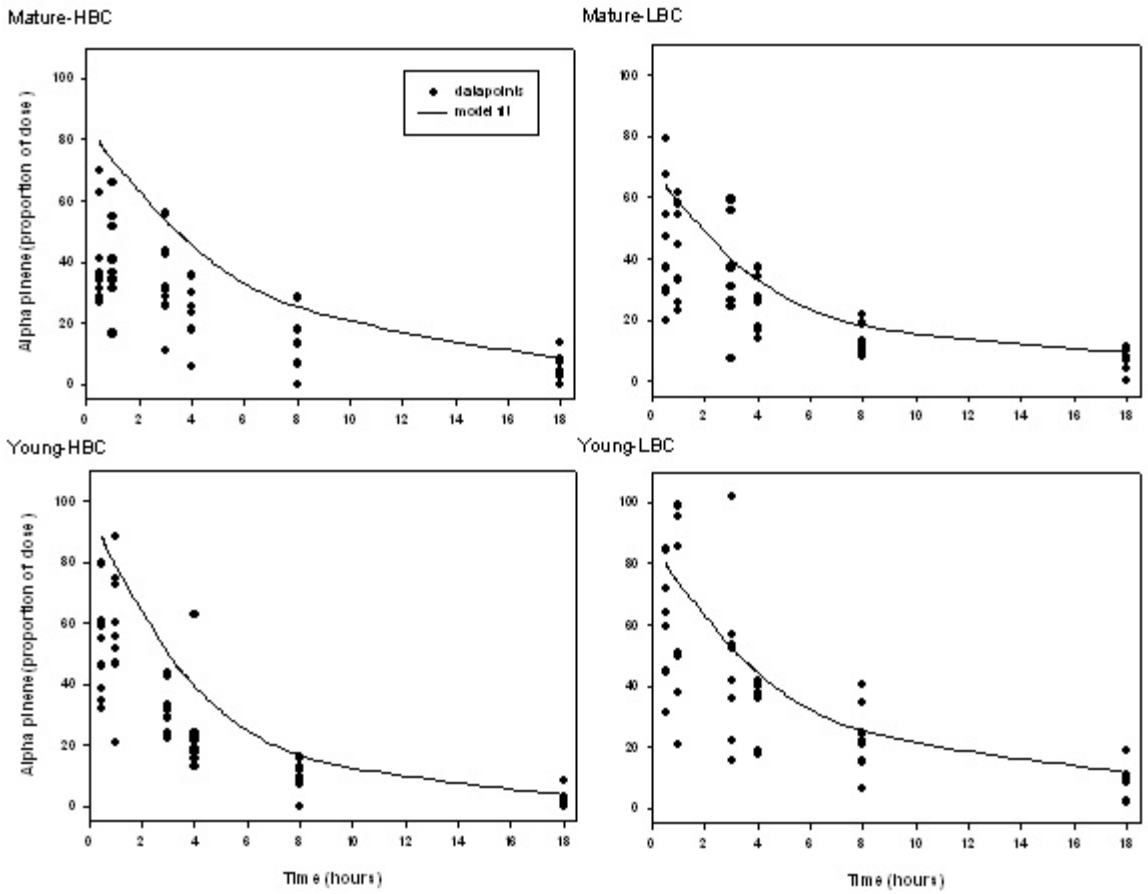
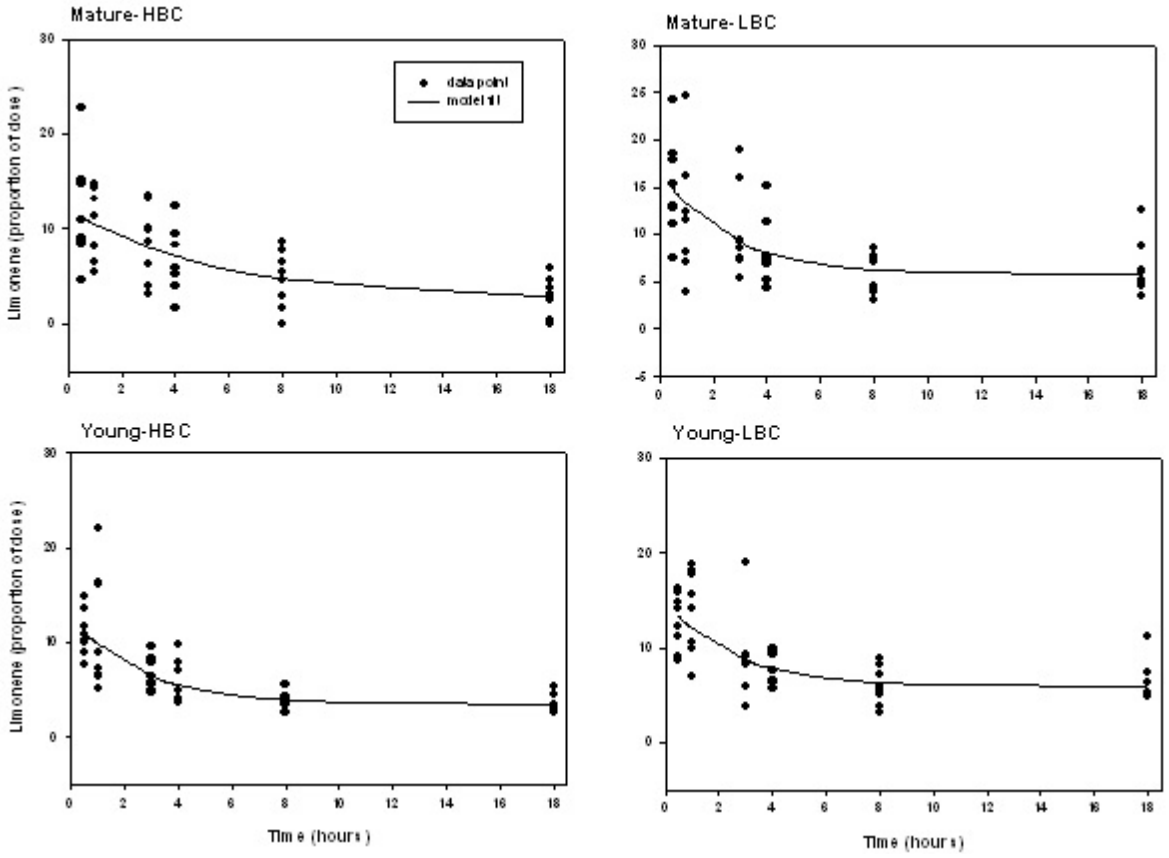


Figure C.8. Concentration of limonene adjusted for dose detected in the whole blood of goats dosed with 4 monoterpenes. Dots represent concentrations over time for each treatment: Mature or Young in high body condition (HBC) or low body condition (LBC). Lines represent fits of the data to an exponential first-order rate of elimination.



APPENDIX D: Animal Care and Use Protocol

University of Idaho
Animal Care and Use Committee

Date: Thursday, July 03, 2003
To: Karen L. Launchbaugh
From: Animal Care and Use Committee
Re: Protocol 2003-46
Influence of age and body condition on consumption of Redberry Juniper (Juniperous pinchotti) by goats

Your animal care and use protocol for the project shown above was reviewed by the Animal Care and Use Committee on Thursday, July 03, 2003.

This protocol was originally submitted for review on: Monday, March 31, 2003
The original approval date for this protocol is: Thursday, July 03, 2003
This approval will remain in affect until: Saturday, July 03, 2004
The protocol may be continued by annual updates until: Monday, July 03, 2006

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Brad Williams

IACUC Representative