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M. Denise Dearing

Effects of Acomastylis rossii tannins on a mammalian herbivore, the North American pika, Ochotona princeps

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Abstract I investigated the effects of tannin consumption, using plant tannins naturally occurring in the diet, on a herbivorous mammal, the North American pika, *Ochotona princeps*. The objectives were to determine if a high-tannin diet influenced protein and dry matter apparent digestibility, fiber digestibility and production of detoxification by-products. Additionally, I examined the possibility that pikas produce salivary tannin-binding proteins, a potential mechanism for avoiding detrimental effects of tannins. My results demonstrate that although pikas constitutively produce salivary tannin-binding proteins, animals consuming a high-tannin diet of *Acomastylis rossii* exhibited lower dry matter, protein and fiber digestion and excreted higher concentrations of detoxification by-products. Thus, *A. rossii* tannins are potential toxins as well as digestibility reducers. I propose a hypothesis coupling detoxification to reduced fiber digestion that is applicable to pikas as well as other mammalian herbivores consuming phenolic-rich diets.

Key words *Acomastylis rossii* · Detoxification · Digestibility · *Ochotona princeps* · Tannins

Introduction

Understanding the effects of plant secondary compounds on mammalian herbivores is a central question in ecology. Tannins, one of the most universal subclasses of secondary compounds, are the focus of many ecological studies (e.g., Feeny 1976; Rhoades and Cates 1976; Bryant 1981; Robbins et al. 1991; Meyer and Richardson 1993). Chemically, tannins are characterized as water soluble polyphenolics (MW 500–3000) that bind proteins (Swain 1979). Several tannins have been documented to deter feeding by mammalian herbivores. However, the physiological mechanisms responsible for the deterrent quality of tannins remain enigmatic and a topic of debate (Bernays 1981; Bernays et al. 1989; Schultz 1989; Mole and Waterman 1985; Appel 1993; Owen-Smith 1993; Owen-Smith et al. 1993). For example, ingestion of tannin-rich diets reduces protein digestion in some mammalian herbivores (Meyer and Richardson 1993; Robbins et al. 1991) but not others (Barry and Manley 1984; Meyer and Karasov, 1989; for a review see Mole and Waterman 1987).

The most prominent and least contestable feature of tannins is that they form soluble and insoluble complexes with proteins in vitro (Swain 1979). This evident *in vitro* reaction between tannins and proteins led to the assumption that when tannins were ingested by a herbivore, they bound either dietary or endogenous protein, thereby decreasing protein digestion and, ultimately, growth rate (Feeny 1976; Rhoades and Cates 1976). For the past 20 years, researchers have focused predominately on the effects of tannins in altering protein digestion, attempting to relate the in vitro protein precipitating ability of tannins to the in vivo effect of reducing protein digestion (Hagerman and Butler 1991; Appel 1993). Although tannin ingestion increases fecal nitrogen excretion in a variety of mammals (Lindroth et al. 1986; Robbins et al. 1987a; Iason and Palo 1991), the mechanism responsible for decreased growth rates appears to be more intricate than simply tannin-protein binding (Mole et al. 1990; Hagerman and Butler 1991).

Tannins have also been implicated in reducing fiber digestion in mammalian herbivores. The binding of tannins to microbial enzymes, especially cellulases, has been suggested as the mechanism responsible for decreasing fiber digestion (Barry et al. 1986). As many mammalian herbivores derive energy from the fermentation of fiber via gut symbionts (Robbins 1993), a reduction in fiber digestion could have a negative impact on

M.D. Dearing[1]
Biology Department, University of Utah,
Salt Lake City, UT 84112 USA
fax: (801) 581-4668; e-mail: dearing@biology.utah.edu

[1]*Current address*:
Department of Wildlife Ecology,
226 Russell Labs, 1630 Linden Drive,
University of Wisconsin,
Madison, WI 53706-1598, USA

fitness. Decreases in fiber digestion by tannins is based primarily on in vitro studies (Waterman et al. 1980; Palo 1985; Palo et al. 1985; Risenhoover et al. 1985). To date, only two species of mammals, sheep and brushtail possums, have shown a decrease in fiber digestion in vivo when consuming a high-tannin diet (Barry and Manley 1984; Foley and Hume 1987; Robbins et al. 1991). The influence of tannins on fiber digestion, however, has not received nearly as much attention as has protein digestion.

Many tannins, both hydrolyzable and condensed, can be absorbed across the alimentary tract, and therefore are considered potential toxins (McLeod 1974; Mole et al. 1990; Clausen et al. 1990; Robbins et al. 1991; Hagerman and Butler 1991; but see Dietz et al. 1994). Although ample evidence supports the action of tannins as toxins, the traditional idea persists that the efficacy of tannins arises from their ability to interfere with protein digestibility. Few studies have assessed the toxic effects of tannins on mammals by measuring detoxification byproducts, e.g., uronic acids (Lindroth and Batzli 1983, 1984; Lindroth et al. 1986) or changes in metabolic rate (Thomas et al. 1988). The majority have tended to examine the effects of tannins on protein and/or dry matter digestion, and only infer that decreases in growth rates or food consumption of animals on phenolic diets may be due to toxic effects (Robbins et al. 1987a; Meyer and Karasov 1989; Iason and Palo 1991).

Some mammals are capable of reducing or eliminating the deleterious effects of tannins through the production of salivary proteins. These salivary proteins have a high affinity for tannins and are thought to function by binding to tannins, thereby forming tannin-protein complexes resistant to digestion (Mehansho et al. 1983; Austin et al. 1989; Robbins et al. 1987a; Hagerman and Robbins 1993). When the production of salivary tannin-binding proteins (TBPs) is blocked, animals on high-tannin diets gain significantly less weight than do controls (Mole et al. 1990).

Few studies have investigated more than one of the several possible effects of tannins on mammalian herbivores. In this study, I investigated the effects of short-term ingestion of a high-tannin plant, *Acomastylis rossii*, on a mammalian herbivore, the North American pika, *Ochotona princeps*. The objectives were to determine if a high-tannin diet affected food intake, apparent digestibility of protein, dry matter or fiber and detoxification load relative to a low-phenolic diet. Additionally, I tested pikas for the production of salivary tannin-binding proteins (TBPs).

Methods

Study organism

North American pikas, *O. princeps*, are small (175 g), herbivorous lagomorphs restricted to talus montane areas in western North America (Broadbrooks 1965). Pikas are unusual in that they simultaneously select two very different diets (Huntly et al. 1986; Dearing 1995); one is consumed immediately ("summer diet"), while the other is stored for winter consumption ("winter diet"). A detailed study on diet selection revealed that the principal difference between the two diets was not nutritional content but rather tannin content (Dearing 1995). Although both diets contain tannins, the concentration of tannins in the winter diet was 2.5–3 times greater than that of the summer diet (Dearing 1995). The differences in tannin contents between the two diets are due to differences in the amounts of one plant species, *A. rossii* (Rosaceae), which is high in tannins. The winter diet contains 66.5% (dry weight, dw) *A. rossii* leaves, but the summer diet contains less than 8.4% (dw). In tannin analyses of *A. rossii* leaves (Dearing 1995), I found that they contain substantial quantities of total phenolics, 204 mg tannic acid equivalents/g dry weight (Folin-Ciocateu assay, Singleton and Rossi 1965) and have a high astringency value of 550 mg quebracho equivalents/g dw (assay of Hagerman and Butler 1978). However, *A. rossii* leaves tested negatively for condensed tannins (assay of Porter et al. 1986; Dearing 1995) and did not exhibit considerable quantities of simple phenolics in high pressure liquid chromatography analyses (M.D. Dearing, unpublished work). Therefore, the category of phenolics causing the high astringency value are assumed to be hydrolyzable tannins.

In addition to tannins, I also tested *A. rossii* for other major classes of plant secondary compounds. *A. rossii* tested negatively for alkaloids and cyanide in analyses I performed as part of another study (Dearing 1995). It is not known to produce terpenes (Gibbs 1974) and when the leaves are crushed, it does not produce the hallmark odor of monoterpenes (M.D. Dearing, personal observation; Gershenzon and Croteau 1991). Based on this information and the results of the three tannin assays described above, I concluded that the secondary compounds in *A. rossii* relevant to this study are hydrolyzable tannins.

Feeding trials

I conducted two similar feeding trials with captive pikas to investigate the effects of *A. rossii* consumption. The first experiment was conducted in August 1992, and the second in November 1993. Four different pikas were used for each experiment. Pikas were live-trapped on Niwot Ridge, Nederland, Colorado. Prior to each experiment, animals were maintained on a diet primarily comprosed of alfalfa (*Medicago sativa*), rabbit chow, and water *ad lib*. and supplemented with fresh apple and native plant materials (*A. rossii*, *Trifolium parryi*). Thus, prior to both experiments, all animals had experience in captivity with experimental foods. Pikas were housed individually in stainless steel rabbit cages (62 cm × 62 cm × 36 cm) with mesh bottoms. The mesh allowed feces and urine to pass through the bottom of the cage into a stainless steel collection pan.

In both experiments, effects of tannin ingestion on pikas were tested as follows. Each pika was given a high-tannin diet for three consecutive days and a control (no-tannin) diet for 3 days. The high-tannin diet consisted of *A. rossii* leaves, whereas the control diet was alfalfa. Ample, measured quantities of either food type were present at all times during the experiment. During the high-tannin diet, leftovers were removed and measured, fresh samples of *A. rossii* were provided daily to control for decreases in tannin concentrations via oxidation. For the control diet, measured quantities of alfalfa were replaced only as needed. During both diets, pikas were supplemented with water *ad lib.* and known quantities of rabbit chow. Thus, pikas were able to regulate the level of tannins ingested. This approach was necessary to maintain animals through the experiment because pikas do not naturally specialize on *A. rossii* and are difficult to keep in captivity.

To account for water loss from the foods during the trials, the dry weight amount of each food type (including chow) consumed was monitored. To determine the dry weight amount of food initially offered to pikas, wet weights were converted to dry weights based on the wet weight to dry weight ratio of control samples of all food types. The wet weight to dry weight ratio for *A. rossii* could vary daily, therefore, dry weights of control samples were calculated daily during the experiment based on two samples collected at the same time as the samples being presented to the pi-

Table 1 Environmental differences between the two experiments

Variable	August 92	November 93
Pikas	4 juveniles	3 adults, 1 juvenile
Chow type	Easily digestible	Standard
Feeding order	High-phenolic first	Control first
Temperature	Ambient, up to 18° C	Cold room, constant 10° C
Light regime	16L:8D	9L:15D
Time in captivity	5 weeks	2–5 months

kas. For the duration of each experiment, both the alfalfa and rabbit chow were obtained from the same batches and were stored in plastic containers. The wet weight to dry weight ratios of alfalfa and rabbit chow were calculated only once during each experiment based on three wet weight samples. At the end of each 3-day diet period, all feces and leftover food were removed and dried to a constant weight at 40°C.

Pikas were weighed before and after each trial. Because the two experiments were performed in different years and under somewhat different conditions (Table 1), the experiments were analyzed separately.

In the first experiment (August 1992), four juvenile pikas (mean weight = 125.9 ± 1.8 g) were used in the feeding trials. Pikas were housed in an empty cabin at the University of Colorado's Mountain Research Station, Nederland, Colorado, at an elevation of 2,893 m. Pikas were kept at ambient temperature which rarely exceeded 18°C; airflow was provided during the day with an oscillating fan. Animals were maintained on a 16L:8D light regime. The alfalfa-based chow fed during the feeding trials was Rabbit-Glo, (Manna Pro Corporation, Los Angeles, Calif.). This is an easily digestible formula designed for weaning rabbits; juvenile pikas preferred it to the standard adult rabbit chow (M.D. Dearing, unpublished work). In this experiment, pikas were first given the high-tannin diet for three days and then given the control diet for three days. *A. rossii* was collected daily and presented fresh. Alfalfa was baled and semi-dry. Pikas had been in captivity for approximately 5 weeks prior to the experiment.

In the second experiment (November 1993), three adult pikas and one juvenile (mean weight = 162.7 ± 15.3 g) were used in the feeding trials. Pikas were housed initially at the Mountain Research Station as described above; however, daytime temperatures in 1993 regularly approached temperatures (20°C and above) detrimental to pika survival (MacArthur and Wang 1973). Therefore, pikas were moved to a cold room at the University of Colorado's Boulder campus. The light and temperature regime were dictated by other ongoing experiments being conducted in the cold room and could not be altered to replicate light and temperature regime of the 1992 experiment. The cold room was a constant 10°C; this temperature is representative of maximum ambient temperatures in the animals' natural habitat during the summertime (Greenland 1989). The light regime was 9L:15D. Animals were fed an adult formula rabbit chow, Rabbit Family Ration (Manna Pro Corporation, St. Louis, Mo.). Although this formula was slightly more nutritious than the wild diet, it was more similar in its nitrogen and fiber contents to the wild diet than the chow used in August 1992 (M.D. Dearing, unpublished work). The *A. rossii* fed to pikas in this experiment was collected in August 1993, and stored at –70°C until just prior to feeding. Freshly thawed samples of *A. rossii* were given daily to pikas during the high-tannin portion of the experiment. Pikas were given control food prior to high-tannin food. Pikas had been in captivity in the cold room for 2–5 months prior to the trial.

I analyzed the two rabbit chows, alfalfa and *A. rossii* for water content, total phenolics (Folin-Ciocateu assay, Singleton and Rossi 1965), nitrogen (CHN Elemental analysis, Perkin-Elmer model 2400) and the following fiber fractions: total fiber (neutral detergent fiber analysis, NDF; Goering and Van Soest 1970) and cellulose-lignin (acid detergent fiber analysis, ADF; Van Soest 1963; Goering and Van Soest 1970). At the end of each 3-day trial, foliage leftovers, either *A. rossii* or alfalfa, were pooled for each animal and analyzed for fiber. Because pikas could forage selectively on *A. rossii* and alfalfa leaf parts (but not chow) with respect to fiber, foliage fiber components were corrected using the following formula:

g foliage fiber ingested = (g foliage fiber initially offered) – (g fiber in leftovers)

This allows for a more realistic estimation of fiber ingested. In the calculations of nutritional and fiber contents of the high-tannin and control diets, I incorporated, the amount of rabbit chow consumed. These calculations were done on a per pika basis.

Fecal collection

Pikas are coprophagic (Severaid 1955) and in addition to the final fecal product they produce specialized feces, "cecotroph" (Harder 1950), which they reingest. The two types of feces are easily distinguishable from one another. Cecotrophs are roughly oval (10 mm long) and black while feces are perfectly spherical (2 mm diam) and brown. The cecotroph is the fermented, highly digestible and nutritious contents of the cecum and is reingested directly from the anus or stored for later consumption (Hornicke and Bjornhag 1980). Storage renders cecotrophs an intermediate in digestion rather than an initial or final product; therefore, they were separated from other feces and not considered in the final calculations of digestibility. Cecotrophs were analyzed for the fiber components as described above. Cecotrophs appeared to be excreted simultaneously with urine, so they were not analyzed for nitrogen content. Total cecotroph production was unknown because cecotroph consumption could not be restricted without severely hindering the animals, thereby affecting normal digestion. From here on, the term "feces" represents only the final fecal product while cecotrophs are referred to specifically.

Fecal samples were collected to estimate dry matter, fiber and nitrogen digestibilities. After each 3-day trial, all feces and cecotrophs were removed from each animal's cage and the collection pans underneath the cage. Samples were dried at 40°C and weighed. Urine-free feces are required to estimate nitrogen digestion (Robbins 1993). To facilitate identification and collection of feces free of urine, all feces and cecotrophs in the pans underneath the animal's cages were collected 8 h prior to the end of the experiment. During the last 8 h of the experiment, collection pans were periodically checked and feces not contaminated with urine were removed. The weight of this subsample was included in the total weight of the feces. After this 8-h period, all feces and cecotrophs were removed from cages and collection pans. The urine-free feces were analyzed for nitrogen content (CHN Elemental Analysis, Perkin Elmer model 2400). Feces were also analyzed for the fiber components previously described.

Digestibilities

Digestibilities were calculated for total dry matter (dry weight biomass consumed), nitrogen, total fiber (NDF) and cellulose-lignin combined (ADF). Digestibilities were calculated as follows:

[(g of component ingested – g of component excreted)/g of component ingested] × 100.

It is extremely difficult to accurately measure individual digestibilities of separate components of a mixed diet in animals consuming a mixed diet, as the components of the feces cannot be accurately separated. Therefore, digestibilities were calculated for the diets as a whole; e.g., in the high-tannin diet, digestibilities were calculated for *A. rossii* and chow combined.

The control and high-tannin diets contained slightly different nitrogen levels resulting in higher protein contents in control diets (Table 2). This difference could present a potentially confounding factor as apparent nitrogen digestibility is a curvilinear function of food nitrogen content (Robbins 1993). To determine whether apparent nitrogen digestibility on experimental diets differed from that predicted by its nitrogen content, I used the linear relationship

Table 2 Components (percent by dry weight) of the diets consumed in digestion trials. Standard errors are in parentheses. *Asterisks* indicate significant differences between diets within a year (*N/A* not available)

Component	August 92		November 93	
	Phenolic	Control	Phenolic	Control
Protein	19.6 (0.06)*	21.0 (0.38)	19.4 (0.25)*	23.3 (0.06)
Total fiber	19.5 (0.11)*	23.4 (1.2)	26.9 (0.78)*	31.8 (0.51)
Cellulose & lignin	10.7 (0.1)*	14.9 (1.1)	17.9 (0.3)*	21.2 (0.4)
Lignin	3.0 (0.1)*	4.2 (0.2)	N/A	N/A
Total phenolics	2.8 (0.1)*	0	6.1 (0.6)	0

between amount of digestible nitrogen (percent nitrogen × apparent nitrogen digestibility) and the percentage of nitrogen in the diet, to generate a regression equation using the data from animals on the control diets in both experiments. With this equation, I generated predicted values of digestible nitrogen for animals on the experimental diets based on the nitrogen content of the diet. Using paired *t*-tests, these predicted values were compared to observed values for animals on high-tannin diets in each experiment.

Quantification of phenolics

Control samples of *A. rossii* for phenolic assays were left in the pika room for the same length of time, approximately 24 h, as *A. rossii* rations fed pikas. Control *A. rossii* samples for the 3-day trial period were pooled for phenolic analyses. Samples were kept at –70°C until assayed. Leaves were ground under liquid nitrogen. Phenolics were extracted in 85% MeOH using a homogenizer (Torti et al. 1995). Extracts were assayed for total phenolics using the Folin-Ciocateu method (Singleton and Rossi 1965) with tannic acid (Sigma lot 1764 KCNT) as a standard.

Detoxification

As an estimate of detoxification, I measured the concentration of total uronic acids in pika urine during the November 1993 experiment, using the methods of Lindroth and Batzli (1983). Urine samples were collected by pipetting urine from collection pans underneath the pika cages. Collection of urine was initiated 48 h into each diet and all urine produced the during the subsequent eight hours was collected. Pans were checked hourly during this eight hour interval. In addition to these two collection periods, I also collected one urine sample from each pika 8 h after the initiation of the high-tannin diet. A few drops of sulfuric acid were added to each sample to terminate bacterial growth (Lindroth and Batzli 1983). Samples were stored at –20°C until assayed. Daily urine outputs were calculated from 8-h samples.

Tannin-binding proteins

To look for the presence of tannin-binding proteins (TBPs) in pika saliva and also to test for the induction of these proteins on high-tannin diets, saliva samples were collected on the last day of each diet in the November 1993 feeding trials. Saliva was collected from all four pikas, by rinsing each pika's mouth with 500–700 µl of distilled water and collecting the rinse using tubing attached to a collection vial and vacuum pump. Saliva was kept at –70°C until assayed. The presence and induction of TBPs were evaluated using electrophoretic methods (Austin et al. 1989; modified by A.E. Hagerman, personal communication). To determine if TBPs are present, a model tannin is added to animal saliva. If TBPs are present in the saliva, these proteins complex the tannin, and when the saliva-tannin mixture is run on a polyacrylimide gel, the mobility of the tannin-binding proteins is altered by the interaction with the tannin. The change in mobility is visualized by comparing the banding patterns of proteins in untreated saliva to those in saliva treated with tannin. If TBPs are present, protein bands disappear, become markedly less pronounced, or shift position in the lane containing saliva treated with tannic acid (Austin et al. 1989). Mule deer (*Odocoileus hemionus*) saliva, obtained from a captive herd at Utah State University, was used as a positive control for the assay (Robbins et al. 1991). A hydrolyzable tannin, tannic acid (Sigma lot 1764 KCNT), was used as the model tannin.

Results

Diet composition

The nutritional contents of the control versus high-tannin diets differed slightly but significantly (Table 2). In both experiments, the control diet had a slightly higher protein content (paired *t*-test, 1992: $t = 5.59$, $P < 0.05$, $n = 4$; 1993: $t = 13.9$, $P < 0.001$, $n = 4$). Total fiber and cellulose-lignin contents were significantly higher in the control diet in both experiments (total fiber: paired *t*-test, 1992: $t = 3.5$, $P < 0.05$, $n = 4$; 1993: $t = 4.04$, $P < 0.05$, $n = 4$; cellulose and lignin: paired *t*-test, 1992: $t = 3.7$, $P < 0.05$, $n = 4$; 1993: $t = 6.1$, $P < 0.05$, $n = 4$). Thus, the control diet was slightly more nutritious with respect to nitrogen but less digestible with respect to cell wall components.

Effect of tannins on food intake

In the August 1992 experiment, there was no significant difference in the total amount of food consumed by pikas on either the control or high-tannin diet (paired *t*-test,

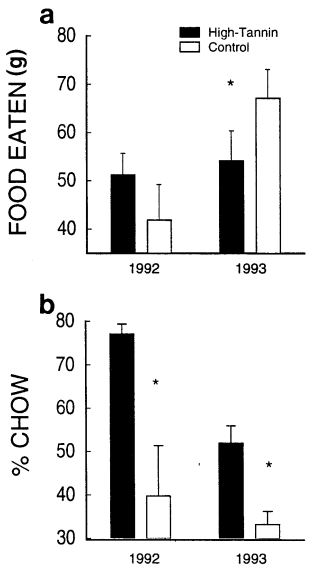

Fig. 1 **a** Total grams of food, including rabbit chow, consumed during each trial. **b** Percent of consumed food consisting of rabbit chow. *Asterisks* indicate significant differences between diets within a year. *Error bars* represent 1 SE

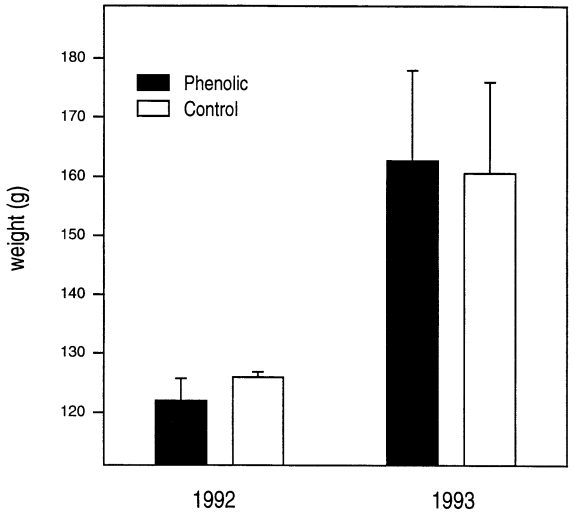

Fig. 2 Pika weights (g) after 3 days on the control diet and 3 days on the high-tannin diets. Standard errors are given in parentheses, $n = 4$ for each year. Differences were not significant

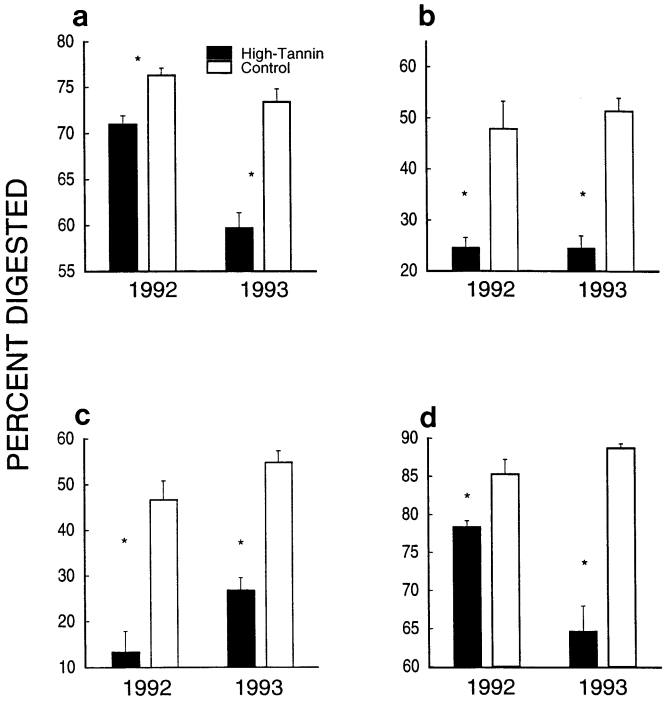

Fig. 3 Digestibilities of **a** dry matter, **b** total fiber (NDF), **c** cellulose and lignin (ADF) and **d** nitrogen. *Asterisks* indicate differences between diets within a year. *Error bars* are 1 SE

$t = 1.8$, $P > 0.05$, $n = 4$; Fig. 1a). However, in November 1993, pikas on the high-tannin diet ate less food overall, including rabbit chow, compared to the control (paired *t*-test, $t = 4.0$, $P < 0.05$, $n = 4$; Fig. 1a). Moreover, in both years, pikas on the high-tannin diet consumed proportionally more chow than when they were on the control diet (paired *t*-tests, 1992: $t = 4.1$, $P < 0.05$, $n = 4$; 1993: $t = 5.2$, $P < 0.05$, $n = 4$; Fig. 1b). Pika weights did not differ significantly between diets in either year (paired *t*-

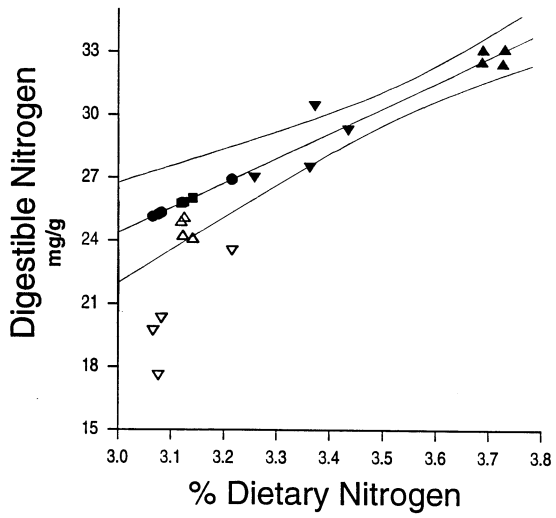

Fig. 4 Regression and 95% confidence interval of digestible nitrogen and dietary nitrogen for pikas consuming control diets only ($y = 1.2084x\text{-}11.95$, $r^2 = 88.7$, $n = 8$; *filled triangles, pointing up* 1992; *down* 1993). Values predicted from the regression line for animals consuming a high-tannin diet are plotted with *filled squares* (1992) and *filled circles* (1993). Observed values are represented by *open up triangles* (1992) and *open down triangles* (1993). The observed digestible nitrogen values of pikas on high-tannin diets are lower than predicted based on dietary nitrogen content

tests: 1992: $t = 1.4$, $P > 0.05$, $n = 4$; 1993: $t = 0.17$, $P > 0.05$ $n = 4$; Fig. 2).

Effects of phenolics on digestibilities

Dry matter digestion was significantly lower on high-tannin diets in both experiments (paired *t*-tests, 1992: $t = 7.3$, $P < 0.01$, $n = 4$; 1993: $t = 6.9$, $P < 0.01$, $n = 4$; Fig. 3a). In 1993, the difference in dry matter digestion between diets was two and a half times greater than in 1992. Total fiber digestion was also significantly reduced on the high-tannin diet (paired *t*-tests, 1992: $t = 6.4$, $P < 0.01$, $n = 4$; 1993: $t = 6.3$, $P < 0.01$, $n = 4$; Fig. 3b). The reduction in fiber digestion was reflected the cellulose-lignin fraction in both experiments (paired *t*-tests, cellulose-lignin: 1992: $t = 3.9$, $P < 0.05$, $n = 4$; 1993: $t = 5.3$, $P < 0.05$; Fig. 3c).

The apparent digestibility of nitrogen declined significantly on the high-tannin diet (paired *t*-tests, 1992: $t = 4.9$, $P < 0.05$, $n = 4$; 1993: $t = 8.4$, $P < 0.005$, $n = 4$; Fig. 3d). The extent of the difference was 3.4 times greater in 1993. Because apparent digestibility of nitrogen decreases with nitrogen concentration in the diet, the absolutely lower concentrations of nitrogen in the high-tannin diets compared to the control diets could explain the lower apparent nitrogen digestibilities of pikas on the experimental diet. However, the decrease in the amount of nitrogen digested on the high-tannin diets was significantly less than that predicted based on its nitrogen content (Paired *t*-test, 1992: $t = 5.1$, $P < 0.01$, $n = 4$; 1993: $t = 4.4$, $P < 0.006$ $n = 4$; Fig. 4).

Table 3 Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of feces and cecotrophs. NDF and ADF fiber contents of feces and cecotrophs within a year were analyzed for differences with two-way ANOVAs. Different *letters* within year and fiber categories indicate significant differences (REGW multiple range tests). Standard errors are given in parentheses

	Feces		Cecotrops	
	Phenolic	Control	Phenolic	Control
1992				
NDF	58.1 (1.1) a	52.2 (2.2) a	38.5 (0.5) b	29.0 (3.1) c
ADF	27.9 (1.9) a	32.2 (1.4) a	11.9 (0.8) b	19.3 (1.9) c
1993				
NDF	48.9 (4.1) a	54.1 (2.3) a	8.7 (0.6) b	17.1 (3.8) b
ADF	33.6 (2.1) a	38.9 (1.7) a	6.8 (0.9) b	14.8 (3.2) c

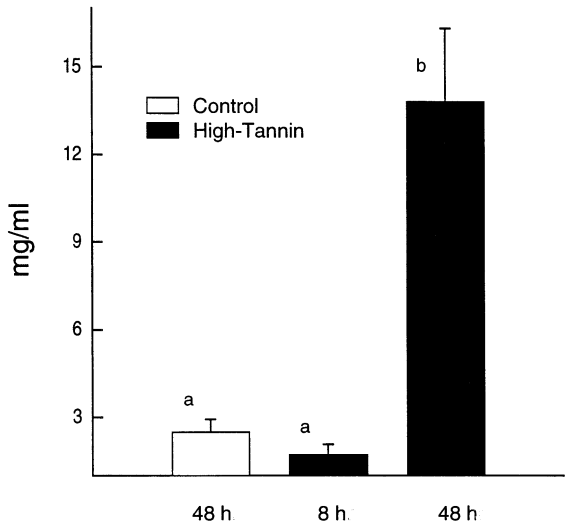

Fig. 5 Uronic acid concentrations in pika urine 48 h into the control diet, and 8 h and 48 h into the high-tannin diet. Samples were collected during experiment 2 in 1993. *Bars* with different *letters* are significantly different. *Error bars* represent 1 SE

Change in cecotroph composition

In both 1992 and 1993, the fiber content of cecotrophs (both NDF and ADF) was always less than that of feces, regardless of whether pikas were fed control or phenolic diets (two-way ANOVA: cecotroph vs. feces: *df* 1, 12, $P < 0.05$; control vs. phenolic, *df* 1, 12, $P < 0.05$; Table 3). There were no significant interactions (*df* 1, 12, $P > 0.05$). There was no effect of diet on fecal fiber content in either year (Ryan-Einot-Gabriel-Welsch Multiple Range Test, Table 3). In contrast, ingestion of a high-tannin diet altered the fiber composition of cecotrophs in both experiments, but, this change was not consistent between years (Table 3). In 1992, total fiber content (NDF) of cecotrophs was higher in pikas consuming high-tannin forage, while cellulose-lignin content (ADF) was lower than in controls. In 1993, the cellulose-lignin component of cecotrophs was lower in pikas on the high-tannin diet. Total fiber content of cecotrophs was lower in 1993 than in 1992.

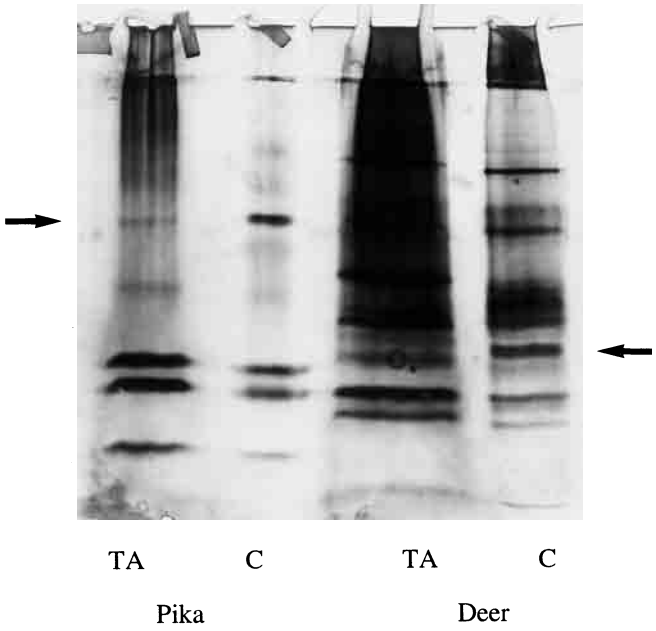

Fig. 6 Results of the tannin-binding assay (Austin et al. 1989). From *left* to *right*, pika saliva with tannic acid, pika saliva control, mule deer saliva with tannic acid, mule deer saliva control. Experimental and control lanes were loaded with equivalent amounts of saliva. The increased background and darker staining of bands in the lanes containing tannic acid is due to a reaction of the tannin with the silver stain that intensifies the sensitivity of the staining. *Left arrow* indicates pika TBPs, *right arrow* indicates deer TBPs

Detoxification of phenolics

There was no significant difference in daily urine production as estimated from eight hour collections on either diet (means: control = 4.4 ± 1.00 ml; high-tannin = 3.4 ±1.35 ml; *t*-test, $t = 1.43$, $P > 0.05$, $n = 4$), therefore, uronic acid concentrations are expressed as mg/ml. There was no apparent increase in the concentration of uronic acid production 8 h after initiation of the high-tannin diet Fig. 5). However, 48 h later, pikas exhibited a fourfold increase in urinary uronic acid excretion (ANOVA, *df* 2, 9, $F = 61.26$, $P = 0.0001$; Ryan-Einot-Gabriel-Welsch Multiple Range Test, $P < 0.05$ for means with different letters, Fig. 5).

Tannin-binding proteins

I obtained saliva samples sufficient to test for the presence of tannin-binding proteins (TBPs) from three of the four pikas. All three pikas exhibited the same TBPs in their saliva (Fig. 6). One of the protein bands visible on the native gels of the pika saliva was markedly less apparent when the saliva was treated with tannic acid even though equal quantities of diluted saliva were loaded into each lane (Fig. 6). It is believed that interaction with tannin restricts protein mobility in electrophoretic gels causing the TBPs band to become less visible, disappear, or run at a different mobility (A.E. Hagerman, personal

communication). The mobility of the pika TBPs was lower than the mobility of the TBPs present in the mule deer saliva used as a positive control. This lower mobility suggests that pika TBPs differ in protein charge or size in comparison to deer TBPs. On SDS-PAGE gels, there was no evidence for change in saliva composition on control versus experimental diets (n = 4 for each diet), suggesting that pika TBPs are constituitive and are not induced by dietary tannins.

Discussion

Despite the production of tannin-binding proteins, pikas ingesting the high-tannin diet experienced numerous physiological consequences compared to pikas consuming the control diet. Several measures of digestibility were lower in animals consuming the high-tannin diet. Moreover, pikas consuming the high-tannin diet showed a fourfold increase in the excretion of detoxification by-products. This increase in by-products strongly suggests that tannins in *A. rossii* are absorbed during digestion, detected as xenobiotics, conjugated with glucuronic acid and excreted in the urine.

Pikas consuming high-tannin diets exhibited decreased apparent protein and fiber digestibilities compared to those on control diets. The fiber and nitrogen contents of the high-tannin diets were absolutely lower than those of the control diets. Since both nitrogen and fiber digestibilities are functions of their dietary levels (Robbins 1993), declines in digestibilities could be artifacts of the lower nitrogen and fiber levels in high-tannin diets rather than the result of phenolic interaction. However, digestible protein on high-tannin diets was demonstrably lower than predicted by dietary nitrogen content alone (Fig. 4), implying that decreased nitrogen digestion resulted from the presence of tannins, not from the lower dietary nitrogen levels in the high-tannin diet. This analysis could not be extended to digestible fiber as the relationship between digestibility and fiber content is unknown. However, the magnitudes of the differences observed in fiber digestion were too extreme to be explained exclusively by differences in dietary fiber content. In other experiments where dietary fiber was increased from 8.5 to 22.5% (ADF), an 11.2% increase in fiber digestion resulted (Hammond and Wunder 1991). In my study, ADF contents varied between diets by less than one-third the of the variation seen in Hammond and Wunder's experiments, yet fiber digestibility differences were 2–3 times greater than in their results. Thus, decreases in fiber digestion in this study were attributed to high levels of tannins in the diet. Moreover, fiber digestibility decreases as the lignin proportion of total fiber increases (Parra 1978). The control diet was slightly but significantly higher in lignin (Table 1). Thus, although the control diet was higher in total fiber contents, its greater degree of lignification should have made it more refractory to digestion, thereby biasing fiber digestibility in favor of the experimental diet.

Tannins are not typically categorized as toxins, yet this and other studies have demonstrated that tannins have the potential to exert toxic effects on mammalian herbivores in that they increase detoxification metabolism (Lindroth and Batzli 1983, 1984; Lindroth et al. 1986; but see Dietz et al. 1994). Although the consideration of tannins as potential toxins complicates existing theories of plant-herbivore interactions, this should not be ignored for simplicity's sake. Detoxification of tannins could play a more prominent role in herbivore diet selection than digestibility-reducing effects since detoxification may exact a greater physiological cost to the herbivore. The relative contributions of toxic versus digestibility-reducing effects of tannins in plant defense remain speculative and an area for future research.

Salivary tannin-binding proteins (TBPs) are purported to reduce the harmful effects of tannins especially with respect to fiber digestion (Robbins et al. 1987b). For example, mule deer secrete TBPs, and fiber digestion is unaffected on tannin-rich forages (Robbins et al. 1987b). Thus, results presented here may seem paradoxical – pikas produce TBPs, yet fiber digestion was reduced on a high-tannin diet. Pika TBPs may function in ameliorating effects of tannins on fiber digestion, but to a lesser extent than do TBPs of mule deer. If the production of TBPs were experimentally blocked, it is possible that pikas consuming high-tannin forages would experience an even greater decrease in fiber digestion.

The efficacy of TBPs in pikas may be lower than in mule deer for several reasons pertaining to differences in their natural history. Because of their smaller body size, pikas consume more food for their body size than mule deer, and because of this, may be less able to saturate dietary tannins with salivary TBPs. Moreover, pikas chew their food essentially only once before digestion and absorption while mule deer ruminate. Rumination repeatedly coats the food bolus with saliva, perhaps providing absolutely more TBPs per gram of food consumed than a nonruminating animal is able to furnish in a single mastication. In addition, TBPs do not bind indiscriminately with tannins (Hagerman and Robbins 1993). Pika TBPs may selectively bind with only a subset of *A. rossii* phenolics. Thus, tannins which remain uncomplexed by TBPs may be responsible for the increase in detoxification metabolism and decrease in fiber digestion. This study did not verify the function of alleged tannin-binding proteins present in pikas. The possibility remains that pika TBPs may be completely ineffective at reducing deleterious effects of tannins.

In pikas on the high-tannin diet, alteration of digestion was apparent at the intermediate stage of digestion, cecotroph formation. The overall effect of tannin ingestion was a decrease in the fiber content of cecotrophs. Based on this result, it may appear as if tannins were enhancing fiber digestion by increasing fiber digestion in the cecum, thereby resulting in lower fiber cecotrophs. However, this was not reflected in the final digestibilities, as fiber digestion decreased on the high phenolic diet.

Table 4 Effects of phenolic-rich diets on fiber digestion. *Column 2* lists the primary site of fermentation. *Column 3* describes the effects of phenolics of fiber digestion. *Column 4* lists NDF digestibility on a phenolic-free diet as an approximate measure of the amount of fermentation of which the animal is capable of under ideal circumstances. TBP indicates whether tannin-binding proteins have been documented for that animal

Organism	Fermentation Site	Phenolic Effect on Fiber Dig.	NDF Digestibility	TBP
Pika[a]	Hindgut	decrease	54.9	Yes
Brushtail[b] possum	Hindgut	decrease	48.1	No
Desert[c] woodrat	Hindgut	no effect	33.6	?
Sheep[d]	Foregut	decrease	42.4	No
Mule deer[d]	Foregut	no effect	37.5	Yes
Elk[e]	Foregut	no effect	54.3	?

[a] M.D. Dearing, this study
[b] Foley and Hume 1987
[c] Meyer and Karasov 1989
[d] Robbins et al. 1991
[e] Mould and Robbins 1982

A model for detoxification decreasing fiber digestion

A decrease in fiber digestion due to tannin ingestion has been documented several times in vitro (Waterman et al. 1980; Palo 1985; Palo et al. 1985; Risenhoover et al. 1985), however, in vivo it has only been demonstrated for sheep, brushtail possums and pikas (Barry and Manley 1984; Foley and Hume 1987; Robbins et al. 1991; M.D. Dearing, this study). An equal number of studies have found no effect of tannins on fiber digestion (Mould and Robbins 1982; Robbins et al. 1987b; Meyer and Karasov 1989). Despite the equivocal evidence, tannins have been largely discounted in reducing fiber digestion.

The binding of tannins to microbial enzymes, especially cellulases, has been proposed as the mechanism responsible for the reduction in fiber digestion of animals on high-tannin diets (Barry et al. 1986). This may be appropriate for foregut fermenters in which tannins contact microbial enzymes shortly after swallowing, however, it seems a less feasible mechanism for hindgut fermenters, such as pikas. In hindgut fermenters, tannins should contact numerous suitable substrates after ingestion long before reaching the hindgut fermentation chamber. It is doubtful that tannins with their affinity for protein binding, would pass through the stomach and lengthy small intestine without complexing with proteins. Although the environmental conditions of the fermentation chamber may cause phenolic reactivation or a subset of phenolics may selectively target cellulases found in the hindgut (Foley and Hume 1987), these suggestions remain unsubstantiated.

I propose a hypothesis to account for tannins as fiber digestibility reducers. This hypothesis does not involve direct contact between tannins and fermentation enzymes, but couples detoxification to decreases in fiber digestibility. Foley (1992) demonstrated that detoxification of phenolics can lead to metabolic acidosis. Faced with maintaining acid-base balance, animals reduce urea production to conserve bicarbonate ions for blood buffering. A decrease in urea production results in less urea being shunted back to the fermentation chamber. Recycled urea is nutrition for microbes, and much of the nitrogen balance of hindgut fermenters is met through reingestion of hindgut fermentation products (Chilcott and Hume 1984). Metabolic acidosis may therefore negatively affect nitrogen balance in hindgut fermenters by reducing the nutritional value of the cecotrophs. I propose that metabolic acidosis could similarly influence fiber digestion. If the microbial population in the fermentation chamber decreases during detoxification-induced acidosis because of decreased urea production, then fiber digestion should be reduced.

Hindgut fermenters span the gamut of abilities in obtaining energy from fiber fermentation (Hume and Sakaguchi 1991). Hindgut fermenters that acquire a significant fraction of energy from fiber fermentation should exhibit the greatest decreases in fiber digestion during detoxification-induced acidosis. This effect would be further exacerbated in those hindgut fermenters with selective retention, for the reasons discussed in the previous section. The impact of tannins on fiber digestion abilities in species that obtain only a small fraction of their energy from their hindgut should exhibit less of an effect and these effects may be so small that they are immeasurable.

The decrease in fiber digestion due to detoxification-induced acidosis need not be exclusive to hindgut fermenters; it could apply to foregut fermenters as well. Many foregut fermenters recycle urea (Robbins et al. 1974; Mould and Robbins 1981). If ingested tannins are toxic and detoxification impacts urea recycling in these animals, then they too are predicted to show a decrease in fiber digestion. However, ruminants may be less likely to be affected by detoxification-induced acidosis because of the location of their fermentation chamber. Rumen microbes may degrade, and thereby detoxify phenolics prior to absorption in the small intestine (Simpson et al. 1969). Moreover, perturbations to a ruminant's fermentation chamber alter fermentation of all diet components, not just fiber (Robbins 1993). Therefore, ruminants may be under stronger selection pressure to minimize effects of toxins on rumen microbes and would be more likely to possess highly effective mechanisms, i.e., TBPs, to decrease effects of tannins on their gut symbionts. In summary, I suggest that the mammalian herbivores whose fiber digestion should be most impacted by tannin ingestion are (1) those in which tannins are toxic and detoxification causes metabolic acidosis, and (2) those which gain a significant fraction of their energy from fiber fermentation. Mammals most likely to meet both of these criteria are a subset of small, hindgut fermenters. Ruminants that consume tannin-rich forage should be less likely to exhibit this trend, since they should possess antitoxicity mechanisms for reasons presented above.

The six studies that have been conducted on effects of tannins on fiber digestion can be interpreted to support this view (Table 4). Pikas and brushtail possums (*Trich-*

osurus vulpecula), two of three species that exhibited decreases in fiber digestion on high-tannin diets are both small herbivores with the ability to digest a significant fraction of the cell wall content in non-tannin foods (M.D. Dearing, this study; Foley and Hume 1987). A high-tannin diet also decreased fiber digestion in sheep (*Ovis aries*) (Barry and Manley 1984; Robbins et al. 1991). Although sheep are foregut fermenters, they do not produce TBPs, and appear to absorb tannins (Robbins et al. 1991).

Abilities of woodrats (*Neotoma lepida*), mule deer (*Odocoileus hemionus*) and elk (*Cervus elaphus*) to digest dietary fiber are not affected by phenolic-rich forage. It has been suggested but not demonstrated, that the phenolics of creosote (*Larrea tridentata*) ingested by woodrats are toxic (Meyer and Karasov 1989). Under the proposed hypothesis, even if phenolics are toxic to woodrats, they would not be expected to exhibit a decrease in fiber digestion as woodrats do not appear to be extensive fiber digesters. They digest less fiber on a non-tannin diet than do pikas and brushtail possums (Table 4; Meyer and Karasov 1989). Because mule deer produce TBPs and ingested tannins are not absorbed (Robbins et al. 1991), it was not surprising that fiber digestion was not affected. It is unknown whether elk produce TBPs.

Clearly more studies are necessary to satisfactorily test my hypothesis. However, the theory provides testable predictions with respect to which herbivores are most likely to exhibit perturbations in fiber digestion due to ingestion of tannins. A caveat: studies investigating effects of tannins on fiber digestion should attempt to use experimental diets with fiber contents that reflect natural dietary fiber contents as unnaturally low fiber contents may eliminate the interaction of detoxification and fiber digestion.

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