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# Effects of simulated and insect herbivory on nitrogen and protein precipitable phenolic concentrations of two legumes

Tiana K. Blackmon[a,b], James P. Muir[a,b], Roger D. Wittie[a,b], David H. Kattes[a,b] and Barry D. Lambert[a,b]

[a]Department of Wildlife, Sustainability, and Ecosystem Sciences, Tarleton State University, Stephenville, TX, USA; [b]Texas A&M Agrilife Research, Stephenville, TX, USA

**ABSTRACT**

Protein-precipitating polyphenolics (PPPs) serve as a plant defense against herbivory, increasing with stress. We studied how varying intensities of simulated and *Melanoplus differentialis* herbivory affected (1) PPP concentration; (2) protein bound by PPP (PB); and (3) N concentration of panicled tick-clover (*Desmodium paniculatum*; PTC) and sericea lespedeza (*Lespedeza cuneata*; SL) leaf regrowth. Leaves of PTC that were submitted to simulated herbivory had lesser ($p \leq .05$ for all significant differences) PPP concentration than the control for most treatments. For PTC, PPP concentration decreased with increasing herbivory intensity for both herbivory types. For SL, PPP was similar between herbivory types for Harvest 1 but not for 2, decreasing as herbivory intensity increased for both herbivory types. Simulated herbivory resulted in lower PB concentrations for PTC and SL compared to the grasshopper herbivory. Nitrogen concentration was similar for PTC and SL between herbivory types but variable among degree of herbivory. Herbivory type affects PPP.

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Condensed tannins; grasshopper; phenolics; *Lespedeza cuneata*; *Desmodium paniculatum*

## Introduction

Phenolics are plant secondary metabolic compounds that may serve as a defensive response to herbivory (Levin 1971; Boudet 2007; Khoddami et al. 2013). Examples include flavonoids, anthocyanins, anthocyanidins, and proanthocyanidins (Khoddami et al. 2013). Proanthocyanidins include polymers of phenolic acids (ellagic acid) called tannins (Levin 1971; Chung et al. 1998; Khoddami et al. 2013). Within plants, tannins vary widely in concentration (Mosjidis et al. 1990), structure (Fahey and Jung 1989; Chung et al. 1998), allocation (Mosjidis et al. 1990; Haring et al. 2007), and other characteristics, which may result from the age of the plant (Stitt and Clarke 1941; Buntin 1991; Cooper et al. 2014), stresses (Feeny 1976; Fales 1984; Tharayil et al. 2011), or season (Stitt and Clarke 1941; Fahey and Jung 1989; Muir et al. 2014).

Tannins are generally classified as hydrolysable or condensed (Chung et al. 1998). Condensed tannins are very reactive compounds with both oxidative and protein-binding activities (Naumann et al. 2013). These activities may be regulated by structural differences, which could affect ruminant protein utilization (Naumann et al. 2013). In addition to proteins, CT binds to compounds including starch, cellulose, and minerals and reduces feed intake and efficiency, growth rate, and protein digestibility (Chung et al. 1998; Ndlovu et al. 2000). Condensed tannins also suppress plant, gastrointestinal, and fecal parasites (Levin 1971; Appel 1993; Haring et al. 2007; Acero et al. 2010; Littlefield et al. 2011).

A study by Pellissier (2013) focused on distinguishing physiological responses of *Abies alba* and *Rubus fruticosus* to physical damage versus responses from the presence of ungulate saliva. More specifically, flavonoid and chlorophyll responses varied according to the treatment applied (clipping and/or saliva). These treatments also affected plants in different ways, with clipped treatments showing the greatest chlorophyll fluorescence. Plants with clipping and saliva applied tended to have lower chlorophyll fluorescence than the either/or treatments, although the opposite became true 9 d after treatment. However, flavonoid and chlorophyll contents were not affected by any of the treatments for most of the days after treatment, with the exception of saliva on Day 4 (chlorophyll content) and clipping + saliva on Day 5 (flavonoid content). These results suggest that the plant does not distinguish between physical damage and the presence of ungulate saliva.

Condensed tannins can deter feeding by some insect herbivores. In some grasshoppers, CT weakly deters feeding (Mole and Joern 1994). When given the choice, grasshoppers will consume tannin-free plants more often than tannin-containing plants (Dini and Owen-Smith 1995). Much of the negative feeding effects of tannins occur in situations of low protein and water, such as in mature leaves which are often avoided (Bernays and Chamberlain 1982). In general, graminivorous grasshoppers are unable to digest tannins, while polyphagous grasshoppers are able to do so with no apparent consequences (Barbehenn 2002). Still, the addition of tannins into the diets of either grasshopper type reduces enzymatic activities, including superoxide dismutase, catalase, ascorbate peroxidase, and glutathione transferase peroxidase, which are antioxidants (Barbehenn 2002). The latter two are involved in tannin defense (Barbehenn 2002).

Sericea lespedeza (SL) is a tanniferous perennial warm-season legume native to Asia (Diggs Jr. et al. 1999). It was brought to the USA in 1896 as a forage and soil stabilizer (Gamble et al. 1996; Cummings et al. 2007); it has since become naturalized to the point that it is considered an invasive weed in much of the USA (Dudley and Fick 2013). Some sources hypothesize that it is allelopathic and its residues may reduce germination rates of seeds from other plants (Dudley

**CONTACT** Tiana K. Blackmon blackmon@vt.edu

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and Fick 2013). It purportedly has antioxidant health benefits when consumed by humans (Kim et al. 2012). It is an effective anthelmintic and reduces methane production in ruminants (Puchala et al. 2012; Burke et al. 2014).

Panicled tick-clover (PTC) is a perennial warm-season legume native to the USA (Diggs et al. 1999). It is common in eastern states, spreading westward to Texas and Nebraska and north to the Canadian border (Isely 2013). Among its natural uses are quail feed (seed) and deer browse (Surrency and Owsley 2001) but it could provide nutritious forage and gastrointestinal nematode suppression in domesticated ruminants (Muir et al. 2008; Cherry et al. 2013). Muir et al. (2008) found that PTC is high in CT and N content, making it a good candidate for agronomic domestication.

The differential grasshopper (*Melanoplus differentialis*) is common to Texas and is a generalist feeder (Lewis 1984; Reinert et al. 2011). This is an important species since it devastates entire landscapes in a short time (Reinert et al. 2011). It has been used in experimental studies since it will eat a variety of plants and is easy to locate and handle (Hodge 1933; Howard 1995). It feeds on SL and PTC and is utilized in CT studies (Young and Cantrall 1955; Hinks et al. 1993; Cross et al. 1997).

Studies comparing simulated herbivory with insect herbivory have not focused on plant phenolic responses. The objectives of this study were to determine how varying intensities of herbivory (simulated and by differential grasshopper) and plant ontogeny affect (1) leaf protein-precipitable polyphenolics (PPPs) concentration; (2) leaf N content; and (3) leaf regrowth PPPs and N contents. Our hypothesis was that there is a difference in plant responses between herbivory types.

## Materials and methods

### Experimental design

We germinated PTC and SL seeds obtained from Texas A&M AgriLife Research, Stephenville, TX, USA (32°15′ N, 98°12′ W, altitude 395 m) in Petri dishes in May 2013. The elevated germination rate of SL led us to plant new SL (cultivar AU Grazer) seed directly into 1.9 L pots, each of which contained Sunshine Mix #4/LA4 potting mix (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia, Canada). We transplanted recently germinated PTC seedlings into pots and potting mix of the same type and amount. We grew plants in a greenhouse environment, which included natural light and irrigation twice daily by an automatic system for a total of 10 mm/d to avoid a water stress response. Plants were blocked by height and then placed randomly into 6 treatments: 50% and 100% mechanical clipping, and adult *M. differentialis* density intensities of 0, 5, 10, and 15 per cage (0.0973 $m^3$), each receiving 2 plants. We acquired grasshoppers in the area surrounding the greenhouse. We maintained grasshopper density by replacing those that expired during the experiment. The experiment began when plants reached 30-cm height (26 July).

### Defoliation

For simulated herbivory, plants were defoliated by hand. The 50% defoliation intensity was determined by the plane of symmetry through the stem and only leaves were removed. For insect herbivory, grasshoppers were allowed to feed on the SL for a 24-h period and on the PTC for a 48-h period. The difference in duration of exposure to the grasshoppers was necessary due to the greater biomass of PTC vis-à-vis SL. Plants were allowed to regrow for 24 d to achieve sufficient regrowth to provide material for laboratory analyses. Half of each treatment was removed from the study for leaf laboratory analysis. The remaining half was exposed to herbivory once more in the same treatment classification as before. Leaves in the 50% defoliation treatment were removed from the same side as the original exposure. Plants were allowed sufficient time for regrowth and harvested for analyses.

### Laboratory analyses

Leaves from each plant were analyzed to determine concentrations of N and PPPs as well as amount of protein bound (PB) by PPP as described by Nauman et al. (2013). Leaves were placed in paper bags and dried at 55°C in a forced-air oven for 48 h. These were then ground in a Thomas Wiley® Mini-Mill (Arthur H. Thomas Co., Philadelphia, PA, USA) to pass a 1-mm screen.

For phenolic extraction, 1 mL of 50% methanol:water (v/v) was added to 50 µg of plant material. This mixture was vortexed, placed on a G10 Gyrotory® shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA) for 30 min, and then centrifuged for 5 min at 16060 × *g*. The supernatant was immediately used for analysis.

For PPP analysis, 250 µL of Buffer A (0.20 M acetic acid, 0.17 M NaCl, pH 4.9), 50 µL of bovine serum albumin (BSA) (10 mg/mL in Buffer A), 50 µL 50:50 (v/v) methanol:water, and 50 µL of plant extract were required for each sample in addition to a blank comprising 800 µL sodium dodecyl sulfate (1% w/v)-triethanolamine (5% w/v) (SDS/TEA) and 200 µL of ferric chloride (0.01 M $FeCl_3$ in 0.01 M HCl) (Naumann et al. 2013). Samples were vortexed and then allowed to incubate for 30 min. Following incubation, samples were centrifuged twice for 5 min each at 16060 × *g*, with the supernatant aspirated and washed with 250 µL of Buffer A each time before one final aspiration. The pellet was dissolved in 800 µL of SDS/TEA and vortexed. Once the pellets were completely dissolved, 200 µL of ferric chloride was added and the samples were allowed to stabilize for 30 min at ambient temperature before the absorbance was read at 510 nm.

PB by PPP was analyzed beginning with crude extraction and PPP analysis to form a pellet. The pellet was washed twice, re-suspended with 500 µL of Buffer A, and vortexed until dissolved. A 500 µL sample of solution was put into pre-weighed foil to analyze for N. Three blanks were used: a buffer blank (500 µL buffer A + pre-weighed foil), a bovine serum albumin blank (500 µL bovine serum albumin + pre-weighed foil), and a plant extract blank (200 µL plant extract + pre-weighed foil). All foils were placed in a forced-air oven set at 55°C and dried until the liquid evaporated then weighed before N analysis in a Vario MACRO C-N Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ, USA). The N concentration was multiplied by 6.25 to calculate bound protein.

### Experimental design and statistical analyses

Four plants were batched per experimental unit to determine concentrations (four replications arranged as blocks). Analysis of variance was completed using Statistix 10 (Analytical Software, Tallahassee, FL, USA). Species by defoliation

interaction was first examined and simple factors analyzed only if these were not significant ($p > .05$). Nitrogen, PPP, and PB by PPP were dependent variables. We used a probability of ≤0.05 throughout when considering significant differences.

## Results

Plants exposed to grasshopper herbivory were defoliated differently compared to the simulated treatments. For both species, grasshoppers consumed the youngest leaf material first, followed by more mature leaves. In general, they consumed inter-vein leaf material. In greater intensity treatments, however, older leaves were defoliated closer in time to younger leaves since there was less material available for each grasshopper.

### Nitrogen

PTC N content was similar between mechanical and grasshopper herbivory, although they differed within herbivory types. Nitrogen content increased by 26% and 40%, respectively, across the 50% and 100% simulated defoliation treatments compared to the control but not across the grasshopper treatments (Table 1). While N content increased 14% between Intensities 1 and 2, it declined 9% from Intensity 2 to 3.

Nitrogen contents were also similar for SL between mechanical and grasshopper treatments. Sericea lespedeza N content decreased for most treatments compared to the control, but remained unchanged for the 100% simulated defoliation (Table 2). Within herbivory type, leaf N content increased 12% between Intensities 1 and 2 but remained unchanged for Intensity 3. With repeated herbivory, N content increased for moderate herbivory (Intensities 1 and 2 and 50% defoliation) but decreased with greater herbivory (Intensity 3 and 100% defoliation).

### Protein-precipitable polyphenolics

PTC PPP contents were lower for mechanical herbivory than grasshopper herbivory, with the exception of 50% defoliation which was similar to Intensity 1. PPP content decreased with increasing herbivory intensity (Table 1). Within insect herbivory, PPP content increased 37% between Intensities 1 and 2 but decreased 12% between Intensities 2 and 3. Similarly, PPP decreased 20% between 50% and 100% defoliation. PPP content increased among all treatments with repeated herbivory, excluding 100% defoliation, which decreased 75%.

Sericea lespedeza PPP was similar between mechanical and grasshopper treatments for Harvest 1 but not 2. PPP content decreased an average 20% as herbivory increased regardless of herbivory type (Table 2). PPP content decreased a maximum of 26% from control to Intensity 1 and a minimum of 15% each from Intensity 2 to 3 and from the control to 50% defoliation. PPPs increased from Harvest 1 to 2 for all treatments except Intensity 1 and 50% defoliation, which decreased by 16% and 24%, respectively.

### PB by PPP

PTC PB was much lower for simulated herbivory than grasshopper herbivory, and 50% defoliation was similar to the control for Harvest 1. PTC PB increased by 17% between grasshopper Intensities 1 and 2 but declined by 14% between Intensities 2 and 3 (Table 1). Simulated treatments also caused a 100% decrease in PB, from Harvest 1 to 2.

PB by PPP was also lower for SL-simulated herbivory treatments than grasshopper herbivory, although all treatments were at least 50% greater among herbivory treatments compared to the control (Table 2). There were no differences in PB between the simulated treatments for the first harvest; PPP in leaves submitted to 100% mechanical defoliation, however, declining by 48%, twice as much as for the 50% defoliation.

**Table 1.** Panicled tick-clover leaf nitrogen, protein-precipitable polyphenolics (PPPs) and protein bound (PB) by PPP chemical response to mechanical or grasshopper defoliation.

| | Nitrogen (%) | | PPPs (g kg$^{-1}$) | | PB by PPPs (g kg$^{-1}$) | |
|---|---|---|---|---|---|---|
| Treatment[a] | 1 | 2 | 1 | 2 | 1 | 2 |
| Control | 2.05$^{c}$ | 1.78$^{b}$ | 20.42$^{a}$ | 22.72$^{a}$ | 242.42$^{c}$ | 171.79$^{b}$ |
| Grasshopper intensity 1 | 2.22$^{b}$ | 1.57$^{c}$ | 13.32$^{d}$ | 23.29$^{a}$ | 310.42$^{b}$ | 262.98$^{a}$ |
| Grasshopper intensity 2 | 2.14$^{b,c}$ | 1.79$^{b}$ | 18.33$^{b}$ | 24.37$^{a}$ | 363.13$^{a}$ | 196.93$^{b}$ |
| Grasshopper intensity 3 | 2.16$^{b,c}$ | 1.43$^{c}$ | 16.15$^{c}$ | 21.44$^{a}$ | 313.89$^{b}$ | 208.45$^{a,b}$ |
| 50% Clip | 2.22$^{b}$ | 1.79$^{b}$ | 14.05$^{d}$ | 15.95$^{b}$ | 239.18$^{c}$ | 110.88$^{c}$ |
| 100% Clip | 2.81$^{a}$ | 2.52$^{a}$ | 11.31$^{e}$ | 2.83$^{c}$ | 186.73$^{d}$ | 0.00$^{d}$ |
| Standard error | 0.0591 | 0.0696 | 0.7340 | 1.4354 | 18.935 | 28.843 |

Note: Letters denote significant differences ($p \leq .05$) within columns based on least significant difference multiple means comparisons.

[a]Grasshopper intensity X refers to the number of grasshoppers (5X) per 0.0973 m$^3$ cage. Two plants were placed in each cage, representing the experimental unit.

**Table 2.** Sericea lespedeza leaf nitrogen, protein-precipitable polyphenolics (PPPs), and protein bound (PB) by PPP response to mechanical or grasshopper defoliation.

| | Nitrogen (%) | | PPPs (g kg$^{-1}$) | | PB by PPPs (g kg$^{-1}$) | |
|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 1 | 2 | 1 | 2 |
| Control | 2.46$^{a}$ | 2.24$^{a,b}$ | 7.10$^{b}$ | 9.05$^{a}$ | 122.16$^{c}$ | 146.13$^{b,c}$ |
| Grasshopper intensity 1[a] | 1.72$^{c,d}$ | 1.77$^{d}$ | 8.97$^{a}$ | 7.52$^{b}$ | 231.09$^{a}$ | 174.74$^{a}$ |
| Grasshopper intensity 2[a] | 1.93$^{b,c}$ | 2.04$^{b,c}$ | 6.98$^{b}$ | 7.34$^{b}$ | 175.17$^{b}$ | 166.89$^{a,b}$ |
| Grasshopper intensity 3[a] | 1.98$^{b}$ | 1.96$^{c,d}$ | 5.92$^{c}$ | 6.73$^{c}$ | 181.85$^{b}$ | 124.18$^{c,d}$ |
| 50% Clip | 1.67$^{d}$ | 2.02$^{b,c}$ | 5.99$^{c}$ | 4.57$^{e}$ | 131.31$^{c}$ | 100.40$^{d}$ |
| 100% Clip | 2.41$^{a}$ | 2.36$^{a}$ | 4.55$^{d}$ | 5.47$^{d}$ | 131.30$^{c}$ | 68.11$^{e}$ |
| Standard error | 0.0987 | 0.1214 | 0.2018 | 0.2745 | 9.3417 | 11.856 |

Note: Letters denote significant differences ($p \leq .05$) within columns based on least significant difference multiple means comparisons.

[a]Grasshopper intensity X refers to the number of grasshoppers (5X) per 0.0973 m$^3$ cage. Two plants were placed in each cage, representing the experimental unit.

## Discussion

Some plant responses were different between simulated and grasshopper herbivory types. Although N was similar for both species, PPP and PB were different between herbivory types.

The increase and subsequent decrease in N content with herbivory intensity is consistent with similar studies (Rooke and Bergström 2007; Schädler et al. 2007; Cooper et al. 2014). Lower N content may be due to N mobilization for leaf production; greater herbivory intensities result in fewer leaves to photosynthesize, so the plant allocates resources to produce more leaves (Culvenor and Simpson 1991; Cooper et al. 2014).

Alternatively, the reduction in N content could be the result of nutrient stress (Bryant et al. 1993). Since the plants grew in pots and were not fertilized, N would have been a limited resource if not for atmospheric N fixation in the plant nodules. This biological N fixation may have been affected by a number of variables, such as exhausted soil nutrients including P, photosynthetic rates, rhizobial genotype, and environmental factors not accounted for in our study (West et al. 2005).

Similar trends between simulated and natural herbivory treatments are consistent with some studies (Lyytikäinen-Saarenmaa 1999) but not others (Baldwin 1990). The tendency for SL N content to decline with increasing herbivory intensity was consistent with findings reported by Cooper et al. (2014), with the exception of the 100% defoliation which was similar to the control. Lower N availability can limit feed efficiency by reducing urea recycling and microbial efficiency in ruminants (Reed 1995) as reported for other forages containing CT (Mangan 1988). In grasshoppers, lower N availability is associated with reduced population densities because N is a limiting nutrient (Loaiza et al. 2011). Additionally, N availability is a major driver of foliar damage by grasshoppers (Loaiza et al. 2011).

PTC PPP contents were inconsistent with a previous study. Cooper et al. (2014) found that PPP content increased from unclipped leaves to those submitted to 25% defoliation before declining for the remaining treatments. These results parallel what we observed following the second harvest, but not the first, suggesting that insect defoliation affects naive plants in a different way. Once-stressed regrowth never attained the PPP of the control plants, while repeatedly stressed regrowth remained above control plant levels with the exception of major defoliation (grasshopper Intensity 3 and 50% and 100% mechanical defoliation).

Donnelly and Anthony (1983) reported greater condensed tannin contents in plants repeatedly defoliated than in plants defoliated once, in line with our observations of PTC. The reduction in PPP with increased defoliation over repeated events is also consistent with a study observing *Quercus* spp., in which leaf PPP on previously defoliated branches decreased compared to control branches (Faeth 1992).

Sericea lespedeza PPP contents following grasshopper herbivory differed from what Cooper et al. (2014) reported for mechanical harvests. Contrary to our findings, they observed a decrease in leaf PPP between 0% and 25% defoliation followed by an increase under more intense herbivory. This suggests differences in plant response between low-intensity natural and simulated herbivory.

The decrease in SL PPP contents between harvests for all species with the exception of Intensity 3 and 100% clip suggests that lower-intensity treatments were not adequate to achieve responses similar to what other studies on SL have observed (Donnelly and Anthony 1983; Muir et al. 2014). The greater intensity treatments that resulted in biomass loss similar to mowing or cutting for hay that previous studies have used as treatments could explain the variation in results (Muir et al. 2014), as could variations in timing of those treatments (Nykänen and Koricheva 2004).

An additional simulated herbivory treatment for both species would be required in this study to directly compare our simulated results with Cooper et al. (2014). The decrease in PPP content for both species from 50% to 100% defoliation, however, is similar, suggesting that there may be distinctive responses in leaf PPP accumulation for herbivory types. This phenomenon has been supported by studies examining other physiological processes (Baldwin 1988; Kessler and Baldwin 2002) and herbivores (Ward and Young 2002). However, some studies contradicted these findings, for example, in the meta-analysis by Nykänen and Koricheva (2004).

PTC PB dynamics as a result of herbivory were consistent with those reported by Cooper et al. (2014) for simulated herbivory of PTC and by Alba-Meraz and Choe (2002) for *Melanoplus* herbivory of other plant species. Protein-binding affinity affects ruminant nutrition since it prevents microbial degradation in the rumen (Silanikove et al. 2001; Min and Hart 2003; Pawelek et al. 2008). PTC PB declined following 100% defoliation in this study as well as in that reported by Cooper et al. (2014) corresponding to the physiologically costly requirements of making protein-binding compounds (Coley et al. 1985; Keinänen et al. 1999), since polyphenolic production is reliant on photosynthesis to fix carbon (Bryant et al. 1983; McDonald et al. 1999). These results do not support some hypotheses which predict that more resources are spent on defenses in high-intensity herbivory conditions (Janzen 1974; Coley et al. 1985). Similarly, McDonald et al. (1999) found that increased $CO_2$ availability increases polyphenolic concentrations in certain trees. Starch, a product of photosynthesis (Sharkey 1985), is also positively correlated with accumulation of certain tannins, including CT (Lawler et al. 1997; McDonald et al. 1999). Even in nutrient-rich conditions, CT are much lower in low light (thus low photosynthetic activity) conditions for *Eucalyptus* spp., possibly mirroring conditions with little to no leaves (Lawler et al. 1997), although severe defoliation increases polyphenolic response in some species (Bryant et al. 1993).

Compared to PTC, SL PB changes as a result of herbivory were inconsistent, with no apparent trends across treatments. For example, both harvest types increased leaf PB content between the control and Intensity 1 and decreased between Intensities 1 and 2, but were similar in simulated treatments in Harvest 1 while they decreased between simulated treatments in Harvest 2. Cooper et al. (2014) found that defoliation resulted in less leaf PB than in undefoliated plants, in contrast to responses to both herbivory types in this study. This may be due at least in part to differences in how simulated herbivory treatments were applied (Baldwin 1990). Cooper et al. (2014) removed leaves a pre-measured distance from the top of the plant to simulate ruminant herbivory, while we harvested equally from all heights to simulate grasshopper herbivory as observed in a pilot study at our greenhouse.

In summary, there were no differences in N response between simulated and grasshopper herbivory for either legume in our study. Plant PPP and PB response to herbivory, however, varied between the two species and herbivory types. These physiological differences could have a nutritional impact on the herbivores that consume them in addition to differences in other biological effects in the herbivores. If producers encounter extensive attacks by grasshoppers on pastures containing these legumes, they should be prepared to compensate livestock rations for the reduced N availability and associated increase in tannin content of forages. Management should be based on production needs – for example, if there is an extensive internal parasite issue with goats, then they should manage the legumes for the increased PPP.

Otherwise, N availability will be more important for production, as in cattle. Our results support studies, such as Korth and Dixon (1997), which provide evidence for distinctive responses to herbivory compared to mechanical wounding. Future research should focus on the possibilities of further distinguishing herbivory types.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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## ORCID

*Tiana K. Blackmon* http://orcid.org/0000-0002-1045-2911
*James P. Muir* http://orcid.org/0000-0003-1775-8072
*Roger D. Wittie* http://orcid.org/0000-0001-8156-9204
*David H. Kattes* http://orcid.org/0000-0002-7063-7942
*Barry D. Lambert* http://orcid.org/0000-0003-1488-3118

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