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EFFECTS OF CONDENSED TANNINS ON THE TOXICITY OF FIREWEED  
(*SENECIO MADAGASCARIENSIS*) TO CATTLE

by

Carolyn L. Wong

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Range Science

Approved:

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UTAH STATE UNIVERSITY  
Logan, Utah

2014

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**ABSTRACT**

Effects of Condensed Tannins on the Toxicity of Fireweed  
(*Senecio madagascariensis*) to Cattle

by

Carolyn L. Wong, Master of Science

Utah State University, 2014

Major Professor: Dr. Frederick D. Provenza  
Department: Wildland Resources

*Senecio madagascariensis*, also known as fireweed, is a noxious weed that has invaded pastures in Hawaii, Australia, and South America. Fireweed contains pyrrolizidine alkaloids (PAs) that, when metabolized into highly reactive pyrroles, are toxic to most grazing mammals. Toxic effects to cattle include irreversible damage to liver cells, liver fibrosis, and loss of liver function, which may lead to jaundice, edema, ascites, and other physiological malfunctions. External effects include rough appearance, diarrhea, prolapsed rectum, lassitude and dullness, photosensitization, and abnormal behavior, many of which can lead to death. Fireweed also can reduce pasture productivity by as much as 30-40%, particularly in Hawaii. As a result of these adverse effects on cattle and pasture production, people are seeking ways to manage fireweed and cattle.

Condensed tannins, which are common in many forage legumes, have a high affinity for binding with other molecules such as protein and alkaloids.

Complementarities among secondary compounds such as condensed tannins and alkaloids can increase intake of forages high in intake-limiting compounds such as alkaloids; however, information is lacking on the protective effects these

complementarities have against toxicity. The effects of condensed tannins on the toxicity of fireweed to cattle were examined in studies *in vitro* and *in vivo*. The *in vitro* studies showed that tannins bound pyrrolizidine alkaloids in cattle rumen fluid and binding was highest when mixed with tannins at 8%, with some effect at 12% as well, by weight of fireweed. The *in vivo* research sought to determine if tannins reduced toxicity in cattle as measured by various behavioral observations and physiological responses. The results were inconclusive as animals from both the Fireweed and Fireweed-Tannin groups demonstrated ill effects. The quantitative data gathered in the form of serum analyses and liver biopsies did not identify a clear protective effect of tannin in the diet, but the results likely were confounded by the unanticipated effects brought on by the method of treatment administration, which involved dosing animals daily with large amounts of plant material. The findings also illustrate how strongly cattle avoid eating fireweed while grazing on pasture. In summary, management to reduce fireweed effects on cattle should focus on improving pasture diversity and resiliency, on multi-species grazing to take advantage of the innate resistance of sheep and goats to fireweed's toxicity, and on ways to enable cattle to utilize fireweed.

**PUBLIC ABSTRACT**

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Utah State University, 2014

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Department: Wildland Resources

Fireweed (*Senecio madagascariensis*) is a noxious and invasive weed affecting pastures in Hawaii, Australia, and South America. Fireweed contains compounds called pyrrolizidine alkaloids that are toxic to most grazing mammals. Toxic effects to cattle include irreversible damage to liver cells, hardening of the liver, and loss of liver function, which may lead to jaundice, swelling, and the accumulation of fluids in the stomach and other physiological malfunctions. External effects include rough appearance, diarrhea, low energy and dullness, photosensitization, and abnormal behavior, many of which can lead to death. Fireweed also can reduce pasture productivity by as much as 30-40%, particularly in Hawaii. As a result of these adverse effects on cattle and pasture production, people are seeking ways to manage fireweed and cattle.

Condensed tannins, which are common in many forage legumes, bind with other molecules such as protein and alkaloids. Complementarities among secondary compounds such as condensed tannins and alkaloids can allow animals to consume more of plant material they would otherwise avoid due to toxicity; however, there is very little information on whether tannins actually protect animals from the toxic effect of

alkaloids like the ones found in fireweed. The effects of condensed tannins on the toxicity of fireweed to cattle were examined in two stages of a research project that included studies in the lab (*in vitro*) and in live animals (*in vivo*). The *in vitro* studies showed that tannins bound pyrrolizidine alkaloids in cattle rumen fluid and binding was highest when mixed with tannins at 8%, with some effect at 12% as well, by weight of fireweed. The *in vivo* studies were inconclusive as animals from both the Fireweed and Fireweed-Tannin group demonstrated ill-effects brought on by the fireweed. The data gathered in the form of blood tests and liver tests did not identify a clear protective effect provided by having tannin in the diet, but the results likely were affected by the way we conducted the research, which involved dosing animals daily with large amounts of plant material. The findings also illustrate how strongly cattle avoid eating fireweed while grazing on pasture. In summary, management to reduce fireweed effects on cattle should focus on improving pasture diversity and resiliency, on multi-species grazing to take advantage of the innate resistance of sheep and goats to fireweed's toxicity, and on ways to enable cattle to utilize fireweed.

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Carolyn L. Wong

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## INTRODUCTION

Plant secondary compounds, such as tannins, alkaloids, terpenes, and saponins, have traditionally been understood to be defenses of plants (Freeland and Janzen 1974). From the perspective of grazing mammals, these compounds can effectively limit intake of plants due to their negative physiological effects (Freeland and Janzen 1974; Kumar and Singh 1984; Molyneux et al. 1988; Provenza 1996; Pfister et al. 2001). Many *Senecio* species, for example, are considered toxic if consumed in too large amounts due to the production of pyrrolizidine alkaloids (PAs) (Cheeke 1988; Stegelmeier 2011). While the PAs themselves are not toxic, the highly reactive metabolites (pyrroles), formed when the PAs are absorbed and metabolized in liver cells, are toxic (Molyneux et al. 1988).

Once ingested, the PAs can follow various metabolic pathways including ester hydrolysis, conversion to *N*-oxides (both considered detoxification pathways), and dehydrogenation to pyrrolic derivatives (associated with cytotoxicity) (Mattocks 1986). Pyrroles are strong alkylating agents able to cross-link DNA thereby hindering cell division and protein synthesis (Cheeke 1988). The DNA cross-linking leads to the damaging effects of pyrroles in the liver as seen in the enlargement of hepatocytes, centrilobular necrosis, megalocytosis of parenchymal cells, karyomegaly, liver fibrosis, bile duct proliferation, veno-occlusion, and loss of liver metabolic function (Cheeke 1989). Subsequent signs of impaired liver function include hyperbilirubinemia, hypoalbuminemia, jaundice, edema, and ascites (Cheeke 1989), all of which can have far-reaching effects on other organ systems.

External signs of PA toxicity may include a rough appearance, diarrhea, prolapsed rectum, ascites, edema of digestive tract tissues, lassitude and dullness, photosensitization, and abnormal behavior (Cheeke 1989; Stegelmeier 2011). Signs of

chronic PA toxicity may not be immediately apparent, but a nutritional stress, such as pregnancy or the onset of lactation, may trigger signs of toxicity in animals that previously appeared to be normal (Stegelmeier 2011). Young animals exposed to PAs from other *Senecio* species have shown delayed hepatic responses that occurred months after exposure (Molyneux et al. 1988). As liver damage is progressive, death from PA toxicosis may occur months or even years later (Stegelmeier 2011). Analyses of serum enzyme concentrations are an effective method of detecting the presence of liver disease (Kahn 2010).

Many species of plants contain PAs, including *Senecio madagascariensis* Poir. (fireweed), which is an invasive species implicated in cattle poisoning in Hawaii, Australia, and South America as well as in Brazil (Cruz et al. 2010). Fireweed contains several specific PAs, all macrocyclic diesters of retronecine and otonecine bases (Gardner et al. 2006). The retronecine-type alkaloids are senecivernine, senecionine, integerrimine, mucronatinine, retrorsine, and usaramine. The onotnecine-types are senkirkine, otosenine, acetylsenkirkine, desacetyldoronine, florosenine and doronine (Gardner et al. 2006). In addition to being hepatotoxic, these specific PAs are also known to be genotoxic and tumorigenic (Fu et al. 2004). The average amount of total PAs collected in Hawaii ranges from 217  $\mu\text{g/g}$  – 1990  $\mu\text{g/g}$  (dry weight basis) and is comparable to PA content in fireweed collected in Australia (Gardner et al. 2006) where there are documented cases of livestock poisonings associated with fireweed (Seaman 1987).

Fireweed often invades disturbed or over-utilized rangelands. Once established, fireweed is difficult to manage because it is a prolific seeder with a high germination rate; it grows rapidly, flowers early, and exhibits a perennial habit in tropical areas (Thorne et al. 2005). Fireweed reduces pasture availability for livestock by an estimated 30-40% in Hawaii (Thorne et al. 2005); estimated losses of about \$2 million dollars annually occur

to the livestock industry in Australia (Watson et al. 1984). Chemical methods of controlling fireweed are generally costly and short-lived and hence only marginally effective. Multi-species grazing can be effective due to the higher tolerance of sheep and goats to PAs (Motooka et al. 2004). Sheep and goats can tolerate an intake of PAs of several times their body weight (Cheeke 1988). On the other hand, horses and cattle are quite susceptible, and over time eating just 5-10% of body weight in dry matter intake can be lethal (Cheeke 1988). Species differences are likely due to lack of PA absorption, degradation and detoxification in the rumen, lack of hepatic formation of pyrroles, and differences in abilities to conjugate and excrete PAs and pyrroles (Cheeke 1989). Multi-species grazing has not been a widely adopted method of control in Hawaii due to a variety of factors including the costs associated with infrastructure improvements to manage these smaller herbivores.

As fireweed is toxic to cattle (Gardner et al. 2006; Cruz et al. 2010), there is considerable interest in exploring alternative ways to avoid negative impacts to pasture productivity and animal health. One alternative is to explore complementarities, or beneficial interactions that effectively minimize negative effects, among plant species with different secondary compounds. These complementarities may enable herbivores to overcome the negative effect of toxins such as PAs. Due to the variation in the types and concentrations of various secondary compounds in plant populations, animals can either learn to avoid them or develop strategies for eating them in ways that negate their harmful effects (Freeland and Janzen 1974; Provenza et al. 2003; Villalba et al. 2004; Meuret and Provenza 2014). Intake of forages with intake-limiting compounds, such as alkaloids, may increase in the presence of tannins in the diet (Lyman et al. 2008, 2011). Lambs can discriminate among forages and selectively consume more of plants high in alkaloids, such as endophyte-infected tall fescue, when fed high-tannin diets (Lisonbee et al. 2009; Villalba et al. 2011; Owens et al. 2012a, 2012b).

At high concentrations condensed tannins, which are common in most shrubs as well as in many forage legumes, can reduce forage intake and animal performance (Kumar and Singh 1984). Negative impacts vary depending on the species of herbivore, rumen microbial populations, quantity and type of tannin ingested and the primary compound characteristics of the diet (Kumar and Singh 1984; McSweeney et al. 2001; Provenza et al. 2003). On the other hand, condensed tannins can also have beneficial effects by reducing internal parasite loads (Min and Hart 2003) and bloat (Waghorn 1990), as well as enhancing by-pass protein effects (Barry et al. 2001), and increasing intake of foods high in terpenes (Mote et al. 2007) and alkaloids (Lyman et al. 2008, 2011). While research on complementarities has demonstrated increased intake and diet preference mediated by tannins, information is lacking on protective effect of these complementary interactions against toxicity.

The objectives of the research were to examine the effects of condensed tannins on the toxicity of fireweed to cattle. To do so, the study determined 1) if tannins bound pyrrolizidine alkaloids in cattle rumen fluid *in vitro* and if so at what tannin concentrations binding was highest, and 2) if tannins reduce toxicity of PAs in cattle *in vivo* as measured by various physiological responses and non-quantified observations of behavioral responses to treatments.



## MATERIALS AND METHODS

The objective of the *in vitro* experiment was to assess the effectiveness of three levels of condensed tannin found in the South-American quebracho tree (*Aspidosperma quebracho*) for binding with PAs, at levels found in fireweed (1447 µg/g), in cattle rumen fluid. Determining whether or not the tannins and PAs bind in rumen fluid *in vitro*, and if so at what level they bind the most PAs, would guide the *in vivo* work to follow.

### ***In vitro* Experiment**

Cattle rumen fluid was collected from a cannulated mature cow maintained on an all-grass hay diet at the USDA – ARS Poisonous Plants Research Laboratory in Logan, UT. Collection procedures were approved by the Utah State University Institutional Animal Care and Use Committee (IACUC #1513). The rumen fluid was mixed with artificial saliva (McDougall's; Tilley and Terry 1963) to produce a 1:4 rumen fluid-to-saliva solution.

All fireweed, which was collected in North Kohala on the Big Island of Hawaii in January 2009, was dried in an oven (70 degrees C) for 3 d and then ground. Fireweed was stored in a climate controlled environment at the PPRL until January 2010 when the *in vitro* research was conducted. The PA concentration of the plant material used in the *in vitro* study was 1,447 µg/g; fireweed collected in Hawaii generally has PA concentrations that range from 217 – 1990 µg/g (Gardner et al. 2006). Fireweed analysis was conducted just prior to the trial and was comparable to that in freshly collected samples.

The commercially available quebracho tannin is a complex of condensed tannins, flavonoids, and other phenolic compounds (Mole and Waterman, 1987). The quebracho tannin used contained approximately 85% tannin (Titus and Provenza unpublished results), and was obtained from Tannin Corporation, Peabody, Massachusetts, USA.

### ***Experimental design***

Forty-eight individual plastic rumen fermentation tubes were randomly assigned to one of four treatments: 0% tannin (control), 4% tannin, 8% tannin, and 12% tannin per weight of fireweed. Each tube received 0.5 g of ground fireweed and the appropriate amount of tannin; 50 ml of rumen solution was added and the head space of each tube was filled with CO<sub>2</sub> and the replicated samples were allowed to ferment in a water bath at 40° C. Five ml aliquots were collected at 0.5, 1, 2, 4, 6 and 24 h elapsed time intervals (n=2). Aliquots were centrifuged and the decanted solution was frozen to stop microbial activity.

To quantitatively measure the amount of tannin-PA binding, the thawed solutions were first filtered through a 0.2 µm filter and then filtered through a 1 kDa Millipore Ultrafiltration filter to remove all compounds of molecular weight greater than 1000 Da. Quebracho tannin is estimated to weigh about 1200 Da, and the MW of the PAs measured ranged from 300-500 Da. Therefore any unbound PA should have passed through the ultra-filter, thereby indicating the amount of tannin-PA binding occurring *in vitro*. The unbound PA in the resulting filtrate was then removed via solid-phase extraction using a Strata X-C polymeric strong cation column (60 mg/3 ml). The columns were pretreated by washing first with methanol and then with deionized water. A measured aliquot of the samples was then added to the columns and vacuum applied to pull the solution through the columns slowly. Columns were then rinsed first with DI water and then with methanol. The PAs were extracted from the columns with ammoniated methanol (10% of an ammonia saturated methanol solution) and the extract dried under a stream of nitrogen at 60°C. The residue was dissolved in 1.0 ml 50% methanol (containing 50 µg/mL atropine) and analyzed via LC/MS (Karam et al. 2011). The LC/MS measured levels of nine specific PAs.

### **Statistical Analyses**

The statistical design for the analysis of variance was a completely randomized two-way factorial. Analyses were run on total PAs and on individual PAs. Effect of tannin treatment, as well as the effect of time and interaction of treatment x time were considered. A homogenous variance model was used for total PAs, which satisfied the assumptions for normality, though there was a slight indication of non-constant variance, likely due to having only two replicates of each sub-sample.

### ***In vivo* Experiment**

The objectives of the *in vivo* experiment were to measure whether the interaction of tannins and PAs provided a protective benefit to cattle by assessing physiological responses detectable in blood serum during the study and observing animal performance and behavior for 4 mo afterward. The procedures were approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC #10-999) and done under veterinary supervision.

### **Experimental design**

Twenty Angus steers, from Haleakala Ranch, Maui, Hawaii (N 20° 49' 56.8626", -156° 18' 57.3588"), weighing 208-217 kg, were randomly assigned to one of four treatment groups: control, fireweed only, tannin only, or fireweed + tannin. The animals were penned at Haleakala Ranch and fed Timothy grass hay for 3 d prior to beginning the feeding trial. Once the trial commenced, all animals were drenched each morning for 30 d (treatment phase) as follows: the control group received ground grass hay (750 g); the fireweed group received ground fireweed that provided 7.5 mg PA/kg body weight (600-1000 g); the tannin group received only a water drench containing a solution of 8% by weight of tannin (per the amount of fireweed provided to the other two groups, but without any plant material); and the fireweed-tannin group received fireweed at 7.5 mg

PA/kg body weight and tannin at 8% of the weight of the fireweed (600-1000 g). The ground plant material and/or tannin were mixed with 8-12 l of water to create a slurry that was administered to the cattle via oral drench. The fireweed for the trial was collected, dried and analyzed in the same manner as the fireweed used in the *in vitro* trial. The quebracho tannin was also obtained from the same source. The basal diet for all animals was Timothy grass hay fed every morning after treatments were administered. Each group initially was given about half a bale of hay each day, but that amount was reduced to about a quarter of a bale each day as the cattle were not eating it all and affecting the administration of treatments the next day if their rumens were full.

Blood serum analyses are useful for evaluating several physiological functions that may indicate the effect of pyrrolizidine alkaloids on animals (Table 1). Blood samples were collected from each animal every 2 wk throughout the treatment phase (wk 1-4) and throughout the monitoring phase (wk 5-21) for a total of 21 wk. Senecio-induced hepatic injury may be initiated shortly following ingestion but measurable physiological damage may take some time to manifest (Molyneux et al. 1988). One blood serum sample for each animal at baseline (pretreatment) and one sample from each month (March, April, May, June, July and August) was analyzed for sodium, potassium, chloride, bicarbonate, Anion gap, glucose, blood urea nitrogen (BUN), creatinine, calcium, magnesium, phosphorus, total protein, albumin, total cholesterol, total bilirubin, alkaline phosphatase (AP), alanine aminotransferase (ALT(SGPT)) and aspartate aminotransferase (AST(SGOT)) (Table 1).

Table 1. Serum variables measured in study animals with reference ranges and potential implications of changes as affected by toxicity in animals:

Variable	Reference Range <sup>a</sup>	Units	Implication
<b>Albumin</b>	2.5-3.8	g/dL	Hypoalbuminemia or low serum albumin is an indicator of chronic liver disease due to decreased functional hepatic parenchyma <sup>a</sup> .
<b>Alanine Aminotransferase (ALT)</b>	6.9-35	μ/L	Elevated levels are considered a direct indicator of damage to liver cells <sup>b</sup> .
<b>Alkaline Phosphatase</b>	18-153	μ/L	Elevated levels may implicate acute and chronic liver disease <sup>c</sup> .
<b>Anion Gap</b>	Not available	mmol/L	High anion gap may indicate titration acidosis, alkalemia, dehydration. Low anion gap may indicate acidemia, decreased albumin. <sup>e</sup>
<b>Aspartate Aminotransferase (AST)</b>	60-125	μ/L	Used to assess hepatic dysfunction and disease <sup>a</sup> Non-specific. Other sources besides the liver, such as skeletal muscle, cardiac muscle, and kidneys can also contribute to high concentrations of AST
<b>Bicarbonate</b>	20-30	mmol/L	Increases with metabolic alkalosis and decreases with metabolic acidosis <sup>a</sup> .
<b>Bilirubin</b>	0-1.6	mg/dL	Used to assess hepatic dysfunction and disease <sup>a</sup> Elevated levels of bilirubin may indicate impaired liver function <sup>b</sup> or hemolytic crises <sup>a</sup> where red blood cells are being destroyed too quickly.
<b>Blood Urea Nitrogen</b>	10-25	mg/dL	Low levels are indicative of decreased hepatic function <sup>a</sup> . High levels are indicative of kidney failure.
<b>Calcium</b>	8-11.4	mg/dL	Increases due to dehydration. Decreases due to hypoalbuminemia <sup>a</sup> .
<b>Chloride</b>	99-107	mmol/L	Linked to levels of sodium <sup>a</sup> .
<b>Cholesterol</b>	62-193	mg/dL	Increases due to hepatic or biliary disease <sup>a</sup> . Decreases in severe cases of liver dysfunction <sup>a</sup> .
<b>Creatinine</b>	0.5-2.2	mg/dL	Elevated creatinine levels may indicate renal dysfunction <sup>a</sup> .
<b>Glucose</b>	40-100	mg/dL	Low levels are indicative of decreased hepatic function <sup>a</sup> .
<b>Magnesium</b>	1.5-2.9	mg/dL	Magnesium decreases are usually due to a dietary deficiency and may be affected by diarrhea <sup>a</sup> .
<b>Phosphorus</b>	5.6-8.0	mg/dL	Phosphate may also increase due to renal failure or decrease with stress <sup>a</sup> .
<b>Potassium</b>	3.6-4.9	mmol/L	May increase with renal failure. Decrease

			may be due to chronic renal dysfunction, vomiting or diarrhea
<b>Protein, total</b>	6.7-7.5	g/dL	Increases due to dehydration, chronic inflammation and paraproteinemia. Decreases due to over-hydration, severe congestive heart failure and other conditons <sup>a</sup> .
<b>Sodium</b>	136-144	mmol/L	Sodium increases with dehydration and restricted water intake and decreases with the loss of sodium source. Ascites, one of the clinical signs of hepatic disease <sup>a</sup> is usually preceded by increased sodium retention by the kidneys <sup>b</sup> .
<sup>a</sup> Kahn 2010; <sup>b</sup> Lawhead & Baker 2009; <sup>c</sup> Tennant & Center 2008; <sup>d</sup> Turk and Casteel 2008; <sup>e</sup> French et al. 2014.			

Liver biopsies also were done at the beginning of the trial, at the end of the 30-d feeding trial, and every 30 d until the end of the 21 wk. Percutaneous biopsies were collected using a 14 gauge Trucut biopsy needle following previously described techniques (Rogers et al. 2001). A 5 cm section of skin about 1/3 down the thoracic cavity over the 12th left rib was shaved and surgically prepped. A small area in the 12th intercostal space was anesthetized using 2 ml of lidocaine and a 2-3 mm incision was made using a scalpel. Then a 14 gauge "Trucut" biopsy needle was directed anteriorly and medially (towards the right olecranon). The biopsy was taken about 3 to 4 cm deep, as determined by the tactile feel as the needle was inserted. After biopsy the skin was closed with a suture. Subsequent biopsies were directed slightly ventral or anterior to the initial site to avoid sampling previously damaged tissues (Rogers et al. 2001). All samples were immediately fixed in 10% neutral buffered formalin and then analyzed at the USDA ARS Poisonous Plants Research Lab for signs of fat deposition, biliary hyperplasia, and necrosis of hepatocytes and/or fibrosis. Hepatic disease can be diagnosed via percutaneous liver biopsy; though generally qualitative, such evaluations provide information that may indicate disease etiology and severity (Kahn 2010).

***Statistical Analyses***

Analyses of variance were conducted on all serum variables using a mixed linear model with random (animals) and fixed (treatment) factors. The statistical design was a completely randomized factorial with repeated measures. The models satisfied the assumptions for normality. Mean comparisons were made using the probability of difference procedure (pdiff) in SAS, and least significant differences (LSD) were calculated from the least square means and SEM when significant interactions occurred (Piepho 2012).

## RESULTS

### *In vitro* Experiment

Treatment and time affected the outcome ( $P < 0.0001$ ). The 8% treatment and elapsed times 6 and 24 h had less free PAs than all of the other treatments and times (Figures 1, 2, and 3).

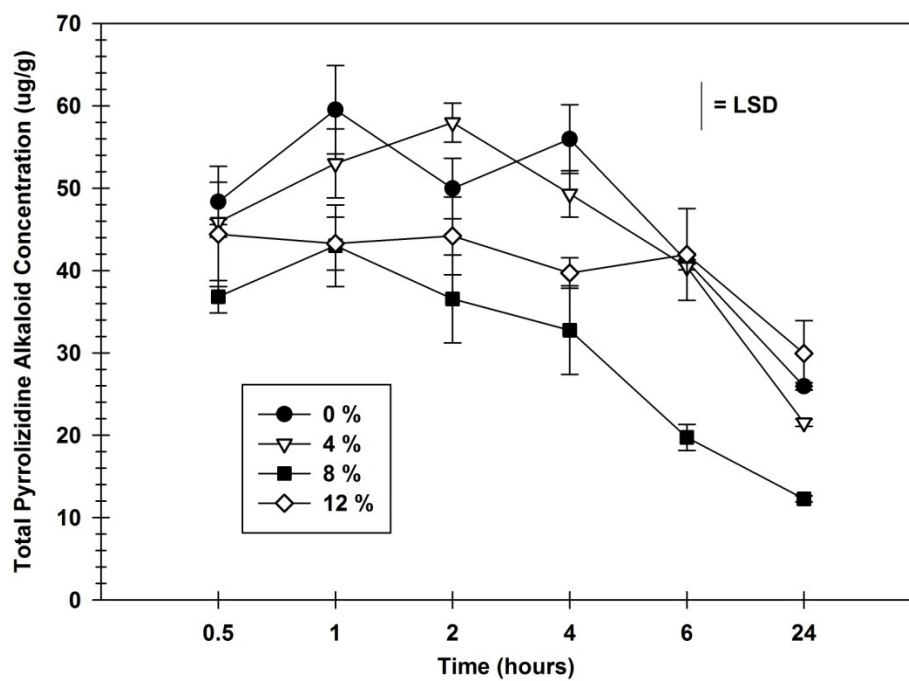


Figure 1. Total PAs among four treatments over time (treatment x time interaction  $P < 0.09$ ).



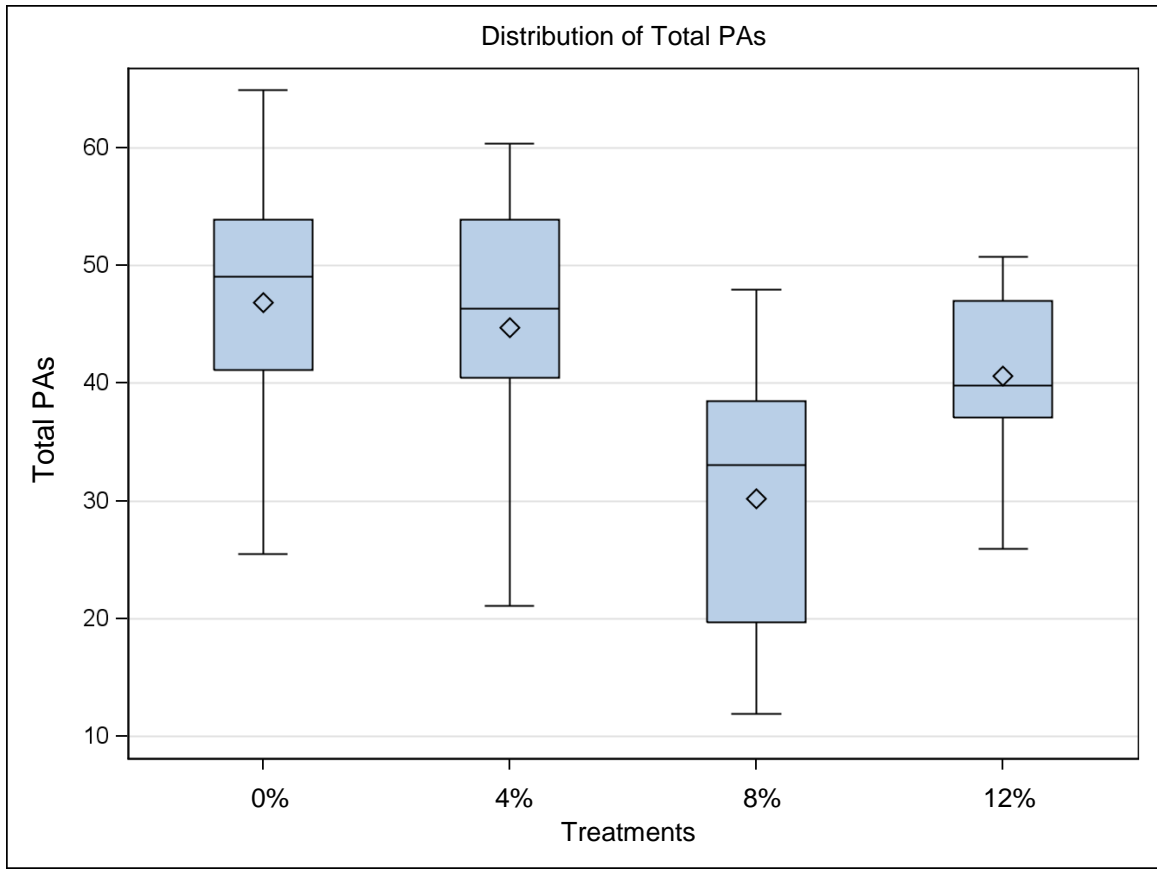


Figure 2. Distribution of PAs by treatment. The 8% treatment had significantly less total PAs than the other three treatments.

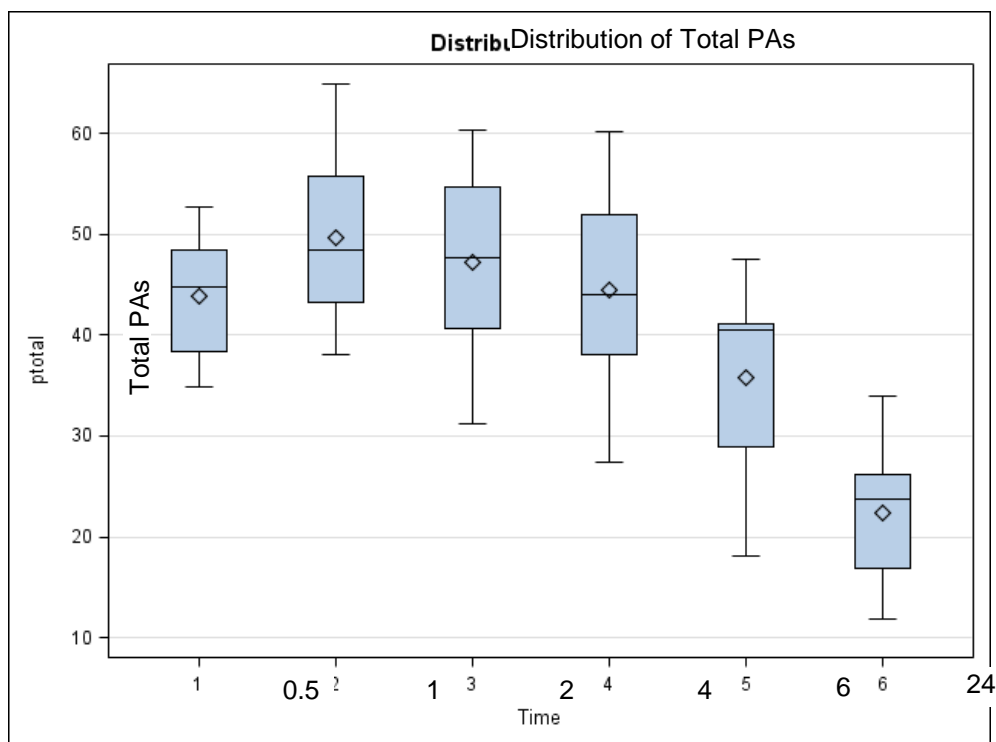


Figure 3. Distribution of total PAs by time (elapsed hours). Times 6 and 24 had significantly less total PAs than the other four times.

### ***In vivo* Experiment**

#### ***Liver Biopsies***

Liver biopsies were done at the beginning of the trial, at the end of the 30-d feeding trial, and every 30 d until the end of the 21 wk. Liver tissues were examined for potential lesions by Dr. B. Stegelmeier, DVM PhD pathologist, who has extensive experience with PA-treated animals. All liver biopsies showed minimal hepatocellular swelling with occasional small microgranulomas in all calves (Figure 4). These granulomas, composed of macrophages with smaller numbers of degenerative hepatocytes and eosinophils, were randomly distributed throughout the liver.

The livers of the animals that died also were examined: two animals from the Fireweed-only group died during the treatment phase of the study, Steer 1 on day 28 and Steer 2 on day 29; a steer (3) from the Fireweed-Tannin group died on day 42 and a third animal (steer 4) from the Fireweed-only group died on day 43 during the monitoring phase of the study. Their livers had similar diffuse hepatocellular swelling, as well as increased numbers of neutrophils and mono-nuclear inflammatory cells in the sinusoids. In some areas the centrilobular hepatocytes were more severely swollen with small areas of necrosis characterized by nuclear pyknosis and disruption (Figure 5). Some of the adjacent hepatic cords were disrupted with focal hepatocyte individualization. Increased numbers of sinusoidal leukocytes were also present and there were multifocal areas of endothelial cell swelling.

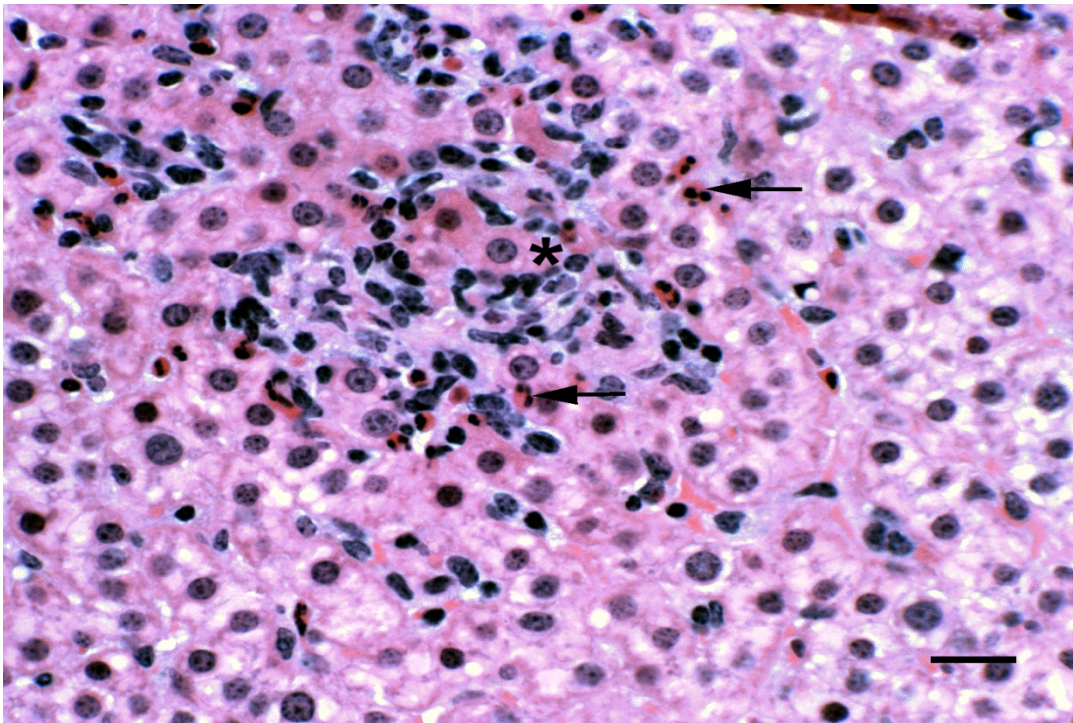


Figure 4. Photomicrograph of the liver of a calf treated with fireweed (7.5 mg total PA/kg BW) and tannin on the initial day of treatment. Notice the small granuloma (\*) with minimal eosinophilic inflammation (arrows). Hematoxylin and Eosin stain bar=30  $\mu$ m.

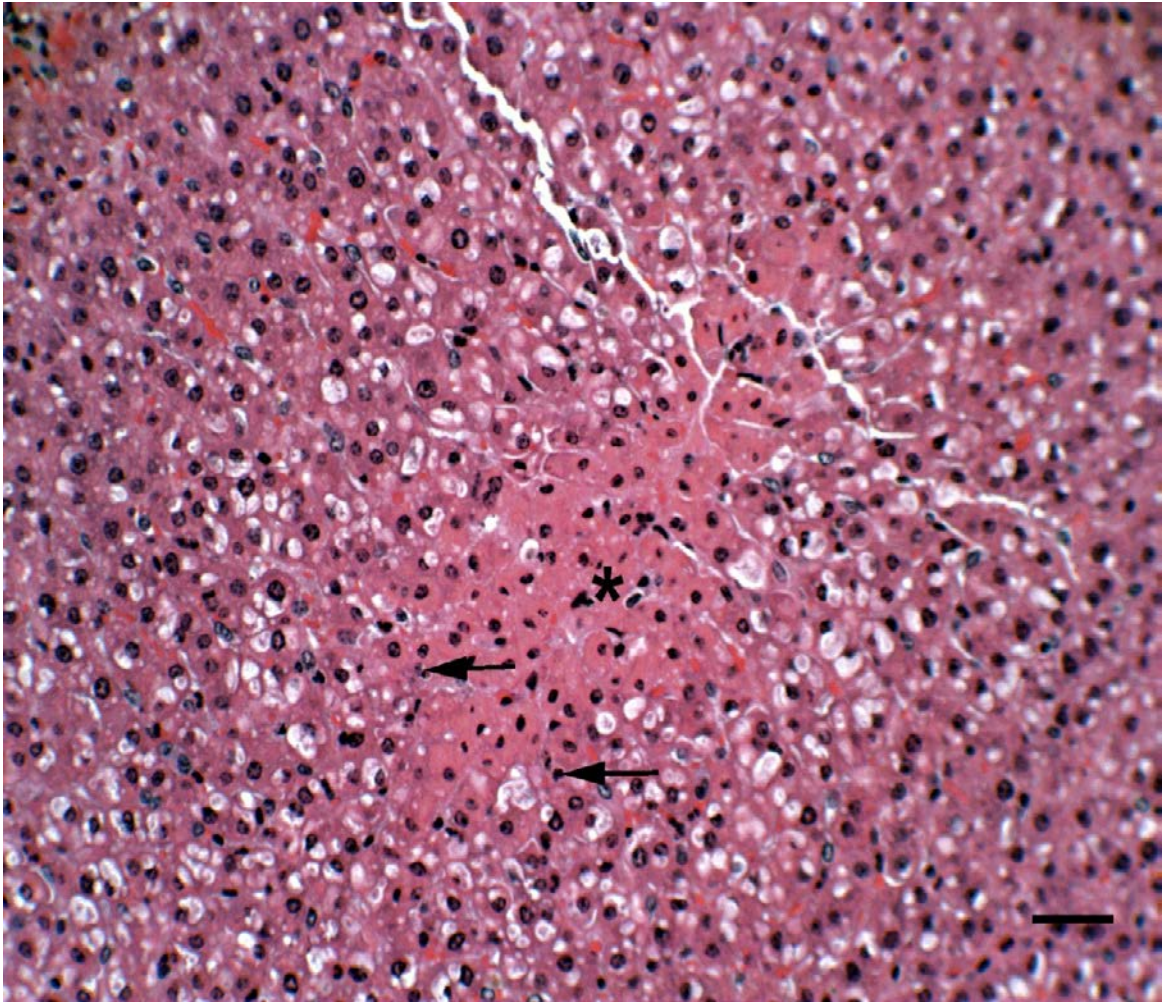


Figure 5. Photomicrograph of the liver of a calf treated with fireweed (7.5 mg total PA/kg BW) that died after 28 d of treatment. Notice the centrilobular hepatocyte degeneration and necrosis (\*) with increased numbers of leukocytes in many sinusoids (arrows). Hematoxylin and Eosin bar=30  $\mu$ m.

### ***Blood Analyses***

**Alanine Aminotransferase (ALT).** ALT is an enzyme found primarily in liver cells. ALT levels typically range from 6.9-35  $\mu$ /L; elevated levels of ALT are considered an indicator of damage to liver cells (Lawhead and Baker, 2009). In the first two



measurements, taken during the treatment phase of the trial, ALT levels were elevated and higher for control, fireweed, and fireweed+tannin than for the Tannin group; once the treatments stopped, there were no differences among groups (treatment x date  $P < 0.0001$ ; Figure 6; Appendix, Table 2).

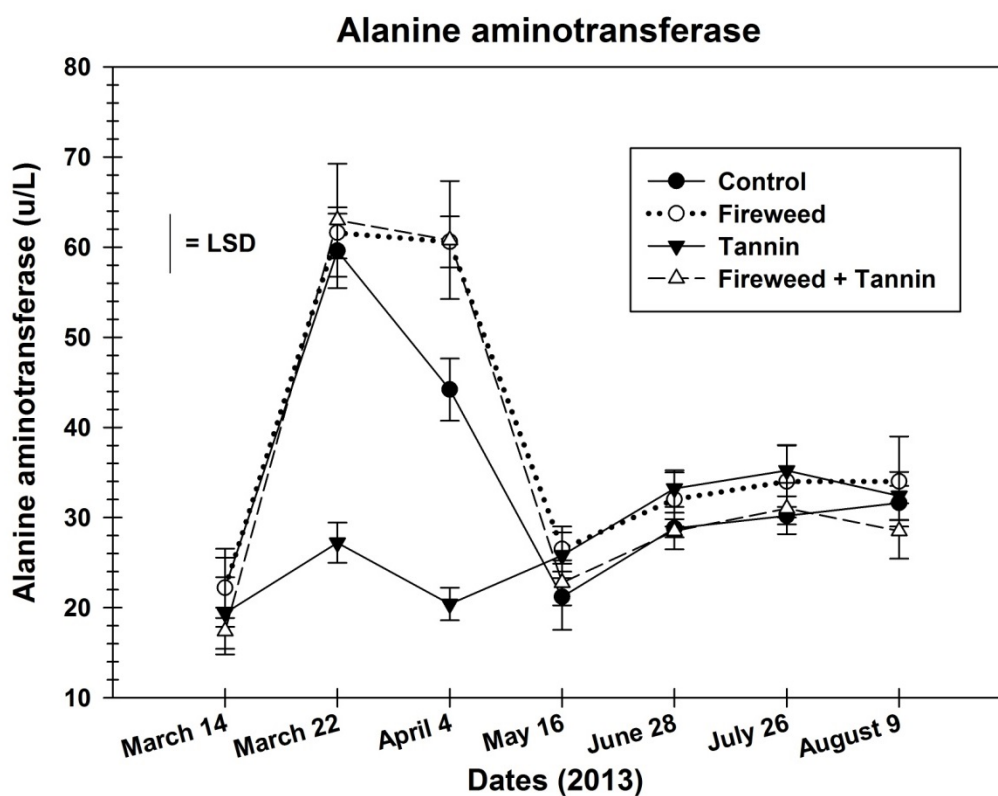


Figure 6. ALT Treatment by Date interaction.

**Anion Gap.** The anion gap is the difference between the concentrations of cations (sodium and potassium) and anions (chloride and bicarbonate) in blood serum (Kraut and Madias 2007). It is considered useful in evaluating acid-base disorders (Kraut and Madias 2007) and in diagnosing metabolic acidosis (Ewaschuk et al. 2003), which affects immune function (Kellum et al. 2004). During the treatment phase of the trial, the anion gaps for the control, fireweed and fireweed-tannin groups was lower than

for the tannin group (treatment x date  $P < 0.0001$ ; Figure 7; Appendix, Table 3). A low anion gap is frequently caused by hypoalbuminemia. Albumin is a negatively charged protein and its loss from the serum results in retention of other negatively charged ions such as chloride and bicarbonate. After the treatment phase, the levels of those three groups rose again and there were no difference between the treatment groups from May through August.

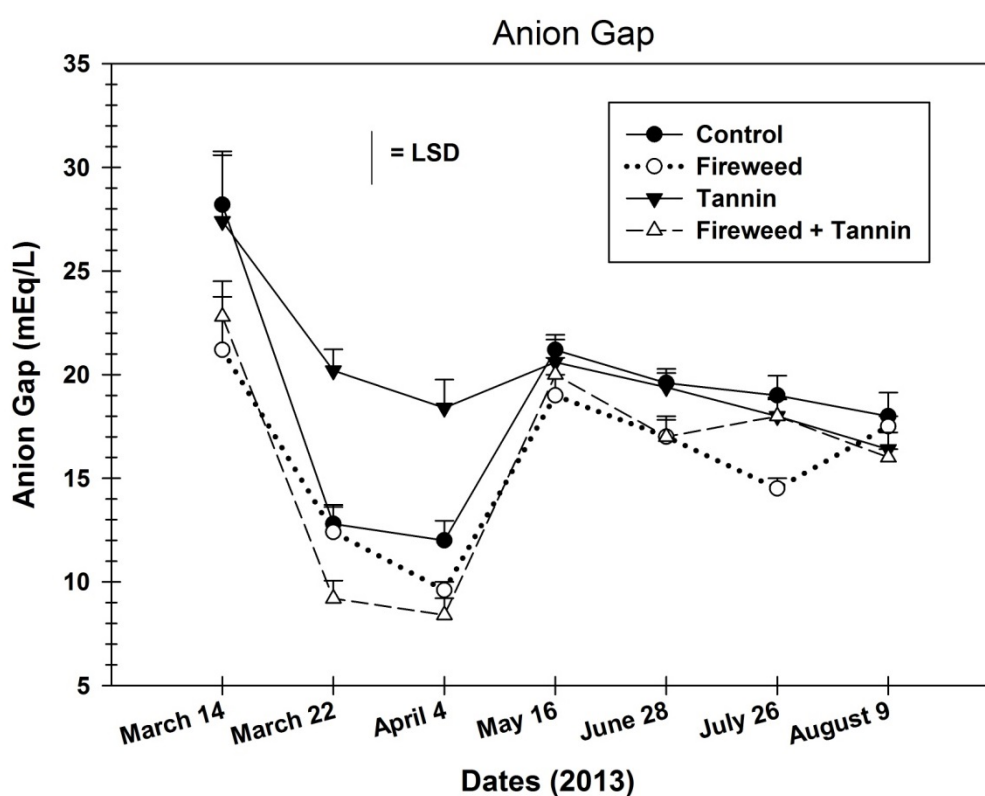


Figure 7. Anion gap Treatment by Date interaction.

**Albumin.** Hypoalbuminemia or low serum albumin is an indicator of chronic liver disease due to decreased function of hepatic parenchyma cells (Kahn 2010). Albumin levels for the fireweed and fireweed tannin groups were lower ( $P = 0.0193$ ; Figure 8) than the control and tannin groups during the beginning of the treatment phase of the trial.

There was no difference between groups during the monitoring phase and all measurements were within the normal range (2.5-3.8 g/dL) (Appendix, Table 4).

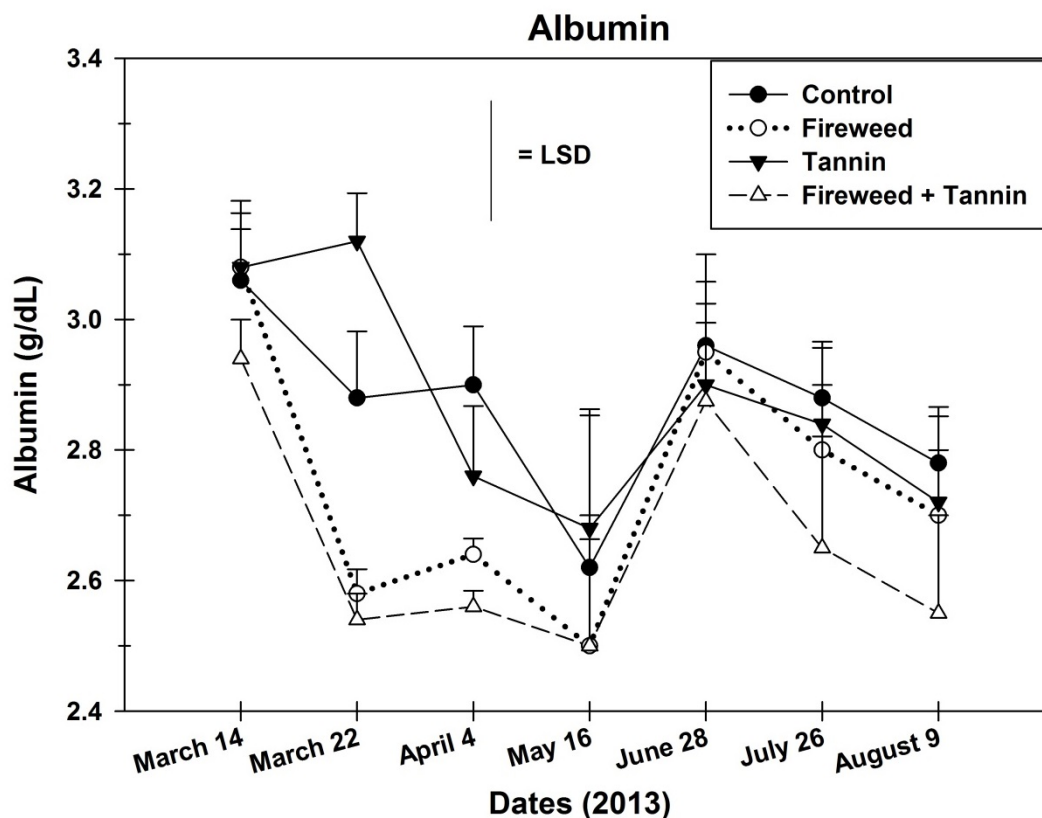


Figure 8 Albumin Treatment by Date interaction.

**Sodium and Chloride.** Sodium increases with dehydration and restricted water intake and decreases with low sodium intake. Chloride levels generally change in parallel with sodium (Kahn 2010). Ascites, one of the clinical signs of hepatic disease (Kahn 2010), is usually preceded by increased sodium retention by the kidneys (Tennant and Center 2008). There were no effects on sodium due to treatment ( $P=0.8634$ ) or treatment x date ( $P=0.6202$ ), but there was an effect due to date ( $P<0.0001$ ). Sodium levels were high and outside the reference range of 136-144 mmol/L in the first

measurement and low in April (Appendix, Table 5). Similarly, chloride was not effected by treatment ( $P = 0.0856$ ) or treatment x date interaction ( $P=0.3253$ ), but there was an effect of date ( $P<0.001$ ). Chloride levels were below the reference range (99-107 mmol/L) in April, May, June and July (Appendix, Table 6).

**Calcium.** Calcium levels increase due to dehydration or may decrease due to hypoalbuminemia (Kahn 2010). There was no effect on Ca due to treatment ( $P = 0.8140$ ) or treatment x date ( $p=0.5372$ ). There was an effect of date ( $P<0.0001$ ). Calcium levels were within the reference range (8-11.4 mg/dL) during the study and were lowest in April and highest in June and July (Appendix Table 7).

**Alkaline Phosphatase.** Acute and chronic liver disease may be implicated by elevated levels of serum AP, which is found in the membranes of liver cells and believed to have a role in membrane transport (Tennant and Center 2008). High levels of AP also occur in the cells lining the biliary ducts and elevated levels of AP in serum may indicate obstruction (Lawhead and Baker 2009). There was no effect on AP due to treatment ( $P = 0.3015$ ) or treatment x date ( $p=0.9935$ ). There was an effect of date ( $P=0.0199$ ); AP levels were within the normal range (18-153  $\mu$ /L) throughout the trial and lowest in April (Appendix, Table 8).

**Aspartate Aminotransferase.** Sequential measurements of AST are used to assess hepatic dysfunction and disease (Kahn 2010). While AST is a sensitive indicator for liver disease, it is also non-specific as other sources besides the liver, such as skeletal and cardiac muscles and kidneys, can also contribute to high concentrations of AST (Kahn 2010). There was no effect on AST due to treatment ( $P = 0.0482$ ) but there was a treatment x date interaction ( $P<0.0001$ , Appendix, Table 9). AST level in the Fireweed-Tannin group was higher than all other groups and outside the normal range (60-125  $\mu$ /L) on March 22. The Fireweed-only group was also higher than the Tannin and Control groups on March 22; AST level in the Tannin group dropped below the



normal range and was lower than the other three groups in April, but by May the levels of all three groups were similar and within the normal range.

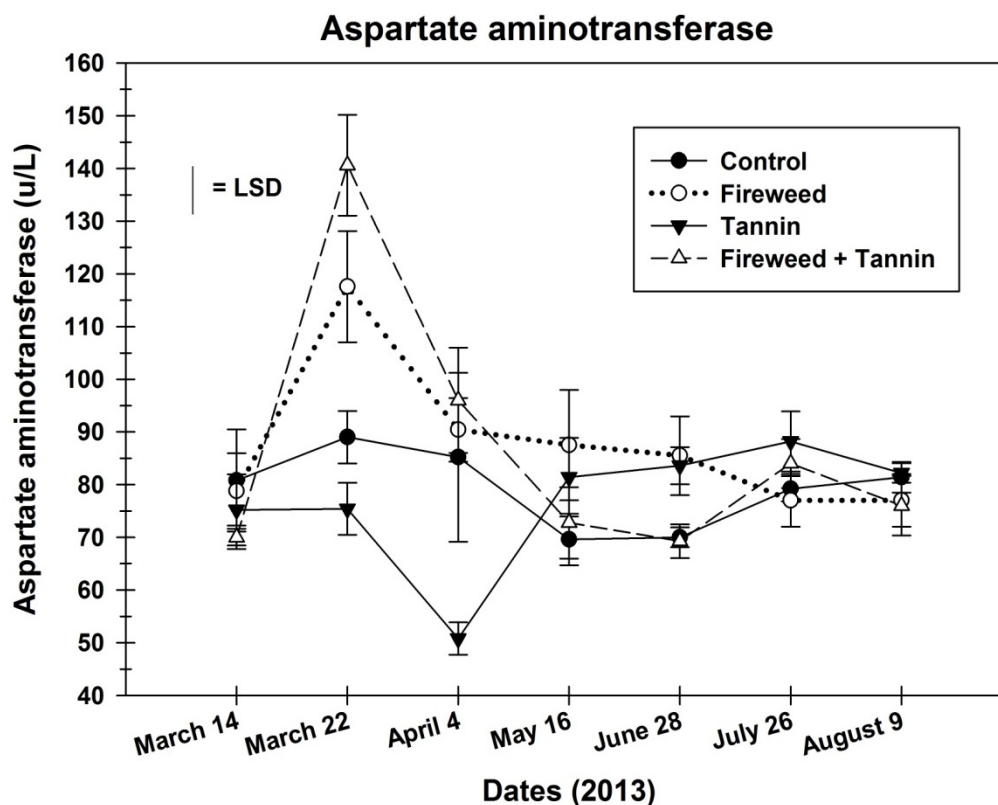


Figure 9. Aspartate Aminotransferase (AST) Treatment by Date interaction.

**Bilirubin.** Elevated levels of bilirubin may indicate impaired liver function (Lawhead and Baker 2009) or hemolytic crises (Kahn 2010) where red blood cells are being destroyed too quickly. The bilirubin levels for the Control, Fireweed, and Fireweed-Tannin groups were higher than for the Tannin group during the treatment phase, but by May, and for the rest of the study, there was no difference among groups (treatment x date  $P < 0.0001$ ; Figure 9; Appendix, Table 10) and all measurements were within the normal range (0-1.6 mg/dL).

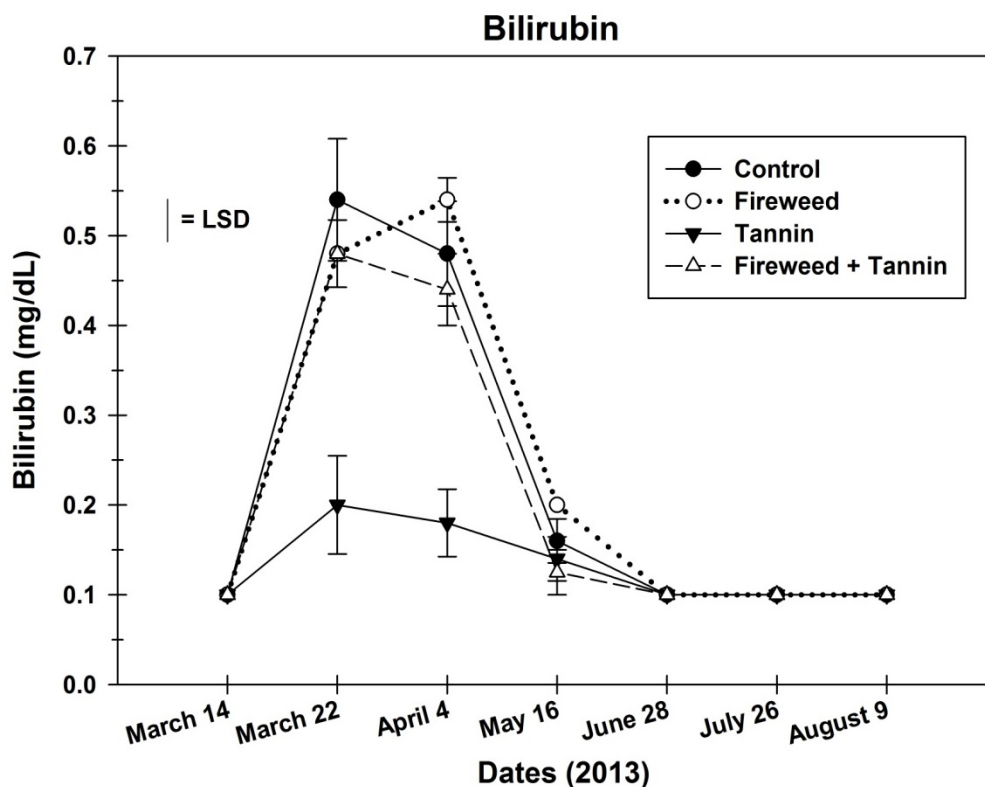


Figure 10 Bilirubin Treatment by Date interaction.

**Blood Urea Nitrogen.** Among other things, decreased BUN concentrations can indicate decreased hepatic function (Kahn 2010). There were no effects on BUN due to treatment ( $P = 0.6286$ ). There was an effect of treatment x date ( $P=0.0542$ , Figure 11). BUN levels in the two tannin groups were lower than the Fireweed-only and control and below normal (10-25 mg/dL) in March and April. In May, June, July, and August the Fireweed-only and Control BUN levels dropped to less than 10 while the two Tannin groups were within the normal range (Appendix, Table 11).

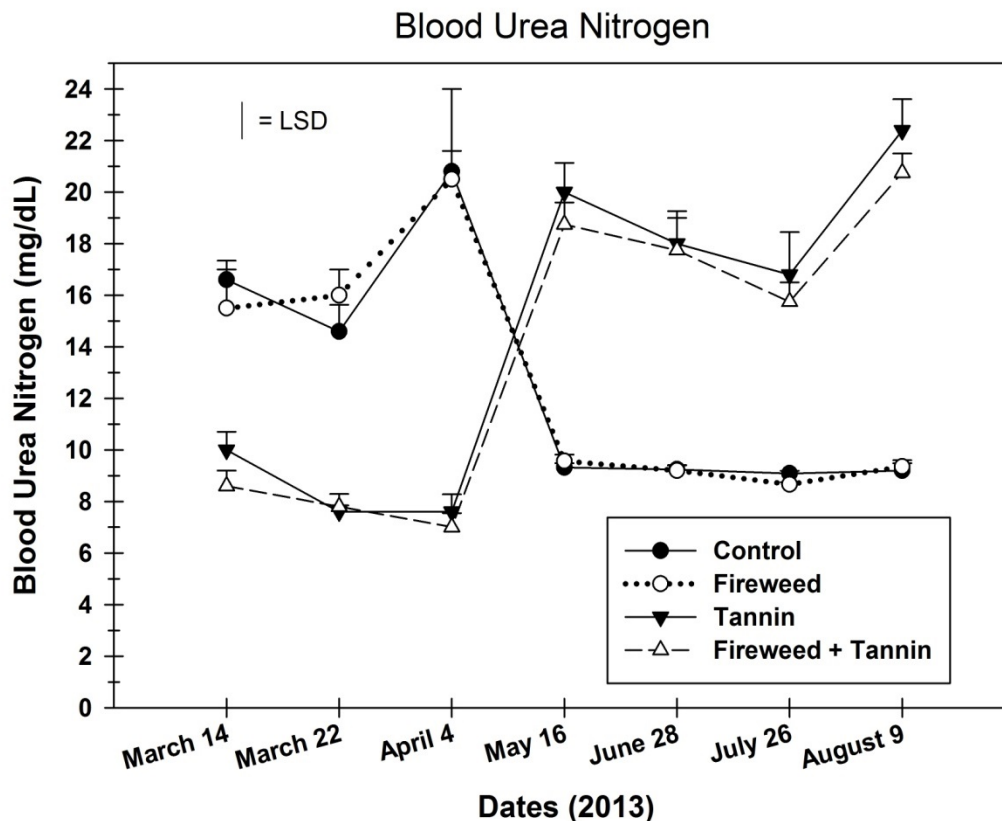


Figure 11 BUN Treatment by Date interaction.

**Cholesterol.** Increases in cholesterol levels may implicate hepatic or biliary disease, while decreases may indicate severe liver dysfunction (Kahn 2010). There were no effects due to treatment ( $P = 0.4902$ ) or interaction between treatment and date ( $P=0.7766$ ). There was an effect of date ( $P<0.0001$ ; Appendix, Table 12) but all levels were within the normal range (62-193 mg/dL).

**Bicarbonate (CO<sub>2</sub>).** High or low levels of bicarbonate can indicate metabolic alkalosis or metabolic acidosis, respectively. Levels of CO<sub>2</sub> were lower for the tannin group during the treatment phase of the study, and higher in the fireweed group in July. There were no differences between groups during May, June, and August (treatment x

date  $P=0.0002$ ; Figure 12; Appendix, Table 13) and levels were mostly within the reference range of 20-30 mmol/L.

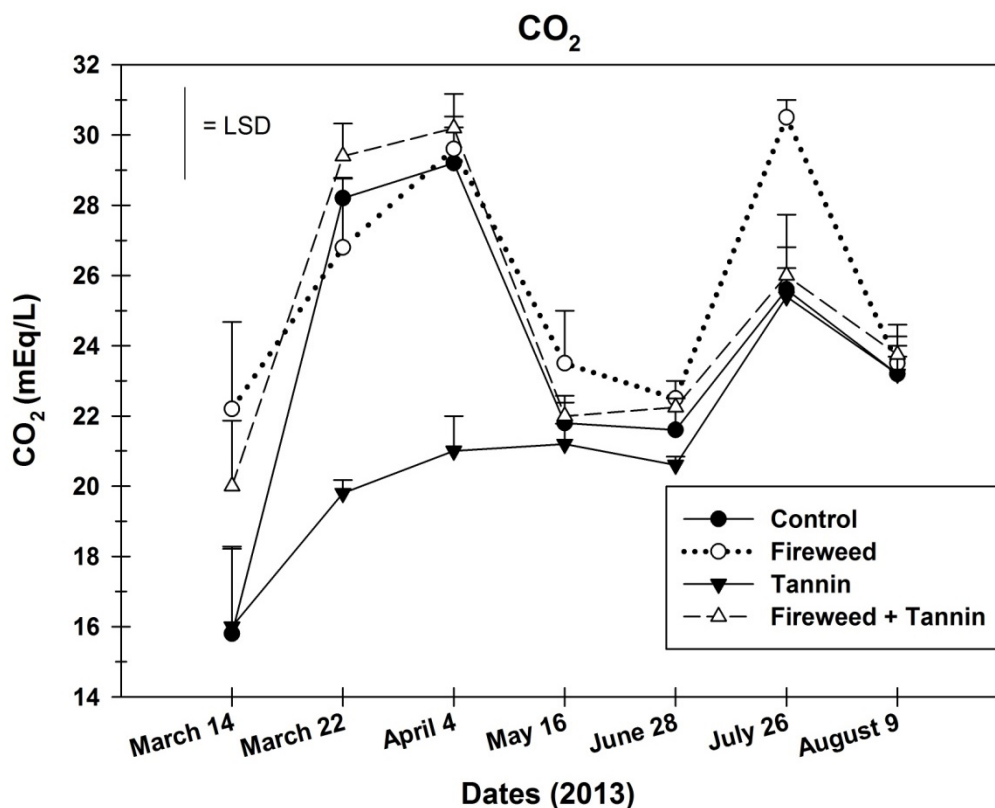


Figure 12 CO<sub>2</sub> Treatment by Date interaction.

**Creatinine.** Elevated creatinine levels may indicate renal dysfunction (Kahn 2010). Creatinine levels were lower for the Fireweed-Tannin and Control groups than for the other two groups at the beginning of the study. The Fireweed-Tannin group was lower than the Control in March; the Fireweed group's creatinine levels rose and were higher than the other three groups in July and were higher than for the Fireweed-Tannin group in August (treatment x date  $P=0.0012$ ; Figure 13; Appendix, Table 14). All measurements were within the normal range (0.5-2.2 mg/dL).

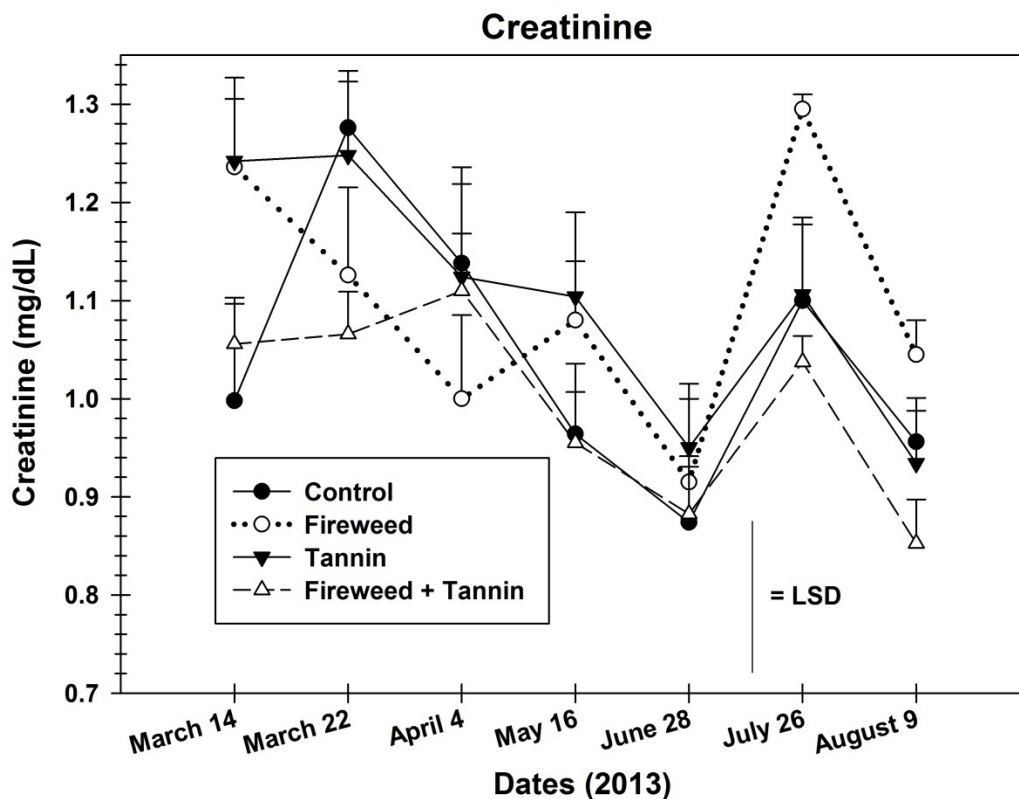


Figure 13. Creatinine Treatment by Date interaction.

**Glucose.** Low glucose concentrations can indicate decreased hepatic function (Kahn 2010). There were no effects on glucose due to treatment ( $P=0.6611$ ) or treatment x date ( $P=0.7116$ ), but glucose levels were higher for the baseline measurement at the beginning of March than for all other dates and outside the normal range (40-100 mg/dL) ( $P<0.0001$ ; Appendix, Table 15).

**Magnesium (Mg).** Decreases in Mg are usually due to a dietary deficiency, which can be caused by diarrhea (Kahn 2010). The Control group had a higher level of Mg than the other three groups at the beginning of the trial; Mg levels were similar among all groups through the treatment phase. The Fireweed group's Mg level was higher than the other three groups in July, but levels were similar otherwise (treatment x

date  $P=0.0325$ ; Figure 14, Appendix, Table 16), and within the reference range of 1.5-2.9 mg/dL.

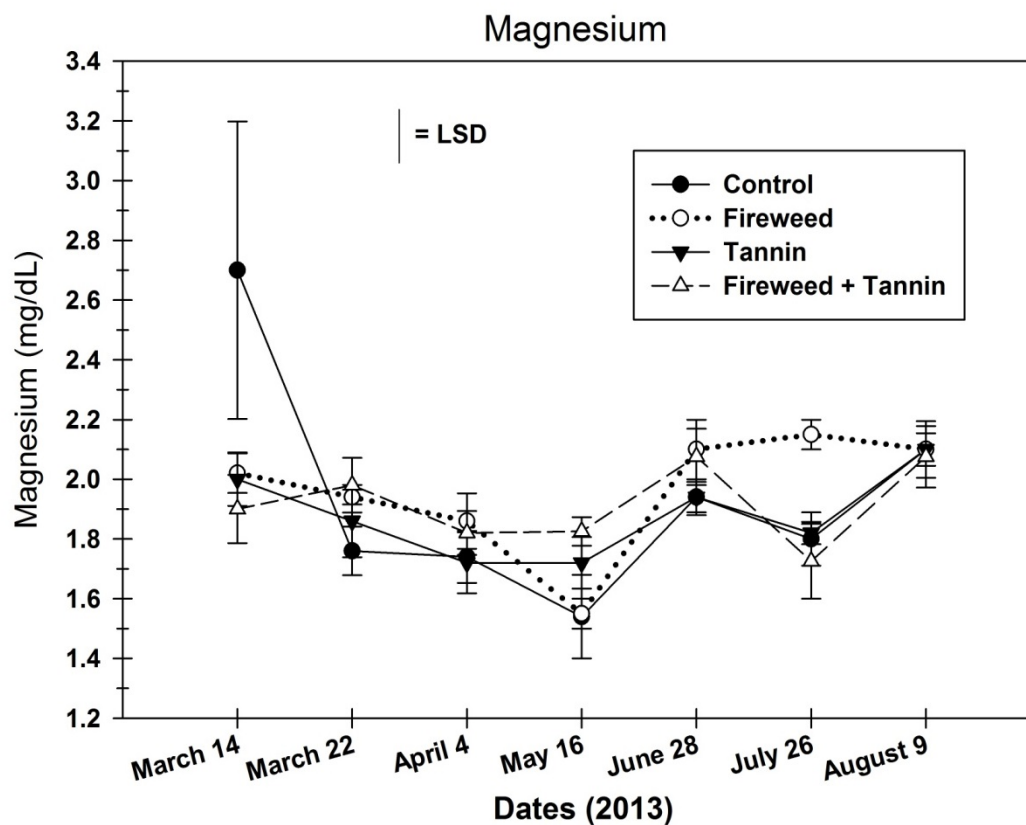


Figure 14. Magnesium Treatment by Date interaction.

**Phosphorus.** Phosphorus may increase due to renal failure or decrease with stress (Kahn 2010). There was no effect on phosphorus due to treatment ( $P=0.3642$ ) or treatment x date ( $P=0.6612$ ), but there was an effect due to date ( $P=0.0004$ ).

Phosphorus levels were slightly higher than normal at the beginning of the treatment phase in March but otherwise within the normal range (5.6-8.0 mg/dL). Measurements in the monitoring phase were lower than in the treatment phase (Appendix, Table 17).

**Potassium.** Increases in potassium may indicate renal failure, while decreases may be due to chronic renal dysfunction or diarrhea (Kahn 2010). There was no effect on potassium due to treatment ( $P=0.2666$ ) or treatment x date ( $P=0.5512$ ), but there was

an effect due to date ( $P < 0.001$ ). Potassium levels were slightly higher than the normal range in the baseline measurement in the beginning of March, but otherwise normal during the course of the trial (3.6-4.9 mmol/L). Measurements in the monitoring phase were lower than in the treatment phase (Appendix, Table 18).

**Protein.** Increases in total protein may be due to dehydration or chronic inflammation while decreases may be caused by over-hydration, severe congestive heart failure (with edema) or other conditions (Kahn 2010). There were no effects on protein due to treatment ( $P = 0.3346$ ), date ( $P = 0.4128$ ) or treatment x date ( $P = 0.3212$ ).

### **Body Weights**

All treatment groups lost weight during the treatment phase of the trial. During the subsequent monitoring phase, all groups gained similar amounts of weight.

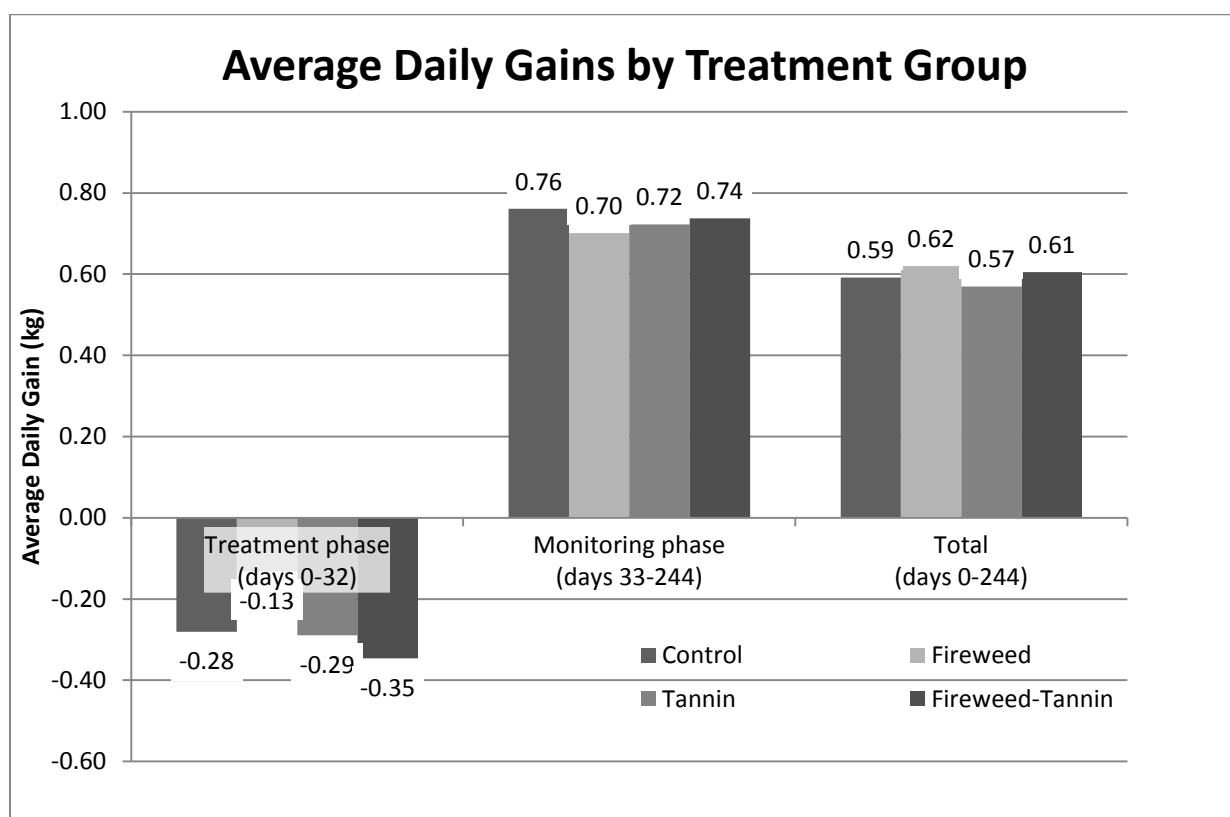


Figure 15. Average daily gains or losses by animals in four treatment groups.

## DISCUSSION

### ***In vitro* Experiment**

The results from the *in vitro* experiment, where four levels of tannins (0%, 4%, 8%, or 12%) were mixed with PA-containing fireweed in rumen solution, suggest tannin at 8% bound the most PAs, with some effect at 12% as well. In either case, the results were variable and not dramatic and it is not clear why there was less effect at 12% than at 8%. The number of sub-samples for each treatment x time was small (n=2), which contributed to variability in the findings (Figure 1).

Measured PAs also dropped off dramatically over time. This phenomenon is likely due to the PAs changing form as they were hydrolyzed or metabolized by the rumen microbes into other forms that were not specifically measured via the LC/MS.

Nonetheless, the data support the hypothesis that when tannins were present in rumen fluid at 8% and 12%, there was less PAs in the rumen solution. Based on the affinity of tannins to bind with other molecules, and previous research on the effects of tannins on altering diet selection (Lyman et al. 2011; Villalba et al. 2011) and increasing intake (Lyman et al. 2008; Lisonbee et al. 2009; Owens et al. 2012a, 2012b) of foods high in alkaloids, it was predicted that there would be some binding of tannins and alkaloids in rumen fluid. If PAs are bound when they pass out of the rumen and move through the gastro-intestinal tract, it was hypothesized that the binding by tannins might confer a protective benefit by less PAs getting absorbed and metabolized in the liver. While PA metabolic pathways have been studied, much less is understood about the interactions of secondary compounds such as PAs and tannins in living animals. The results from the *in vitro* experiment were used to design and conduct the *in vivo* experiment.



### ***In vivo* Experiment**

The study examined if tannins reduced the toxicity of PAs in cattle as measured by various physiological responses and observations of animal performance and behavior. Based on the fact that both the Fireweed and the Fireweed-Tannin groups showed signs of illness due to the treatments, and three out of the five animals in the Fireweed group and one out of five animals in the Fireweed-Tannin group died, the animals were clearly affected by the fireweed but a protective effect of tannins could not be identified.

Animals exposed to high doses of PAs may experience serum biochemical changes that include massive elevations in AST and AP and increased amount of bilirubin, all of which indicate damage to liver cells, impaired liver function, and acute liver disease (Stegelmeier 2011). The present study found slightly above normal levels of AST in both the Fireweed and the Fireweed-Tannin groups in March. By the end of the treatment phase of the study, AST levels were similar for the Fireweed, Fireweed-Tannin, and Control groups. There was no effect due to treatment or treatment x date for AP. Levels of bilirubin were similar, but within the normal range, for the Fireweed, Fireweed-Tannin, and Control groups, and their levels were all significantly higher than the Tannin group during the treatment phase.

Studies of the effects of *Cynoglossum officinale*, which contains heliotridine-type pyrrolizidine alkaloids, on cattle also revealed increases of AST activities and serum bile acid and total bilirubin concentrations (Baker et al. 1991). However, animal response to PAs is highly dependent on PA dose and rate of ingestion. Calves fed *Senecio riddellii* by gavage for 20 d at a daily dose of 45 mg of total PA/kg developed clinical signs and serum enzyme changes typical of intoxication (Molyneux et al. 1991). However, calves receiving riddelliine at 4.5 mg/kg/d for 20 d had neither serum enzyme changes nor clinical signs of PA toxicity. In another study, calves fed *Senecio riddellii* to provide 10

mg of PA/kg for 20 d did not develop clinical signs of toxicity and had no serum enzyme changes. However, feeding the plant at doses that provided 15 to 20 mg of PA/kg/d or more (gavaged or fed in capsules for the same time period) resulted in high mortality (Johnson et al. 1985). Johnson et al. (1985) also reported that toxicity was dependent on the rate at which the dose was consumed and that mortality was not necessarily dependent on the cumulative dosage. Unpublished results of a toxicity study conducted at the USDA-ARS Poisonous Plants Research Lab in 2011 showed elevated levels of AST and ALT in calves dosed with Hawaii fireweed at 15 mg PA/kg BW (Jim Pfister, personal communication). Significant and counter-intuitive differences in physiological responses between individuals receiving various levels of *Senecio jacobea* also was reported by Thorpe and Ford (1968) where a calf receiving a relatively low dose of PAs succumbed much earlier than others receiving the same or even greater amounts. Thorpe and Ford's 1968 study and the study by Ford et al. (1968) demonstrate the large animal to animal variation in response to PAs. Ford et al. (1968) found no correlation between liver pathology and the patterns of serum enzyme changes, which they speculate may be due to specific enzyme localization within cellular components and the rate at which those cellular components are damaged relative to the dosage of hepatotoxin and the stage of disease process.

The method of administering the plant material may have affected the Fireweed, Fireweed-Tannin, and Control groups relative to the Tannin group. It was difficult to drench plant material into such large calves and at times the animals vomited some of the slurry back up. There was concern that filling their rumens with so much finely ground material and water so quickly might result in pulmonary tract infection due to aspiration of fluids and/or rumen stasis. The two groups that received fireweed had more severe physiological response, including diarrhea and lethargy, than the control group. Additionally, the fireweed only group differed from the fireweed-tannin group in

that they also experienced constipation and straining, labored breathing and weakness. By the end of the trial, all individuals in all treatment groups exhibited nasal discharge and cough at some point during the treatment phase, undoubtedly due to the daily drenching.

Two animals from the Fireweed-only group died during the treatment phase; Steer 1 died on day 28 and Steer 2 died on day 29 of the 30-d treatment phase. Prior to dying, Steer 1 showed signs of bloat and was relieved via a stomach tube. About an hour after treatment, the steer was bloated again, was frothing at the mouth and had a distended head showing signs of distress and difficulty breathing. Steer 1 died during the night of Day 28. Steer 2 showed similar symptoms as Steer 1 although bloating was not as severe. When his conditions worsened overnight, the decision was made to euthanize him, but before euthanization could occur, the steer died. An unofficial necropsy was conducted on Steer 2. Rigor mortis set in within 30 min. The abdomen was distended and there was a slight rectal prolapse. The steer was slightly bloated with froth at the mouth. His gall bladder was filled with dark, thick mucus-like liquid, oily and blackish-yellow in color. Other organs appeared normal in form and color. The lungs were not examined. In hindsight, the cause of death of these animals could have been the secondary effects of pneumonia or bloat or impaction of the gut.

During the monitoring phase of the study, a steer (3) from the Fireweed-Tannin group died on day 42 and a third animal (steer 4) from the Fireweed-only group died on day 43. Steer 3 was the first to show signs of respiratory distress during the treatment phase; he improved after a 3-d course of antibiotics. After the treatments were completed on day 30, Steer 3 was turned out to pasture and was observed grazing with the other steers. On day 42, the livestock manager noted the steer had lost condition and was very thin and emaciated. He decided to euthanize the animal and an unofficial necropsy noted compaction in the omasum with slightly jaundiced and emaciated mucus

membranes. The gall bladder was distended with oily, dark, yellow black mucoid fluid. His liver showed changes similar to the earlier biopsies with the exception of some centrilobular hepatocellular degeneration, necrosis with sinusoidal leukocytosis, endothelial change and some hepatocyte individualization. Other internal organs observed during the removal of the liver appeared normal in form and color. Steer 4 also was emaciated and thin, in poor condition, reluctant to graze, and recumbent. A decision was made on day 43 to euthanize this animal and another unofficial necropsy was conducted. No jaundice or impaction was noted. Gall bladder was of normal size and bile was normal color and consistency. No other abnormal observations were noted during removal of liver samples.

The serum chemistry of the groups drenched with plant material (Fireweed, Fireweed-Tannin, and Controls) differed from the group drenched with Tannin in several ways – ALT, anion gap, bilirubin, CO<sub>2</sub> – which suggests that the former may have experienced more stress and illness due to drenching with large amounts of wet plant material as opposed to only water and tannin (effectively a “tea”). No serum changes commonly associated with PA intoxication were observed in any of the fireweed-treated animals and the results of the liver biopsies also were inconclusive as the changes were not uniquely different in the Fireweed and Fireweed-Tannin groups. Illness due to PAs is difficult to determine and diagnoses are often based on characteristic histological changes alone (Stegelmeier 2011). Poisoning from PAs can be difficult to identify definitively as symptomatology strongly depends on the species, age and condition of animal and the amount and species of PA-containing plant being ingested and duration of exposure (Huxatable 1980; Mattocks 1986; Stegelmeier 2011). The physical effects observed in the Fireweed and Fireweed-Tannin groups are certainly notable, but are difficult to compare quantitatively. It remains unclear whether the animals that died from

ruminal tympany or a pulmonary infection or secondary effects due to an impaired immune system or more likely a combination of all of the above.

Consistent with these findings, while all animals lost weight during the treatment phase of the trial and they all gained weight during the monitoring phase of the trial (Figure 5), the behavior of the Fireweed, Fireweed-Tannin, and Control groups differed from the Tannin group when they were all put out to pasture for the monitoring phase of the study. The Fireweed, Fireweed-Tannin and Control groups didn't graze right away, whereas the Tannin group began to graze immediately.

Cattle do not voluntarily consume large amounts of fireweed so it was not possible to conduct a trial where they voluntarily consume fireweed with or without tannin in their diet. Administering large amounts of ground fireweed by oral drench was difficult and may have introduced complicating factors of stress and/or pulmonary tract infection into the study which likely confounded some of the results. The results may have been different had another method of administering PAs been used. It remains to be determined if supplemental tannins, or the addition of a forage high in tannins to the diet, positively affect intake of fireweed and blood serum parameters associated with toxicity. Future studies would benefit from using another method of administering fireweed, using smaller calves, and quantitatively monitoring behavioral observations and monitoring a broader set of physiological indicators over a longer period of time including red and white blood cell counts and other indicators of renal, lung and immune function.

## MANAGEMENT IMPLICATIONS

The *in vitro* data support the hypothesis that tannins in rumen fluid at 8% and 12% reduced free PAs in rumen fluid. While the *in vitro* results appeared promising, it was much harder to test the protective effect of tannins on PAs *in vivo* for the reasons described above. The studies described here focused on toxicity of fireweed to cattle in the presence of tannins. It would be worthwhile to determine if intake of fireweed by cattle could be increased when cattle eat plants that contain tannins.

Fireweed's toxicity is under-studied because cattle usually avoid eating it. In 2011, for instance, a 30-d grazing trial was conducted to determine if cattle would increase consumption of fireweed as other desirable forage declined in the pasture. Throughout the trial, cattle totally avoided eating fireweed. Even as forage quality and quantity declined, they showed a remarkable ability to avoid fireweed, even rejecting fireweed that they had accidentally bitten. Forage availability was reduced through grazing until cattle were forced to consume very coarse and fibrous, low-quality kikuyu grass (*Pennisetum clandestinum*) stolons, but they refused to eat fireweed. This grazing study suggests that cattle will only select fireweed under extreme grazing conditions when fireweed dominates a pasture and there is essentially no alternative forage.

### **Grazing Fireweed with Livestock**

Since fireweed is such an aggressive invasive, capable of significantly reducing pasture productivity and extremely avoided by and indeed potentially harmful to cattle and horses, alternative grazing management strategies must be used to sustainably graze fireweed-dominated pastures and reduce fireweed abundance.

One such strategy is multi-species grazing. Multi-species grazing can be effective due to the higher tolerance of sheep and goats to PAs (Motooka et al. 2004). Sheep and goats can tolerate an intake of PAs of several times their body weight

(Cheeke 1988); on the other hand, horses and cattle are quite susceptible, and over time eating just 5-10% of body weight in dry matter intake can be lethal (Cheeke 1988).

Species differences are likely due to lack of PA absorption, degradation and detoxification in the rumen, lack of hepatic formation of pyrroles, and differences in abilities to conjugate and excrete PAs and pyrroles (Cheeke 1989).

Multi-species grazing has not been a widely adopted method of control in Hawaii due to a variety of factors including the costs associated with infrastructure improvements and necessary management changes in keeping these smaller herbivores. However, anecdotal observations of ranchers suggest that appropriate numbers of sheep and goats will utilize fireweed infested pastures and that over time, that grazing pressure can effectively reduce fireweed populations. Thus the combination of innate tolerance and small ruminant selection of fireweed appears to present opportunities for management not provided by grazing cattle alone. Thus, management to reduce fireweed effects on cattle and pasture productivity should focus on multi-species grazing to take advantage of the innate resistance of sheep and goats to fireweed's toxicity to improve pasture diversity and resiliency. In addition, provided with appropriate complimentary forages, cattle may be able to safely utilize fireweed (Meuret and Provenza 2014), though that is a subject for further study.

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APPENDIX

Table 2 ALT

<b>Alanine Aminotransferase (ALT) (<math>\mu</math>/L)</b>								
<b>LSD = 6.78</b>								
<b>Reference Range: 6.9-35 u/L</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed+Tannin SE
3/14/2013	22.20	4.33	22.20	3.35	19.40	3.97	17.40	2.58
3/22/2013	59.60	4.13	61.60	2.84	27.20	2.22	63.00	6.26
4/4/2013	44.20	3.46	60.60	2.82	20.40	1.81	60.80	6.55
5/16/2013	21.20	3.68	26.50	2.50	25.80	2.54	22.75	2.50
6/28/2013	28.80	1.02	32.00	3.00	33.20	2.03	28.50	2.02
7/26/2013	30.20	0.97	34.00	4.00	35.20	2.85	31.00	2.86
8/9/2013	31.60	1.91	34.00	5.00	32.40	2.66	28.50	3.07

Table 3 Anion Gap

<b>Anion Gap (mmol/L)</b>								
<b>LSD = 2.41</b>								
<b>Reference Range: None</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed+Tannin SE
3/14/2013	28.20	2.58	21.20	2.56	27.40	3.19	22.80	1.71
3/22/2013	12.80	0.92	12.40	1.21	20.20	1.02	9.20	0.86
4/4/2013	12.00	0.95	9.60	0.40	18.40	1.36	8.40	0.81
5/16/2013	21.20	0.49	19.00	1.00	20.60	1.33	20.00	0.82
6/28/2013	19.60	0.68	17.00	1.00	19.40	0.68	17.00	0.82
7/26/2013	19.00	0.95	14.50	0.50	18.00	0.89	18.00	0.82
8/9/2013	18.00	1.14	17.50	0.50	16.40	0.81	16.00	0.41

Table 4 Albumin

<b>Albumin (g/dL)</b> <b>LSD = 0.22</b> <b>Reference Range: 2.5-3.8 g/dL</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed+Tannin SE
3/14/2013	3.06	0.10	3.08	0.06	3.08	0.10	2.94	0.06
3/22/2013	2.88	0.10	2.58	0.04	3.12	0.07	2.54	0.04
4/4/2013	2.90	0.09	2.64	0.02	2.76	0.11	2.56	0.02
5/16/2013	2.62	0.23	2.50	0.20	2.68	0.18	2.50	0.16
6/28/2013	2.96	0.10	2.95	0.15	2.90	0.09	2.88	0.15
7/26/2013	2.88	0.09	2.80	0.10	2.84	0.12	2.65	0.17
8/9/2013	2.78	0.09	2.70	0.10	2.72	0.13	2.55	0.15

Table 5 Sodium

<b>Sodium (mmol/L)</b> <b>LSD = 2.02</b> <b>Reference Range: 136-144 mmol/L</b>		
Date	Mean	SE
3/14/2013	147.40	0.71
3/22/2013	139.25	0.48
4/4/2013	135.35	1.23
5/16/2013	138.50	0.38
6/28/2013	138.13	0.31
7/26/2013	140.25	0.38
8/9/2013	140.00	0.39

Table 6 Chloride

<b>Chloride (mmol/L)</b> <b>LSD = 1.69</b> <b>Reference Range: 99-107 mmol/L</b>		
Date	Mean	SE
3/14/2013	104.00	0.60
3/22/2013	99.55	0.51
4/4/2013	95.75	0.86
5/16/2013	96.19	0.42
6/28/2013	98.00	0.47
7/26/2013	96.13	0.46
8/9/2013	99.69	0.68



Table 7 Calcium

<b>Calcium (mg/dL)</b> <b>LSD = 0.26</b> <b>Reference Range: 8.0-11.4 mg/dL</b>		
Date	Mean	SE
3/14/2013	9.43	0.09
3/22/2013	9.35	0.09
4/4/2013	8.98	0.12
5/16/2013	9.39	0.11
6/28/2013	9.91	0.08
7/26/2013	9.83	0.10
8/9/2013	9.39	0.07

Table 8 Alkaline Phosphatase (AP)

<b>Alkaline Phosphatase (<math>\mu</math>L)</b> <b>LSD = 31.6</b> <b>Reference Range: 18-153 <math>\mu</math>L</b>		
Date	Mean	SE
3/14/2013	100.80	7.07
3/22/2013	110.15	7.27
4/4/2013	76.75	5.95
5/16/2013	108.81	15.65
6/28/2013	124.50	17.35
7/26/2013	122.75	14.69
8/9/2013	123.25	19.09

Table 9 AST

<b>Aspartate Aminotransferase (AST) (<math>\mu</math>L)</b> <b>LSD = 9.19</b> <b>Reference Range: 60-125 <math>\mu</math>L</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed +Tannin SE
3/14/2013	80.80	9.70	78.80	7.17	75.20	6.76	70.00	2.21
3/22/2013	89.00	4.99	117.60	10.54	75.40	4.93	140.60	9.56
4/4/2013	85.20	16.04	90.40	6.06	50.80	3.07	96.00	10.00
5/16/2013	69.60	4.88	87.50	10.50	81.40	7.46	72.75	6.76
6/28/2013	70.00	1.87	85.50	7.50	83.60	3.53	69.25	3.20
7/26/2013	79.20	2.40	77.00	5.00	88.20	5.72	84.00	4.67
8/9/2013	81.40	2.93	77.00	5.00	82.20	1.83	76.00	5.66

Table 10 Bilirubin

<b>Bilirubin (mg/dL)</b> <b>LSD = 0.04</b> <b>Reference Range: 0-1.6 mg/dL</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed +Tannin SE
3/14/2013	0.10	0.00	0.10	0.00	0.10	0.00	0.10	0.00
3/22/2013	0.54	0.07	0.48	0.04	0.20	0.05	0.48	0.04
4/4/2013	0.48	0.06	0.54	0.02	0.18	0.04	0.44	0.04
5/16/2013	0.16	0.02	0.20	0.00	0.14	0.02	0.13	0.03
6/28/2013	0.10	0.00	0.10	0.00	0.10	0.00	0.10	0.00
7/26/2013	0.10	0.00	0.10	0.00	0.10	0.00	0.10	0.00
8/9/2013	0.10	0.00	0.10	0.00	0.10	0.00	0.10	0.00

Table 11 BUN

<b>Blood Urea Nitrogen (BUN) (mg/dL)</b> <b>LSD = 1.54</b> <b>Reference Range: 10-25 mg/dL</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed +Tannin SE
3/14/2013	16.60	0.75	15.50	1.50	10.00	0.71	8.60	0.60
3/22/2013	14.60	1.03	16.00	1.00	7.60	0.24	7.80	0.49
4/4/2013	20.80	0.80	20.50	3.50	7.60	0.68	7.00	0.55
5/16/2013	9.32	0.16	9.56	0.26	20.00	1.14	18.75	0.85
6/28/2013	9.24	0.17	9.20	0.20	18.00	1.26	17.75	1.25
7/26/2013	9.08	0.11	8.66	0.09	16.80	1.66	15.75	0.75
8/9/2013	9.20	0.28	9.35	0.25	22.40	1.21	20.75	0.75

Table 12 Cholesterol

<b>Cholesterol (mg/dL)</b> <b>LSD = 9.56</b> <b>Reference Range: 62-193 mg/dL</b>		
Date	Mean	SE
3/14/2013	100.40	3.80
3/22/2013	91.00	3.50
4/4/2013	82.50	3.65
5/16/2013	92.19	4.00
6/28/2013	111.06	4.30
7/26/2013	112.25	3.83
8/9/2013	102.00	3.58

Table 13 Bicarbonate (CO<sub>2</sub>)

<b>Bicarbonate (mmol/L)</b> <b>LSD = 2.49</b> <b>Reference Range: 20-30 mmol/L</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed +Tannin SE
3/14/2013	15.80	2.42	22.20	2.48	16.00	2.28	20.00	1.87
3/22/2013	28.20	0.58	26.80	1.96	19.80	0.37	29.40	0.93
4/4/2013	29.20	1.02	29.60	0.93	21.00	1.00	30.20	0.97
5/16/2013	21.80	0.58	23.50	1.50	21.20	0.58	22.00	0.58
6/28/2013	21.60	0.93	22.50	0.50	20.60	0.24	22.25	0.25
7/26/2013	25.60	1.21	30.50	0.50	25.40	0.81	26.00	1.73
8/9/2013	23.20	0.49	23.50	0.50	23.20	1.07	23.75	0.85

Table 14 Creatinine

<b>Creatinine (mg/dL)</b> <b>LSD = 0.15</b> <b>Reference Range: 0.5-2.2 mg/dL</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed +Tannin SE
3/14/2013	1.00	0.11	1.24	0.07	1.24	0.08	1.06	0.04
3/22/2013	1.28	0.06	1.13	0.09	1.25	0.08	1.07	0.04
4/4/2013	1.14	0.08	1.00	0.09	1.12	0.11	1.11	0.06
5/16/2013	0.96	0.07	1.08	0.06	1.10	0.09	0.96	0.05
6/28/2013	0.87	0.06	0.92	0.09	0.95	0.07	0.88	0.06
7/26/2013	1.10	0.08	1.30	0.02	1.11	0.08	1.04	0.03
8/9/2013	0.96	0.04	1.05	0.04	0.93	0.05	0.85	0.04

Table 15 Glucose

<b>Glucose (mg/dL)</b> <b>LSD = 14.01</b> <b>Reference Range:</b>		
Date	Mean	SE
3/14/2013	105.55	10.60
3/22/2013	77.20	4.00
4/4/2013	57.15	1.86
5/16/2013	59.88	1.32
6/28/2013	65.50	1.84
7/26/2013	58.69	2.97
8/9/2013	62.63	1.78

Table 16 Magnesium

<b>Magnesium (mg/dL)</b> <b>LSD = 0.21</b> <b>Reference Range: 1.5-2.9 mg/dL</b>								
Dates	Control mean	Control SE	Fireweed mean	Fireweed SE	Tannin Mean	Tannin SE	Fireweed +Tannin mean	Fireweed +Tannin SE
3/14/2013	2.70	0.50	2.02	0.07	2.00	0.09	1.90	0.11
3/22/2013	1.76	0.08	1.94	0.02	1.86	0.12	1.98	0.09
4/4/2013	1.74	0.09	1.86	0.09	1.72	0.10	1.82	0.07
5/16/2013	1.54	0.14	1.55	0.05	1.72	0.09	1.83	0.05
6/28/2013	1.94	0.05	2.10	0.10	1.94	0.06	2.08	0.09
7/26/2013	1.80	0.09	2.15	0.05	1.82	0.04	1.73	0.13
8/9/2013	2.10	0.09	2.10	0.00	2.10	0.05	2.08	0.10

Table 17 Phosphorus

<b>Phosphorus (mg/dL)</b> <b>LSD = 0.57</b> <b>Reference Range: 5.6-8.0 mg/dL</b>		
Date	Mean	SE
3/14/2013	7.84	0.17
3/22/2013	8.12	0.16
4/4/2013	7.52	0.23
5/16/2013	7.83	0.23
6/28/2013	7.55	0.23
7/26/2013	6.83	0.16
8/9/2013	6.79	0.20

Table 18 Potassium

<b>Potassium (mmol/L)</b> <b>LSD = 0.19</b> <b>Reference Range: 3.6-4.9 mmol/L</b>		
Date	Mean	SE
3/14/2013	5.00	0.09
3/22/2013	4.51	0.04
4/4/2013	4.52	0.07
5/16/2013	4.07	0.05
6/28/2013	4.06	0.07
7/26/2013	4.03	0.05
8/9/2013	3.98	0.05