

## RESEARCH ARTICLE

# Induced and constitutive responses of digestive enzymes to plant toxins in an herbivorous mammal

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### SUMMARY

Many plants produce plant secondary compounds (PSCs) that bind and inhibit the digestive enzymes of herbivores, thus limiting digestibility for the herbivore. Herbivorous insects employ several physiological responses to overcome the anti-nutritive effects of PSCs. However, studies in vertebrates have not shown such responses, perhaps stemming from the fact that previously studied vertebrates were not herbivorous. The responses of the digestive system to dietary PSCs in populations of Bryant's woodrat (*Neotoma bryanti*) that vary in their ecological and evolutionary experience with the PSCs in creosote bush (*Larrea tridentata*) were compared. Individuals from naïve and experienced populations were fed diets with and without added creosote resin. Animals fed diets with creosote resin had higher activities of pancreatic amylase, as well as luminal amylase and chymotrypsin, regardless of prior experience with creosote. The experienced population showed constitutively higher activities of intestinal maltase and sucrase. Additionally, the naïve population produced an aminopeptidase-N enzyme that was less inhibited by creosote resin when feeding on the creosote resin diet, whereas the experienced population constitutively expressed this form of aminopeptidase-N. Thus, the digestive system of an herbivorous vertebrate responds significantly to dietary PSCs, which may be important for allowing herbivorous vertebrates to feed on PSC-rich diets.

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Key words: digestive enzymes, plant–herbivore interactions, plant secondary chemicals.

### INTRODUCTION

Plants produce a wide array of plant secondary compounds (PSCs) to deter feeding by herbivores (Dearing et al., 2005). Many of these compounds, such as tannins or flavonoids, may bind to plant protein, preventing its digestion, and thus lowering the nutrient availability to the animal (Min et al., 2003). Additionally, if these compounds are present in high enough concentrations, they may bind and inhibit digestive enzymes produced by the herbivore, further limiting nutrient availability (Min et al., 2003).

To counteract the effects of PSCs on digestive enzymes, herbivores may employ one or more strategies. First, herbivores may alter the environment of their gut to prevent protein precipitation, perhaps through an alkaline-biased pH (Berenbaum, 1980). Second, herbivores can maintain baseline activity through increased synthesis of digestive enzymes to compensate for activity lost to inhibition (Jongsma and Bolter, 1997). Additionally, herbivores might produce variants of digestive enzymes that are less inhibited by PSCs (Jongsma and Bolter, 1997). Such responses have been well documented in herbivorous insects (Jongsma and Bolter, 1997). In contrast, many studies on terrestrial vertebrates have documented little response of digestive enzymes to PSCs, with some even exhibiting decreases in the activities of intestinal or pancreatic enzymes (Ahmed et al., 1991; Glick and Joslyn, 1970; Longstaff and McNab, 1991; Mariscal-Landín et al., 2004; van Leeuwen et al., 1995).

The differences in the responses observed in insects *versus* vertebrates may stem from the fact that the vertebrates tested (domestic rats, pigs and chickens) are all evolutionarily naïve to

high concentrations of PSCs as none are typically herbivorous, whereas all the insects examined are herbivorous. The physiological responses of the digestive systems of herbivorous insects are thought to have evolved with the defense compounds of their host plants (Jongsma and Bolter, 1997). Likewise, the detoxification systems of vertebrate herbivores are believed to have evolved in response to the ingestion of PSCs (Dearing et al., 2005; Freeland and Janzen, 1974). Thus, investigations in wild herbivores may give better insight into the physiological and evolutionary responses of the digestive systems of vertebrates to PSCs.

We investigated the digestive responses of vertebrates to PSCs in a wild mammalian herbivore. Bryant's woodrat (*Neotoma bryanti* Merriam 1887) is a small, herbivorous mammal found primarily in California, USA (Patton et al., 2007). *Neotoma bryanti* feeds primarily on cactus (*Opuntia occidentalis*) and sage (*Salvia* spp.) in the coastal regions of its range (Atsatt and Ingram, 1983), whereas it consumes creosote bush (*Larrea tridentata*) in desert habitats. Creosote bush is interesting from a dietary perspective because it is a relatively recent dietary addition given that it has only been present in the southwestern deserts of the USA since the end of the last glacial period (*ca.* 17,000 years ago) (Hunter et al., 2001). Thus, this *Neotoma* species has coastal populations that are evolutionarily and ecologically naïve to creosote bush ('naïve population'), as well as desert populations that have had >10,000 years of contact with creosote bush and its PSCs ('experienced population'). These populations vary in the types of PSCs they encounter in the wild. The naïve population feeds mainly on plants that produce low-molecular-weight defense compounds that are unlikely to bind

dietary or enzymatic proteins (Abreu et al., 2008; Atsatt and Ingram, 1983; Stintzing and Carle, 2005). Alternatively, creosote bush leaves produce a complex resin consisting of various phenolics, flavonoids and saponins (Mabry et al., 1977), chemical classes that have been shown to bind and inhibit digestive enzymes (Cheeke, 1971; Fontana Pereira et al., 2011). Although interactions between digestive enzymes and specific compounds in creosote resin remain unstudied, creosote resin does bind protein and inhibit proteolytic enzymes (Rhoades, 1977). Because of the differences in plant chemistry that each of these populations naturally consume, their physiological responses to protein-binding PSCs are expected to vary.

We collected individuals from each of these populations and fed them diets containing creosote bush resin. We investigated for the presence of induced responses, predicting that when feeding on creosote resin individuals would: (1) increase their gut pH, (2) increase the mass-specific activity of digestive enzymes and (3) produce variants of digestive enzymes that are less inhibited by creosote resin. Additionally, we examined for constitutive responses, in which we predicted the experienced population to maintain: (4) a higher gut pH, (5) higher mass-specific enzyme activities and (6) a constant production of enzyme variants that were less inhibited by creosote resin in comparison with the naïve population.

## MATERIALS AND METHODS

### Animal collection and maintenance

Individuals of the naïve population were collected in July 2006 from Casper's Wilderness Area, CA (33°31'N, 117°33'W). Individuals of the experienced population were collected in April 2009 outside Palm Desert, CA (33°68'N, 116°36'W), in the Sonoran Desert. All animals were trapped with Sherman live traps. Woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48×27×20 cm) under a 12h:12h light:dark cycle, with 28°C ambient temperature and 20% humidity.

### Dietary treatments and diet preparation

Prior to experimentation, animals were maintained on a diet of pelleted high-fiber rabbit chow (Teklad formula 2031, Harlan Laboratories, Madison, WI, USA). During experimentation, animals were fed the same rabbit chow formulation except in a powdered form to prevent caching of food. Five individuals from each population served as control animals and were fed powdered rabbit chow in cages with feeder-hoods for 8 days. Nine woodrats (four from the naïve population, five from the experienced population) were fed the control diet for 3 days followed by the same diet with increasing amounts of creosote resin (1 and 2% creosote resin for 2 and 3 days, respectively). This protocol was used to permit time for the induction of digestive enzymes (Deren et al., 1967). A diet with 2% creosote is tolerated without body mass loss in naïve animals. Body mass and dry matter intake (DMI) of all animals was measured daily. DMI was calculated as the difference between the amount of food presented and what remained each day, after samples were dried at 50°C for 3 days.

To prepare creosote resin diets, creosote leaves were collected from trapping sites and frozen at –20°C prior to resin extraction. Resin was extracted by soaking leaves in acetone (1:6, wet leaf mass:volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotovap until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48h to remove any remaining acetone. Extracted resin was stored at –20°C prior to use.

Creosote diet was prepared by dissolving the appropriate amount of resin in a volume of acetone equal to 25% of the dry mass of ground rabbit chow to which it was added. Control diet (0%) was prepared by adding an identical ratio of acetone, without creosote resin. Acetone was evaporated from all diets in a fume hood, and complete evaporation was confirmed gravimetrically.

Following diet treatments, animals were euthanized under CO<sub>2</sub> and immediately dissected. Pancreatic tissue was removed, weighed and frozen on dry ice. The luminal contents of the small intestine were removed, pH was measured with an Omega Soil pH electrode (PHH-200) and then contents were frozen. The intestine was then flushed with ice-cold saline, cut in half length-wise, weighed and frozen. Tissues were kept at –80°C until analysis.

### Pancreatic and luminal enzyme assays

Activities of pancreatic and luminal amylase were measured by modification of the 3,5-dinitrosalicylate method (Dahlqvist, 1962). For pancreatic amylase, several pieces of pancreas were thawed and homogenized for 30s using 10 ml g<sup>–1</sup> tissue of amylase homogenizing buffer [5 mmol l<sup>–1</sup> phosphate buffer, pH 6.9, containing 7 mmol l<sup>–1</sup> NaCl, 3 mmol l<sup>–1</sup> taurocholic acid, 0.27% (w/v) Triton X-100, 1 mmol l<sup>–1</sup> benzamidine and 2 mmol l<sup>–1</sup> hydrocinnamic acid]. For luminal amylase, lumen contents of the intestine were vortexed in 10 parts of amylase homogenizing buffer and then centrifuged for 2 min at 7000g to collect the supernatant. Diluted 100 µl aliquots of either pancreas homogenate or luminal supernatant were incubated with 100 µl of 2% potato starch (Sigma-Aldrich S2630, St Louis, MO, USA) at 37°C for 3 min. The reaction was terminated by the addition of 200 µl dinitrosalicylate reagent. The tubes were immersed in boiling water for 10 min and cooled with tap water. Blank samples contained exactly the same reagents, but dinitrosalicylate was added before substrate to deactivate enzymes and prevent reaction, and then were handled in the same way as other samples. Aliquots of 150 µl were transferred to 96-well plates, and absorbance at 530 nm was determined using a BioTek PowerWave HT microplate spectrophotometer (Broadview, IL, USA).

For analysis of pancreatic chymotrypsin and trypsin, several pieces of pancreatic tissue were homogenized for 30s using 10 ml g<sup>–1</sup> tissue of chymotrypsin/trypsin homogenizing buffer [50 mmol l<sup>–1</sup> Tris/HCl buffer, pH 8.2, containing 3 mmol l<sup>–1</sup> taurocholic acid and 0.27% (w/v) Triton X-100]. To activate zymogens, homogenate samples were incubated with 0.3% enterokinase (Sigma-Aldrich E0632) in 50 mmol Tris/HCl buffer (pH 8.2) containing 20 mmol l<sup>–1</sup> CaCl<sub>2</sub> for 20 min at 37°C. Preliminary trials indicated that this treatment gave reproducible maximal activation of the proteolytic zymogens. Samples were centrifuged for 2 min at 7000g to remove a white suspension that sometimes appears in solution. Chymotrypsin activities were measured by the amount of p-nitroaniline released by hydrolysis when incubating 160 µl of homogenate supernatant and 800 µl of 1 mmol l<sup>–1</sup> N-glutaryl-L-phenylalanine-p-nitroanilide (GPNA) solution at pH 7.6 for 10 min at 37°C. Aliquots of 16 µl supernatant mixed with 144 µl distilled water were assayed to measure trypsin activity using 800 µl 1 mmol l<sup>–1</sup> benzoyl-arginine-p-nitroanilide (DL-BAPNA) solution as substrate at pH 8.2 for 10 min at 37°C. For both chymotrypsin and trypsin assays, reactions were terminated with 160 µl of 30% acetic acid solution. Blank samples contained exactly the same reagents but acetic acid was added before the substrate to deactivate enzymes and prevent reaction, and then they were handled in the same way as other samples. The liberated amounts of p-nitroaniline were estimated by transferring 200 µl of the final reaction to a 96-well

plate and reading the absorbance at 410 nm (Erlanger et al., 1961), and enzyme activity was calculated using a p-nitroaniline standard curve.

Luminal chymotrypsin and trypsin activities were measured similarly. Lumen contents were vortexed in 10 parts of chymotrypsin/trypsin homogenizing buffer and then centrifuged for 2 min at 7000g to collect the supernatant. Further activation by enterokinase was not required for luminal contents. For chymotrypsin, 160 µl of supernatant was added to 800 µl of 1 mmol l<sup>-1</sup> GPNA. For trypsin, 80 µl of supernatant and 80 µl of distilled water were added to 800 µl of 1 mmol l<sup>-1</sup> DL-BAPNA. All other steps were identical to the technique for measuring pancreatic enzyme activities.

### Intestinal enzyme assays

Intestinal enzyme assays were carried out on the proximal half of the small intestine. We assayed disaccharidase (maltase, sucrase) activity using a modification of a previously developed colorimetric method (Dahlqvist, 1984). Briefly, tissues were thawed and homogenized in 350 mmol l<sup>-1</sup> mannitol in 1 mmol l<sup>-1</sup> N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)-KOH, pH 7.0. Intestinal homogenates (30 µl) diluted with 350 mmol l<sup>-1</sup> mannitol in 1 mmol l<sup>-1</sup> Hepes-KOH were incubated with 30 µl of 56 mmol l<sup>-1</sup> maltose or 56 mmol l<sup>-1</sup> sucrose in 0.1 mol l<sup>-1</sup> maleate and NaOH buffer, pH 6.5, at 37°C for 20 min. Next, 400 µl of a stop-develop reagent (GAGO-20 glucose assay kit, Sigma-Aldrich) was added to each tube, vortexed and incubated at 37°C for 30 min. Lastly, 400 µl of 6 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> was added to each tube to stop the reaction. Several 200 µl aliquots of the final reaction were transferred to a 96-well plate, and the absorbance was read at 540 nm.

We used L-alanine-p-nitroanilide as a substrate for aminopeptidase-N. To start the reaction we added 10 µl of the homogenate to 1 ml of assay mix (2.0 mmol l<sup>-1</sup> L-alanine-p-nitroanilide in one part of 0.2 mol l<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer no. 1, pH 7 and one part of deionized H<sub>2</sub>O). The reaction solution was incubated for 20 min at 37°C and then the reaction was terminated with 3 ml of ice-cold 2 mol l<sup>-1</sup> acetic acid. Several 200 µl aliquots of the final reaction were transferred to a 96-well plate, and absorbance was measured at 384 nm.

### Inhibition of enzyme activity by creosote resin

We added creosote resin to enzyme activity reactions to monitor enzyme inhibition. We were unable to measure the inhibition of maltase and sucrase activity, as the stop-develop reagent used for these enzyme assays utilizes the purified enzymes glucose oxidase and peroxidase. We could not design a protocol that inhibited maltase or sucrase activity, but did not also inhibit the color-developing enzymes. For all other enzymes (amylase, trypsin, chymotrypsin and aminopeptidase-N), tissues were incubated with a series of concentrations of creosote resin directly following homogenization. Resin concentrations were determined specifically for each enzyme based on the concentrations that gave significant inhibition of enzyme activity. Resin was dissolved in 85% methanol, added to tissue homogenates and allowed to sit for 10 min. Control tubes contained only tissue homogenate and 85% methanol. Tissue homogenates were centrifuged for 1 min at 7000g to remove a suspension created by the resin solution. Supernatants were used to run enzyme assays as described above. Relative enzyme activity was calculated by dividing the activity of resin-treated homogenates by the activity of control homogenates.

### Statistics

Body mass, small intestinal pH and DMI were compared using separate two-way ANOVAs, with population and diet treatment as main effects. Additionally, absolute organ masses were compared using diet and population as main effects and body mass as a covariate. Enzyme activities were compared with two-way ANOVAs, using population and diet treatment as main effects, as well as investigating DMI as a covariate. If the covariate of DMI was insignificant, it was removed from the model. Significant differences between treatment groups were detected using Tukey's honestly significant difference (HSD) test. Relative enzyme activity was compared using repeated-measures ANOVA with population and diet treatment as main effects. Statistical analyses were conducted in JMP (SAS Institute, 2010).

## RESULTS

### Body and organ masses, intestinal pH and food intake

Individuals from the naïve population were larger than those from the experienced population (Table 1). There were no population, diet treatment or interaction effects on the pH of small intestinal contents (Table 1). Because of a limited amount of digesta, intestinal pH for one animal was not measured. Creosote caused animals to consume significantly less food, and this decrease was more exaggerated in the naïve population (Table 1). However, DMI was not a significant covariate in analysis of any enzyme activity, and so it was removed from all analyses. There were no significant effects of diet or population on organ masses when controlling for body mass (Table 1).

### Pancreatic and luminal enzyme activities

The response of pancreatic and luminal enzymes to creosote resin varied across enzymes. Addition of creosote resin to the diet significantly increased mass-specific amylase activity of both populations (Table 2, Fig. 1A). Naïve individuals feeding on creosote showed 4.3× higher amylase activity in the pancreas compared with controls, and experienced individuals showed 2.6× higher activity. However, this response did not differ between populations, as no significant interaction between population and diet treatment was observed (Table 2). There were no significant effects of population or diet treatment on pancreatic chymotrypsin or trypsin activity (Table 2, Fig. 1B,C). Two of the luminal enzymes assayed (amylase and chymotrypsin) showed higher activity in individuals fed creosote (Table 2, Fig. 2A,B). Because of a limited amount of digesta, we were unable to measure luminal trypsin activity for one animal. There was no significant effect of population or diet on luminal trypsin activity (Table 2, Fig. 2C).

### Intestinal enzyme activities

The experienced population exhibited 1.8× higher maltase activity and 1.5× higher sucrase activity compared with the naïve population (Table 2, Fig. 3A,B). However, diet did not significantly alter disaccharidase activity in either population (Table 2). Additionally, there were no significant effects of population or diet treatment on intestinal aminopeptidase-N activity (Table 2, Fig. 3C).

### Inhibition of enzyme activity by creosote resin

For all enzymes, creosote resin significantly inhibited enzyme activity (resin concentration effect:  $P < 0.0001$  for all enzymes; Fig. 4, supplementary material Fig. S1). For amylase, chymotrypsin and trypsin, there were no effects of population or diet treatment on enzyme inhibition by creosote resin *in vitro* (supplementary material Fig. S1). Relative aminopeptidase-N activity was not significantly

Table 1. (A) Means ±1 s.e.m. and (B) summary of ANOVAs for body mass, intestinal pH and dry matter intake (DMI), and ANCOVAs for organ masses

A) Variable	Naïve		Experienced	
	Control	Creosote	Control	Creosote
Sample size	5	4	5	5
Body mass (g)	161.5±11.9	159.1±14.4	127.0±12.0	120.3±9.1
Intestinal pH	7.98±0.08	7.99±0.16	7.80±0.09	7.94±0.10
DMI (g d <sup>-1</sup> )	10.59±0.48	6.99±0.41	9.49±0.41	8.92±0.63
Pancreas mass (g)	0.37±0.03	0.33±0.06	0.30±0.03	0.23±0.03
Intestine mass (g)	3.26±0.18	2.99±0.24	2.29±0.15	2.36±0.21
B) Source of variation	<i>F</i>	d.f.	<i>P</i>	
Body mass				
Population	9.61	1,15	<b>0.007</b>	
Diet	0.15	1,15	0.71	
Interaction	0.03	1,15	0.86	
Intestinal pH				
Population	1.06	1,14	0.32	
Diet	0.44	1,14	0.52	
Interaction	0.45	1,14	0.51	
DMI				
Population	0.66	1,15	0.43	
Diet	16.99	1,15	<b>&lt;0.001</b>	
Interaction	8.95	1,15	<b>0.009</b>	
Pancreas mass				
Population	0.46	1,14	0.51	
Diet	1.38	1,14	0.26	
Interaction	0.15	1,14	0.70	
Covariate (body mass)	3.73	1,14	0.07	
Intestine mass				
Population	3.99	1,14	0.07	
Diet	0.10	1,14	0.75	
Interaction	1.57	1,14	0.23	
Covariate (body mass)	10.26	1,14	<b>0.006</b>	

Significant differences are in bold.

altered by diet treatment in the experienced population ( $F_{1,8}=0.0008$ ,  $P=0.98$ ; Fig. 4A). In the naïve population, though, individuals feeding on creosote produced an aminopeptidase-N enzyme that was less inhibited ( $F_{1,7}=14.56$ ,  $P=0.006$ ; Fig. 4B), and very similar to that of the experienced population.

DISCUSSION

Herbivores can mediate the inhibitory impact of PSCs on their digestive process through several mechanisms. They can alter gut pH, increase enzyme activities or produce variants of digestive enzymes less subject to inhibition. These responses have not been extensively documented in vertebrate herbivores. In this study, we compared evolutionarily and ecologically naïve herbivores with experienced ones to investigate whether experience played a role in the ability to respond to PSCs. Indeed, the consumption of PSCs alters digestive enzyme activity and inhibition rates in a wild mammalian herbivore. How these responses varied as a function of the particular enzyme as well as the animal’s experience with PSCs and the organismal implications are described.

A highly alkaline gut pH is thought to be an important mechanism by which larval insects avoid inhibition of digestive enzymes (Berenbaum, 1980). However, no significant differences in the pH of intestinal contents between populations or diet treatments were observed. Vertebrates may be limited in their ability to increase gut pH as a defense against PSCs, as their digestive enzymes usually operate optimally within pH ranges of 6 to 7.5 (Blair and Tuba, 1963; Maze and Gray, 1980), whereas some insect digestive enzymes can perform optimally up to a pH of 12 (Wolfson and

Murdock, 1990). However, it is noteworthy that the intestinal pH of *N. bryanti* (~7.9) is far higher than that found in laboratory rodents (pH 5–6) (McConnell et al., 2008). Further surveys of intestinal pH in rodents with different dietary habits may be warranted.

Pancreatic amylase activity increased in *N. bryanti* individuals feeding on creosote resin. Previous experiments on digestive enzymes in terrestrial vertebrates have not shown changes in mass-specific activity of pancreatic amylase, but showed hypertrophy of the pancreas after feeding on tannins for 4 weeks, resulting in greater activity summed across the pancreas (Ahmed et al., 1991). In our study, diet had no effect on pancreas size, perhaps because creosote diet treatments were ingested for only 5 days. The increase in mass-specific amylase activity seen in woodrats feeding on creosote resin may represent an acute response to toxins, and longer exposure might result in pancreatic hypertrophy along with the loss of any mass-specific differences in amylase activity.

Luminal activities of pancreatic enzymes may be a better measure than pancreatic activities by which to investigate physiological responses to enzyme-binding toxins. Prior to acting on nutrients, enzymes stored within pancreatic tissue must be secreted into the intestinal lumen, as well as activated by enterokinase (in the case of trypsin and chymotrypsin) (Stevens and Hume, 2004). Woodrats feeding on creosote resin showed higher amylase and chymotrypsin activity in the intestinal lumen compared with control-fed animals. These data, coupled with those for pancreatic enzyme activity, allow inference into the storage and release of pancreatic enzymes in response to dietary toxins. Based on the lumen data, when fed creosote resin, woodrats appear to increase the secretion of



Table 2. ANOVA results for enzyme activities

Enzyme	<i>F</i>	d.f.	<i>P</i>
<b>Pancreatic enzyme activities</b>			
Amylase			
Population	1.04	1,15	0.32
Diet	16.13	1,15	<b>0.001</b>
Interaction	1.39	1,15	0.26
Chymotrypsin			
Population	0.09	1,15	0.77
Diet	0.06	1,15	0.81
Interaction	0.11	1,15	0.74
Trypsin			
Population	0.0004	1,15	0.98
Diet	0.02	1,15	0.88
Interaction	0.43	1,15	0.52
<b>Luminal enzyme activities</b>			
Amylase			
Population	0.05	1,15	0.82
Diet	6.18	1,15	<b>0.025</b>
Interaction	0.02	1,15	0.88
Chymotrypsin			
Population	0.001	1,15	0.97
Diet	9.07	1,15	<b>0.009</b>
Interaction	1.35	1,15	0.26
Trypsin			
Population	2.26	1,14	0.15
Diet	2.87	1,14	0.11
Interaction	0.23	1,14	0.64
<b>Intestinal enzyme activities</b>			
Maltase			
Population	5.55	1,15	<b>0.032</b>
Diet	0.001	1,15	0.97
Interaction	0.34	1,15	0.57
Sucrase			
Population	4.78	1,15	<b>0.045</b>
Diet	0.51	1,15	0.48
Interaction	0.03	1,15	0.86
Aminopeptidase-N			
Population	0.11	1,15	0.74
Diet	0.10	1,15	0.76
Interaction	0.12	1,15	0.90

Significant differences are in bold.

chymotrypsin into the intestine. However, the lack of difference in pancreatic chymotrypsin activity suggests that the pancreas maintains an even and balanced level of stored chymotrypsinogen (the precursor to chymotrypsin). Thus, dietary toxins may increase the turnover of this enzyme within the pancreas. In contrast, the increases in both pancreatic and luminal amylase activity suggest that in response to toxins, the pancreas not only secretes more amylase, but also stores excess amylase, which may be lost if the pancreas undergoes hypertrophy following long-term exposure to dietary toxins as in other vertebrates (Ahmed et al., 1991).

Additionally, these results suggest that modulation of the secretion rates of pancreatic enzymes may be an important mechanism for physiological responses. Secretion of pancreatic enzymes is tightly regulated by the gastrointestinal hormones cholecystokinin and secretin (Hadley and Levine, 2007). In fact, trypsin inhibitors found in soybeans induce secretion of cholecystokinin from intestinal tissue, which then induces trypsin production and secretion (Savelkoul et al., 1992). However, many studies investigating responses of digestive enzymes to changes in diet composition (usually nutrients) measure activity only within pancreatic tissue and not the lumen (Ciminari et al., 2001; Kohl et al., 2011). Future studies should integrate enzyme activities of pancreatic tissue with

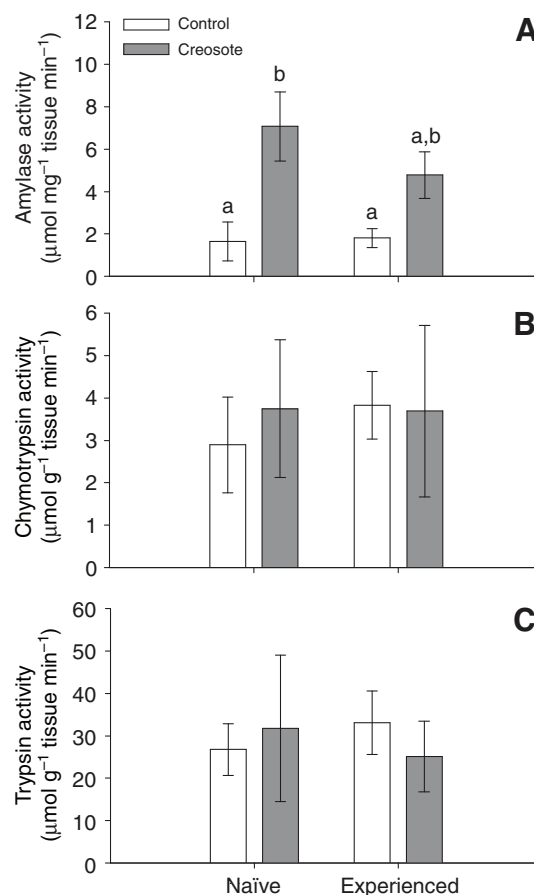


Fig. 1. Mean mass-specific activities of (A) amylase, (B) chymotrypsin and (C) trypsin within pancreatic tissue of woodrats with different ecological and evolutionary experience with, as well as short-term exposure to, PSCs. Bars represent  $\pm 1$  s.e.m. Different letters above bars indicate significant differences between treatments (Tukey's HSD).

that of the intestinal lumen when investigating responses of pancreatic enzymes to changes in dietary substrates or toxins.

Activities of intestinal carbohydrases showed constitutive differences between populations such that the naïve population had lower activity than the experienced population. This is surprising, given that activities of maltase and sucrase in wild and laboratory rodents are quickly and drastically modulated in response to changes in diet (Karasov and Hume, 1997). Modulation of intestinal disaccharidases seems to be dependent on glucocorticoid signals released by the adrenal gland (Lebenthal et al., 1972). It could be that signaling pathways do not exist to convey information on dietary toxin presence to promoters of disaccharidase expression. If true, experienced animals may have solved the problem of maltase and sucrase inhibition through higher constitutive activity of these enzymes to deal with a natural diet rich in enzyme inhibitors.

Woodrats seemed to respond to inhibition of aminopeptidase-N by altering the enzyme variant produced, rather than modulating activity of this enzyme. In naïve populations feeding on a control diet, the aminopeptidase-N enzyme was significantly inhibited by the addition of creosote. However, when naïve individuals were fed a creosote-containing diet, they produced an aminopeptidase-N enzyme that was less inhibited by creosote and showed inhibition rates similar to those of all experienced individuals, regardless of diet. These results are similar to those in insects showing that after

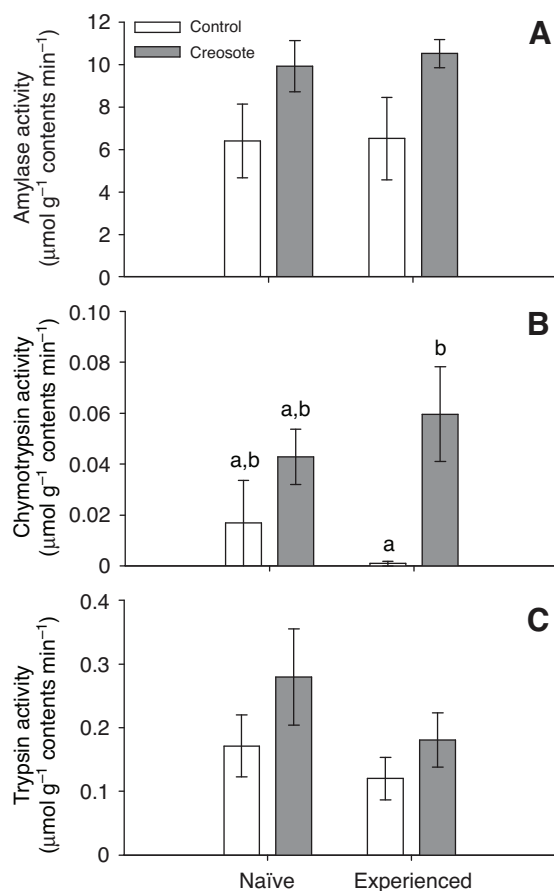


Fig. 2. Mean mass-specific activities of (A) amylase, (B) chymotrypsin and (C) trypsin from intestinal lumen contents of woodrats with different ecological and evolutionary experience with, as well as short-term exposure to, PSCs. Bars represent  $\pm 1$  s.e.m. Different letters above bars indicate significant differences between treatments (Tukey's HSD).

feeding on plants containing protease inhibitors, insects produce variants of proteases that are insensitive to inhibition (Jongsma et al., 1995). This change in variants is achieved through transcriptional induction of insensitive protease genes belonging to complex, multigene families (Bown et al., 1997). However, the aminopeptidase-N gene is only represented by one copy in the mouse genome (Waterson et al., 2002). Still, the possibility of gene duplication and differentiation within *N. bryanti*, and differential transcriptional regulation between populations, cannot be discarded, as new gene families can arise between closely related mammals (Demuth et al., 2006). Another possible mechanism of inducing enzymes tolerant to protease inhibitors is through post-translational modifications. For example, protein glycosylation significantly decreases binding by tannins (Sarni-Manchado et al., 2008). Future work addressing these mechanisms will enlighten whether *N. bryanti* has convergently or uniquely solved the challenge of protease inhibition.

#### Functional implications

It is interesting that naïve populations are able to physiologically respond to a completely novel toxin, creosote resin. Naïve populations of *N. bryanti* prefer to feed on plants that largely contain low-molecular-weight PSCs (Abreu et al., 2008; Atsatt and Ingram, 1983; Stintzing and Carle, 2005) that are unlikely to inhibit digestive enzymes. However, coastal live oak (*Quercus agrifolia*), which is

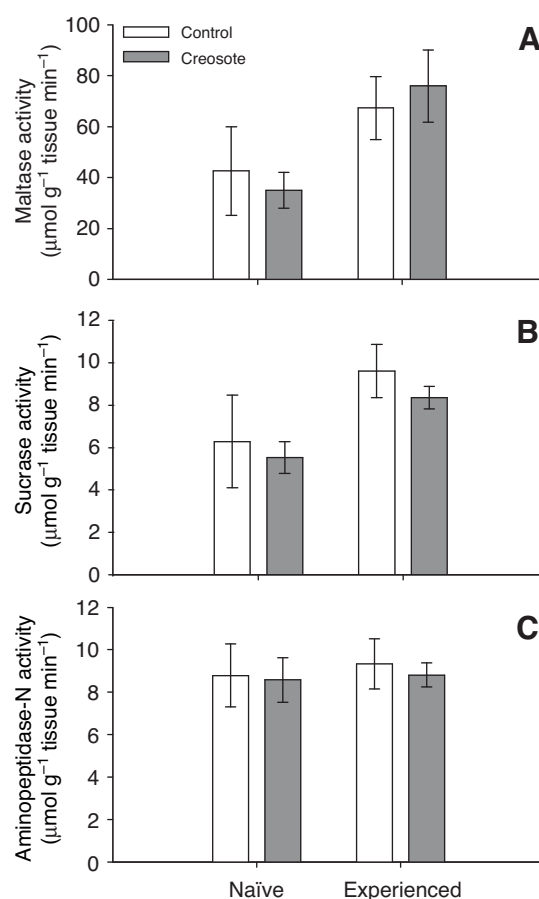


Fig. 3. Mean mass-specific activities of (A) maltase, (B) sucrase and (C) aminopeptidase-N from intestinal tissue of woodrats with different ecological and evolutionary experience with, as well as short-term exposure to, PSCs. Bars represent  $\pm 1$  s.e.m.

rich in phenolics and tannins, also grows within the native habitat of naïve *N. bryanti* (Atsatt and Ingram, 1983). Naïve *N. bryanti* are able to ingest and maintain body mass on a diet consisting only of oak (Skopec et al., 2008), but do not choose to consume oak when other food sources are available (Atsatt and Ingram, 1983). The physiological mechanisms by which naïve *N. bryanti* are able to respond to novel creosote toxins may be due to an ability to detect and respond to the phenolics and tannins present in both oak and creosote. These generic responses might allow naïve *N. bryanti* to feed on oak in times of intense intraspecific competition or during low abundance of preferred plant species.

Overall, the responses exhibited by digestive enzymes to creosote resin are expected to facilitate digestion and nutrient assimilation of poor-quality diets. We do not have digestibility data from *N. bryanti* feeding on control or creosote diets. However, the possibility exists that, because of the long-term exposure of desert populations of *N. bryanti* to creosote, their combination of both induced and constitutive responses might allow increased digestibility of creosote rich diets over naïve populations. Indeed, creosote resin does not alter energy digestibility in another experienced species, the desert woodrat (*N. lepida*) (Mangione et al., 2004). Digestibility trials comparing populations of *N. bryanti* on day 1 of a creosote diet treatment (presumably before induction of digestive enzymes) may also be necessary to address whether the constitutive differences found in this study are functionally significant.

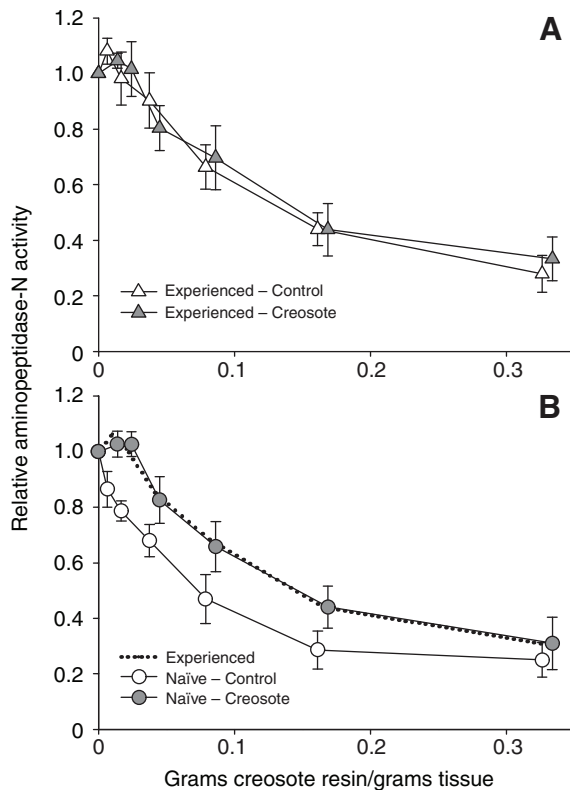


Fig. 4. Relative aminopeptidase-N activity with increasing creosote resin concentrations added *in vitro* for the (A) experienced population and (B) naïve population with differing dietary treatments. Dotted line in B represents the mean of all experienced individuals for comparison. Bars represent  $\pm 1$  s.e.m.

The results presented in this study are the first to show increases in digestive enzyme activities of terrestrial vertebrates in response to plant toxins, independent of changes in organ mass. We argue that the evolutionary and ecological experience with PSCs may be required to respond to such toxins. However, this hypothesis must be tested in other systems to determine whether this pattern is a general strategy for coping with PSCs. To better study the role of evolutionary experience in determining responses to PSCs, studies must be conducted to survey clades where herbivory has evolved several times independently, thus allowing for phylogenetic or taxonomic correction [e.g. liolaemid lizards (Espinoza et al., 2004), cyprinid minnows (German et al., 2010) or a wider survey of cricetid rodents (Samuels, 2009)]. Additionally, this question can be investigated at higher trophic levels. For example, insects are able to sequester PSCs into various organs or cuticular structures to then act as anti-predatory agents (Price et al., 1980). Thus, insectivores may come into contact with enzyme-inhibiting toxins, and may employ similar or novel mechanisms to overcome this challenge. These studies, combined with those conducted in insects, will allow better understanding the role of evolutionary history in determining interactions between toxins and the digestive system.

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