

## Effects of Fruit Toxins on Intestinal and Microbial $\beta$ -Glucosidase Activities of Seed-Predating and Seed-Dispersing Rodents (*Acomys* spp.)

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### ABSTRACT

Plant secondary compounds (PSCs) have profound influence on the ecological interaction between plants and their consumers. Glycosides, a class of PSC, are inert in their intact form and become toxic on activation by either plant  $\beta$ -glucosidase enzymes or endogenous  $\beta$ -glucosidases produced by the intestine of the plant-predator or its microbiota. Many insect herbivores decrease activities of endogenous  $\beta$ -glucosidases to limit toxin exposure. However, such an adaptation has never been investigated in nonmodel mammals. We studied three species of spiny mice (*Acomys* spp.) that vary in their feeding behavior of the glycoside-rich fruit of *Ochradenus baccatus*. Two species, the common (*Acomys cahirinus*) and Crete (*Acomys minous*) spiny mice, behaviorally avoid activating glycosides, while the golden spiny mouse (*Acomys russatus*) regularly consumes activated glycosides. We fed each species a nontoxic diet of inert glycosides or a toxic diet of activated fruit toxins and investigated the responses of intestinal and microbial  $\beta$ -glucosidase activities. We found that individuals feeding on activated toxins had lower intestinal  $\beta$ -glucosidase activity and that the species that behaviorally avoid activating glycosides also had lower intestinal  $\beta$ -glucosidase activity regardless of

treatment. The microbiota represented a larger source of toxin liberation, and the toxin-adapted species (golden spiny mouse) exhibited almost a fivefold increase in microbial  $\beta$ -glucosidase when fed activated toxins, while other species showed slight decreases. These results are contrary to those in insects, where glycoside-adapted species have lower  $\beta$ -glucosidase activity. The glycoside-adapted golden spiny mouse may have evolved tolerance mechanisms such as enhanced detoxification rather than avoidance mechanisms.

**Keywords:** *Acomys*, digestion, glycosides, gut microbes, plant-animal interactions, plant secondary compounds.

### Introduction

Plants produce various types of secondary compounds (PSCs) to deter consumption by animals (Dearing et al. 2005). Glycosides, a class of PSC, are stored in inactive forms by attaching a glucose molecule to a toxic moiety (the aglycone) through a  $\beta$ -glycosidic linkage (Morant et al. 2008). Glycosides in their intact forms are typically physiologically inert but can often be activated by  $\beta$ -glucosidase enzymes produced by plants. These enzymes are usually compartmentalized to avoid release of toxic aglycones within the plant and instead interact with glycosides upon physical disturbance, such as mastication by animals (Morant et al. 2008). The intestinal tracts of animals also contain  $\beta$ -glucosidase enzymes, which are produced by the gut tissue and symbiotic microbes. These  $\beta$ -glucosidase enzymes benefit animals by digesting nutritional compounds such as cellobiose and oligosaccharides (Yapi et al. 2009; Sharf et al. 2010). Additionally, the enzyme lactase phlorizin hydrolase is an intestinal  $\beta$ -glucosidase enzyme that hydrolyzes the milk sugar lactose (Karasov and Douglas 2013). However, these endogenous  $\beta$ -glucosidase enzymes, including lactase phlorizin hydrolase, can also hydrolyze plant glycosides, allowing the aglycones to be absorbed through intestinal tissue (Day et al. 2000; Németh et al. 2003). Thus, the hydrolysis of glycosides by endogenous enzymes represents a mechanism by which animals might inadvertently increase the toxicity of their diets.

In order to avoid the release of toxic compounds, many insects reduce activities of midgut  $\beta$ -glucosidases in response to diets containing glycosides (Lindroth 1988; Desroches et al. 1997; Ferreira et al. 1997; Pankoke et al. 2010, 2012). It is also thought that glycosides are nontoxic to birds due to their low activities of endogenous  $\beta$ -glucosidases (Struempf et al. 1999). However, such a physiological adaptation has never been

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investigated in mammals that naturally consume glycosides. Additionally, the gut microbiota play a large role in the ecology and evolution of their hosts (McFall-Ngai et al. 2013) and have a greater capacity to hydrolyze glycosides than host intestinal tissue (Nakano and Gregory 1995). However, the response of gut microbial  $\beta$ -glucosidase activities to dietary glycosides has not been studied in natural systems.

We investigated the response of endogenous  $\beta$ -glucosidase activities to dietary glycosides in three species of spiny mice (*Acomys* spp.) that differ in their consumption of the glycoside-rich fruit of sweet mignonette (*Ochradenus baccatus* Delile). The principal defense compounds in *O. baccatus* are glucosinolates (Lotan and Izhaki 2013), which contain a sulfur atom between the glucose and aglycone group and must be activated by myrosinase or thioglucoside glucohydrolase enzymes (Kliebenstein et al. 2005). The vertebrate gut apparently has little to nil capacity to hydrolyze glucosinolates (Lessner et al. 2015), and so there is little to no opportunity for vertebrates to limit exposure to these toxic compounds via changes in enzyme activities. However, in addition to glucosinolates, *O. baccatus* produces a number of typical O-glycosides (Barakat et al. 1991; Hussain et al. 2014), which can be acted on by  $\beta$ -glucosidase enzymes (Ketudat Cairns and Esen 2010). Physiological adaptations in the activities of  $\beta$ -glucosidase enzymes have not yet been investigated in vertebrates, though this idea has been suggested (Majak 1991).

Previous work on this system has demonstrated that three species of spiny mice (*Acomys* spp.) vary in their behavioral and physiological adaptations to the toxins of *O. baccatus*. The toxic components of *O. baccatus* fruit are compartmentalized, such that the glycosides are stored in the fruit pulp and glucosidase enzymes are stored in the seeds (Samuni-Blank et al. 2012). Thus, toxic aglycones are released only if a seed predator crushes the seeds while consuming the pulp. In behavioral trials we previously reported that two species of *Acomys*, the common spiny mouse (*Acomys cahirinus* Geoffrey) and Crete spiny mouse (*Acomys minous* Bate), are not likely to activate glycosides, as they prefer to consume only the pulp or seed, respectively, and discard the other component (Samuni-Blank et al. 2013a, 2013b). The golden spiny mouse (*Acomys russatus* Wagner) crushes the seed and pulp of *O. baccatus* together and is therefore exposed to activated glycosides (Samuni-Blank et al. 2013a). Thus, these species interact with *O. baccatus* very differently, with the common spiny mouse acting as seed disperser and the Crete and the golden spiny mice acting as seed predator. Further, when these species are fed diets of mashed fruit and seeds (thus activating the toxins), the common and Crete spiny mice drastically reduce food intake and lose 15%–20% of their body mass, while the golden spiny mouse exhibits higher food intake and only loses ~10% of body mass (Samuni-Blank et al. 2013b, 2014). These results suggest that the golden spiny mouse has physiological adaptations for coping with these toxins.

We fed spiny mice nontoxic diets containing only the pulp of *O. baccatus* (pulp diet) or toxic diets containing pulp and crushed seeds (mash diet), which results in activation of glycosides. We measured the intestinal and microbial activities of

two  $\alpha$ -glucosidases (maltase, sucrase) and two  $\beta$ -glucosidases (salicinase, amygdalinase). We predicted that the toxin-adapted species, the golden spiny mouse, would show lower constitutive intestinal and microbial  $\beta$ -glucosidase activities compared to other spiny mice species. We also predicted that intestinal and microbial  $\beta$ -glucosidase activities would decrease in all species in response to the toxic diet. These predictions are consistent with what has been observed in insects (Lindroth 1988; Desroches et al. 1997; Ferreira et al. 1997; Pankoke et al. 2010, 2012). We also measured  $\alpha$ -glucosidase activities (maltase, sucrase) with the expectation that these activities would not be affected by diet, given that they do not interact with glycosides. However, some glycosides have the potential to inhibit digestive enzymes (Silva et al. 2006), and, thus, measurements of  $\alpha$ -glucosidases were useful to investigate the possibility of universal changes in enzyme activities, even in nontarget enzymes.

## Methods

### Animals

All samples were collected from animals used in other studies (Samuni-Blank et al. 2013b). While these animals had been in captivity for a period of time, our findings are likely still ecologically relevant. First, the differential feeding behavior of species was observed in both wild and captive-bred individuals (Samuni-Blank et al. 2012, 2013a, 2013b), suggesting that the differential tolerance to fruit toxins does not change with captivity. Second, interspecific differences in microbial communities are often maintained when animals are brought into captivity (Fraune and Bosch 2007; Kohl et al. 2014b). Briefly, individuals of all three species were housed individually in standard mouse cages (21 cm  $\times$  31 cm  $\times$  13 cm) in a temperature-controlled room ( $25^\circ \pm 2^\circ\text{C}$ ) under a 12L:12D cycle. Animals were fed carrots and rodent chow (Koffolk 19510, Tel Aviv, Israel). During the diet trial, animals were fed either a pulp diet (rodent chow mixed with homogenized *Ochradenus baccatus* pulp, which contains only inert, intact glycosides) or a toxic mash diet (rodent chow mixed with pulp and crushed seeds; myrosinase in the seeds activates the pulp toxins). Animals were given a diet of 25% fruit (pulp or mash) for 1 d, followed by 50% fruit (pulp or mash) for 3 d. Sample sizes for the experiment were as follows: common spiny mouse: 8 pulp, 8 mash; Crete spiny mouse: 6 pulp, 7 mash; golden spiny mouse: 8 pulp, 8 mash. Following the trial, animals were euthanized with  $\text{CO}_2$  and immediately dissected. Entire contents were extruded from the small intestine, placed on dry ice to freeze, and later stored at  $-80^\circ\text{C}$ . Next, the small intestine was cut in half longitudinally. Half of the small intestine was placed on dry ice to cause flash freezing and was later frozen at  $-80^\circ\text{C}$  for storage, while the other half was placed in RNAlater for other experiments. Tissues were transported on dry ice to the University of Utah (Salt Lake City) for enzyme analysis. All protocols were approved by the Committee of Animal Experimentation of the University of Haifa (permit 096/08) and the University of Utah Institutional Animal Care and Use Committee (protocol 10-01013).

### Enzyme Assays

We assayed glucosidase activities using a modification of a previously developed colorimetric method (Dahlqvist 1984). Enzyme activities were measured either in intestinal tissue or from gut contents, which estimates microbial  $\beta$ -glucosidase activity (Banks et al. 1994; Nakano and Gregory 1995; Hylla et al. 1998). We measured the activities of two  $\alpha$ -glucosidase enzymes that hydrolyze nutrients, maltase and sucrase, to investigate the possibility of universal effects of glycosides on digestive enzymes. Additionally, we measured the hydrolase activities against two  $\beta$ -glycosides, amygdalin and salicin. It should be noted that amygdalinase and salicinase activities are not the direct measurements of activities of specific enzymes but rather the capacity for a number of glucohydrolase enzymes to hydrolyze these substrates. These substrates have been widely used to estimate the  $\beta$ -glucosidase activity toward plant-derived glycosides (Adewusi and Oke 1985; Lindroth 1988; Ferreira et al. 1997; Lessner et al. 2015). Briefly, intestinal tissue or luminal contents were thawed and homogenized in 350 mM mannitol in 1 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)-KOH, pH 7.0. Homogenates (30  $\mu$ L) diluted with 350 mM mannitol in 1 mM Hepes-KOH were incubated with 30  $\mu$ L of 56 mM maltose, sucrose, amygdalin, or salicin in 0.1 M maleate and NaOH buffer, pH 6.5, at 37°C for 20 min. Next, 400  $\mu$ L of a stop-develop reagent (GAGO-20 glucose assay kit; Sigma-Aldrich, St. Louis, MO) was added to each tube, vortexed, and incubated at 37°C for 30 min. Last, 400  $\mu$ L of 12 N H<sub>2</sub>SO<sub>4</sub> was added to each tube to stop the reaction. Blank tubes were used to control for endogenous glucose present in tissues. These blank tubes contained the same reagents and tissues as those used for measuring activity, but the substrates were added after the addition of H<sub>2</sub>SO<sub>4</sub> to prevent the enzymatic reactions from occurring. Several 200- $\mu$ L aliquots of the final reactions were transferred to a 96-well plate, and the absorbance was read at 540 nm using a BioTek (Broadview, IL) PowerWave HT microplate spectrophotometer. Protein content was measured using a Bradford assay with a standard curve generated using bovine serum albumin.

### Statistics

Activities of  $\beta$ -glucosidase were transformed using a  $(x + 1)^{0.5}$  transformation (McDonald 2014). Enzyme activities were compared with two-way ANOVAs, using diet and species as main effects, with JMP 12.0 and an  $\alpha$  value of 0.05. Differences among groups were investigated using Tukey's HSD test. Using these same animals, we have previously demonstrated that these species respond differently to the toxic and nontoxic diets in terms of food intake and changes in body mass (Samuni-Blank et al. 2013b). Given that food intake and fasting can influence digestive enzyme activities (McNeill and Hamilton 1971), we included the following variables as covariates: total food consumption over the 4-d trial normalized to body mass<sup>3/4</sup> and percent change in body mass. Also, to investigate whether differences in  $\beta$ -glucosidase activities were specific or perhaps due to universal differences in activities of all glucosidases, we conducted ANCOVAs for salicinase and amygd-

dalase using maltase activity as a covariate. Nonsignificant covariates ( $P > 0.05$ ) were removed from the final analyses.

### Results

Measurements of  $\beta$ -glucosidase enzyme activities did not covary with normalized food intake, percent change in body mass, or maltase activities, and thus all covariates were removed from the final models. Intestinal activities of all glucosidases differed significantly between species, with the golden spiny mouse exhibiting the highest activities (table 1; fig. 1). Intestinal activities of  $\alpha$ -glucosidases (maltase, sucrase) and amygdalase showed no significant diet effect. There was a significant effect of diet on intestinal salicinase activity (table 1; fig. 1), such that individuals fed the activated mash diet exhibited salicinase activity that was 34%–69% lower than those fed the pulp diet.

There were no significant differences between species or diet treatments on the microbial activities of  $\alpha$ -glucosidases (maltase, sucrase) or the  $\beta$ -glucosidase amygdalase (table 1; fig. 2). Interestingly, microbial salicinase activity showed a highly significant diet  $\times$  species interaction. When feeding on the mash diet, the microbial salicinase activity of the common and Crete spiny mice was 31%–66% lower compared to that of those fed the nontoxic pulp diet, whereas the golden spiny mice fed the toxic mash diet exhibited a 4.8-fold higher microbial salicinase activity compared to animals fed the pulp diet.

### Discussion

The physiological responses of  $\beta$ -glucosidase enzymes to dietary glycosides have been studied in a number of insect systems. This study represents the first investigation into these responses in nonmodel species of mammals. We predicted that the toxin-adapted species, the golden spiny mouse (Samuni-Blank et al. 2013a), would show lower constitutive intestinal and microbial  $\beta$ -glucosidase activities and that all species would decrease intestinal and microbial  $\beta$ -glucosidase activities in response to dietary glycosides, that is, the activated mash diet. Many of our predictions were not supported. In contrast to our prediction for across-species differences, the golden spiny mouse had the highest activities of intestinal and microbial  $\beta$ -glucosidases. Although intestinal salicinase activity declined as predicted when ingesting the toxic diet, there was no significant effect of diet on amygdalinase activity. Furthermore, microbial  $\beta$ -glucosidase activity declined in the common and Crete spiny mice consuming the mash diet; however, it increased in the golden spiny mouse. Thus, our hypothesis that the golden spiny mouse may limit toxin exposure by lowering rates of intestinal hydrolysis was not supported. Rather, this species may exhibit tolerance mechanisms, such as enhanced hepatic detoxification. We discuss these findings below, as well as potential ecological implications.

#### Intestinal $\beta$ -Glucosidases

The golden spiny mouse showed the highest intestinal  $\beta$ -glucosidase activities (salicinase, amygdalinase). This species

Table 1: ANOVA results for intestinal and microbial enzyme activities

	Intestine			Microbiota		
	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>
Maltase:						
Diet	1.62	1, 39	.21	2.06	1, 39	.16
Species	12.33	2, 39	<b>&lt;.0001</b>	1.73	2, 39	.19
Diet $\times$ species	1.36	2, 39	.27	.07	2, 39	.92
Sucrase:						
Diet	.17	1, 39	.68	.28	1, 39	.60
Species	17.89	2, 39	<b>&lt;.0001</b>	3.01	2, 39	.06
Diet $\times$ species	.46	2, 39	.63	.02	2, 39	.98
Salicinase:						
Diet	4.26	1, 39	<b>.046</b>	1.52	1, 39	.23
Species	3.51	2, 39	<b>.039</b>	4.57	2, 39	<b>.016</b>
Diet $\times$ species	.70	2, 39	.50	6.45	2, 39	<b>.004</b>
Amygdalase:						
Diet	2.51	1, 39	.12	.03	1, 39	.87
Species	4.87	2, 39	<b>.013</b>	2.12	2, 39	.13
Diet $\times$ species	.09	2, 39	.91	.52	2, 39	.60

Note. Salicinase and amygdalase activities were transformed using a  $(x + 1)^{0.5}$  transformation before statistical analysis. Bolded items are statistically significant ( $P < 0.05$ ).

also showed the highest activities of intestinal  $\alpha$ -glucosidases (maltase, sucrase). This pattern could suggest that all intestinal glucosidases are under universal regulation in these species. However,  $\beta$ -glucosidase activities did not covary with maltase activity. In addition, the mash diet decreased intestinal salicinase activities but had no effect on maltase or sucrase activities. Thus, it seems more likely that activities of intestinal  $\beta$ -glucosidases are regulated separately from  $\alpha$ -glucosidases.

The golden spiny mouse exhibited higher intestinal  $\beta$ -glucosidase activity than the other spiny mouse species, which should serve to increase the bioavailability of toxic aglycones. This result did not support our predictions and is contrary to what has been reported for beetle and lepidopteran larvae, where adaption to dietary glycosides is associated with reduced constitutive  $\beta$ -glucosidase activities (Desroches et al. 1997; Lindroth 1988). However, due to the seed-crushing behavior of the golden spiny mouse, its intestinal tissue and enzymes are more likely to encounter toxic aglycones rather than intact glycosides. Thus, the avoidance mechanism of low intestinal  $\beta$ -glucosidase activity may be at best irrelevant in the golden spiny mouse and at worst disadvantageous because  $\beta$ -glucosidases digest some nutritional compounds such as lactose, cellobiose, and oligosaccharides (Yapi et al. 2009; Scharf et al. 2010). The effects that these changes in enzyme activities might have on the digestion of nutrients require further investigation. The golden spiny mouse may have instead evolved tolerance mechanisms, such as enhanced hepatic detoxification, to cope with high levels of dietary glycosides. The avoidance mechanism is instead observed in the common spiny mouse, which exhibits the lowest activities of intestinal  $\beta$ -glucosidases and also behaviorally avoids toxins by separating the seed and pulp. In this case, low activities of  $\beta$ -glucosidases may reduce its exposure to toxic aglycones.

In all species, we observed a significant decrease in intestinal salicinase activity on the activated mash diet. Again, this response was independent of regulation of maltase and sucrase and so is likely to be a targeted response to dietary glycosides. Lowering intestinal  $\beta$ -glucosidase activities might prevent further exposure to toxins. For this mechanism to be effective,  $\beta$ -glucosidase activities would need to be downregulated in response to glycosides or aglycone molecules. While downregulation of  $\beta$ -glucosidase activities in response to aglycones has not been studied in terms of toxic glycosides, there is precedence for it in other systems. Both dietary pyridoxine-glucoside and the aglycone pyridoxine cause decreases in intestinal  $\beta$ -glucosidase activity in laboratory rats (Nakano and Gregory 1995) and guinea pigs (Banks et al. 1994). Interestingly, the aglycone pyridoxine is more potent at reducing intestinal  $\beta$ -glucosidase activities than pyridoxine-glucoside (Nakano and Gregory 1995), perhaps due to higher rates of absorption (Németh et al. 2003). Concentrations of PSCs in the blood are thought to be important for determining animals' physiological and behavioral responses (McLean and Duncan 2006; Torregrossa and Dearing 2009). Thus, the higher concentrations of easily absorbable, toxic aglycones in the activated mash may underlie the lower intestinal  $\beta$ -glucosidase activities in spiny mice fed this diet. It would be interesting to investigate the mechanisms of regulation of  $\beta$ -glucosidase activity in response to purified glycosides and aglycones in an ecologically relevant context.

#### Microbial $\beta$ -Glucosidases

The activity of intestinal microbial  $\beta$ -glucosidase activity per unit protein was 3.5–6 times that of intestinal tissue. These results are similar to findings in rats, where intestinal microbial

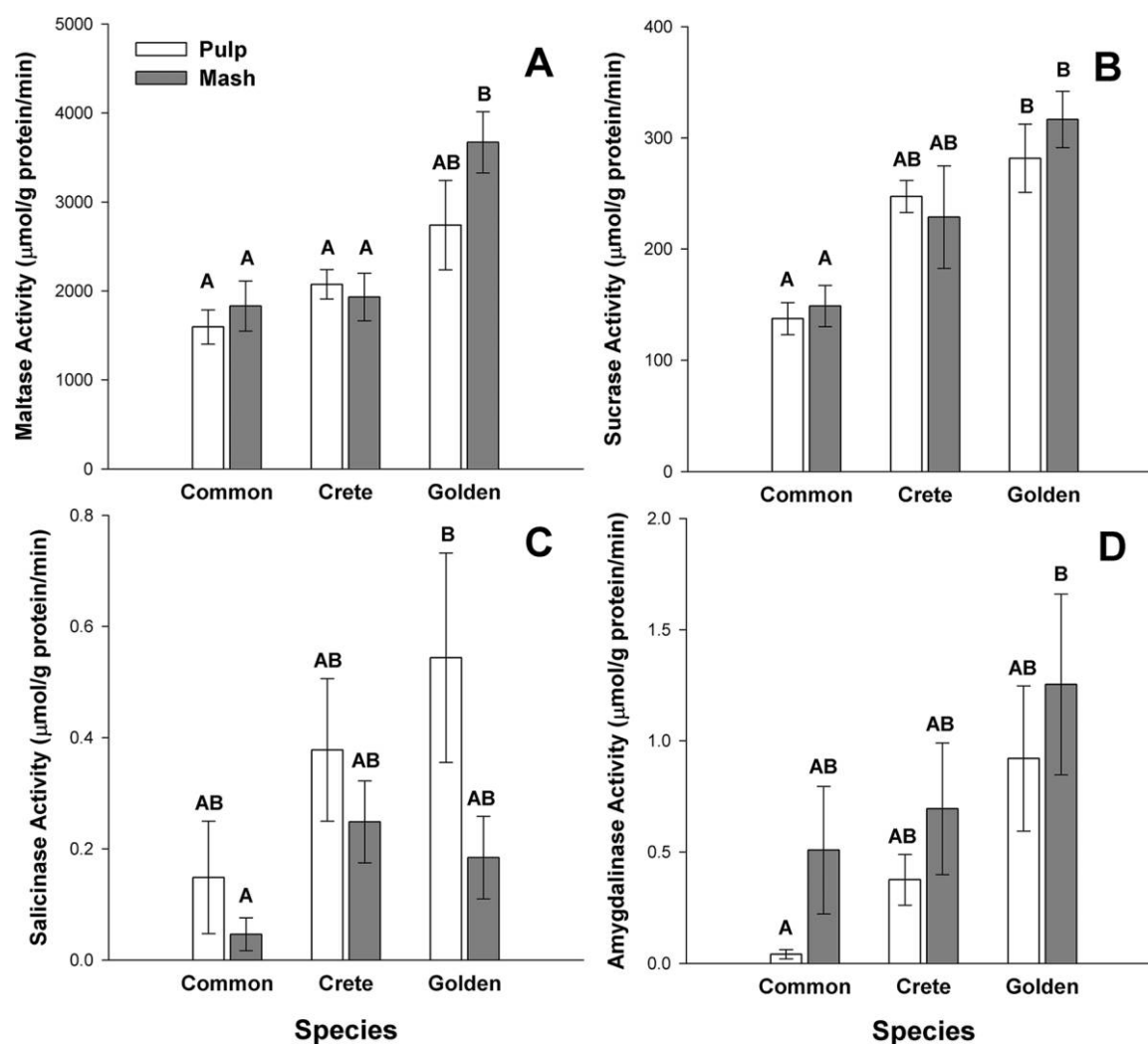


Figure 1. Mean activities of maltase (A), sucrase (B), salicinase (C), and amygdalinase (D) from host intestinal tissues of common (*Acomys cahirinus*), Crete (*Acomys minous*), and golden (*Acomys russatus*) spiny mice. Mice were fed either a nontoxic pulp diet or a toxic mash diet. Bars represent means  $\pm$  SEM. Graphs depict original data, though statistical analyses for salicinase and amygdalinase were conducted on transformed data. Bars not sharing letters are significantly different as determined by Tukey's HSD test.

activity was  $\sim 10$  times higher than intestinal tissue (Nakano and Gregory 1995). These results are remarkable given that the small intestine harbors the lowest density of microbial cells across the gut (Kohl et al. 2014a). The techniques used here have been widely used to estimate both intestinal and microbial  $\beta$ -glucosidase activities (Hylla et al. 1998; Banks et al. 1994; Nakano and Gregory 1995). It could be argued that activity detected in the gut lumen may be driven by the sloughing off of intestinal cells with active enzymes. However, we are confident that we primarily measured microbial activity. First, previous studies have utilized fluorescent staining of the gut lumen of rodents to reveal that it is overwhelmingly dominated by bacteria and not intestinal cells (Johansson et al. 2008). Second,  $\beta$ -glucosidase activities from the intestine and the lumen showed different responses to glycosides, such that intestinal salicinase activity decreased on the toxic diet while luminal activity increased. If luminal activity were primarily driven by intestinal

activity, we would predict the same direction of change in these two measurements. However, the possibility exists that luminal activities were partially driven by intestinal enzymes or sloughed mucosal cells. Studies with germ-free mice (those lacking a microbiota) would elucidate the proportion of luminal activity generated by microbes.

In our experiment, we observed a strong diet  $\times$  species interaction in microbial salicinase activity, such that the common and Crete spiny mice exhibited lower activity when feeding on the mash diet, while the golden spiny mouse exhibited higher activity. Results from the golden spiny mouse follow previous research showing induction of microbial  $\beta$ -glucosidase by both pyridoxine-glucoside and the aglycone pyridoxine (Banks et al. 1994; Nakano and Gregory 1995). Induction of microbial  $\beta$ -glucosidase in a toxin-adapted species may facilitate the microbial metabolism of aglycone molecules (Keppler and Humpf 2005). However, in the common and Crete spiny mice we see lower

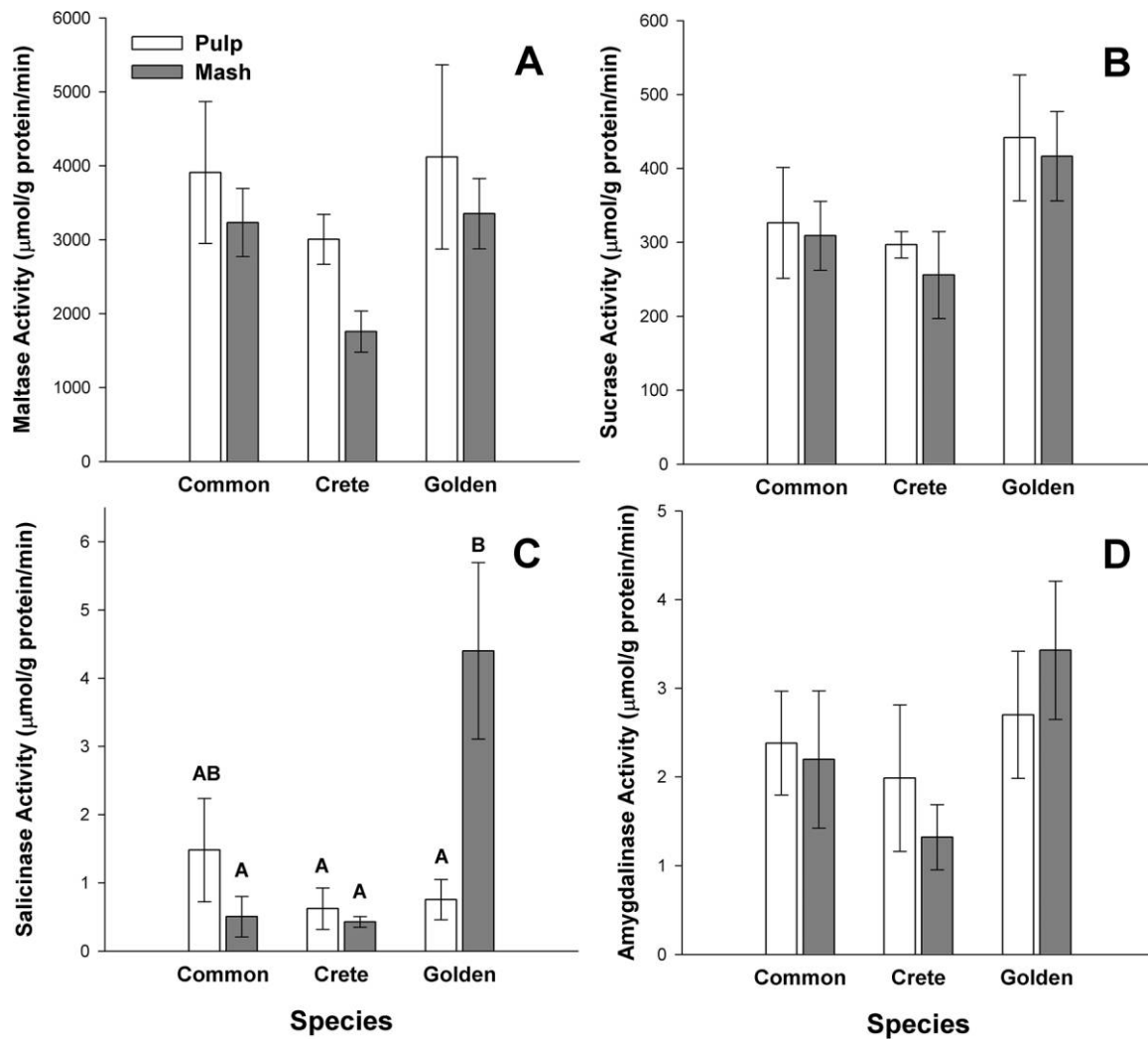


Figure 2. Mean activities of microbial maltase (A), sucrase (B), salicinase (C), and amygdalinase (D) in common (*Acomys cahirinus*), Crete (*Acomys minous*), and golden (*Acomys russatus*) spiny mice. Mice were fed either a nontoxic pulp diet or a toxic mash diet. Bars represent means  $\pm$  SEM. Graphs depict original data, though statistical analyses for salicinase and amygdalinase were conducted on transformed data. Bars not sharing letters are significantly different as determined by Tukey's HSD test.

microbial  $\beta$ -glucosidase activity when feeding on the mash diet. This reduction in activity may benefit the host by limiting hydrolysis of glycosides, allowing them to be excreted in the feces. Hosts exert strong selection on which bacteria flourish in the gut (Rawls et al. 2006), and host experience with plant toxins exerts a selective force on gut microbial community structure (Kohl and Dearing 2012). Thus, each species may house microbial populations that facilitate either tolerance or avoidance of glycosides, a possibility that warrants further study.

### Conclusions

Species that behaviorally avoid the liberation of glycosides (by consuming only seeds or only pulp) also avoid endogenous hydrolysis by intestinal and microbial enzymes. The golden

spiny mouse readily consumes activated glycosides and shows less physiological avoidance to hydrolysis. Thus, it is likely that the golden spiny mouse has adapted tolerance mechanisms to deal with these toxic compounds. Such adaptations may influence the feeding behavior of the studied species, thus altering their ecological role as either seed dispersers or seed predators.

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