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Bitter avoidance in Guinea Pigs (*Cavia porcellus*) and Mice (*Mus musculus* and *Peromyscus leucopus*)

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Abstract

Rejection of bitter substances is common in many species and may function to protect an animal from ingestion of bitter-tasting toxins. Since many plants are bitter, it has been proposed that high tolerance for bitterness would be adaptive for herbivores. Earlier studies conducted on herbivorous guinea pigs (*Cavia porcellus*) have been used to support this proposal. We tested guinea pigs with bitter plant secondary metabolites (salicin, caffeine, quinine hydrochloride) and bitter protein hydrolysates (two types of hydrolyzed casein, hydrolyzed soy) in a series of two-choice preference tests. For comparison, we tested two non-herbivorous mouse species (*Mus musculus* and *Peromyscus leucopus*). Guinea pigs did show weaker avoidance of QHCl than did the mice, confirming predictions generated from earlier work. However, guinea pigs had similar responses to caffeine as did *Peromyscus*. Both of these species showed weaker avoidance responses than *Mus* to 10 mM caffeine. For salicin, guinea pigs were the only species to avoid it at 10 mM and their preference scores at this concentration were significantly lower than for the two mice species. Guinea pigs avoided all of the protein hydrolysates more strongly than the other species. Responses to the protein hydrolysates did not reflect the patterns observed with the simple bitter compounds, suggesting that other properties of these complex stimuli may be responsible for guinea pig avoidance of them. Our results suggest caution in accepting, without further empirical support, the premise that guinea pigs (and herbivores in general) have a generalized reduced bitter sensitivity.

Keywords

Cavia; *Mus*; *Peromyscus*; two-choice test; hydrolysate; plant secondary metabolite

The tendency to reject bitter tastants (defined herein as stimuli perceived as bitter by humans) is often assumed to be an adaptation that protects animals from consuming toxic foods (Bachmanov & Beauchamp, 2007; Chandrashekar, Hoon, Ryba, & Zuker, 2006; Garcia & Hankins, 1975; Meyerhof, 2005). Given variability in species' responses to bitter and that many edible plants contain bitter compounds (Drewnowski & Gomez-Carneros, 2000), Jacobs (1978) suggested that it might be maladaptive for herbivores to have low

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tolerance for bitter compounds, which would drastically reduce their dietary options. Extending this hypothesis to other trophic levels, Glendinning (1994) argued that bitter taste thresholds should have co-evolved with and be reflective of the frequency of bitter compounds and the relative toxin loads of an animal's typical dietary environment (also see Ruxton & Kennedy, 2006). In empirical tests, guinea pigs (*Cavia porcellus*) have served as an exemplar to support the contention that herbivores have "poorly developed" bitter taste (Lindemann, 1996, p. 736), which was based on guinea pig performance in two-bottle tests with sucrose octaacetate (SOA) and quinine sulfate (Jacobs, 1978) and in trials with chow treated with denatonium benzoate, denatonium saccharide, limonene, L-phenylalanine, naringin, quebracho, QHCl, and SOA (Nolte, Mason, & Lewis, 1994).

The purpose of the work described herein was to expand the number and type of bitter tastants with which a model herbivore, the guinea pig, has been preference tested, and to conduct testing such that results could be compared directly to other species. We asked how the guinea pig would respond to three bitter plant secondary metabolites (salicin, caffeine, and quinine), given the prevailing expectation that herbivores have an elevated bitter tolerance in comparison to non-herbivores (here, *Mus musculus* and *Peromyscus leucopus*). Testing with quinine hydrochloride (QHCl), a prototypical stimulus that has been used in much of the published work on bitter taste, had the additional advantage of providing a meaningful comparison to earlier studies. Additionally, we presented novel protein hydrolysates (two animal- and a plant-based mixture(s) of peptides and amino acids having bitter components) to the three species. We predicted that herbivores avoid degraded food-based proteins, whether they are animal- or plant-based, more strongly than do non-herbivores. An earlier study with two different hydrolysates found that herbivores showed stronger avoidance than did the omnivores (Field, Bachmanov, Mennella, Beauchamp, & Kimball, 2009). We expected that the results of the work described here—a comparison of species' responses to a variety of novel bitter stimuli that addresses whether guinea pigs show greater tolerance of bitter stimuli relative to the non-herbivores—would inform future mechanistic predictions on how or why particular species respond to bitterness as they do.

Methods

Species and maintenance conditions

We purchased 15 guinea pigs (7 females) and 18 *M. musculus* ("laboratory mice," outbred CD1 strain, 9 female) from Charles River Laboratories (Wilmington, MA), and 16 *P. leucopus* ("white-footed mice," 8 female) from the *Peromyscus* Genetic Stock Center (Univ. of South Carolina, Columbia, SC). Animals were housed individually on a 12L:12D schedule (lights on at 0730 h) and had ad libitum access to chow throughout testing (Rodent Diet 8604, Harlan Teklad, Madison, WI; Guinea Pig Chow 5025, Dyets, Inc., Bethlehem, PA).

Test stimuli—Salicin (Bosche Scientific, New Brunswick, NJ), caffeine (Alfa Aesar, Ward Hill, MA) and QHCl (Sigma-Aldrich, St. Louis, MO) were diluted in deionized water (referred to as "water") to make the following concentrations: salicin (1 mM, 10 mM, 100 mM), caffeine (3 mM, 10 mM, 50 mM), and QHCl (0.02 mM, 0.2 mM, 2 mM). Concentrations were chosen based on published and pilot data to evoke a range of responses from no or weak avoidance to strong avoidance. In preference tests, each of these compounds was paired against an alternative choice ("vehicle") of water. For hydrolyzed protein tests, we used two types of casein (HC1, HC2) and a soy (Hsoy) hydrolysate (DMV International, Delhi, NY). Hydrolysates were characterized by the manufacture as having the following properties: HC1 (Product CE90GBT), 19% degree of hydrolysis (DH), average molecular weight 1300 Daltons; HC2 (Product CE90STL), 39% DH, ave. mol. wt. 380 Da;

Hsoy (Product SE70M), 21% DH, ave. mol. wt. 833 Da. Hydrolysates were made into 2%, 10%, and 15% w/v emulsions by adding the hydrolysates to 0.3% w/v xanthan gum (Sigma-Aldrich, St. Louis, MO) solutions, which have no or minimal flavor to mice (Bachmanov, Reed, Tordoff, Price, & Beauchamp, 2001). Subjects could choose between a particular hydrolysate emulsion and the 0.3% xanthan gum vehicle, except for HC1 at 2% concentration, which did not contain gum and was tested against a water alternative. Subjects were naïve to test stimuli used in this study.

Two-bottle preference test procedures

Mouse “bottles” were constructed from 25-ml polystyrene serological pipettes. Two bottles were placed on top of each cage so that the sipper tubes were accessible to the subjects (for details, see Bachmanov, Reed, Beauchamp, & Tordoff, 2002; Tordoff & Bachmanov, 2001). Guinea pigs were tested with 950-ml Macrolon bottles fitted with a rubber stopper through which a 10-cm bent stainless steel sipper tube was placed (Catalog #s: M32, TD-300, S-100; Ancare Corp., Bellmore, NY). Two bottles were attached to the outside back grid of each cage so the sipper tubes protruded into the cages, approximately 8 cm apart.

Both bottles were filled with water for 4 d to acclimate the subjects. Stimuli were then presented in the following order: salicin, caffeine, QHCl, HC1, Hsoy, and HC2. Each stimulus was paired with its vehicle and concentrations were presented in ascending order for 2 d each, with 1 d of water-water access between the different stimuli. Technical issues (e.g., leakage, lack of solubility) prevented inclusion of the two higher concentrations of Hsoy and HC2, as well as andrographolide, a plant metabolite presented between caffeine and QHCl.

The position of the stimulus relative to the vehicle was randomized starting with the salicin, and then alternated daily to counterbalance for any positional biases. Mouse intake was estimated by the reduction in volume after 24 h. For the guinea pigs, the difference in bottle weight after 24-h access was used to estimate consumption. Measurements were made during the middle of the light period. Procedures were approved by the Monell Chemical Senses Center IACUC (ACC #1120).

Analyses

Preference scores (“scores”) were calculated as the proportion of the total fluid consumed (vehicle + stimulus) that comprised the test fluid. The two 24-h scores for each concentration of a stimulus were averaged for each subject to produce a mean score for the 2 d of testing. Scores were arcsin square root transformed (Sokal & Rohlf, 1995, p. 419–422) for statistical tests. For missing data, the second day’s measure at that concentration was also discarded for that individual, since the water v water days (not shown) indicated that individuals in all species often had strong positional biases. Degrees of freedom, therefore, varied among tests.

We used repeated measures analysis of variance (RM ANOVA) models for salicin, caffeine, QHCl and HC1 to determine if differences in scores could be attributed to species (between-subjects factor), stimulus concentration (within-subjects factor) or to any interactions. Species differences at particular concentrations were assessed using Tukey Honestly Significant Differences (HSD) post-hoc tests. For each of the Hsoy and HC2 low concentrations, one-way ANOVAs were performed. Using an RM ANOVA (between factor: species; within factor: hydrolysate), scores at the 2% concentrations of the hydrolysates were compared to determine whether hydrolysate type (HC1, Hsoy, HC2) could explain data patterns. An alpha level of 0.05 was used for ANOVAs and HSD tests.

For all stimuli, we conducted t-tests against a hypothesized mean score of 0.5, which would indicate equal consumption of a stimulus and its vehicle. “Preference” and “avoidance” were operationally defined as scores that were statistically significantly above or below 0.5, respectively. For t-tests, we adjusted the alpha level using the Dunn-Sidak correction for multiple comparisons to $\alpha'=0.0037$ (14 tests on each species’ data).

Results

For all tested stimuli, species differences were statistically significant (ANOVAs; Table 1). For salicin, caffeine, QHCL and HC1, effects of concentration and the interaction between species and concentration were also statistically significant (Table 1). Species differences at each concentration and results for the other hydrolysates are shown in Figure 1.

All species were indifferent to 1 mM of salicin (i.e., scores did not significantly differ from 0.5) and avoided the salicin at 100 mM (i.e., scores significantly lower than 0.5). At the 10 mM concentration, guinea pigs avoided salicin, while both species of mice were indifferent to it. Relative to each other, the species showed similar scores for the low and high concentrations (Tukey’s HSD P -values >0.05). At the intermediate concentration, the laboratory and white-footed mice had similar scores (Tukey’s HSD $P=1.0$), which were higher than the guinea pig mean score (Tukey’s HSD P -values <0.001).

All species were indifferent to caffeine at 3 mM and avoided it at 10 mM and 50 mM. All species had similar scores at the lowest and highest concentrations (Tukey HSD P s >0.05). At 10 mM, the laboratory mice had lower scores (Tukey HSD P s <0.021) than either of the other species, which had similar scores (Tukey HSD P >0.05).

Guinea pigs and white-footed mice were indifferent to 0.02 mM QHCL, while the laboratory mice avoided it. At 0.2 mM, guinea pigs remained indifferent to the QHCL, while mice of both species avoided it. At 2 mM, all species avoided the QHCL. Scores of the mouse species did not differ statistically from each other (Tukey HSD P s >0.05) at any concentration. The guinea pig scores were not statistically different (Tukey HSD P s >0.05) from either mouse species at 0.02 mM. At 0.2 mM, guinea pigs had higher scores than either mouse species (Tukey HSD P -values <0.002). At the highest concentration, guinea pig scores were higher than those of the laboratory mice (Tukey HSD $P=0.006$), while scores of the white-footed mice were intermediate and did not differ statistically from the other species.

For the first hydrolysate, at all concentrations, the guinea pigs avoided the HC1, the laboratory mice preferred it, and the white-footed mice were indifferent to it. Regardless of concentration, laboratory mouse scores were higher than those of the other species, while guinea pig scores were lower than those of the other species (Tukey HSD P -values <0.012).

Data from the 2% concentrations of Hsoy and HC2 were consistent with the HC1 patterns. Guinea pigs avoided both the Hsoy and HC2, laboratory mice preferred them, and white-footed mice were indifferent to them. Pair-wise comparisons between the species’ scores (Tukey HSD P -values <0.002) indicated that the guinea pigs had the lowest scores, white-footed mice had intermediate scores and laboratory mice had the highest scores. Hydrolysate type did not explain variance in the 2% hydrolysate scores ($F_{2,76}=1.43$, $P=0.246$), while species could explain the variance ($F_{2,76}=109.13$, $P<0.001$; Tukey’s HSD P -values <0.001) and there was no interaction between them ($F_{4,76}=0.50$, $P=0.736$).

Discussion

Expanding the number of bitter stimuli with which guinea pigs have been tested revealed that these herbivores do not always avoid bitter stimuli at higher concentrations than do non-

herbivores. Guinea pigs avoided salicin at 10 mM, while the mouse species were indifferent towards it. Salicylic acid commonly mediates several plant pathogen defense pathways (e.g., Hammerschmidt & Smith-Becker, 1999) and may warn plant eaters of diseased plant material. Recent sequencing of the guinea pig genome found a putative ortholog to the human *TAS2R16* gene

(www.ensembl.org/Cavia_porcellus/Gene/Summary?g=ENSCPOG00000007419; accessed Nov. 11, 2009), for which salicin has been identified as a ligand (Bufe, Hofmann, Krautwurst, Raguse, & Meyerhof, 2002).

Guinea pigs also avoided the protein hydrolysates, while white-footed mice were indifferent to them and laboratory mice preferred them. Proteolysis has been proposed as an integral step in the activation of induced plant defenses (Delauré, Van Hemelrijck, De Bolle, Cammue, & De Coninck, 2008). Peptides and amino acids common to a variety of protein hydrolysates could indicate an increased probability of dangerous or suboptimal forage for herbivores.

For the other stimuli tested in our study, guinea pigs were only less tolerant than both mouse species of the QHCl. QHCl tests confirmed previous findings that guinea pigs avoid QHCl around 2 mM concentration (Warren & Pfaffmann, 1959) and that they show indifference toward concentrations that many other species avoid in two-bottle tests (Glendinning, 1994; Jacobs, 1978). For caffeine, although guinea pigs did have significantly higher scores for the 10 mM concentration than did the laboratory mice, the guinea pig scores were virtually identical to those of the white-footed mice. Both QHCl and caffeine have yet to be shown to be ligands for bitter receptors encoded by known genes, so molecular mechanisms for perception and response to these stimuli are still unknown. Why, in an evolutionarily based framework, guinea pigs would be remarkably tolerant of QHCl, yet respond unremarkably to other compounds relative to other non-herbivorous mammals, can only be speculated upon at this point. With quinine being derived from *Cinchona* bark, which is a tree with a natural range in South America where wild guinea pigs also originated, perhaps a high tolerance for quinine was adaptive for foraging on *Cinchona* seedlings.

It is unclear whether our findings are an exception to the general pattern of guinea pigs being tolerant of most bitter tasting substances or if the assumption of greater bitter tolerance of guinea pigs, relative to non-herbivorous species, is premature. Our findings were unexpected given previous studies (Jacobs, 1978; Nolte et al., 1994). Fasting guinea pigs for 18 h before offering bitter-treated chow in no-choice tests in the Nolte et al. study may have artificially inflated consumption, due to the disruption of naturally frequent, small meals (Hirsch, 1973). Presentation of bitter compounds in a mixture containing salts, sugars and fats (i.e., chow) in the Nolte et al. study may also have altered perception of bitterness (Breslin & Beauchamp, 1995; Koriyama, Wongso, Watanabe, & Abe, 2002; Lawless, 1979; Ley, 2008).

Our study, with its more natural, feeding setup could not discriminate among the possible mechanisms responsible for our subjects' behaviors. For example, the testing duration permitted postingestive cues, in addition to sensory cues, to influence preferences. For this example however, we would expect to see a generalization in responses from earlier to later stimuli that shared similar sensory components if postingestive effects had been strong and malaise had been the primary cause for avoiding a stimulus. There was no suggestion that strong avoidance at high concentrations caused low scores for subsequent stimuli. Conversely, if the hydrolysates had been universally rewarding, we would expect increases in scores as concentration increased for HCl and between hydrolysates, which was not observed. Our results were consistent with sensory cues playing a primary role in evoking the observed avoidance and preference responses.

For the complex stimuli (hydrolysates), however, our design did not discriminate between the possible sensory cues responsible for our results. Unlike the single compound, odorless plant metabolites, the hydrolysates, do have strong odors and complex flavors. In informal observation of the guinea pigs, all but two animals were seen sniffing and tasting the first hydrolysate (HC1, 2%) at its initial presentation. Subjects typically put their lips to the spout 2–3 times before pulling away and switching to the alternative spout. For higher concentrations and other hydrolysate types, some individuals seemed to be deterred by olfaction alone, while others would sample the hydrolysate, after which they often made rapid retreats, gapes and limb flails, which are aversive patterns demonstrated by a number of species (Berridge, 2000). Other qualities of the hydrolysates, in addition to or even rather than bitterness, may have caused the guinea pigs' strong avoidance of these stimuli. The results for the hydrolysates did not evoke the same response pattern as the simple bitter compounds (salicin, caffeine, QHCl), in which preference scores decreased at higher concentrations. Our results do indicate that a hydrolysate from plant-based protein is treated similarly to hydrolysates originating from animal-based proteins. Our use of protein hydrolysate, which has shown promise as a deer repellent (Kimball & Nolte, 2006; Kimball, Nolte, & Perry, 2005), is an initial step toward determining how food acceptability is altered with hydrolysate addition. Our study provides preference data for two standard bitter tastants, salicin and caffeine, that have not yet been tested on guinea pigs, white-footed mice or this outbred *Mus* strain (QHCL has also never been tested on *Peromyscus* and CD1s). Our results suggest caution in accepting, without further empirical support, the premise that guinea pigs (and herbivores in general) have a generalized reduced bitter sensitivity.

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Figure 1.

Preference scores (bars=confidence intervals) for salicin, caffeine, quinine hydrochloride (QHCl), two types of hydrolyzed casein (HC1 and HC2), and hydrolyzed soy (HSoy) in guinea pigs, laboratory mice and white-footed mice. Taste compounds are presented from left to right in the order they were tested. Asterisks indicate significant differences from a preference score of 0.5 (one-sample t-tests, $P < 0.0037$; alpha-level corrected for multiple comparisons). For each concentration, species means marked by different letters significantly differ ($P < 0.05$; Tukey's HSD post-hoc tests).

Table 1

Summary of ANOVA Results for Preference Scores for Taste Stimuli

Stimulus	Effect	df	F	P
Salicin	Species	2, 47	5.28	0.009
	Concentration	2, 94	148.87	<0.001
	Species $\times$ Concentration	4, 94	15.80	<0.001
Caffeine	Species	2, 46	7.24	0.002
	Concentration	2, 92	181.46	<0.001
	Species $\times$ Concentration	4, 92	5.16	<0.001
QHCl	Species	2, 46	34.60	<0.001
	Concentration	2, 92	55.78	<0.001
	Species $\times$ Concentration	4, 92	5.78	<0.001
HC1	Species	2, 46	179.93	<0.001
	Concentration	2, 92	8.19	<0.001
	Species $\times$ Concentration	4, 92	4.89	0.001
HSoy	Species	2, 41	63.15	<0.001
HC2	Species	2, 43	51.80	<0.001

Note. Repeated Measures ANOVA were used for salicin, caffeine, QHCl and HC1 (between-subject factor: species; within-subject factor: concentration); One-way ANOVAs were used for HSoy and HC2.