Original Article

Bitter-Induced Salivary Proteins Increase Detection Threshold of Quinine, But Not Sucrose

Laura E. Martin¹, Kristen E. Kay¹ and Ann-Marie Torregrossa^{1,2,*}

¹Department of Psychology, University at Buffalo, Buffalo, NY 14216, USA and ²Center for Ingestive Behavior Research, University at Buffalo, Buffalo, NY 14216, USA

Correspondence to be sent to: Ann-Marie Torregrossa, University at Buffalo, Buffalo, NY 14216, USA. e-mail: amtoreg@buffalo.edu

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Abstract

Exposures to dietary tannic acid (TA, 3%) and quinine (0.375%) upregulate partially overlapping sets of salivary proteins which are concurrent with changes in taste-driven behaviors, such as rate of feeding and brief access licking to quinine. In addition, the presence of salivary proteins reduces chorda tympani responding to quinine. Together these data suggest that salivary proteins play a role in bitter taste. We hypothesized that salivary proteins altered orosensory feedback to bitter by decreasing sensitivity to the stimulus. To that end, we used diet exposure to alter salivary proteins, then assessed an animal's ability to detect quinine, using a 2-response operant task. Rats were asked to discriminate descending concentrations of quinine from water in a modified forced-choice paradigm, before and after exposure to diets that alter salivary protein expression in a similar way (0.375% quinine or 3% TA), or 1 of 2 control diets. Control animals received either a bitter diet that does not upregulate salivary proteins (4% sucrose octaacetate), or a nonbitter diet. The rats exposed to salivary protein-inducing diets significantly decreased their performance (had higher detection thresholds) after diet exposure, whereas rats in the control conditions did not alter performance after diet exposure. A fifth group of animals were trained to detect sucrose before and after they were maintained on the 3% TA diet. There was no significant difference in performance, suggesting that these shifts in threshold are stimulus specific rather than task specific. Taken together, these results suggest that salivary proteins reduce sensitivity to quinine.

Key words: saliva, taste, threshold detection

Introduction

Obesity is a prevalent disease with long-term psychological, health, and economic impacts (Division of Nutrition, Physical Activity, and Obesity, Centers for Disease Control and Prevention [CDC]), so the incorporation of vegetables into daily diet is of increasing importance. However, many people find plant-based, healthy diets to be undesirable especially in contrast to fat, sugar, and salt-heavy obesogenic diets (Chapman and Maclean 1993; Brown et al. 2000). As plants are often accompanied by an unpleasant, bitter taste (Drewnowski and Gomez-Carneros 2000; Barratt-Fornell and Drewnowski 2002), understanding how to increase the acceptance

of bitter taste could help to increase the attractiveness of healthy food. Bitter taste has been subject to substantial research focusing primarily on bitter receptors (Adler et al. 2000; Chandrashekar et al. 2000; Shi et al. 2003), their ligands (Bufe et al. 2002, 2005; Pronin et al. 2004; Meyerhof et al. 2005, 2010), and neural representation (Di Lorenzo and Monroe 1990; Dahl et al. 1997; Clarke and Ossenkopp 1998; Di Lorenzo and Victor 2003; Roussin et al. 2008; Martin and Sollars 2017; Schier and Spector 2019). However, there is mounting evidence that saliva can affect taste perception, especially for bitters. Stimuli are dissolved in saliva in order to reach taste receptors; saliva is an incredibly complex solution, containing

hundreds of proteins, and changing under numerous physiological conditions (Katsukawa et al. 1999; Katsukawa and Ninomiya 1999; Neyraud et al. 2006; Quintana et al. 2009; Cabras et al. 2010, 2012; Morzel et al. 2012, 2017).

It has been hypothesized that salivary proteins (SPs) act as a defense mechanism against potentially harmful chemicals found in many plants, which allows animals to consume those plants while minimizing negative physiological effects. This work has primarily focused on a group of SPs called proline-rich proteins, which are hypothesized to increase acceptance of tannic acid (TA). TA is a fairly ubiquitous class of plant secondary compounds (found in beer, wine, tea, beans, nuts, unripe fruit, etc.), which can be harmful when found at high concentrations (Mehansho et al. 1983, 1987b; Chung et al. 1998; Skopec et al. 2004; Shimada 2006; Lamy et al. 2010).

There is a growing body of work demonstrating that saliva content alters taste in both animals and humans. Mice with a subset of SPs upregulated show an increased preference for a TA solution (Glendinning 1992). Rats with SPs upregulated by exposure to TA or quinine show increases in bitter acceptance in 24-h tests, as well as increases in brief access licking (Torregrossa et al. 2014; Martin et al. 2018). It has also been demonstrated that the presence of SPs decreases chorda tympani nerve signaling in response to quinine (Martin et al. 2018).

In humans, SP profiles are related to taste sensitivity and food preference. SP profiles are different in children with limited food selection than children with no such eating difficulties (Morzel et al. 2017). Amylase activity has been correlated to the changes in the perception of starch-thickened food (Ferry et al. 2006) as well as hedonic ratings of fat and sweetness (Méjean et al. 2015), and SP profiles are also correlated with fat and salt taste sensitivity (Mounayar et al. 2014; Stolle et al. 2017) and liking for salt (Méjean et al. 2015). There are links between SPs and 6-n-propylthiouracil (PROP) taster status (Cabras et al. 2012), and oral administration of certain SPs can facilitate increases in PROP intensity (Melis et al. 2013). In infants, SP profiles are predictive of the acceptance of a urea-containing solution (Morzel et al. 2014), and finally, those considered sensitive to the taste of caffeine have differences in SP profile from those considered insensitive (Dsamou et al. 2012; Mounayar et al. 2014). This work supports that SP profile, and specific SPs, can play a role in altering taste (Martin et al. 2019). It is unclear, however, how taste is altered. Most of these measures involve hedonic evaluation of the stimulus/food, and could therefore involve a change in sensitivity to the stimulus (detection) or a change in the hedonic evaluation as more positive or negative. In other words, it is unclear whether SPs change the perception of the stimulus as tasting more or less palatable, or if they decrease/increase sensitivity to the stimulus.

Experimental work on SPs and taste are limited because there are no perfect ways to experimentally manipulate the SP profile. In our previous work, we used donor saliva for our electrophysiology preparations (Martin et al. 2018) but there are limitations to that method in awake and behaving animals, such as the presence of their own saliva and the volumes of donor saliva needed to deliver the stimuli. Injections of isoproterenol have been used (Glendinning 1992) to increase SP expression but the necessary doses of the drug make the animals ill in addition to altering SP profile, limiting the utility of that treatment. The simplest way to alter SP profile is through diet exposure, which requires one to control for bitter diet experience. Here we test the hypothesis that altered SPs increase the palatability of bitter diets by decreasing sensitivity to them by examining detection thresholds for quinine in rats before and after they were exposed to 1 of 4 diets; a TA or quinine-containing diet,

which upregulate similar sets of SPs (Torregrossa et al. 2014; Martin et al. 2018), a sucrose octaacetate (SOA) diet, which activates a bitter receptor (Brockhoff et al. 2007) but has no effect on SPs (Martin et al. 2019), or a nonbitter control diet that does not alter SP profile (Torregrossa et al. 2014; Martin et al. 2018). In addition, we assessed the stimulus specificity of the effect, by examining sucrose detection thresholds for rats before and after SPs were upregulated by the TA-containing diet.

Materials and methods

Subjects

Subjects were 30 adult male Long Evans rats (Charles River Breeding Laboratory), weighing 150–250 g at study onset. The colony room was maintained at 20 ± 2 °C with a 12:12 h light/dark cycle. All training, testing, and sample collections were performed during the lights on phase. All animal procedures were approved by University at Buffalo Animal Care and Use Committees.

Water restriction

Training and testing took place 5 days/week (from Sunday to Thursday). Water was removed from cages on Saturday so animals were water deprived for 22 h prior to testing on Sunday, and water bottles were returned on Thursday after testing. This way, animals were allowed ad lib access to water for 48 h in between training/testing sessions.

Timeline

Animals were divided into 5 groups ($n = 6/\text{group}$). Four of the groups were trained and tested for the detection threshold of quinine. The fifth group was trained to detect sucrose. After all animals had successfully completed their training (see methods discussed later) and the first testing phase, they entered the induction phase, where they were given an experimental diet (3% TA, 0.375% quinine) or a control diet (4% SOA, nonbitter control) for 14 days. After the induction phase, animals were all returned to the control diet overnight and reacquainted with the detection task the following day. Refresher training for the second test consisted of 2 days of the detection task at the training concentration (0.4 mM quinine or 0.6 M sucrose), after which all animals performed at 80% or above. Animals were then retested (see Table 1).

Testing apparatus

Training and testing were conducted in a specialized stimulus delivery system that we refer to as the gustometer. The gustometer, which has been previously described and used to assess taste sensitivity in rats (St John and Spector 1996; Geran and Spector 2000; Spector and Kopka 2002; Bales et al. 2015; Blonde et al. 2015; Spector et al. 2015), is a modified skinner box housed in a sound attenuation chamber. Briefly, animals are placed in a small, plastic, wire-bottom chamber. The front panel of the chamber has 3 slots where the animal can access 3 response balls: the sample (center) ball is a borosilicate ball that spins on a horizontal axis, and the 2 reinforcement (left and right) balls are stationary. Solution is dispensed onto the sample ball, from 1 of 14 tubes, which are driven by computerized syringe pumps. The sample ball is attached to a computer-controlled retractable arm. After the sampling period, the arm is retracted and the ball is washed with deionized (DI) water, dried with pressurized air and repositioned. Water is dispensed from PE tubing run through the 2 stationary reinforcement balls, available

Table 1 Timeline of experimental phases

Phase	Days	Stimuli
Saliva collection training	14	30 mM citric acid in 1 M sucrose
Baseline saliva collection	4	
Stationary training	3	DI H ₂ O
Side training	8	DI H ₂ O or 0.4 mM quinine, 0.6 M sucrose
Alternation training	15–20	DI H ₂ O, 0.4 mM quinine, 0.6 M sucrose
Random training I	13–15	DI H ₂ O, 0.4 mM quinine, 0.6 M sucrose
Random training II	8–11	DI H ₂ O, 0.4 mM quinine, 0.6 M sucrose
Preinduction testing	20	DI H ₂ O, 0.4–0.00006 mM quinine, 0.6–0.0002 M sucrose
Induction	14	3% TA, 0.375% quinine, 4% SOA, nonbitter control
Retraining	2	DI H ₂ O, 0.4 mM quinine, 0.6 M sucrose
Retesting	20	DI H ₂ O, 0.4–0.00006 mM quinine, 0.6–0.0002 M sucrose

Figure 1 Schematic of the quinine detection task. During the initiation phase, the animal must lick the sample ball twice within 250 ms to initiate a trial. During the sampling phase, the animal is allowed 3 s or 10 licks of a randomly presented sample fluid. During the decision phase, the animal is given 5 s to lick at either the right or the left response ball depending on the stimulus sampled. There are 3 possible contingencies; if the animal makes a correct response, it is allowed 5 s or 25 licks of H₂O from the response ball. If the animal makes no response within 5 s, or an incorrect response, the animal is given no H₂O and a 20 s time-out where it is not allowed to initiate a new trial. Following the contingency phase, there is a 6 s intertrial interval.

at the left and right slots. Licks on all balls are recorded by force transducers. To limit any sounds that could be used by the animals as a non-taste cue (turret shifting, unique pump clicking, etc.), white noise is played during all training and testing sessions. A stainless steel shield is positioned between the animals and the stimulus delivery system to limit visual cues. An exhaust fan is positioned directly above the sample ball, to minimize olfactory cues.

Trial structure

Training and testing methods were based on Bales et al. (2015) and Blonde et al. (2015). Detection for both quinine and sucrose was conducted in water restricted rats, using a 2-response operant task, with a modified descending method of limits procedure for stimulus presentation (Figure 1). The animal sampled a stimulus solution and responded by licking at the appropriate response ball, according to the stimulus (quinine/sucrose or water) presented. Across test sessions, the concentration of the stimulus was decreased until the animals could no longer discriminate it from water (0.4, 0.1, 0.025, 0.006, 0.002, 0.0005, 0.0002, 0.00006 mM quinine or 0.6, 0.15, 0.075, 0.0375, 0.01875, 0.01, 0.005, 0.002 M sucrose). A single concentration of the test stimulus was presented in any given session, and animals participated in only 1 session per day. Sessions lasted 30 min.

At the beginning of the session, the house light was turned on, and the sample ball was washed and then positioned in front of its slot. The rat could initiate a trial by licking the sample ball twice within 250 ms, and all subsequent licks would dispense the sample solution (approximately 5 μ L/lick). Sample solutions were presented randomly during the sessions. During the sampling period, rats were allowed to sample either 3 s or 10 licks of solution. The sample ball was then retracted, and the rat was given 180 s to respond at a reinforcement ball. The time allowed to respond at the reinforcement

balls was progressively shortened during training, with its final testing duration at 5 s. If the animal responded at the correct reinforcement ball, it was allowed 5 s or 25 licks of DI water. If the animal responded at the incorrect ball or took too long to respond, it was given no water and was punished with a 20 s time-out where it could not initiate a new trial. The intertrial interval was 6 s.

Training and testing procedure

We used a 2-response operant task to determine the detection thresholds of quinine and sucrose. Animals were trained to associate the presence of a stimulus on the sample ball with water delivery on 1 of the reinforcement balls (stimulus-paired side); alternatively, the presence of water on the sample ball was associated with water being delivered on the opposite reinforcement ball (water-paired side). This task required a water restricted rat to respond to the presence of a taste stimulus by licking the reinforcement ball on the stimulus-paired side, and to respond to water by licking on the water-paired side. When trained rats responded at chance levels (50% correct) we consider the concentration below threshold.

During the first phase of training, stationary training (Table 1), animals learned that fluid could be received at the sample ball and at each of the reinforcement balls. For 1 day each, animals were allowed ad lib access to the left, right, or center ball, whereas access to the other 2 balls was blocked with an attached shutter.

During the second phase of training, side training (Table 1), animals were allowed access to the taste solution at the center ball, and access to 1 of the reinforcement balls, whereas the other was blocked with a shutter. At trial initiation, animals were presented with the sample solution; if the animal sampled the stimulus at the center ball, they were then given 180 s to respond at the open reinforcement ball, before the trial ended. Across 8 sessions, animals were given 4 pairings of each of the sample fluids (quinine/sucrose

or water) with the assigned response side. Correct response assignments were counterbalanced, such that half the group had a taste stimulus paired to right-side responses, and the other half had water paired with right-side responses. During side training, animals were not punished with a time-out.

After side training, animals moved to alternation training (Table 1). Animals sampled a taste stimulus and water during the same session, and had access to both reinforcement balls. Animals were presented with the same sample repeatedly, until they made a predetermined number of correct responses (correct responses were not required to be consecutive), at which point the other fluid was presented. Animals started with a stimulus change after 8 correct responses, which was lowered across sessions (to 6, then 4, then 2). At each point, every animal was required to respond with 80% accuracy before they could move to the next response criterion. During the alternation phase, animals were allowed 15 s to respond, and incorrect or nonresponses were punished with a 20 s time-out.

During the last phase of training, randomized training (Table 1), sample fluids were presented randomly, such that animals had a 50% chance of receiving water or a taste stimulus (quinine or sucrose) throughout the session. After animals reached/maintained 80% accuracy on the task, the decision time duration was shortened from 15 to 10 s (random training I, Table 1), then from 10 to 5 s (random training II, Table 1). After random training was completed, with all animals performing at 80% or better on the task, animals were subjected to a “water control test” where assignments of each syringe were maintained (quinine syringes were still labeled quinine in the program), but all solutions were replaced with DI water. If animals relied on taste to inform their decisions, then, without any taste information, performance on the water control test would be at approximately 50%. If, however, animals used other cues (visual, auditory, etc.), then their performance would appear more accurate. All animals were found to perform at approximately 50% on the water control test, and so none were dropped from the study.

During testing, animals alternated each day between “test” sessions and “stimulus control” sessions. Stimulus control sessions used the training concentration of the taste stimulus (quinine, 0.4 mM, sucrose, 0.6 M), to maintain at least 80% accuracy in the task. This concentration was used for all rats, as it is well above threshold. Performance for all rats during stimulus controls days was comparable (approximately 85–90%) suggesting that if there was any shift in perceived intensity at this concentration it was not sufficient to alter the animal's ability to perform the task. During test sessions, the concentration of the test stimulus was lowered (quinine: 0.4–0.6 $\log_{10}$ units each session, sucrose: 0.18–0.30 $\log_{10}$ units per session) until animals reached chance levels of performance (50% correct responding). Following this, animals were retested at concentrations slightly above and at 50% correct responding. Finally, animals were given another water control test, to ensure that their performance was based solely on taste cues.

Diets

Animals were individually housed in custom-designed Plexiglas shoebox cages, equipped with a feeding niche containing a spill-resistant food cup, as well as a rack for a water bottle. Food intake was measured by weight (g). During training and preinduction testing phases, animals were allowed 18 h ad lib access to a nonbitter control diet, which does not alter SP profile (Torregrossa et al. 2014; Martin et al. 2018). Following the completion of the first testing phase (preinduction testing), animals entered the induction phase, where they were placed on 1 of 4 diets. Diets were as follows: 4%

TA by weight, 0.375% quinine, 4% SOA, or continuation of the nonbitter control. Each diet is isocaloric to the custom control diet (Torregrossa et al. 2014; Martin et al. 2018), as the bitter stimulus is added to the recipe while an equal percentage of cellulose is removed from the recipe. Animals were maintained on these diets for 14 days to ensure SP upregulation, and were then switched back to the control diet for the retesting phase.

Saliva collection

A total of 4 control saliva samples were collected, and the latter 2 were used to establish baseline SP densities. Following these baseline collections, saliva samples were collected from all animals once/week on Fridays, approximately 18 h after water had been replaced following the final testing session. Saliva was collected and processed as described previously (Torregrossa et al. 2014; Martin et al. 2018). Briefly, saliva was collected from awake, trained animals. Training consisted of repeated pairings (approximately 25 pairings/session, 14 sessions) of 2 mL of 30 mM citric acid in 1 M sucrose with a pipette tip placed under the tongue. This solution, which served as an unconditioned stimulus (US), was pipetted into the mouth in 200 μ L increments. The pipette tip, which served as a conditioned stimulus (CS), was inserted into the side of the mouth and was moved over and under the tongue during CS/US pairings. By the end of saliva collection training, animals were conditioned to salivate by merely inserting the pipette into the mouth. Following the training period, saliva samples were collected from rats by inserting a 200 μ L pipette with an empty wide orifice tip under the tongue and gently drawing up small volumes of saliva. This method allows us to collect uncontaminated whole saliva. Saliva samples ranging from 50 to 100 μ L were immediately placed on ice in 10 μ L of 10 $\times$ Halt Protease/Phosphatase Inhibitor Cocktail (Thermo Scientific). All samples were then frozen for later analysis.

Saliva processing and gel electrophoresis

Samples were processed and gels were run as previously described (Martin et al. 2018). Briefly, saliva samples were defrosted and mixed with equal volumes of 0.2% Trifluoroacetic acid (Sigma-Aldrich). Samples were centrifuged at 2000 $\times$ g for 15 min at 4 $^{\circ}$ C to remove cells and debris. The supernatant was then aspirated. Total protein concentration was determined by the bicinchoninic acid (BCA) protein assay method (Pierce Protein Biology Products).

For gel electrophoresis, equal volumes of whole saliva were mixed with one-third volume of 4 $\times$ Invitrogen sample buffer with reducing agent, heated at 82 $^{\circ}$ C for 10 min and resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Thermo Scientific) with 3-(N-morpholino) propanesulfonic acid buffer. Molecular mass markers (Bio-Rad, Ref LC5800) were run simultaneously with the samples in each gel to calibrate molecular masses of each protein band. Gels were fixed in 40% methanol in 10% acetic acid for 30 min followed by staining with Coomassie Brilliant Blue R-250 (0.1% stain in 25% methanol and 10% acetic acid) for 1 h, then destained in 10% acetic acid according to published protocols (Beeley et al. 1991; Sarni-Manchado et al. 1998). Bands were captured using Azure c400 Gel Imaging System. Densitometric analysis was performed using Azurespot Analysis Software (Azure Biosystems).

Data analysis

Proportion correct for each animal was calculated as the number of trials with correct responses at a single stimulus concentration, divided by the total number of trials taken for that same concentration of quinine or sucrose. Trials were pooled across all testing sessions

for each concentration. Animals took between 130 and 160 trials for each concentration. Trials with nonresponses were not included in the calculation. In this way, individual differences in the number of trials taken can be controlled for. A curve was fit to each animal's performance data with a sigmoidal 3-parameter logistic function $\left[\frac{a - 0.5}{1 + 10^{(x-c)*b}} \right] + 0.5$, where the curve parameters, a , b , and c , were defined as the asymptotic maximum performance as determined by the mean of the stimulus control sessions, the slope of the curve, and the midpoint concentration between asymptotic maximum and minimum (EC_{50}), respectively. The variable x represented stimulus concentration, which was log transformed for the analysis. Quinine threshold task data were analyzed with analysis of variance (ANOVA). If main effects were found, further analyses were performed with Bonferroni-corrected t -tests.

To determine detection thresholds, performance data for each group at each concentration (starting with 0.4 mM, the highest concentration) was compared to 50% (chance performance) in a series of Bonferroni-corrected single sample t -tests. Detection threshold was considered the lowest concentration that was significantly different from 50% correct responding. Performance data were additionally compared between quinine- and TA-induced groups across testing (preinduction testing vs. retesting) to explore differences in the efficacy of the diets to alter detection threshold.

For the sucrose detection task, data were compared in a paired t -test. Preinduction testing and retesting values were compared. For graphical representation only, performance for each group at each concentration was averaged across all animals for that group.

Band densities for all saliva samples were calculated as a change from the animal's own baseline (average of control collections). For the preinduction testing phase, densities were compared in a 1-way ANOVA with induction diet as a between-groups measure. For the induction and retesting phases, densities were compared in a repeated-measures ANOVA with diet as a between-groups measure and time as a repeated measure. Significant differences were followed with Bonferroni-corrected t -tests to verify an increase in expression over controls.

Results

Protein expression

After preinduction testing (testing on the control diet), there was no change from baseline in the expression of any SP that we measured ($F(3,24) = 0.407$ – 2.317 , $P = 0.749$ – 0.101), and there were no group differences in protein expression (see Table 2, Figure 2). Saliva samples were collected at the end of each week, during all phases (training, preinduction testing, induction, and retesting). We did this

in the case SP induction was driven by the gustometer testing; if so, frequent collection would allow us to pinpoint what phase of the test correlated with altered SP profile. As this was not an issue, only samples from the end of preinduction testing, induction, and retesting were analyzed and are presented here. We quantified the protein level of 7 bands previously reported to be altered with TA or quinine exposure (Table 2, 37, 35, 25, 23, 19, 18.5, and 14 kDa) (Torregrossa et al. 2014; Martin et al. 2018).

SP expression at the beginning and end of retesting was compared in a 2-way ANOVA; diet treatment significantly upregulated SPs (main effect of diet; Table 3). Protein expression did not change during the retesting phase as there was no effect of time ($F(1,24) = 0.051$ – 3.401 , $P_s = 0.824$ – 0.078). Protein expression measures for SOA and control diet were not significantly different from each other at any band, when compared with post hoc Bonferroni-corrected t -tests ($t(20) = 0.083$ – 1.93 , $P_s = 0.935$ – 0.089), therefore these groups were combined to represent our control expression in further analyses. Post hoc analyses also demonstrated that compared to the control groups, TA-upregulated animals had higher densities of all of the bands we have previously demonstrated to be altered with TA diet exposure (Torregrossa et al. 2014, 35, 23, 19, 18.5, 14 kDa: $t(42) = 6.039$ – 2.888 , $P = 0.001$ – 0.043) and quinine upregulated all of the bands that we have previously demonstrated are upregulated by a quinine diet (Martin et al. 2018, 37, 35, 25, 18.5, 14 kDa: $t(32) = 5.501$ – 2.885 , $P = 0.001$ – 0.047).

Performance for preinduction testing

During the preinduction phase, there was no significant difference in the performance (proportion of correct responses) between any group of animals trained to detect quinine ($F(3,20) = 0.440$, $P = 0.727$). In addition, the parameters of the psychometric functions fit to each of the 4 groups during the preinduction testing phase showed that the asymptotes, slopes, or EC_{50} for all groups show no group differences (a : $F(3,20) = 1.889$, $P = 0.166$, b : $F(3,20) = 1.243$, $P = 0.322$, c : $F(3,20) = 1.074$, $P = 0.384$), indicating no baseline differences in performance. To approximate the detection threshold, we compared average performance for each group at each concentration to chance (50%) in Bonferroni-corrected single sample t -tests. Performance values were compared at descending concentrations, beginning with 0.4 mM quinine, until animals failed to perform above chance. For preinduction testing, the lowest concentration that was significantly different from chance was 0.0005 mM ($t(5) = 2.96$ – 6.08 , $P = 0.007$ – 0.042) for all groups, likewise all groups performed no better than chance at 0.0002 mM quinine ($t(5) = 1.164$ – 1.786 , $P = 0.124$ – 0.894).

Table 2 Mean (SE) normalized densitometry units of protein bands during the preinduction testing phase at each of the listed masses (kDa) with between-groups ANOVA comparisons

	kDa	TA	Q	SOA	Control	Effect of diet	
						$F(df)$	P value
Preinduction testing	37	1.26 (0.38)	0.91 (0.26)	0.93 (0.66)	0.65 (0.22)	0.438 (3,24)	0.728
	35	1.08 (0.21)	1.17 (0.21)	0.89 (0.28)	1.46 (0.25)	0.824 (3,24)	0.494
	25	1.24 (0.22)	1.35 (0.26)	1.25 (0.20)	1.14 (0.19)	0.119 (3,24)	0.948
	23	2.75 (0.57)	2.59 (1.42)	2.09 (0.64)	2.16 (0.56)	0.155 (3,24)	0.925
	19	1.95 (0.57)	1.81 (0.56)	1.55 (0.53)	2.16 (0.66)	0.138 (3,24)	0.936
	18.5	2.29 (0.44)	2.83 (0.71)	1.72 (0.64)	2.08 (0.72)	0.483 (3,24)	0.697
	14	1.45 (0.33)	1.34 (0.08)	1.98 (0.33)	1.20 (0.29)	0.877 (3,24)	0.467

SE, standard error.

Figure 2 A representative SDS-PAGE of saliva samples from a single rat from each group. This saliva was collected during the baseline control period, preinduction testing, induction, and retesting. Saliva was loaded 12% Tris-tricin gel and labeled with Coomassie R-250 staining. Molecular weight markers (M) in kDa are indicated on the left margin.

Performance for retesting

Animals that completed the quinine detection task were compared in a 2-way ANOVA with preinduction testing and retesting performance as within-groups measure and induction diet as a between-groups measure. There were no diet effects on the asymptote (a : $F(3,20) = 0.687$, $P = 0.571$) or EC_{50} (c : $F(3,20) = 2.064$, $P = 0.139$, Figure 3, Table 4). Diet exposure increased the slope (b : $F(3,20) = 3.897$, $P = 0.025$), and there was a significant interaction ($F(3,20) = 14.546$, $P = 0.001$, Figure 3, Table 4). Post hoc tests revealed that rats fed TA and quinine diets showed a significant increase in the slope (TA: $t(5) = 2.624$, $P = 0.047$, Q: $t(5) = 2.884$, $P = 0.034$) after diet exposure. There was no change in the slope for either the SOA or the control group (SOA: $t(5) = 0.727$, $P = 0.507$, Control: $t(5) = 1.214$, $P = 0.279$).

Again, to assess detection threshold, animals' performances at descending concentrations were compared to chance (50%) in Bonferroni-corrected single sample t -tests. The lowest concentration significantly

different from chance remained 0.0005 mM for both SOA and control ($t(5) = 3.177$, $P = 0.034$ and $t(5) = 2.722$, $P = 0.042$, respectively). The detection threshold for TA exposed rats shifted to 0.1 mM quinine ($t(5) = 6.673$, $P = 0.010$, 0.025 mM: $t(5) = 0.5866$, $P = 0.579$), and to 0.025 mM for quinine-exposed rats ($t(5) = 3.190$, $P = 0.024$, 0.006 mM: ($t(5) = 1.121$, $P = 0.313$). To directly compare the magnitude of change after the TA or quinine diets, we used an ANOVA to examine diet (factor) and the effect of induction (preinduction testing to retesting, as a repeated measure). There was no significant interaction between diet and induction at either 0.1 mM ($F(1,11) = 0.376$, $P = 0.552$) or 0.025 mM ($F(1,11) = 4.140$, $P = 0.067$), although there was a trend for TA to decrease performance more than quinine.

Sucrose detection

As a single group performed the sucrose detection task, t -tests were used to compare the preinduction testing and retesting curve parameters. No significant differences were found in asymptote ($t(5) = 1.029$, $P = 0.350$), slope ($t(5) = 1.612$, $P = 0.187$), or EC_{50} ($t(5) = 0.175$, $P = 0.868$, Figure 4, Table 4), indicating that bitter diet experience/SP upregulation did not alter performance.

Discussion

There is a growing body of work that strongly implicates SPs in the increased acceptance of bitter taste. Animals with altered SP profiles show increased preference, 24 h food consumption, and unconditioned licking to bitters (Glendinning 1992; Melis et al. 2013; Torregrossa et al. 2014; Martin et al. 2018), whereas in humans, sensitivity to the taste of bitters is linked to SP profile (Cabras et al. 2012; Dsamou et al. 2012; Melis et al. 2013; Morzel et al. 2014). However, much of this work suggests that palatability is altered without determining how it is altered. Traditional measures of acceptance like total intake, rate of feeding, and brief access licking are informed by both sensitivity to the stimulus and hedonic evaluation. When these measures are changed, it is possible that the stimulus is qualitatively unchanged but hedonically reevaluated (i.e., better or worse), or that the stimulus is hedonically different *because* it is qualitatively different (i.e., weaker or stronger) (Loney et al. 2012). Here we used an operant task where the measure of the animal's behavior is not based on the hedonic evaluation of the stimulus but instead on the animal's ability to detect the stimulus (Bales et al. 2015). We hypothesized that threshold detection of quinine is impaired in animals with diet-upregulated SPs, and that these SPs affect sensitivity to bitters, but not other taste qualities.

SPs were upregulated by exposure to quinine or TA but not SOA. The concentrations of bitter in these diets were chosen on the basis of similar first day intake (i.e., rats reduce intake of 3% TA, 0.375% quinine, and 4% SOA diets to the same degree on first presentation). We acknowledge that this is not a perfect control for intensity or the perceptual experience of the diet, however, SOA is an aversive stimulus that activates bitter taste receptors (Kratz et al. 1978; Meyerhof et al. 2010) and therefore serves as a control for bitter pre-exposure. We do not currently know why SOA does not alter the salivary proteome. The mechanism for diet-induced SP upregulation is unknown. The SOA data suggest that oral bitter exposure alone is not sufficient, and unfortunately we do not know enough about the receptors activated by our compounds to hypothesize on receptor populations involved. Quinine is considered a promiscuous ligand, activating 9 of the known TAS2Rs (4, 7, 10, 14, 39, 40, 43, 44, and 46) (Meyerhof et al. 2010). TA also activates TAS2R39 and additionally TAS2R5. SOA activates TAS2R46, but it has not been

Table 3 Mean (SE) normalized densitometry units of protein bands during both the induction and retesting phases at each of the listed masses (kDa) with between-groups ANOVA comparisons

kDa	Phase	TA	Q	SOA	Control	Effect of diet	
						F(df)	P-value
37	Induction	0.64 (0.14)	2.04 (0.62) ^a	0.78 (0.44)	0.73 (0.16)	3.649(3,24)	0.027
	Retesting	0.69 (0.13)	2.13 (0.57) ^a	1.39 (0.92)	1.63 (0.35)		
35	Induction	3.39 (0.64) ^a	3.08 (0.45) ^a	1.05 (0.25)	0.71 (0.15)	9.858(3,24)	<0.001
	Retesting	3.25 (0.30) ^a	2.88 (0.66) ^a	1.00 (0.28)	1.39 (0.47)		
25	Induction	1.03 (0.16)	3.15 (1.46) ^a	1.48 (0.36)	0.70 (0.13)	3.007(3,24)	0.05
	Retesting	0.99 (0.24)	2.47 (0.88) ^a	1.08 (0.04)	0.97 (0.20)		
23	Induction	8.08 (2.96) ^a	0.61 (0.05)	1.66 (0.31)	1.98 (0.62)	3.803(3,24)	0.011
	Retesting	6.53 (2.74) ^a	1.07 (0.24)	1.30 (0.49)	1.30 (0.26)		
19	Induction	2.81 (0.49) ^a	1.21 (0.35)	1.46 (0.27)	1.40 (0.20)	6.328(3,24)	0.003
	Retesting	2.31 (0.45) ^a	1.23 (0.31)	0.46 (0.21)	1.19 (0.23)		
18.5	Induction	5.49 (1.08) ^a	4.49 (1.05) ^a	1.77 (0.45)	0.99 (0.28)	3.040(3,24)	0.048
	Retesting	4.41 (1.69) ^a	3.93 (1.72) ^a	1.15 (0.34)	1.04 (0.41)		
14	Induction	1.50 (0.39) ^a	2.13 (0.39) ^a	0.91 (0.10)	0.86 (0.28)	3.732(3,24)	0.025
	Retesting	1.51 (0.28) ^a	2.57 (0.97) ^a	1.07 (0.20)	0.87 (0.22)		

SE, standard error.

^aRepresents significantly different from control saliva values (SOA and Control). **Figure 3** Mean ($\pm$ SE) proportion correct as a function of stimulus concentration for quinine. The solid lines represent performance during the preinduction testing phase, whereas dotted lines represent performance during the retesting phase. Asterisks represent significant within-group differences between preinduction testing and retesting. Animals were tested, then exposed to 3% tannic acid (**A**), 0.375% quinine (**B**), 4% sucrose octaacetate (**C**), or a nonbitter control (**D**), and retested. The curves were fit to the data based on a 3-parameter logistic function (see Data analysis). SE, standard error.

Table 4 Mean (SE) curve parameters for detection testing within experimental groups

	TA	Quinine	SOA	Control	Sucrose-TA
Preinduction testing					
Asymptote (<i>a</i>)	1.00 (0)	1.00 (0)	1.00 (0)	0.95 (0.02)	0.87 (0.01)
Slope (<i>b</i>)	-0.47 (0.04)	-0.48 (0.06)	-0.76 (0.26)	-0.85 (0.24)	-1.49 (0.98)
EC ₅₀ (<i>c</i>)	-1.34 (0.11)	-1.53 (0.19)	-1.31 (0.14)	-1.69 (0.22)	-1.88 (0.07)
Adjusted R ²	0.84 (0.04)	0.87 (0.03)	0.87 (0.03)	0.88 (0.03)	0.90 (0.02)
Retesting					
Asymptote (<i>a</i>)	0.94 (0.03)	0.91 (0.03)	0.98 (0.01)	0.92 (0.02)	0.89 (0.01)
Slope (<i>b</i>)	-1.18 (0.26)	-1.86 (0.49)	-1.14 (0.36)	-1.36 (0.30)	-1.30 (0.16)
EC ₅₀ (<i>c</i>)	-1.16 (0.10)	-1.40 (0.09)	-1.27 (0.16)	-1.68 (0.22)	-1.75 (0.03)
Adjusted R ²	0.86 (0.03)	0.94 (0.02)	0.88 (0.04)	0.91 (0.02)	0.92 (0.02)

SE, standard error.

Figure 4 Mean ($\pm$ SE) proportion correct as a function of stimulus concentration for sucrose. The solid line represents performance accuracy during the preinduction testing phase, whereas dotted line represents performance during the retesting phase. Animals were tested, then exposed to 3% tannic acid, and retested. The curves were fit to the data based on a 3-parameter logistic function (see Data analysis). SE, standard error.

tested on most of the TAS2Rs that are activated by TA and quinine (Brockhoff et al. 2007). There is overlap between quinine and TA, suggesting a possible role for TAS2R39. However, this receptor is considered somewhat promiscuous and appears to weakly favor natural compounds (Meyerhof et al. 2010), and we do not know if it is activated by SOA (a man-made bitter compound). In addition, there are other characteristics of the stimuli that might cause the change in protein production; for example, there could be other sensory properties to the stimuli (astringency), the presence of a metabolite of the stimulus or even a possible shift in the oral microbiome.

Our work does support the hypothesis that the SPs, once expressed, alter taste detection. 1) Animals with upregulated SPs (quinine, TA) had steeper slopes after dietary treatment, whereas the slopes for controls (SOA, non-bitter) did not change. The slope is a measure of the rate of change in detectability, with a steeper slope indicating that the stimulus moved from undetectable (50% or chance performance) to consistently detectable (approximately 90% or maximal performance) more quickly (Bartoshuk 1975). 2) Animals that were quinine or TA exposed had higher detection thresholds. Quinine-exposed animals moved from a detection threshold of 0.0005 mM quinine to

0.025 mM, whereas the TA-induced detection threshold increased to 0.1 mM quinine. Although this suggests that TA-induced SPs may have had a larger effect on quinine detectability than quinine-induced SPs, there was no difference in the performance of the 2 groups when they were directly compared. 3) The shift in detection threshold was not due to bitter experience alone, as pretreatment with SOA did not alter performance in the task. 4) The effects on detection profile were measured while the animals were maintained on the control diet, reducing the possibility of a contrast effect with their home-cage diet. Together these data suggest that SPs were contributing to the change in detection threshold and are consistent with previous findings that SPs can alter chorda tympani signaling in bitter-naïve rats (Martin et al. 2018), and that bitter-naïve mice with upregulated SPs find a TA solutions more acceptable (Glendinning 1992).

We hypothesize that the change in SP expression is altering bitter detection. However, although quinine is not typically described as having oral sensory properties other than bitter, we cannot rule out a role for SPs in altering nonbitter oral signals from the stimuli. For example, tannin has been described as both bitter and astringent (Simons et al. 2003), so perhaps there is a role for astringency in the detection shift.

Although there was a significant change in slope after SP induction, there were no differences in asymptotic performance. In addition, there was no lateral shift in the curve, as the EC_{50} scores, which measure the concentration at which performance on the task is half-maximal, are not different for any group. This suggests that addition of the SPs masked/inhibited the animal's ability to perform the task only at the lowest concentrations (Keast and Breslin 2003). This is consistent with previously published data showing that at high concentrations of quinine, SP-upregulated animals did not lick differently from controls (Martin et al. 2018). However, at low-to-intermediate concentrations, SP-upregulated animals licked more to quinine. These data support the hypothesis that SPs are upregulated as a protective measure against potentially toxic bitters. It has been suggested that the role of some SPs is to bind to toxic bitters (e.g., TA), and in the case of TA, the SPs stay bound through the gut to decrease the absorption of the compound and therefore protect the animal from toxicity (Mehansho et al. 1987a, 1987b; Austin et al. 1989; Bernays et al. 1989; Chung et al. 1998; da Costa et al. 2008). Correspondingly, at low concentrations, the animal would perceive less of the bitter taste, indicating that the food source is safe to eat. At higher concentrations, the SP effect on both absorption in the gut and on bitter taste could be overwhelmed. This again would be protective to the animal, indicating that the high concentration of bitter makes the food source unsafe to consume.

We repeated the threshold detection task using sucrose as the stimulus in place of quinine to verify that the bitter-upregulated SPs would act only to alter performance with bitter stimuli and not alter the animals' ability to perform the task. We found no differences in performance on the task on any measure, supporting our hypothesis that SPs act to alter taste in a quality-specific manner. Thus far we have made use of only 1 bitter stimulus (quinine), and have focused previous efforts to clarify the SP effect on quinine and TA (Torregrossa et al. 2014; Martin et al. 2018). This leaves us hesitant to speculate about the range of bitter tastants and the overall specificity of this effect.

Conclusion

Here we suggest that the increased acceptance of bitter stimuli that is driven by SPs is achieved through weakening detection of the stimulus. More work is necessary to explore the mechanism of the shift in detection, for example, identifying if it is receptor- or stimulus-mediated as well as identifying the particular proteins driving the change. In addition, we would like to expand the range of bitter stimuli used to clarify the broadness of the SP effect on taste. Ultimately, SP-mediated taste changes represent an ideal therapeutic target, as SPs may alter taste independently from diet experience. Once individual proteins are identified as responsible for the change in taste, they could be used to increase consumption of healthy foods.

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Conflict of interests

The authors declare no conflict of interest.

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