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Do gastrointestinal taste receptors contribute to associative learning and foraging behavior?¹

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ABSTRACT: Foraging behavior is an expression of learning, context, and experience arising from integration of sensory information obtained during feeding with postingestive consequences of food ingestion. Although it has been well established that gustatory and olfactory systems of the mouth and nose provide sensory information to the consumer (in the form of flavor), sweet and bitter taste receptors have recently been identified in the intestinal tract of humans and rodents. It remains possible that sensory information generated in the gut could contribute to the learning process. Thus, a series of experiments was conducted to determine if classical associative learning occurs when the conditional stimulus circumvents oronasal presentation via direct delivery to the gut or peritoneal cavity. Mice receiving an intragastric infusion of 5 mM sodium saccharin immediately followed by LiCl administration demonstrated a significant decrease in preference for 5 mM saccharin in 4 consecutive 23h, 2-bottle preference tests versus water ($P = 0.0053$). Saccharin was highly preferred in mice receiving intragastric (IG) saccharin only or interperitoneal (i.p.) injection of LiCl only.

This reduced preference indicated that mice “tasted” saccharin infused into the gut. However, efforts to replicate with a reduced infusion volume failed to result in decreased preference. To understand if there were alternative pathways for oral detection of infused saccharin, mice received intragastric infusions (5.4 mM) and i.p. injections (10.8 mM) of sodium fluorescein. Fluorescence was observed from the tongues and esophagi of mice infused with volumes of 0.5 mL or more or injected with volumes of 0.25 mL or greater. Interperitoneal injections of 5 mM saccharin in mice resulted in reduced preference for 5 mM saccharin presented orally in 2-bottle preference tests ($P = 0.0287$). Oral delivery of a 500-fold less concentration of saccharin (0.01 mM) during conditioning resulted in a similar preference expression as shown in the initial IG experiment. These results demonstrate that although compounds may be tasted in the mouth absent of oral contact, associative learning is attenuated. Therefore, intestinal taste receptors are unlikely to participate directly in learning and recognition of foods during foraging events.

Key words: conditioned taste aversion, foraging behavior, gastric taste, herbivory, reflux

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INTRODUCTION

Taste, smell and somatosensation are essential chemosensory processes foraging animals use to identify

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beneficial nutrients, non-edible miscellany, and potentially deleterious toxins. Ingested foods are mechanically and chemically digested in the stomach or rumen, liberating individual compounds that may not have been detected orally. From the perspective of mammalian diet selection, detection of these compounds by taste receptors in the intestinal tract could provide valuable sensory information.

Although studies with herbivores are more prevalent (Provenza et al., 1992; Hobbs, 1996; Moore and Foley, 2005; Manier and Hobbs, 2006), mammalian diet selection is strongly influenced by integration of cue and consequence across many foraging guilds (Forbes, 1998; Brodie, 1999; Baker et al., 2007; Webb et al., 2008). To

represent mammals in general, experiments with a mouse model were designed to determine if novel compounds presented in the intestine could provide information useful for decisions regarding diet selection in mammals. To separate taste per se from multiple post-ingestive feedbacks, the current study used a non-nutritive sweet taste stimulus (i.e., saccharin) in a well-established methodology for ingestive behavior and associative learning; namely, conditioned taste aversion (CTA; Garcia et al., 1955). Evidence of CTA learning resulting from saccharin infused into the stomach (intra-gastric; IG) paired with the toxic effects of LiCl would indicate that taste receptors in the gut convey gustatory information to the brain.

Results of our initial experiment suggested that, indeed, a lithium-induced aversion to IG saccharin was evident in mice upon oral presentation of a saccharin solution. Regurgitation in a mouse model seemed unlikely, although artificial reflux produced by the experimental delivery system could not be ruled out. When replicating

these findings, we modified the IG delivery procedures and evaluated an alternative pathway for gustatory information to reach the brain (i.e., taste receptors in the oral cavity via circulating blood). These experiments similarly relied on CTA to evaluate preferences for a novel taste cue presented IG, intraperitoneally (i.p.), or orally in association with i.p. LiCl to test the hypothesis that chemosensory input from gastrointestinal (GI) taste receptors modifies the taste response (Table 1).

METHODS

All experimental protocols were approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee.

Subjects

Male outbred CD-1 mice (Charles River Labora-

Table 1. Experimental schedule¹

Experiment	Treatment identifier	n	Conditioning		Preference testing	
			d 1, 3, 5 ^{2,3}		d 8 to 11	
			Conditioning stimulus	Unconditional stimulus ⁴		
1	IG	5	1.0 mL of 5 mM Saccharin ⁵	None	5 mM Saccharin	None
	IG+LiCl	5	1.0 mL of 5 mM Saccharin ⁵	LiCl	vs.	
	LiCl	8	None	LiCl	Water	
2	10IG	9	0.5 mL of 10 mM Saccharin ³	None	5 mM Saccharin	10 mM Saccharin
	10IG+LiCl	9	0.5 mL of 10 mM Saccharin ³	LiCl	vs.	vs.
	10LiCl	9	None	LiCl	Water	Water
3	Dye	3	0.25, 0.5, 0.75, 1.0, 1.25, or 1.50 mL of 5.4 mM	None	NA	NA
	Control	2	Fluorescein ⁶	None		
4			None			
	i.p. Dye	5	0.125, 0.25, 0.375, 0.5, 0.625, or 0.75 mL 10.8 mM	None	NA	NA
	Control	1	Fluorescein ⁷	None		
5						
	IP	8	1.0 mL of 5 mM Saccharin	None	5 mM Saccharin	None
	IP+LiCl	8	1.0 mL of 5 mM Saccharin	LiCl	vs.	
	IPLiCl	8	None	LiCl	Water	
6	IPSaccon ⁸	8	1.0 mL of 5 mM Saccharin	LiCl		
	5Oral+LiCl	8	25 mL of 5 mM Saccharin	LiCl	5 mM Saccharin	None
	0.01Oral+LiCl	8	25 mL of 0.01M Saccharin	LiCl	vs.	
	0.01Oralsaccon ⁸	8	25 mL of 0.01M Saccharin	LiCl ⁷	Water	
	0.01Oralex ^p	8	25 mL of 0.01M Saccharin	None		
	Oral	8	None	None		

¹IG = intra-gastric; LiCl = lithium chloride; IP = interperitoneal (i.p.); Sac = saccharin; Con = concurrent delivery; Oral = oral presentation of conditioning stimulus; Exp = experienced (subjects familiarized with saccharin); NA = not applicable (no preference testing).

²d 2, 4, 6, and 7 were rest days.

³Fluorescein infusion or injection was conducted on d 1 only. Excised tissues examined by light microscopy.

NA = not applicable

⁴Lithium chloride (LiCl) was delivered by intraperitoneal (i.p.) injection 30 min after delivery of conditioning stimulus.

⁵The conditional stimulus was delivered directly to the stomach by intra-gastric infusion by a syringe pump.

⁶Delivery rates varied according to final volume (i.e., 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mL); 3 mice per volume.

⁷Injected volumes were 0.125, 0.25, 0.375, 0.5, 0.625, and 0.75 mL; 5 mice per volume.

⁸LiCl was mixed directly with the saccharin solution in Exp.5 and given immediately before solution presentation in Exp. 6.

tories, Wilmington, MA) were used in all experiments. Subjects were housed in individual cages (28 cm × 12.5 cm × 12 cm) in a temperature-controlled room at 23°C on a 12-h light (12-h dark cycle) and had free access to the Teklad Rodent Diet 8604 (Harlan, Madison, WI).

Intragastric Catheter Surgery

Mice were deeply anesthetized with an i.p. injection of a ketamine hydrochloride (42.8 mg/kg), xylazine hydrochloride (8.6 mg/kg) and acepromazine (1.5 mg/kg) mixture (10 mL/kg) and anesthesia was maintained with 1% isoflurane. The abdomen of each mouse was shaved from the sternum to approximately 5 cm caudal of the sternum and the shaved area was cleaned with alternate gauze swabs of 70% alcohol and iodine disinfectant (Betadine, Purdue Pharma L.P., Stamford, CT). An incision along the midline was made with scissors (~1.5 cm below sternum). On both sides of the incision, the surrounding skin was separated from the underlying muscle layer using a needle holder. A shorter incision was made in the abdominal muscles to display the abdominal cavity. The stomach was removed from the abdominal cavity and laid on a sterile cotton swab. A purse suture was made in the fundus of the stomach using 6-0 silk suture. The heat-flared end of a micro-renathane catheter was inserted into the stomach via a small puncture into the center of the area enclosed by the purse suture, which was pulled closed and tied off. The stomach was replaced in the abdominal cavity and a small hole was made in the abdominal muscle ~1 cm from right side of incision with #7 curved forceps. The catheter was pulled through the opening and the abdominal muscle opening closed with Surgi-Lock liquid suture (Meridian Animal Health, Omaha, NE). The catheter was routed under the skin to the back of the neck and the stomach incision was closed with 5-0 silk suture. A back mount (Plastics One, Roanoke, VA) was attached to the muscle layer on each side of the mount using 5-0 silk suture. The catheter was connected to side port of the back mount and the neck incision was tightly closed with 5-0 suture followed by 18 mm wound clips. All incisions were treated with triple antibiotic (Neosporin, Johnson & Johnson, New Brunswick, NJ). Mice were prophylactically treated with an antibiotic (2.0 mg/kg Gentamicin intramuscularly) and given postoperative treatment (1.0 mg/kg Buprenorphine subcutaneously) for pain.

Several days before, and for several days immediately after surgery, mice were fed a mixture of chocolate Ensure (Abbott Laboratories, Abbott Park, IL) and ground Teklad Rodent Diet 8604 chow (Harlan) to facilitate digestion and BW maintenance. Mice were given 5 to 12 d to recover from surgery during which food and water was available ad libitum.

Apparatus

Mice infused IG were conditioned in groups of 4 (i.e., 2 IG infusion subjects and 2 controls). Conditioning was conducted in a 22.5 cm × 24 cm × 20 cm polycarbonate cage. For IG infusions, polyethylene tubing connected a multi-syringe infusion pump (Harvard Apparatus, Holliston, MA) to the input port of a 22-gauge swivel (Instech Laboratories, Inc. Plymouth Meeting, PA) clamped to a ring stand. The output port of the swivel was attached to the input port of the back mount of each mouse with polyethylene tubing surrounded by a stainless-steel spring (PlasticsOne) for protection. Control mice were placed in an identical polycarbonate cage placed next to the infusion pump to permit access to environmental (e.g., odor or auditory) cues occurring during infusion. Fecal matter was removed promptly during infusion sessions and cages were cleaned with 70% isopropyl alcohol and allowed to dry between each set of mice.

Exp. 1

At the beginning of the experiment, mice were 8 wk old and had a mean BW of 39.5 ± 0.6 g. Mice implanted with IG catheters were acclimated to the infusion apparatus with 5-min infusions of 0.5 mL of water for 3 consecutive d followed by 2 d of rest. Food was removed from all groups at 1600 h on the day before conditioning days and returned at 1600 h after conditioning. Mice were randomly assigned to 3 treatment groups.

Conditioning occurred between 0930 h and 1130 h. Mice in the **IG+LiCl** (n = 5) and **IG** (n = 5) groups were infused with 0.1 mL/min of 5 mM sodium saccharin (Sigma Aldrich, St. Louis, MO) over 10 min for a total of 1 mL of sodium saccharin infused. Thirty minutes after intragastric infusion of 5 mM sodium saccharin, mice in the IG+LiCl treatment received ~1.4 mL i.p. injection of 150 mM LiCl (230 mg/kg). The IG treatment group received an IG infusion of a taste stimulus alone. The **LiCl** treatment group (n = 8) was placed in a polycarbonate cage identical to the infusion cage and 30 min later was injected with ~1.4 mL of 150 mM LiCl 230 mg/kg. Mice in the LiCl treatment group were not implanted with IG catheters. Mice were conditioned on 3 d with a day of rest in between each conditioning day (Table 1).

After 2 d of rest, mice were given 4 consecutive 23-h, 2-bottle preference tests (5 mM saccharin vs. water) in their home cages. The position of the saccharin was counterbalanced among all groups and switched daily. Solutions were offered in 30-mL syringes that were modified to accept a standard stainless steel sipper tube (Allentown Caging Equipment Co., Allentown, NJ). The syringes were mounted on the front of the cage with the drinking spouts penetrating so that the tips were ~ 4.2

cm apart and ~ 4.6 cm above the cage floor.

Exp. 2

In light of research suggesting that intraluminal pressure reaches maximal accommodation without distension in the mouse stomach at approximately 0.5 mL (Dixit et al., 2006), Exp. 1 was repeated with a smaller infusion volume (decreased to 0.5 mL from 1.0 mL). At the beginning of the experiment, mice ($n = 32$) were 8-wk old and had a mean BW of 33.2 ± 0.3 g. To accommodate the change in volume, the concentration of the sodium saccharin solution was increased to 10 mM (from 5 mM in Exp. 1) to maintain identical saccharin doses between the 2 experiments. Mice in the **10IG+LiCl** ($n = 9$) and **10IG** ($n = 9$) groups were infused with 10 mM sodium saccharin (50 μ L/min for 10 min) for a total of 0.5 mL. Mice in the **10LiCl** group ($n = 9$) were exposed to identical environmental conditions, but did not receive surgery. The 2-choice preference test procedure was identical to Exp. 1, with the addition of 10 mM saccharin versus water in a second, separate series of 4 consecutive 23-h, 2-bottle preference tests.

Exp. 3

The previous experiments raised the possibility that large volumes of solutions infused into the stomach might stimulate oral taste by esophageal reflux. To test this directly, we infused various volumes of the fluorescent dye, sodium fluorescein (5.4 mM; prepared in 0.01 M PBS) into the stomach and looked for its appearance in the oral cavity.

Twenty mice from Exp. 2 were used. Food was removed from all groups at 1600 h on the day prior the day of infusion. Two mice received neither surgical treatment nor fluorescein infusion to act as a control for autofluorescence. For the remaining 18 mice, 5.4 mM sodium fluorescein (Sigma Aldrich) was infused so as to deliver over a 10-min period these volumes: 0.25, 0.5, 0.75, 1.0, 1.25, and 1.50 mL. Three mice were infused at each volume. After infusion, mice were returned to their home cages for 90 min and were then euthanized by CO₂ asphyxiation.

Although the mouse was still intact, an observer blind to the experimental treatments recorded the presence of fluorescence in the oral cavity detected with a hand-held ultraviolet. The anterior tongue (i.e., rostral of the intramolar eminence), esophagus, and heart were harvested and stored separately in 0.01 M PBS for 24 h. Esophagi and hearts were halved to expose the interior tissue and all tissues were mounted on glass slides for examination under light microscopy (4 \times magnification) using a fluorescein filter set. Images were captured using

a Nikon digital camera (DXM1200C) attached to a Nikon Eclipse 80i microscope (Nikon Inc., Melville, NY). The exposure times of the camera of the microscope were set for the brightest fluorescence (e.g., tongue 1/30 s, esophagus 1/25 s, and heart 1/12 s) and all images of specific tissues were taken at those exposure times.

Exp. 4

To determine if fluorescein was being transported throughout the body via the circulatory system, fluorescein was injected directly into the peritoneal cavity. At the beginning of the experiment, mice ($n = 31$) were 9-wk old and had a mean BW of 39.6 ± 0.4 g. Food was removed from all groups at 1600 h on the day before the day of injection. To compensate for the small size of the peritoneal cavity, the fluorescein concentration was doubled (10.8 mM) and these volumes were delivered by i.p. injection: 0.0, 0.125, 0.25, 0.375, 0.5, 0.625, and 0.75 mL. With the exception of a single control mouse (no injection), 5 mice were infused at each volume. After i.p. injection, mice were returned to their home cages for 90 min and were then euthanized by CO₂ asphyxiation. Tissues were evaluated for fluorescence as in Exp. 3.

Exp. 5

To determine if the circulatory system provided a route to oral detection of saccharin, Exp. 1 was repeated except that saccharin was delivered by i.p. injection and training occurred in the home cages of the mice. In addition, a 300 mM LiCl concentration was employed to reduce the injection volume because saccharin was also being delivered i.p. in this experiment. At the beginning of the experiment, mice ($n = 32$) were 8-wk old and had a mean BW of 33.1 ± 0.3 g. Food was removed from all groups at 1600 h on the day before the day of injection. The **IP+LiCl** group ($n = 8$) received a 1 mL i.p. injection of 5 mM saccharin followed 30 min later by a second i.p. injection of 300 mM LiCl (230 mg/kg; 0.55 mL for a 30 g mouse). The **IP** treatment group ($n = 8$) received an i.p. injection of 5 mM saccharin alone. The **IPLiCl** treatment group ($n = 8$) was injected with 300 mM LiCl (230 mg/kg; 0.55 mL for a 30-g mouse). A fourth treatment was added to Exp. 5. The **IPSaccon** group ($n = 8$; concurrent delivery of saccharin and LiCl) received a 1 mL i.p. injection of a 5 mM saccharin and LiCl (230 mg/kg) solution such that delivery of the taste stimulus and unconditional stimulus were simultaneous.

Exp. 6

In the final experiment, we verified the aversive response to oral presentation of 5 mM saccharin paired

with lithium toxicosis and evaluated the aversive response to a 500-fold smaller concentration of saccharin (0.01 mM). The experiment was performed to offer a potential explanation for the results of Exp. 1 based on the results of Exp. 2 to 5. If saccharin infused into the gut was reaching the oral cavity, one might expect that it would be dilute in comparison with the concentration originally infused (Exp. 1) or injected (Exp. 5). Experiment 1 was repeated except there was no food restriction, saccharin was presented orally during conditioning, and conditioning occurred in the home cage of each mouse. At the beginning of the experiment, mice ($n = 40$) were 8-wk old and had a mean BW of 29.6 ± 0.2 g. Water was removed from all groups at 1600 h on the day before the days of conditioning to encourage the mice to drink during the 30-min saccharin exposure. Mice in the **0.01Oral+LiCl** ($n = 8$) and **5Oral+LiCl** ($n = 8$) groups were presented orally with 25 mL of 0.01 mM or 5 mM sodium saccharin, respectively, for 30 min followed by i.p. injection of 150 mM LiCl. Mice in the **0.01Oralsaccon** ($n = 8$; concurrent delivery of saccharin and LiCl) group were given an i.p. injection of 150 mM LiCl followed immediately by an oral presentation of 25 mL of 0.01 mM sodium saccharin for 30 min. Mice in the **Oral** ($n = 8$) and **0.01Oralex** ($n = 8$; experienced with saccharin) groups were presented orally with 25 mL of distilled water or 0.01 mM sodium saccharin, respectively, for 30 min, but did not receive any LiCl treatment during conditioning. Preference testing was identical to Exp. 1.

Data Analyses

Intakes of 2-bottle test taste solutions were measured (to the nearest 0.1 g) daily. Intakes were not corrected according to BW. Data for each experiment were analyses separately and examination of residuals indicated that use of ANOVA was valid in each case. For Exp. 1, 2, and 5, preference scores were calculated as the ratio of saccharin solution to total fluid (saccharin + water) intake. Scores were analyzed by repeated measures ANOVA using the MIXED procedure (SAS Inst. Inc., Cary, NC). Treatment group and the interaction were fixed effects. Mice were considered random effects. Multiple comparisons of least square means were made using the false discovery rate controlling procedure (Benjamini and Hochberg, 1995). Residuals were tested for assumptions (location, normality, and independence) using the UNIVARIATE procedure of SAS.

To draw comparisons across multiple experiments, control group means from appropriate experiments were employed as hypothetical means. "Difference scores" were calculated as 5-mM preference scores of mice receiving saccharin paired with lithium (i.e., IG+LiCl, IP+LiCl, 5Oral+LiCl, and 0.01Oral+LiCl) in Exp. 1,

5, and 6 minus the mean preference score of the corresponding unconditioned treatment (i.e., IG, IP, and Oral). Thus, difference scores represented the change in 5-mM saccharin preference relative to the unconditioned response. Scores were analyzed by 1-way ANOVA using the MIXED procedure in SAS. Multiple comparisons of least square means were made using the false discovery rate controlling procedure. Comparisons of the initial BW for mice in each group were analyzed by mixed-model ANOVA with treatment as a fixed effect and subject a random effect.

RESULTS

Exp. 1

There was no difference in initial BW of the 3 groups ($P > 0.1$). Preference scores were impacted by treatment ($P = 0.0053$) and position ($P = 0.0029$; position refers to the right or left position of the saccharin tube as placed in the test cage). No other effects were significant ($P > 0.05$). In 23 h, 2-bottle tests of 5 mM saccharin vs. water, the IG+LiCl group had a reduced preference for saccharin in comparison with both the IG ($P = 0.0061$) and LiCl ($P = 0.0024$) groups, which expressed a strong preference for the saccharin in comparison with water (Figure 1). This level of decreased preference was maintained throughout the 4 d of 2-bottle tests; thus, there was no evidence of extinction. The reduced preference for saccharin displayed by the IG+LiCl group suggested that mice "tasted" saccharin infused into the gut and associated this taste with lithium toxicosis.

Exp. 2

There was no difference in initial BW for all groups ($P > 0.1$). Preference for 5 mM saccharin in comparison with water did not differ among the treatment groups ($P = 0.134$; Figure 2a); or for 10 mM ($P = 0.369$; Figure 2b). These results suggest that mice could not "taste" the reduced volume of saccharin infused directly into the gut.

Exp. 3

The results obtained by observing fluorescence on the tongues of intact mice were almost identical to the results seen under light microscopy, so we present only the latter here. Auto-fluorescence, a natural condition in tissue samples, was observed in all tissue samples (including tissue never exposed to fluorescein), but a strong and unmistakable fluorescent signal was observed from the tongues and esophagi of mice infused with a volume of 0.5 mL of fluorescein or more. However, no fluorescence was observed from the heart tissues (Fig-

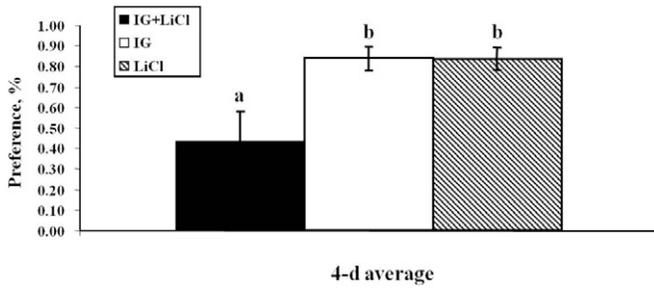


Figure 1. Preference scores (Exp. 1) of mice trained to associate 1.0 mL of intragastric (IG) infusions of 5 mM saccharin with intraperitoneal (i.p.) lithium chloride (IG+LiCl; black bar) relative to mice given IG saccharin alone (IG; white bar) or i.p. LiCl alone (LiCl; cross-hatched bar) mice. ^{a,b}Means without a common letter differ ($P < 0.05$).

ure 3). These results suggest that solutions of 0.5-mL volume or more infused into the mouse stomach may leak through the esophageal sphincter and enter the oral cavity by traveling through the esophagus.

Exp. 4

The results obtained by observing fluorescence on the tongues of intact mice were almost identical to the results seen under light microscopy, so again we present only the latter here. Contrary to the results of Exp. 3, fluorescence

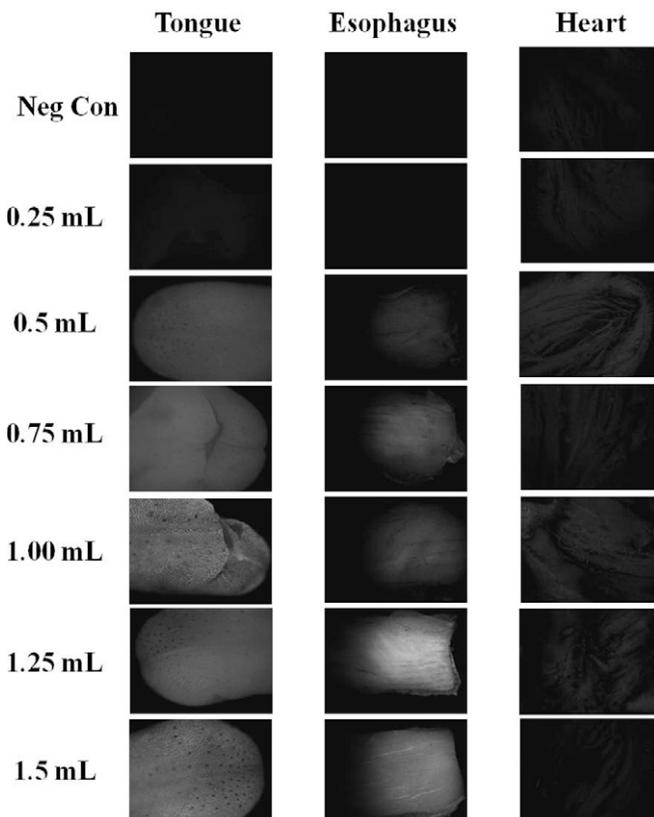


Figure 3. Examples of stained mouse tissue after 10-min intragastric (IG) infusion of various volumes (0.0, 0.25, 0.5, 0.75, 1.0, 1.25, or 1.5 mL) of 5.4 mM fluorescein. Note that fluorescence was observed in the esophagus and the oral cavity if the volume of the infusion was 0.5 mL or greater. No fluorescence was observed in heart tissues.

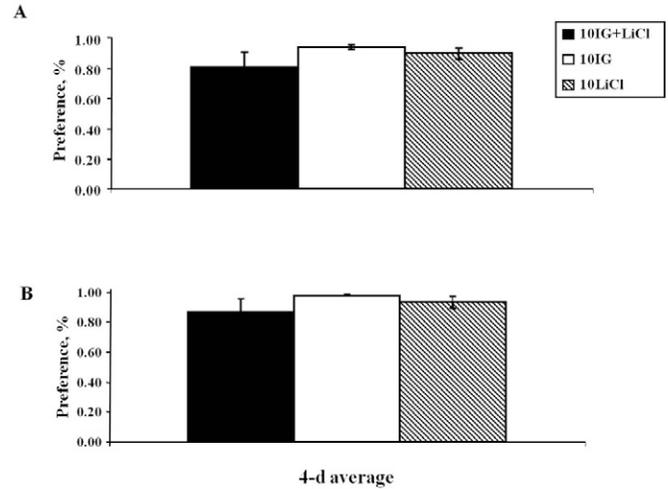


Figure 2. Preference scores of mice in Exp. 2. Mice were trained to associate 0.5 mL of intragastric (IG) infusions of saccharin with intraperitoneal (i.p.) lithium chloride (IG+LiCl; black bar) relative to mice given IG saccharin alone (IG; white bar) or i.p. LiCl alone (LiCl; cross-hatched bar). Top panel: tests using 5 mM saccharin; bottom panel: tests using 10 mM saccharin. There were no differences in preferences among the groups ($P > 0.05$).

was detected with a hand-held ultraviolet light in shallow tissue extremities (i.e., toes and ears). Auto-fluorescence was observed in all tissue samples (including tissue never exposed to fluorescein) but a strong and unmistakable fluorescent signal was observed from the tongues and esophagi of mice injected with volumes of 0.25 mL of fluorescein (equivalent to IG infusions of 0.5 mL) or more (Figure 4). These results suggest that solutions of 0.5-mL volume or more injected into the mouse peritoneal cavity travel to the oral cavity through the blood.

Exp. 5

There was no difference in initial BW of the 3 groups ($P > 0.1$). Preference scores were impacted by treatment ($P = 0.0287$) and day ($P = 0.0012$). No other effects were observed ($P > 0.05$). In 23 h, 2-bottle tests of 5 mM saccharin vs. water, the IP+LiCl (91%) and IP-Saccon (89%) groups had reduced preferences for saccharin in comparison with the IP ($P = 0.0465$ and $P = 0.0151$, respectively) group, which expressed a strong preference (96%) for the saccharin in comparison with water (Figure 5). The IPSaccon (89%) group had a reduced preference for saccharin in comparison with the IPLiCl ($P = 0.0198$) group, which also expressed a strong preference (96%) for the saccharin in comparison with water (Figure 5). This level of decreased preference was maintained throughout the 4 d of 2-bottle tests; thus, there was no evidence of extinction. Although the magnitude of the decrease in saccharin was small, reduced preference for saccharin in the IP+LiCl and IPSaccon treatments demonstrate that mice “tasted” saccharin injected into the peritoneal cavity and associated this taste with lithium toxicosis.

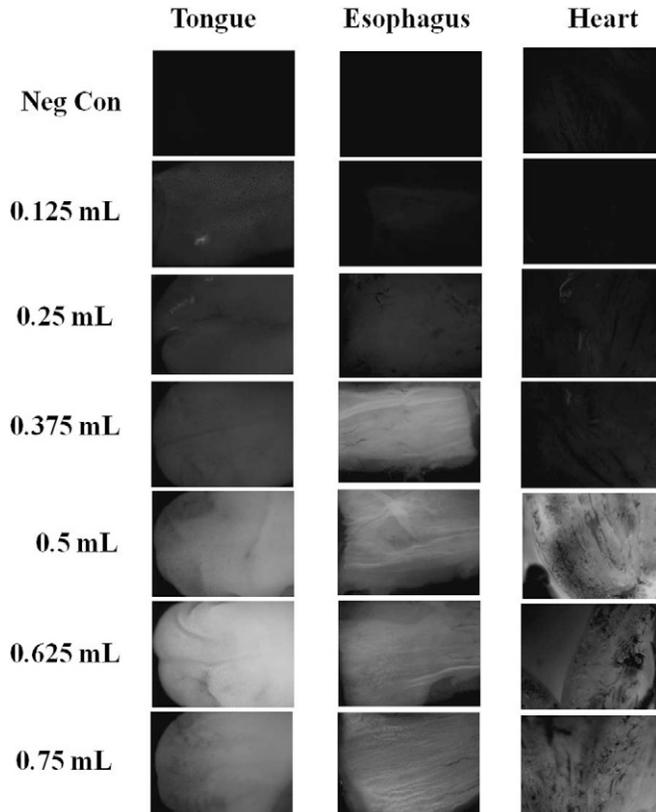


Figure 4. Examples of stained mouse tissues after intraperitoneal (i.p.) injections of various volumes (0.0, 0.125, 0.25, 0.375, 0.5, 0.625, and 0.75 mL) of 10.8 mM fluorescein. Note that fluorescence was observed in the esophagus and the oral cavity if the volume of the injection was 0.25 mL or greater and in the heart if the volume of the injection was 0.25 mL or greater.

Exp. 6

There was no difference in initial BW of the 5 groups ($P > 0.1$). Analyses of preference responses indicated a treatment \times day interaction ($P = 0.0015$). This can be attributed to changing responses across the 4-d testing period among the 0.01Oralsaccon and 0.01Oral+LiCl treatment groups (Figure 6). Day 1 scores in these groups were less than d 2, 3, and 4 indicating extinction occurs fairly rapidly for subjects receiving a low saccharin con-

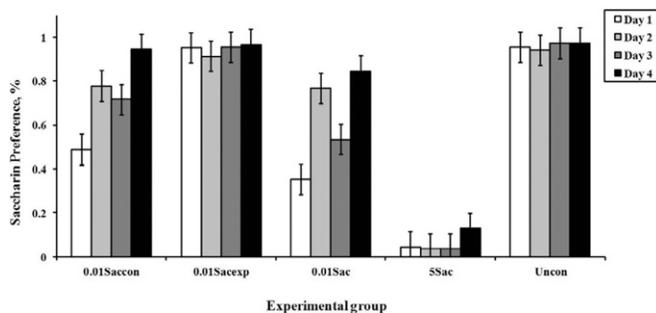


Figure 6. Preference scores by day (Exp. 6) for 5 mM saccharin in comparison with distilled H₂O of mice trained to associate oral presentation of either 0.01 mM (0.01Sac) or 5 mM (5Sac) saccharin with intraperitoneal (i.p.) LiCl after a 30-min delay or concurrently (0.01Saccon) relative to mice experiencing oral presentation of 0.01 mM saccharin (0.01Sacexp) or distilled H₂O (Uncon).

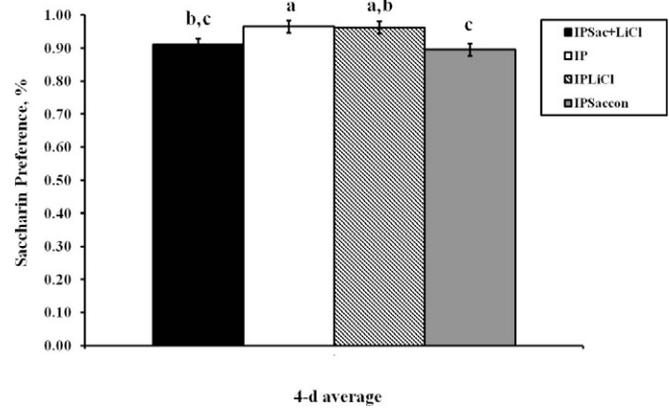


Figure 5. Preference scores (Exp. 5) of mice trained to associate 1.0-mL intraperitoneal (i.p.) injections of 5 mM saccharin with i.p. LiCl after a 30-min delay (IP+LiCl; black bar) or concurrently (IPSaccon; gray bar) relative to mice given i.p. injections of saccharin alone (IP; white bar) or i.p. LiCl alone (IPLiCl; cross-hatched bar) mice. ^{a-c}Means without a common letter differ ($P < 0.05$).

centration during conditioning. The main effects, treatment ($P < 0.0001$) and day ($P < 0.0001$), were the only other significant effects in the model. In 23 h, 2-bottle tests of 5 mM saccharin vs. water, the Oral (96%), and 0.01Oralexp (95%) groups had greater preferences for saccharin in comparison with the 0.01Oralsaccon ($P = 0.0031$ and $P = 0.0051$, respectively) and 0.01Oral+LiCl groups ($P < 0.0001$ and $P < 0.0001$, respectively), which expressed decreased preferences (73% and 62%, respectively) for the saccharin in comparison with water in 2-choice tests (Figure 6). The 5Sac+LiCl group expressed a significant aversion to 5 mM saccharin in comparison with water and had a significantly less preference (6%) than all other groups ($P < 0.0001$).

Comparisons among Experiments

Analysis of difference scores obtained from multiple experiments indicated the magnitude of decreased saccharin preference followed the order: 5Oral+LiCl $>$ 0.01Oral+LiCl = IG+LiCl $>$ IP+LiCl (Figure 7). Decreased preference for 5 mM saccharin in the 0.01Oral+LiCl group was similar to the reduction in preference for the IG+LiCl group observed in Exp. 1 ($P = 0.15$) and may indicate a similarity between the apparent taste of 5 mM saccharin infused IG and a small (0.01 mM) saccharin concentration presented orally.

DISCUSSION

Sensory cues of food are integrated with post-ingestive consequences of consumption by way of associative learning processes (Provenza et al., 1992). A classic illustration of this can be seen in possum feeding preferences for eucalyptus. Eucalyptus terpenes (cues) regulate intake as a result of learned association with the toxic effects of

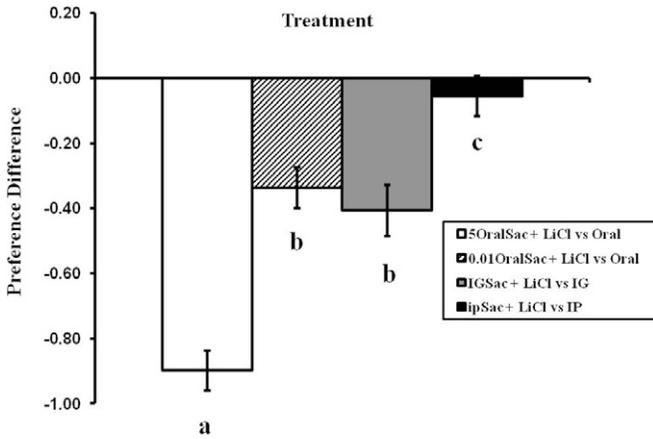


Figure 7. Preference score reductions of conditioned mice in relation to unconditioned mice (control groups) from each conditioned taste aversion experiment. Mice were trained to associate oral presentations of 5 mM saccharin with intraperitoneal (i.p.) LiCl after a 30-min delay (5Oral+LiCl; white bar; vs. Oral), 0.01 mM saccharin with i.p. LiCl after a 30-min delay (0.01Oral+LiCl; cross-hatched bar; vs. Oral), 1.0-mL IG infusions of 5 mM saccharin with i.p. LiCl (IG+LiCl; gray bar; vs. IG), or 1.0-mL i.p. injections of 5 mM saccharin with i.p. LiCl after a 30-min delay (IP+LiCl; black bar; vs. IP). ^{a-c}Means without a common letter differ ($P < 0.05$) in preference compared with controls among the groups.

diformlyphloroglucinol compounds (consequences) also present in eucalyptus leaves (Lawler et al., 1999). In fact, this example clearly demonstrates that compounds giving rise to consequences of forage consumption are rarely the same as those serving as cues (Provenza and Balph, 1990). Preferences are similarly formed when sensory cues are associated with beneficial consequences of ingestion (e.g., nutrients). Importantly, this interplay of cues and consequences (i.e., palatability) has implications far beyond diet selection. Landscape heterogeneity (Manier and Hobbs, 2006), ecosystem function (Hobbs, 1996), and herbivore population dynamics (Moore and Foley, 2005; Wang et al., 2006), among other landscape-level processes, are influenced by foraging behaviors arising from detection and ingestion of phytochemicals.

Mammals, from small rodents to large herbivores, are equipped with anatomical and biochemical attributes permitting detection, use, and detoxification of forage. Integration of gustatory and visceral information is made possible by the confluence of neurons in the solitary tract of the nucleus, allowing for learned preferences and aversions (Provenza, 1995a). Such affective processes, long characterized in rodents (Swank et al., 1996; Thiele et al., 1996; Houpt et al., 1997), are also well recognized in large herbivores (Provenza, 1995b). Thus, information regarding integration of sensory and post-ingestive information obtained in model rodents is relevant to other mammals and their interactions with their foraging environments.

Role of Intestinal Taste Receptors

For obvious reasons, olfactory and taste receptors

present in the nose and mouth have been considered primary participants in sensory evaluation of forage items. Until recently, it was commonly thought that G-coupled protein taste receptors were restricted to the mouth in mammals. However, it is now clear that they also exist in the GI mucosa of humans and rodents (Furness et al., 1999; Rozengurt, 2006; Sternini et al., 2008). In particular, GI G-coupled protein T1R sweet and T2R bitter taste receptors and parts of their second messenger pathways have been identified (Dyer et al., 2005; Wu et al., 2005; Rozengurt, 2006; Margolskee et al., 2007; Rozengurt and Sternini, 2007; Sternini, 2007; Hass et al., 2010) and activation of these pathways in GI cells has been demonstrated (Rozengurt and Sternini, 2007). Information transmitted from taste receptors in the intestinal tract appears to initiate neural activation in the amygdala, hypothalamus, nucleus of the solitary tract and other brain regions related to gustatory processes (Hao et al., 2008, 2009).

Although researchers have been investigating the potential roles of these intestinal taste receptors, the reason for their presence in the intestinal tract remains uncertain. Sweet and bitter compounds acting on GI bitter taste receptors modify taste response (Tracy et al., 2004; Glendinning et al., 2008) and GI motility (Glendinning et al., 2008). Recent work suggests that gustatory information is transmitted to the brain regarding taste qualities associated with a conditioned taste aversion (Tracy et al., 2004; Tracy and Davidson, 2006). These experiments employed nutrients (i.e., maltodextrin and corn oil) with post-ingestive effects of their own as conditioned stimuli. It is not known which taste receptors are activated by polycose and corn oil, although it has been shown that polycose does not act on the sweet receptor (Treesukosol et al., 2009; Zukerman et al., 2009). Thus, it is unclear if secondary post-ingestive effects of these nutrients were associated with the toxic effects of the primary unconditioned stimulus or if these compounds served as conditional stimuli via chemical signals originating in the gut. Although saccharin activates intestinal sweet taste receptors (Margolskee et al., 2007), it is not known to have nutrient-like conditioning effects, particularly at the concentrations employed here. It is important that the test compound used have no post-ingestive consequences of its own so that later preference testing is not influenced by these effects. Put another way, it has not been established whether chemosensory input from GI taste receptors alone is sufficient to modify the taste response to a substance infused directly into the gut (i.e., stomach or small intestine). Uncertainty regarding the role of taste receptors in the gastrointestinal tract suggests that no functions should be considered implausible until adequately tested. This information is critical to understanding palatability and understanding herbivore

responses to their phytochemical environments.

Gastric Taste Aversion

Mice were used in this study because intragastric receptors have been well characterized in this model and the testing apparatus is best described in its use with rodents (Sclafani, 2004). When intragastric infusion of 5 mM saccharin was paired with i.p. injection of LiCl, mice expressed reduced preference for 5 mM saccharin in 2-bottle tests. When viewed in comparison with the results of Exp. 2, the results of Exp. 1 do not lend weight to the hypothesis that saccharin can be tasted by receptors in the GI tract. However, based on the results of this experiment alone, it could be interpreted as evidence that a taste compound with little or no post-ingestive consequence can be detected by gastrointestinal taste receptors and processed by the brain in a manner similar to taste receptor feedback from the oral cavity. Because delays in processing of sensory cues can be detrimental to the learning process, such a detection system would be expected to operate on the same temporal scale as the oronasal receptor systems. Furthermore, rapid recognition of the sensory cues would be required for cessation of feeding on toxic foods at future encounters. Thus, mammals could benefit from concurrent sensory input directly from the intestinal tract when assessing diets.

Many mammals, particularly laboratory rodent species, are likely incapable of emesis (Andrews and Horn, 2006). Although gastric distension by itself did not appear to serve as an unconditioned stimulus, sufficient back pressure could have resulted in reflux into the oral cavity. Concerned with unintentional delivery of the taste stimulus to the oral cavity via reflux, the volume infused into the gut was halved for Exp. 2. Mice did not express an aversion to saccharin using the reduced infusion volume. Two plausible mechanisms may explain these results. First, a critical volume was exceeded, above which experimentally-induced reflux forced the taste solution into the oral cavity via the esophagus. In the second mechanism, increased osmolality of the greater tastant concentration promoted rapid adsorption and delivery to the oral cavity via circulating blood. Exp. 3 to 6 explored these 2 potential mechanisms for compounds to reach the oral cavity. In humans, saccharin is tasted on the tongue shortly after entering the blood stream (Fishberg et al., 1933). In rats, an aversion to the taste of saccharin has been conditioned after intravenous injections of saccharin being paired with exposure to gamma radiation (Bradley and Mistretta, 1971) suggesting that rats can “taste” saccharin after intravenous injection. However, gamma radiation paired with i.p. injections of saccharin did not result in reduced preferences in a different study (Scarborough and McLaurin, 1961). Importantly, the be-

havioral data indicate that the “taste” of 5 mM saccharin presented intragastrically is not the same as an oral presentation, once it reaches the oral cavity. Preference scores from IG+LiCl mice in Exp. 1 only show indifference (~43%) in their preference for oral 5 mM saccharin, whereas 5Oral+LiCl mice show a strong aversion (6%) to oral 5 mM saccharin.

Reflux as a Pathway to the Oral Cavity

We evaluated gastric reflux as a potential pathway for an infused taste stimulus to reach the oral cavity by infusing mice with 5.4 mM fluorescein and examining various body tissues under light microscopy. Fluorescence was detected on the interior surface of the esophagus and the anterior tongue, but not the heart, with infusion volumes greater than 0.25 mL. In Exp. 3, no fluorescence was observed from heart tissues, suggesting that a tastant infused into the stomach of a mouse is unlikely to reach the oral cavity by transport through the blood. These results suggested that stomach distension caused by infusion volumes of 0.5 mL or greater may force fluids through the esophageal sphincter, the esophagus itself, and into the oral cavity. However, these results do not fundamentally establish that dye present on these tissues was a result of reflux. In fact, injection of 10.8 mM fluorescein in the peritoneal cavity did result in observed fluorescence in heart tissues. Importantly, fluorescence was detected in esophageal tissue of mice injected with 0.5 mL of fluorescein in both Exp. 3 and 4, but this volume of saccharin did not evoke a behavioral response in Exp. 2. The major difference between Exp. 3 and 4 was that an unmistakable fluorescent signal was observed from the heart tissues of mice injected i.p. with volumes of 0.5 mL or more. It is not clear why injection with 0.25 mL fluorescein resulted in a fluorescent signal from heart tissue but tongues and esophagi were stained regardless of the method of delivery. It is possible that tongue and esophageal tissue are more sensitive to the dye. The dichotomy of the results in Exp. 3 and 4 suggest that there are other potential pathways from the gut to the oral cavity. Earlier studies provided evidence that this pathway could involve circulating blood (Fishberg et al., 1933; Bradley and Mistretta, 1971).

Blood as a Pathway to the Oral Cavity

Experiment 5 was designed to evaluate circulating blood as a pathway for oral taste sensation. We paired i.p. injections of 5 mM saccharin with both delayed (30 min) and simultaneous presentations of LiCl. A small but statistically significant decrease in saccharin preference was demonstrated when i.p. saccharin was paired with either simultaneous or delayed exposure to LiCl. There

was no decrease in saccharin preference for IPLiCl control group despite being injected with a hypertonic solution of LiCl. A previous study pairing i.p. saccharin with X-ray radiation in rats demonstrated a more dramatic reduction in saccharin intake, but these differences were not statistically significant (Scarborough and McLaurin, 1961). The timing of saccharin adsorption and illness onset was shown to be a critical aspect of i.p. saccharin aversions. In an earlier study, a delay of approximately 120 min between initial i.p. delivery of 2% saccharin and LiCl injection was needed to produce a strong aversion (Bellingham and Lloyd, 1987). Thus, delivery of the conditional stimulus to oral taste receptors via circulating blood is a relatively slow process in the context of food consumption.

Although intragastric infusion of 5 mM saccharin paired with i.p. injection of LiCl reduced preference for 5 mM saccharin during expression testing, this reduced preference was not as pronounced as the aversion produced by oral presentation of 5 mM saccharin. This difference may represent conditioned stimulus/unconditioned stimulus delay, where information regarding saccharin taste was not immediately processed, or a concentration effect, where intragastric 5 mM saccharin was interpreted as being less in concentration than oral 5 mM saccharin. The magnitude of reduced saccharin preference in Exp. 1 (IG) relative to preference reduction in Exp. 5 (i.p.) suggests a concentration effect. Both the 0.01 Oral+LiCl and IG+LiCl groups expressed a similar decrease in preference for 5 mM saccharin, whereas only a minor reduction in preference was observed in the IP+LiCl group. However, it is important to note that the decreased preference for 5 mM saccharin expressed in IG+LiCl mice remained relatively stable over the 4 d of 2-bottle testing, whereas the decreased preference among 0.01 Oral+LiCl mice moved rapidly toward extinction. This difference could arise from differing qualitative taste properties in the mouth of 0.01 mM saccharin delivered directly and 5 mM saccharin arriving indirectly. Considering the effects observed from i.p. saccharin presentation, experimentally-induced gastric reflux represents the most likely route for rapid presentation of saccharin in the oral cavity at a reduced concentration. Furthermore, the resulting aversion was attenuated as compared with oral presentation of the tastant.

Conclusion

It is imperative that mammals maximize intake of primary plant metabolites and minimize toxin ingestion when selecting among natural forages. To accomplish this, they rely on associative and cognitive processes to recognize and respond behaviorally to the phytochemicals they encounter (Provenza et al., 1992). These results demon-

strate that taste stimuli liberated in the GI tract may result in recognition via oral sensory activation. When that route to oral taste receptors is adsorption and delivery via circulating blood, the significant delay will attenuate formation of an aversion. Similarly, when that route is regurgitation (among species capable of emesis) or artificially-induced reflux (as in our experimental model), dilution of tastant concentration will also attenuate the aversion, albeit to a lesser extent. Although taste cues liberated in the gut may ultimately be detected by taste receptors residing in the oral cavity, impediments to formation of necessary preferences and aversions to forage items render this alternative mechanism inadequate for learning. Ultimately, the current study does not support the hypothesis that "intestinal taste" contributes to palatability and foraging behavior.

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