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Citric Acid and Quinine Share Perceived Chemosensory Features Making Oral Discrimination Difficult in C57BL/6J Mice

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Abstract

Evidence in the literature shows that in rodents, some taste-responsive neurons respond to both quinine and acid stimuli. Also, under certain circumstances, rodents display some degree of difficulty in discriminating quinine and acid stimuli. Here, C57BL/6J mice were trained and tested in a 2-response operant discrimination task. Mice had severe difficulty discriminating citric acid from quinine and 6-*n*-propylthiouracil (PROP) with performance slightly, but significantly, above chance. In contrast, mice were able to competently discriminate sucrose from citric acid, NaCl, quinine, and PROP. In another experiment, mice that were conditioned to avoid quinine by pairings with LiCl injections subsequently suppressed licking responses to quinine and citric acid but not to NaCl or sucrose in a brief-access test, relative to NaCl-injected control animals. However, mice that were conditioned to avoid citric acid did not display cross-generalization to quinine. These mice significantly suppressed licking only to citric acid, and to a much lesser extent NaCl, compared with controls. Collectively, the findings from these experiments suggest that in mice, citric acid and quinine share chemosensory features making discrimination difficult but are not perceptually identical.

Key words: bitter, mice, psychophysics, sour, taste, T2R

Introduction

Many chemicals elicit taste sensations, and based on molecular, electrophysiological, and psychophysical findings, these have been categorized into perceptual qualities that are commonly referred to as basic tastes. The number of basic taste qualities is debated and possibly varies across species. When human subjects were given a test taste solution and instructed to verbally categorize each stimulus as ''bitter,'' ''salty,'' ''sour,'' ''sweet,'' or ''no taste'' (Meiselman and Dzendolet 1967) or instructed to categorize the test solutions with one of 4 standard solutions (McAuliffe and Meiselman 1974), there was more overlap of assigning sour and bitter labels relative to other qualitative adjectives. There is also evidence in the literature suggesting that, under certain circumstances, rodents display some degree of difficulty in discriminating quinine and acid stimuli (described by humans as bitter and sour, respectively). Response profiles obtained from 3 groups of rats trained to discriminate NaCl, from HCl, sucrose, or quinine, respectively, showed rats responded as if HCl were quinine-like and HCl-like, and as if quinine were quinine-like and to a lesser extent HCl-like (Morrison 1967). Similarly, although rats could be trained to discriminate each compound (either sucrose, NaCl, quinine, or citric acid) from the remaining 3 compounds, there was some minor drop in performance when discrimination involved lower concentrations of citric acid and quinine (Grobe and Spector 2008). From studies in which taste aversions were conditioned in hamsters, rats, and mice, there is some evidence of behavioral generalization among acids and ''bitter'' salts (Nowlis et al. 1980; Ninomiya et al. 1984a; Yamamoto et al. 1988; Frank and Nowlis 1989). Overall, the psychophysical findings in the literature suggest that although rats and humans can clearly discriminate sour from bitter stimuli, there appears to be some degree of commonality in the taste percepts elicited by such compounds at least at some concentrations.

It has been shown electrophysiologically and through calcium imaging that some rat taste bud cells respond to multiple taste stimuli including quinine and HCl (e.g., Sato and Beidler 1997; Gilbertson et al. 2001; Caicedo et al. 2002). It appears that whether neural patterns in the periphery clearly distinguish between acids and ''bitter-tasting'' compounds is

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dependent on from which gustatory nerve activity is recorded. In the chorda tympani (CT) nerve, a branch of the facial nerve that innervates taste buds in the anterior tongue, some fibers that respond optimally to acids including HCl and citric acid also show sensitivity to quinine in the rat (Frank et al. 1983), hamster (Frank et al. 1988), chimpanzee (Hellekant et al. 1997b), and mouse (Ninomiya et al. 1984b). This is also observed in the rat geniculate ganglion (Lundy and Contreras 1999; Breza et al. 2006, 2007, 2010) but not in all studies (Sollars and Hill 2005). In contrast, there appear to be fibers in the glossopharyngeal (GL) nerve, which innervates taste buds in the posterior tongue, that respond to quinine and other bitter-tasting compounds but not to acids and vice versa (Ninomiya et al. 1984a; Frank 1991; Hellekant et al. 1997a). Although the posterior tongue taste receptors are critical for the normal initiation of gapes, a reflex-like oromotor response, to quinine in rats (Travers et al. 1987; Grill et al. 1992; King et al. 2000; King et al. 2008), the glossopharyngeal nerve does not appear to be necessary for taste quality discrimination. For example, transection of the GL, which has classes of fibers that best discriminate between acids and bitter-tasting compounds, has no effect on a quinine versus citric acid aversion (St John 1997) or a variety of other taste discriminations (see St John and Spector 1998; Spector 2003). The fact that the branches of the seventh cranial nerve have been shown to be critical for taste quality discrimination (see St John and Spector 1998; Spector 2003), coupled with some degree of overlap in the response profiles of fibers in the CT nerve when stimulated by quinine and acids, may underlie the sour–bitter confusion observed in behavioral studies. In the rodent brainstem taste nuclei, there is also evidence of some degree of covariance in the responses of neurons to quinine and acids, but this varies across studies and species (e.g., Travers and Smith 1979; Giza and Scott 1991; Boughter and Smith 1998; Verhagen et al. 2003; Lemon & Smith 2005; McCaughey 2007; Geran and Travers 2009; Lemon and Margolskee 2009). Of particular relevance to the present study, responses to quinine and citric acid in the mouse nucleus of the solitary tract (NST) are highly correlated, suggesting that this species should have particular difficulty perceptually discriminating between these 2 stimuli.

To what extent the taste perception elicited by acids and bitter compounds are distinct from one another in mice, a popular animal model used in genetic engineering experiments involving taste, is unclear. In the present study, adopting a strategy used by Spector and Kopka (2002) to examine discriminability among bitter-tasting compounds, we used a 2-response operant procedure to test whether mice can discriminate citric acid from quinine. In a second experiment, a conditioned taste aversion (CTA) procedure was used to assess how mice trained to avoid quinine or citric acid generalized their avoidance to other taste stimuli in a brief-access test. These 2 experiments, respectively, addressed the questions of (i) whether citric acid and quinine have distinct perceived chemosensory features that make them discriminable and (ii) whether citric acid and quinine share perceived chemosensory features that make them similar, to mice. If citric acid and quinine are perceptually identical to mice, then the animals should be unable to learn to distinguish between them in a taste discrimination task. Furthermore, if the 2 compounds were indiscriminable, then mice should completely cross-generalize conditioned aversions between the 2 stimuli.

Materials and methods

Experiment 1: stimulus discrimination

Subjects

Twenty adult male wild-type C57BL/6J (B6) mice (The Jackson Laboratory) with mean body mass of 23.14 g (±0.47) upon arrival, were assigned to one of 2 groups. Group 1 was initially trained to discriminate citric acid from sucrose. Group 2 was initially trained to discriminate citric acid from quinine. All mice were experimentally naive at the start of the experiments. The mice were individually housed in polycarbonate tub cages in a room where the temperature, humidity, and lighting (12:12 h light:dark) were automatically controlled. Mice were given laboratory chow (Purina Laboratory Chow 5001) and deionized reverse-osmosis water ad libitum except where noted. Following at least 7 days of habituation to the laboratory environment, the mice were placed on a restricted water-access schedule in which fluid was available only during the training and testing sessions on Monday through Friday. Water bottles were replaced on the home cages of the mice after their testing session on Friday and removed again on Sunday, no more than 23 h before testing. While on the water-restriction schedule, mice that dropped below 85% of their free-drinking body weight received 1 ml supplemental water 1 h after the end of the testing session. Testing and training took place during the lights-on phase. All procedures were approved by the Florida State University Animal Care and Use Committee.

Taste stimuli

Test stimuli consisted of 3 concentrations each of quinine hydrochloride (0.495, 0.822, 1.52 mM; Sigma Chemical Co.), citric acid (10.0, 17.4, 33.8 mM; BDH Chemicals), sucrose (0.287, 0.504, 0.914 M; BDH Chemicals), NaCl (0.351, 0.488, 0.829 M; Mallinckrodt Chemicals), and 6-*n*-propylthiouracil (PROP; 1.0, 2.0, 4.0 mM; Sigma Chemical Co.). All taste solutions were prepared daily with deionized reverse-osmosis water and reagent grade chemicals and presented at room temperature. It took several hours to ensure PROP was dissolved into solution; thus, on testing days when PROP was one of the test stimuli, all taste solutions were prepared at least 12 h prior to testing.

Quinine, citric acid, sucrose, and NaCl were chosen as representatives of the prototypical taste quality categories that humans, respectively, describe as bitter, sour, sweet, and

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salty. Evidence in the literature suggests differential processing of ionic and nonionic bitter tastants (e.g., Danilova and Hellekant 2003; Frank et al. 2004; Geran and Travers 2006); thus, in addition to quinine, which is an ionic bitter tastant, PROP, a nonionic bitter taste compound, was included as a test stimulus. Based on mouse data from licking responsiveness to quinine, citric acid, sucrose and NaCl (Dotson et al. 2005) and PROP (Nelson et al. 2003) in a brief-access procedure, concentrations were chosen for use in the discrimination experiment. Stimulus concentrations that produced comparable degrees of licking avoidance for the aversive test compounds and those that conversely produced elevated lick rates for sucrose were chosen from the dynamic range of the concentration-response functions.

Apparatus

The mice were trained and tested in a modified version of a specially designed computer-controlled testing apparatus referred to as a gustometer (Spector et al. 1990; Eylam and Spector 2002). Mice were placed in the testing cage of the apparatus, which was enclosed in a sound-attenuating chamber. A background broadband masking noise produced by a speaker (8 Ohm 2 watt 3A05Z8; Quam) and a ventilation fan in the chamber was used to minimize extraneous auditory cues. Taste solutions and the water used as the reinforcer were placed in pressurized reservoirs located outside the chamber. The solutions in these reservoirs were rotated daily. Solenoid valves were computer controlled and regulated the delivery of controlled amounts of fluid from the fluid reservoirs to the drinking spout. The mouse was trained to lick the centrally positioned sample spout by extending its tongue through a slot located in the front wall of the testing cage. Following the completion of the dry-spout licking requirement (see below), the appropriate taste stimulus filled the shaft of the sample spout. Each subsequent lick delivered ~2 μl of the stimulus solution into the fluid column. Water reinforcement was delivered from 2 stationary horizontally oriented response spouts located on each side of the central spout slot. Contact with the correct response spout during the decision phase resulted in the delivery of ~2 μl/lick of the water reinforcer.

Trial structure

The trial began with the "sample phase." The mouse had to lick the dry spout 2 times within 250 ms to initiate a trial; this ensured that the animal was engaged in active licking. The fluid stimulus was presented through the sample spout for 2 s or 5 licks (whichever came first), after which the sample spout was rotated away from the reach of the mouse. During the "decision phase," the mouse had 10 s (limited hold) to respond by licking one of the 2 response spouts. As soon as the mouse licked one of the 2 response spouts, the "reinforcement phase" began. If the correct response spout was licked, the mouse could receive up to 15 licks of or 4-s access to (whichever came first) the reinforcer fluid (water). If an incorrect choice or no response was made within the allocated time, the mouse received a 30-s time-out during which no fluid was presented. After the reinforcement or punishment phase, the sample spout was rotated over a funnel, rinsed with water, dried with pressurized air, and rotated back into position behind the slot. This intertrial interval was approximately 6 s. The mice were allowed to complete as many trials as possible during the daily 25-min sessions.

Training

"Spout Training" consisted of the presentation each of only one spout (sample spout or one of the 2 response spouts) in the gustometer and allowing the mice to lick water freely for 30 min each day (see Tables 1 and 2).

"Side Training" involved training the mice to associate one of the response spouts with the presentation of either 17.4 mM citric acid or 0.504 M sucrose (Group 1) or 17.4 mM citric acid or 0.833 mM quinine (Group 2), delivered through the sample spout. The sample spout and one of the response spouts were available, whereas the other response spout was retracted and its access slot covered. The sample solution and the assigned response spout were alternated between days. During this phase of training, the reinforcement period was 30 s or 15 licks, whichever came first.

"Alternation" involved the presentation of one stimulus repeatedly until the correct response was made a predetermined number of times. The other stimulus was then presented until an equivalent number of correct responses were made. This alternation continued over 6 sessions with the criterion for the number of correct responses required before switching stimuli decreasing every second day. The stimulus alternated after 4 correct responses during the first 2 sessions, after 2 correct responses for the third and fourth sessions and after 1 correct response for the fifth and sixth sessions. The reinforcement period remained as 30 s (or 15 licks). A time-out was introduced and the limited hold was decreased in this phase of training.

"Discrimination Training I" involved the presentation of taste stimuli in randomized blocks. This was followed by "Discrimination Training II" and "Discrimination Testing" which involved the introduction of 2 more concentrations of each taste test compound, decreasing the limited hold from 15 to 10 s, and decreasing the reinforcement period from 30 to 4 s. After training, all mice were tested for their ability to discriminate citric acid from sucrose (Group 1) and citric acid from quinine (Group 2).

Discrimination testing

After successful completion of the citric acid versus sucrose discrimination task (Group 1), citric acid was replaced with another taste compound. After several sessions of a discrimination task, the discrimination stimuli were changed. To measure and maintain stimulus control, we included a series of 5

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Table 1 Training schedule for Group 1

Days	Phase	Time-out (s)	Limited hold (s)[a]	Stimuli	Schedule
3	Spout training	None	None	Water	Constant
6	Side training	None	180	Citric acid or sucrose (1 concentration)	Constant
6	Alternation[b]	10, 20, 30[c]	15	Citric acid or sucrose (1 concentration)	Alternated after *x* correct responses
25	Discrimination Training I	30	15	Citric acid vs. sucrose (1 concentration)	Semirandom[d]
47	Discrimination Training II and Testing	30	10	Citric acid vs. sucrose (3 concentrations)	Semirandom[d]

[a]Limited hold refers to the time the mouse was given to make a response.
[b]A stimulus was presented repeatedly until a predetermined number of correct responses was made. It was not necessary that the correct responses be successive.
[c]On the first 2 days of alternation training, the time-out was 10 s, which was increased to 20 s for days 3 and 4 and increased again to 30 s for days 5 and 6.
[d]Stimuli were presented in randomized blocks.

Table 2 Training schedule for Group 2

Days	Phase	Time-out (s)	Limited hold (s)[a]	Stimuli	Schedule
3	Spout training	None	None	Water	Constant
6	Side training	None	180	Citric acid or quinine (1 concentration)	Constant
6	Alternation[b]	10, 20, 30[c]	15	Citric acid or quinine (1 concentration)	Alternated after *x* correct responses
20	Discrimination Training I	30	15	Citric acid vs. quinine (1 concentration)	Semirandom[d]
3	Spout training	None	None	Water	Constant
6	Side training	None	180	Citric acid or sucrose (1 concentration)	Constant
6	Alternation	10, 20, 30[c]	15	Citric acid vs. sucrose (1 concentration)	Alternated after *x* correct responses
50	Discrimination Training I	30	15	Citric acid vs. sucrose (1 concentration)	Semirandom[d]
11	Discrimination Training II and Testing	30	10	Citric acid vs. sucrose (3 concentrations)	Semirandom[d]

[a]Limited hold refers to the time the mouse was given to make a response.
[b]A stimulus was presented repeatedly until a predetermined number of correct responses was made. It was not necessary that the correct responses be successive.
[c]On the first 2 days of alternation training, the time-out was 10 s, which was increased to 20 s for days 3 and 4 and increased again to 30 s for days 5 and 6.
[d]Stimuli were presented in randomized blocks.

sessions interspersed between each "test" discrimination. These "stimulus control" sessions involved retesting of the citric acid versus sucrose discrimination. The discrimination sessions were conducted as outlined in Table 3 with 3 concentrations of each stimulus presented. The broad range of stimulus concentrations that produced comparable degrees of licking avoidance for the test compounds were chosen to help render intensity and the non–taste-related physical properties of the stimuli as irrelevant cues for discrimination.

Mice that were trained to discriminate citric acid versus quinine (Group 2) were subsequently retrained in the discrimination task using citric acid and sucrose (Table 2).

Water control testing

Following the last Discrimination Test, 2 "Water Control Test" sessions were conducted in which all reservoirs were filled with purified water. Three reservoirs were assigned to one response spout and the other 3 to the other response spout. This was done to exclude the possibility that extraneous cues contributed to responses during discrimination testing.

Data analysis

Discrimination performance was evaluated using the overall proportion of correct responses. During training, performance on all sessions was analyzed, but during testing, weighted mean performance was assessed by collapsing all trials across both stimuli and concentrations on Tuesdays to Fridays, yielding an average weekly performance value for each animal. Monday sessions were regarded as "refresher" sessions, and data on these days were not included for analysis. Only trials in which a response was made were included for analysis. Overall group performance was tested

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Table 3 Order of stimulus discrimination pairings

Days	Stimulus 1	Stimulus 2
5	Citric acid	Sucrose
15	Quinine	Sucrose
5	Citric acid	Sucrose
10	NaCl	Sucrose
5	Citric acid	Sucrose
10	PROP	Sucrose
5	Citric acid	Sucrose
15	Citric acid	PROP
5	Citric acid	Sucrose
15	Citric acid	Quinine
2	Water	Water

against chance (50%) using one-sample *t*-tests. For water control testing, the normal approximation of the binomial distribution (one-tailed test) was used to determine any positive deviation of performance from chance. The P value ≤ 0.05 was considered significant in all statistical tests.

Experiment 2: conditioned taste aversion generalization

Subjects

Thirty-one male wild-type C57BL/6J (B6) mice (The Jackson Laboratory) with a mean body mass of 23.33 g (±0.31) upon arrival were used. All mice were experimentally naive at the start of the experiments. The mice were housed in conditions as described for Experiment 1.

After the mice were habituated to the laboratory environment for at least 7 days, they were placed on a restricted water access schedule in which fluid was only available during testing and training sessions. Testing and training took place during the lights-on phase. During brief-access testing, mice that fell below 85% of their free-drinking weight during the water-restriction schedule received 1 ml supplemental water 1 h after the end of the testing session. During the restricted home cage fluid access schedule (see below), mice were presented with fluid for 15 min in the morning and water for 30 min in the afternoon to allow for rehydration. All procedures were approved by the Florida State University Animal Care and Use Committee.

Chemical stimuli

For the CTA acquisition phase, 0.495 mM quinine hydrochloride (Sigma Chemical Co.) and 10.0 mM citric acid (BDH Chemicals) were used as the conditioned stimuli. LiCl (Sigma Chemical Co.) and NaCl (Mallinckrodt Chemicals) served as the unconditioned stimuli. During brief-access testing, test stimuli consisted of 2 concentrations of quinine hydrochloride (0.495, 0.822 mM; Sigma Chemical Co.) and citric acid (10.0, 17.4 mM; BDH Chemicals), 1 concentration of sucrose (0.504 M; BDH Chemicals), and 1 concentration of NaCl (0.488 M; Mallinckrodt Chemicals). All taste solutions were prepared daily with deionized reverse-osmosis water and reagent grade chemicals and were presented at room temperature.

Concentrations were chosen to complement those used in Experiment 1 that were originally based on mouse data from licking responsiveness to quinine, citric acid, sucrose, and NaCl (Dotson et al. 2005) as measured in a brief-access test.

Apparatus

The training and testing in the brief-access procedure was conducted in a lickometer commonly referred to as the Davis rig (Davis MS-160, DiLog Instruments) described previously elsewhere (Smith 2001; Glendinning et al. 2002). A mouse is placed in the test chamber of the apparatus. The mouse has access to a single sipper tube containing a taste stimulus, recessed by approximately 5 mm behind a slot. Positioned above the sample slot, a small fan directs a current of air past the drinking spout to minimize potential olfactory cues from the stimulus.

The one-bottle intake tests were conducted in the home cages of the mice. Fluids were presented in modified 25-ml graduated pipettes designed to reduce spillage, as described previously (Eylam and Spector 2002). These were presented by inserting the sipper tube between the metal bars of the cage lid and stabilizing them with a clip attached to the shelf above.

Training and testing

The mice were placed on a restricted water-access schedule for the first 4 days that involved training with water in the brief access test. Water bottles were removed the day before, no more than 23 h before the session, and were returned to their home cages after the last session. A mouse was placed in the test chamber of the Davis rig. A motorized shutter opened presenting the mouse access to a single sipper tube containing water. The mouse initiated a trial by licking the spout. Days 1 and 2 of training involved presenting water via a stationary spout for 30-min sessions. On days 3 and 4, seven sipper tubes of water were presented one at a time. Each trial was 5 s, followed by a 7.5-s intertrial interval during which time the tube was changed via a motorized block. A water rinse (5-lick maximum) presentation was interposed between each 5-s trial. The mice were able to initiate as many trials as possible during the daily 25-min sessions.

Following 4 days of brief access test training and 2 days of ad libitum water, water bottles were again removed from the home cages the day before the conditioning phase. The conditioning phase is outlined in Table 4. The first 4 days involved habituating the animal to the restricted fluid access schedule. Each mouse received water from an intake tube in its home cage for 15 min at the same time each morning. At the same time each afternoon, between 4 h and 4 h 20 min after the morning session, mice were presented with water for 30 min to allow for rehydration. After 4 days of restricted

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Table 4 Conditioning schedule

	4 Habituation days	Pairing 1	2 Days	Pairing 2	2 Days	Pairing 3
AM session	15 min H_2O	CS (15 min) → LiCl or NaCl	15 min H_2O	CS (15 min) → LiCl or NaCl	15 min H_2O	CS (15 min) → LiCl or NaCl
PM session	30 min H_2O	30 min H_2O	30 min H_2O	30 min H_2O	30 min H_2O	30 min H_2O

fluid access habituation, mice were assigned to one of 4 groups according to the conditioned stimulus (CS; 0.495 mM quinine or 10.0 mM citric acid) and the unconditioned stimulus (US; 0.1 M LiCl or 0.1 M NaCl) they would receive. There were no significant differences between groups based on body mass, mean licks/trial, or number of trials during the last 2 days of brief access training nor were there any significant differences in mean intake during the 4 days of restricted fluid access habituation.

After mice were assigned to their treatment groups ($n = 7, 8$/group), conditioning trials were conducted during which the mice were presented with the assigned CS for the 15-min morning session immediately followed by the US, an intraperitoneal injection (3.0 mEq/kg body weight) of 0.1 M LiCl (to induce visceral malaise) or 0.1 M NaCl (to serve as a control). During the afternoon session, mice were presented with water for 30 min to allow for rehydration. Each conditioning trial was separated by 2 days of restricted water access (15 min access to water in the morning and 30 min access to water in the afternoon). After the third conditioning trial, water bottles were replaced on the home cage for 2 days. Mice that drank less than 0.1 ml of their respective CS had ~0.1 ml orally infused with a syringe before receiving the US injection. This schedule has also been successfully used previously in mice (Eylam et al. 2003; Dotson and Spector 2007).

Data analysis

To assess the acquisition of the aversion, intake across the 3 conditioning trials were compared using paired *t*-tests with Bonferroni correction (i.e., Days 1 vs. 2, Days 1 vs. 3, and Days 2 vs. 3). For data from the brief-access test, a Taste/Water Lick Ratio was derived by dividing the mean number of licks to each taste stimulus by the mean licks to water on the prior brief access test day. The Taste/Water Lick Ratio value for each test solution was compared between the LiCl-injected and NaCl-injected groups via Bonferroni-corrected *t*-tests. Significantly lower Taste/Water Lick Ratios for a test stimulus in the LiCl-injected mice relative their NaCl-injected controls indicate conditioned avoidance.

Results

Experiment 1: stimulus discrimination

Group 1: citric acid versus sucrose

The mice that were trained to discriminate citric acid from sucrose were able to learn the task. Performance was at close to 80% accuracy during the last week of this phase (last week of testing: $t_{(9)} = 21.042$; $P < 0.001$; null hypothesis; P(correct response) = 0.5). During the citric acid versus sucrose sessions that were interposed between the discrimination test sessions to maintain stimulus control, weekly mean performance was at least 72% and averaged 75.5 ± 0.01%. A one-way analysis of variance (ANOVA) with repeated measures did not reveal significant differences among these interposed citric acid versus sucrose sessions ($F_{4,36} = 1.624$, $P = 0.128$). The average performance across these 5 weeks of citric acid versus sucrose discrimination was used to compare with performance of the last week of each of the other discrimination pairs.

After Group 1 successfully completed the citric acid versus sucrose discrimination task, the animals were tested on a series of other discriminations (Figure 1). Mice had severe difficulty discriminating citric acid from quinine and discriminating citric acid from PROP with performance only slightly, but significantly, above chance (50%). Bonferonni-corrected paired *t*-tests revealed that performance during citric acid versus sucrose sessions was significantly higher than that for both citric acid versus PROP ($t_{(9)} = 7.469$; $P = 0.001$) and citric acid versus quinine (Figure 1; $t_{(9)} = 13.446$; $P < 0.001$).

In contrast, citric acid versus sucrose discrimination performance did not significantly differ from that for quinine versus sucrose or NaCl versus sucrose. Performance on the citric acid versus sucrose discrimination was significantly higher than that for PROP versus sucrose ($t_{(9)} = 4.434$; $P = 0.025$), but as evident from Figure 1, this was due to a slight drop in performance during week 2 of the PROP versus sucrose test. In fact, when week 1 performance was compared, there was no significant statistical difference. Furthermore, PROP versus sucrose performance was significantly higher than that for both citric acid versus PROP ($t_{(9)} = 5.426$; $P = 0.006$) and citric acid versus quinine ($t_{(9)} = 7.979$; $P < 0.001$). Thus, compared with other discrimination pairs, performance was poor during the testing of the discrimination of citric acid from PROP or quinine.

Group 2: citric acid versus quinine

Group 1 mice that were trained to discriminate citric acid from sucrose, acquired the discrimination task (Figure 2, upper panel). In contrast, Group 2 mice, in which we attempted to train a citric acid from quinine discrimination, did not acquire the task (Figure 2 middle panel; last session: $t_{(9)} = 0.187$; $P = 0.856$; null hypothesis; P(correct

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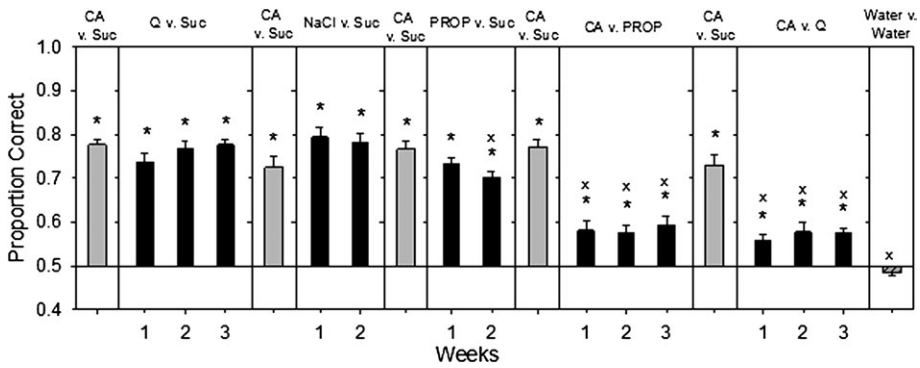

Figure 1 Group means ± standard error (SE) data plotted across all test phases for mice initially trained to discriminate citric acid from sucrose. Gray bars denote mean ± SE performance during citric acid versus sucrose sessions that served as stimulus control sessions (hatched bar). CA, citric acid; Suc, sucrose; Q, quinine. *Significantly different from 0.5 and [x]significantly different from mean citric acid vs. sucrose performance; $P < 0.05$.

responses) = 0.50). Group 2 mice that failed to discriminate citric acid from quinine were subsequently trained to discriminate citric acid from sucrose. Although the performance of Group 2 improved at a slower rate than that of Group 1, presumably because of their earlier training history with the difficult citric acid versus quinine discrimination task, all the mice learned to discriminate sucrose and citric acid at ~74% accuracy during the last week (Figure 2, lower panel). This is comparable to performance of Group 1, demonstrating that these animals were able to learn a taste discrimination task (last week of testing: $t_{(9)} = 9.679$; $P < 0.001$; null hypothesis; P(correct response) = 0.5). For both groups of mice, performance often dropped on Monday sessions (Figure 2). Data from all days of training were included for analysis but for discrimination testing, Monday sessions were regarded as refresher sessions and were not included for analysis described above.

Water control test

During the water control test, no mouse responded significantly above chance (50%; all P values > 0.05) with performance for Group 1 averaging 48.30 ± 0.01% and for Group 2 averaging 52.05 ± 0.03%. Thus, there is no evidence to suggest that the mice could perform the discrimination task in the absence of chemical cues.

Experiment 2: stimulus generalization

Conditioned taste aversion testing

Both LiCl-injected groups demonstrated evidence of an acquired aversion to their respective CS during the conditioning phase. Paired t-tests with Bonferonni adjustments revealed lower quinine intake on the second ($t(7) = 6.677$; $P < 0.01$) and third conditioning days ($t(7) = 6.539$; $P < 0.01$), compared with quinine intake on the first day, for mice that received LiCl injections. There was no significant difference between intake on days 2 and 3. In contrast, quinine intake did not differ between conditioning days for mice that received NaCl injections (Figure 3; $P > 0.05$ for all paired comparisons of intake on conditioning days 1, 2, and 3). Similarly, citric acid intake on the second ($t(7) = 8.461$; $P < 0.01$) and third ($t(7) = 6.693$; $P < 0.01$) conditioning days was significantly lower than intake on the first conditioning day for mice that received LiCl injections. Again, there was no significant difference between intake on days 2 and 3. Paired t-tests failed to reveal significant differences for intake of animals that received NaCl injections across the 3 days (Figure 3; $P > 0.05$ for the 3 comparisons). This indicates that there was a significant decrease in CS intake in the LiCl-injected groups and confirms the effectiveness of the conditioning procedures.

Brief-access testing

For mice conditioned to avoid 0.495 mM quinine, 2-sample t-tests with Bonferroni adjustments revealed that LiCl-injected mice had significantly lower Taste/Water Ratios than NaCl-injected mice for both concentrations of quinine and citric acid but not for NaCl nor for sucrose (Table 5). This suggests that, relative to controls, mice conditioned to avoid 0.495 mM quinine displayed avoidance of the CS and generalized the aversion to citric acid but not to NaCl and sucrose (Figure 4).

Mice conditioned to avoid 10.0 mM citric acid, however, did not display cross-generalization to quinine (Figure 4). Two-sample Bonferroni-adjusted t-tests revealed that LiCl-injected mice had significantly lower Taste/Water Ratios than NaCl-injected mice for both concentrations of citric acid and, to a lesser extent, for NaCl but not for any of the other test stimuli (Table 5).

Discussion

The findings from these experiments suggest that, in B6 mice, citric acid and quinine substantially share chemosensory features but that they are not perceptually identical. In the first experiment, mice that were successfully trained to

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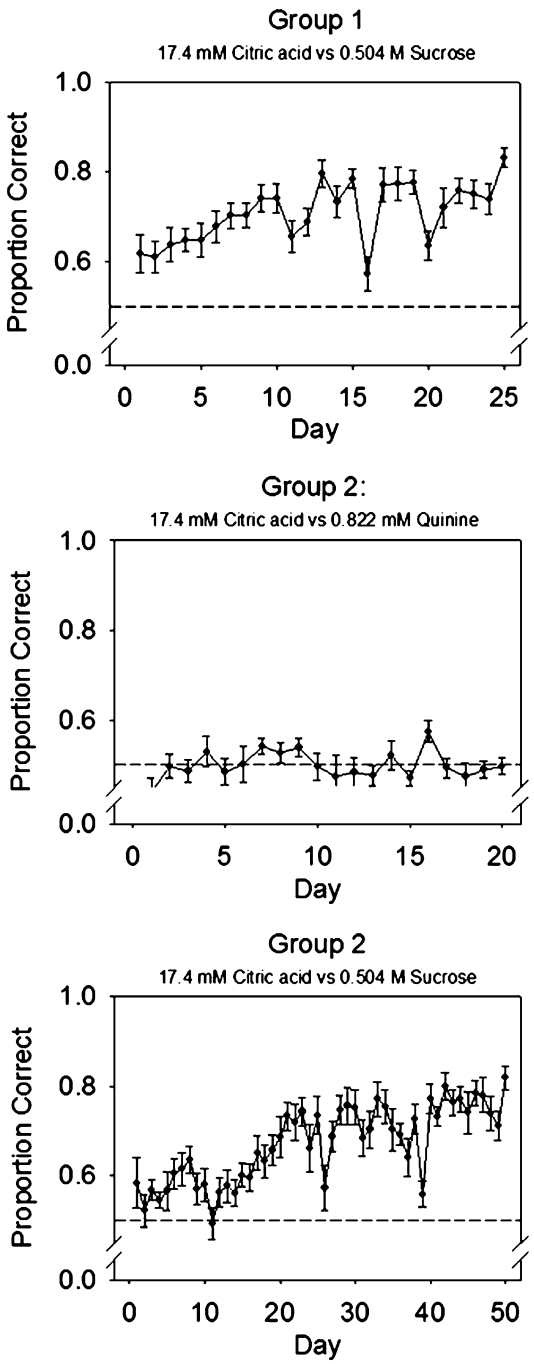

Figure 2 Upper panel: group means ± standard error (SE) for daily sessions during Discrimination Training I, in which Group 1 mice were trained to discriminate 17.4 mM citric acid from 0.504 M sucrose. Middle panel: group means ± SE for daily sessions during Discrimination Training I, in which Group 2 mice were trained to discriminate 17.4 mM citric acid from 0.822 mM quinine. Lower panel: group means ± SE for daily sessions during Discrimination Training I, in which Group 2 mice, that failed to discriminate citric acid from quinine, were subsequently trained to discriminate 17.4 mM citric acid from 0.504 M sucrose.

discriminate citric acid from sucrose showed significantly lower performance when tested on the citric acid versus PROP, and citric acid versus quinine discriminations, as

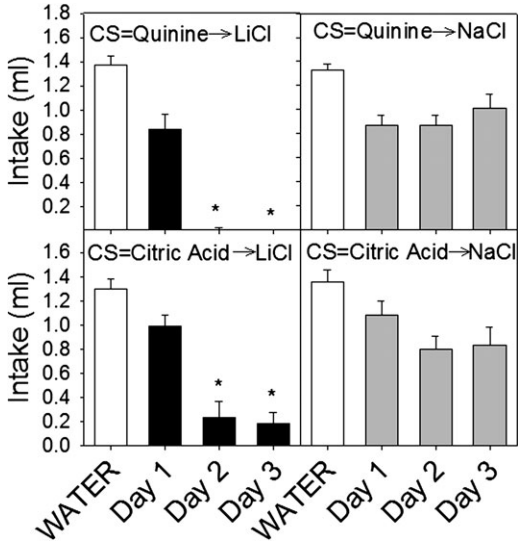

Figure 3 Group means ± standard error of water intake (white bars) and CS intake (quinine upper panel, citric acid lower panel) on conditioning days 1, 2, and 3. For mice injected with LiCl (left panel, black bars) and NaCl (right panel, gray bars). *Significantly different from intake on conditioning day 1.

compared with the other stimulus pairs tested, but were nonetheless above chance. In contrast, these same mice were able to competently discriminate sucrose from citric acid, NaCl, quinine, and PROP. When citric acid and quinine were used to train another group of mice in the discrimination procedure, performance remained at chance (50%), indicating that the mice did not learn the task. However, these same mice that failed to discriminate citric acid from quinine were subsequently able to learn to discriminate citric acid from sucrose. Thus, the failure for mice to initially acquire the citric acid versus quinine discrimination was attributable to the taste stimuli used, rather than the task itself.

To further address the question of whether citric acid and quinine share perceived chemosensory features, the conditioned taste aversion generalization paradigm was used. Mice that were conditioned to avoid quinine by pairings with LiCl injections subsequently suppressed licking responses to quinine and citric acid but not to sucrose or NaCl in a brief-access test relative to NaCl-injected control animals. In contrast, mice that were conditioned to avoid citric acid did not generalize avoidance to quinine. The fact that mice did not completely cross-generalize conditioned aversions between the 2 stimuli suggests that citric acid and quinine have overlapping features but are not perceptually the same.

In some sense, the outcomes of these experiments could be viewed as reflecting a "sour–bitter confusion" and are consistent with the presence of some neurons in the rodent gustatory system that respond to both acids and quinine as observed in electrophysiological studies. For example, in the CT nerve, a branch of the facial nerve that innervates the taste buds of the anterior tongue, fibers that respond optimally to some acids also show sensitivity to quinine in the

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Table 5 Two-sample *t*-test values with Bonferroni adjustments comparing Taste/Water Lick Ratio values for each test solution between the LiCl-injected and NaCl-injected groups

	S	*N*	Q(l)	Q(m)	CA(l)	CA(m)
Quinine	$t(13) = 0.486$, $P = 1.000$	$t(13) = 0.580$, $P = 1.000$	$t(13) = 3.760$, $P = 0.014$	$t(13) = 3.748$, $P = 0.014$	$t(13) = 3.860$, $P = 0.012$	$t(13) = 6.481$, $P < 0.001$
Citric acid	$t(14) = 1.902$, $P = 0.468$	$t(14)=3.151$, $P = 0.042$	$t(14) = 1.144$, $P = 1.000$	$t(14) = 1.973$, $P = 0.412$	$t(14) = 3.325$, $P = 0.030$	$t(14) = 4.806$, $P = 0.002$

rat (Frank et al. 1983), hamster (Frank et al. 1988), and mouse (Ninomiya et al. 1984a). This has also been confirmed in the rat geniculate ganglion (Lundy and Contreras 1999; Breza et al. 2006, 2007, 2010). In contrast to the CT nerve, the GL, which innervates the taste buds of the posterior tongue and responds robustly to bitter compounds (e.g., Oakley 1967; Pfaffmann et al. 1967; Shingai and Beidler 1985; Yamamoto et al. 1988; Dahl et al. 1997; Danilova and Hellekant 2003), has units that differentially respond to quinine and citric acid in several rodent species (Hanamori et al. 1988; Ninomiya and Funakoshi 1989; Frank 1991). Although taste receptors found in the posterior tongue appear to be critical for aversive reflexes to quinine in rats (Travers et al. 1987; Grill et al. 1992; King et al. 2000), the GL does not appear to be necessary for normal taste quality discrimination in the rat (St John and Spector 1998; Spector 2003). In fact, rats with GL transection do not show significant decreases in a citric acid versus quinine discrimination task (St John 1997). Thus, it appears the units in the GL that can discriminate acids from bitters may be serving some role other than taste stimulus identification.

A variety of analytical techniques have been used to assess the degree to which neural responses to representative taste stimuli in the central gustatory system covary. These include multidimensional scaling, in which the similarity between the neural responses evoked by a set of stimuli in a group of sampled neurons is represented by distance in a 3D (or n dimensional) space, and simple correlation procedures in which the relationship between the responses evoked by a pair of stimuli is determined for a group of neurons. At least in the rodent brainstem taste relays, there is some evidence of overlap in the patterns of neuronal responses to acids and quinine, reminiscent of that seen in the CT neve, but the degree of this overlap varies across species and studies (e.g., Travers and Smith 1979; Giza and Scott 1991; Boughter and Smith 1998; Verhagen et al. 2003; Lemon and Smith 2005; McCaughey 2007; Geran and Travers 2009; Lemon and Margolskee 2009). The cases in which there is some covariance between neural responses to bitter-tasting stimuli and acids would predict some degree of perceptual similarity as seen here. Yet, although the patterns of responsiveness to quinine and acids in the brainstem taste nuclei of mice and other rodents appear to be quite similar, they are not necessarily identical (e.g., Nakamura and Norgren 1993). Indeed, Geran and Travers (2006) found that in the rostral NST of the rat, the correlation of the responses to quinine and citric acid was only moderate at best ($r = 0.47$) and did not reach statistical significance, and the correlation between PROP responses and citric acid was, if anything, actually negative ($r = –0.30$; but did not reach significance). Interestingly, in this same study, a multidimensional scaling analysis indicated a greater similarity in responses to quinine and citric acid when applied to the anterior tongue as compared with stimulation of the posterior tongue, which is consistent with the response profiles of the nerve innervating these lingual taste bud fields as described above. Also, it has been shown by Fos-like immunoreactivity (FLI) that

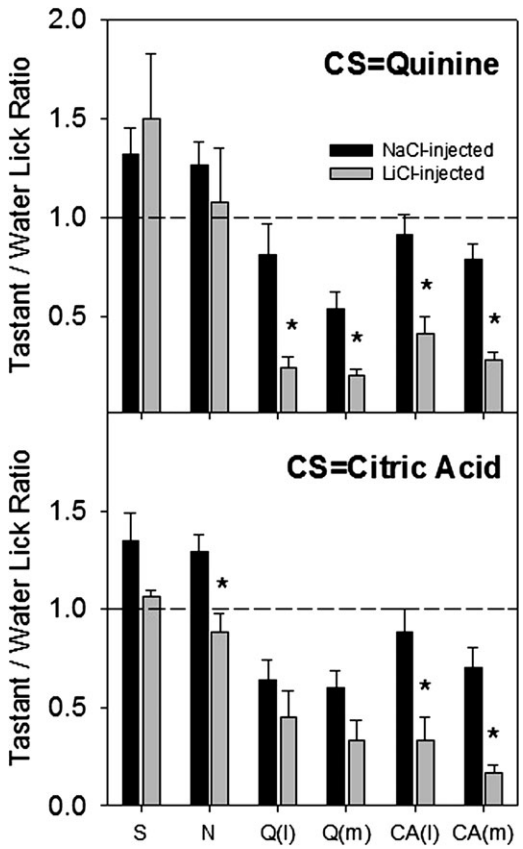

Figure 4 Group mean Taste/Water Ratios ± SE in 5-s trials, to 0.504 M sucrose (S), 0.488 NaCl (N), 0.495 mM quinine (Q(l)), 0.822 mM quinine (Q(m)), 10.0 mM citric acid (CA(l)), and 17.4 mM citric acid (CA(m)). Data for mice presented quinine (top panels) or citric acid (bottom panels) as the CS and injected with LiCl (black bars) or NaCl (gray bars). *Significant difference between LiCl- and NaCl-injected mice for a particular stimulus.

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although quinine and citric acid elicit similar numbers of FLI neurons in the NST of rats, the topographic distributions elicited can be distinguished (Travers 2002). Given that it is unclear whether all taste-responsive neurons contribute to all gustatory functions, care must be exercised in interpreting the relevance of these neuronal response profiles to discriminative performance in psychophysical tasks. Nonetheless, the fact there are neurons in the gustatory system that respond to both acids and bitter compounds is at least consistent with the ''sour–bitter confusion'' observed in behavioral studies. Because rats can discriminate quinine from citric acid with a relatively high degree of competence, notwithstanding some degradation of performance at weaker concentrations (St John 1997; Grobe and Spector 2008), the degree to which quinine and citric acid share perceptual features appears to be much greater in B6 mice. Indeed, in the NST of B6 mice, responses to quinine and those to citric acid or HCl have been shown to be highly correlated across sampled neurons (McCaughey 2007; Lemon and Margolskee 2009).

At the level of taste receptor cells, there appears to be separate candidate taste receptor mechanisms for compounds that humans describe as sour (e.g., proteins encoded by the *Pkd13* and *Pkd2l1* genes [Huang et al. 2006; Ishimaru et al. 2006; LopezJimenez et al. 2006]) and those described as bitter (e.g., genes in the *Tas2r* cluster region [e.g., Adler et al. 2000; Matsunami et al. 2000; Bachmanov et al. 2001; Nelson et al. 2005; Behrens and Meyerhof 2010]). Furthermore, cells expressing PKD2L1, an ion channel thought to be involved with acid sensing in taste receptor cells, are not coexpressed with T2R proteins (encoded by *Tas2r* genes) (Huang et al. 2006). In mice, the genetic ablation of taste receptor cells expressing PKD2L1 eliminates CT responses to acid taste stimuli but not responses to stimuli evoking bitter taste (Huang et al. 2006). On the other hand, some investigators have found that some rodent taste receptor cells display calcium responses to application of both bitter and acid stimuli (Sato and Beidler 1997; Gilbertson et al. 2001; Caicedo et al. 2002; Tomchik et al. 2007). Nevertheless, even if there are independent mechanisms for the coding of signals generated by bitter and acid stimuli at the receptor level, as early as the peripheral afferent, and further downstream, these signals appear to converge, at least partially, potentially underlying the difficulty mice have discriminating quinine and PROP from citric acid.

In the present study, although the mice that were initially trained to discriminate citric acid from sucrose had severe difficulty discriminating citric acid from quinine and PROP, they were able to do so above chance. This finding suggests that there are some distinguishable chemosensory cues associated with these stimuli that maintain some degree of discriminability, but these are likely too weak or lack salience to support better performance in this task. Although unlikely at these concentrations, we cannot dismiss the possibility that discrimination among these stimuli may be derived from nontaste cues such as trigeminal sensory nerve activation (Wang et al. 1993; Pittman and Contreras 1998). Acids can elicit salivation (Eshel and Korczyn 1978; Kawamura and Yamamoto 1978; Matsuo and Yamamoto 1989) and can be detected by nasal inhalation (Settle et al. 1986), possibly by olfaction and/or via intranasal trigeminal afferents. Furthermore, the trigeminal system possesses solitary chemoreceptor cells that express T2R receptors (Finger et al. 2003; Ohmoto et al. 2008) and respond to stimulation by bitter-tasting compounds (Finger et al. 2003). It has previously been shown that rats with bilateral transections of the CT and greater superficial petrosal nerves are still able to discriminate citric acid from quinine in a 2-response operant procedure (St John 1997). These rats were surprisingly competent at discriminating these compounds even though it appears the input of the GL does not contribute to taste discrimination (see Spector 2003). It is possible that these rats were able to make use of nontaste cues to discriminate the 2 compounds. In the present study, for mice, these nontaste cues may not have been as salient for the discrimination task but perhaps more effective in the conditioned taste aversion procedure. In the taste aversion generalization paradigm, mice had access to much larger stimulus volumes both during conditioning and testing compared with the psychophysical discrimination procedure in which stimulus sampling was limited to 5 licks. Moreover, stimulus delivery in the gustometer was designed to minimize orthonasal olfactory cues.

Although we cannot entirely rule out the possibility that discriminative responding in Experiment 1 was guided by the motivational properties of the taste solutions, rats and mice can effectively use taste stimuli as discriminative cues in operant tasks even when there is overlap in the hedonic characteristics of the stimuli, strongly suggesting that responding is under the control of the perceived quality of the stimuli (e.g., Spector et al. 1997; St John and Spector 1998; Geran et al. 2002; Eylam and Spector 2005; Delay et al. 2007; Dotson and Spector 2007; Grobe and Spector 2008). In the context of the current experimental design, mice were able to competently discriminate sucrose from both NaCl and citric acid even though the latter 2 compounds do not likely have the exact same hedonic properties.

There is some evidence in the literature demonstrating that ionic and nonionic bitter-tasting ligands do not activate identical taste-responsive neural units perhaps indicating differential processing of these 2 classes of bitter stimuli (e.g., Dahl et al. 1997; Danilova and Hellekant 2003; Frank et al. 2004; Geran and Travers 2006). Given that in our study, performance was poor for the discrimination of citric acid from both quinine and PROP, it appears that whether the bitter tastant is ionic or nonionic is not a critical factor.

Mice conditioned to avoid quinine generalized their response to citric acid but not vice versa. Asymmetrical generalization of conditioned taste aversions has been reported in the literature for other taste stimuli that share

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chemosensory characteristics but that are not identical (Herness and Pfaffman 1986; Frank et al. 2003; Dotson and Spector 2007). In some cases, generalizations from mixtures to single components appear to be stronger than vice versa (Frank et al. 2003). If citric acid generates a relatively pure taste perception (e.g., the equivalent of what humans refer to as sour) and quinine elicits a mixture of taste qualities (i.e., a citric acid-like sensation in addition to other qualitative percepts), then this could potentially lead to the experimental outcomes presented here. The additional perceptual components associated with quinine could be taste based (e.g., the equivalent of bitter taste in humans) or could be sensations arising from stimulation in the olfactory or trigeminal systems. These are not mutually exclusive possibilities. Nonetheless, given the ability of citric acid to stimulate both the olfactory and trigeminal systems as noted above, one could argue that it generates a more complex chemosensory perception than quinine. Furthermore, mice conditioned to avoid citric acid also generalized their response to NaCl, whereas this was not observed in the group conditioned to avoid quinine (although the degree of the NaCl avoidance was weak). Thus, the asymmetrical generalizations of the aversions should have been in the opposite direction—quinine aversions should have been specific to quinine and citric acid aversion should have generalized to quinine. Another possibility is that the lowest concentration of citric acid had a greater intensity than the highest concentration of quinine used in the CTA experiment. If this were true, the failure for the citric acid aversion to be expressed to the quinine test stimuli used could be due to an intensity generalization decrement in responding (Nowlis 1974; Spector and Grill 1988). A resolution to this issue awaits further investigation.

Asymptotic performance to the sucrose versus citric acid training compounds in the discrimination task of the current study averaged ~75%. This is modest compared with asymptotic performance observed in a similar task in rats (St John and Spector 1998; Geran et al. 2002; Spector and Kopka 2002) that may be attributable to species differences. But this is also modest compared with the performance observed previously in mice (Eylam and Spector 2005; Dotson and Spector 2007). A possibility for this slight difference might be the taste compounds used. In a prior study (Dotson and Spector 2007), when NaCl and sucrose were used as the training stimuli, asymptotic performance averaged ~85%. When NaCl and KCl were used (Eylam and Spector 2005), asymptotic performance averaged ~80%. It may be that certain taste compounds are more salient for discrimination training. Nevertheless, the asymptotic performance to the sucrose versus citric acid training stimuli obtained in the current study were clearly above chance and allowed for the comparison of discrimination performance between pairs of test compounds.

Overall, the findings from these experiments suggest that, in mice, citric acid and quinine substantially share chemosensory features making discrimination difficult, but that perceptually, these compounds are not identical. Taking into account the current findings and evidence in the literature, it appears that there are varying degrees of sour–bitter confusion across different species, with mice displaying considerable difficulty discriminating between compounds representing these 2 taste qualities. There is behavioral evidence that human subjects (Meiselman and Dzendolet 1967; McAuliffe and Meiselman 1974) and rats (Morrison 1967; Grobe and Spector 2008) can display ''sour–bitter confusion'', especially at lower concentrations, but not to the extent observed in the present study with B6 mice. Thus, we should bear in mind these species differences and not expect the mouse model to completely emulate the human or even the rat, gustatory system. In fact, here we only tested B6 mice and given the notable strain differences among mice in taste phenotypes (e.g., Bachmanov et al. 1996; Harder et al., 1996; Eylam and Spector 2004; Sclafani and Glendinning 2005; Bachmanov and Beauchamp 2008; Murata *et al.* 2009), it is possible that other mouse genotypes might not generate the same results as produced in our study. Finally, if these outcomes generalize to a more extensive panel of acids and compounds that humans report as bitter, it will raise the provocative and vexing possibility that what humans call sourness and bitterness may not actually be entirely independent taste qualities in at least B6 mice.

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