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Differential bitterness in capsaicin, piperine, and ethanol associates with polymorphisms in multiple bitter taste receptor genes

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Abstract

To date, the majority of research exploring associations with genetic variability in bitter taste receptors has understandably focused on compounds and foods that are predominantly or solely perceived as bitter. However, other chemosensory stimuli are also known to elicit bitterness as a secondary sensation. Here we investigated whether *TAS2R* variation explains individual differences in bitterness elicited by chemesthetic stimuli, including capsaicin, piperine and ethanol. We confirmed that capsaicin, piperine and ethanol elicit bitterness in addition to burning/stinging sensations. Variability in perceived bitterness of capsaicin and ethanol were significantly associated with *TAS2R38* and *TAS2R3/4/5* diplotypes. For *TAS2R38*, PAV homozygotes perceived greater bitterness from capsaicin and ethanol presented on circumvallate papillae, compared to heterozygotes and AVI homozygotes. For *TAS2R3/4/5*, CCCAGT homozygotes rated the greatest bitterness, compared to heterozygotes and TTGGAG homozygotes, for both ethanol and capsaicin when presented on circumvallate papillae. Additional work is needed to determine how these and other chemesthetic stimuli differ in bitterness perception across concentrations and presentation methods. Furthermore, it would be beneficial to determine which TAS2R receptors are activated in vitro by chemesthetic compounds.

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Conflict of interest

The authors have no conflict of interest to report.

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1. Introduction

It is generally accepted bitterness is an evolutionarily important mechanism used to protect mammals against ingesting toxins and poisons in foods (Glendinning 1994; Drewnowski and Gomez-Carneros 2000). Mammals detect bitterness following the activation of a specific class of G-protein coupled receptors, known as TAS2Rs (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000; Mueller et al. 2005; Behrens et al. 2007). The specific number of bitter receptor genes varies across species (Go et al. 2005), with humans having 25 different *TAS2R* genes (Adler et al. 2000; Chandrashekar et al. 2000; Behrens and Meyerhof 2006) that cluster on chromosomes 5, 7 and 12 (Adler et al. 2000; Matsunami et al. 2000). Similarities between *TAS2R* genes indicates they have diversified over time (Conte et al. 2002; Shi et al. 2003; Fischer et al. 2005; Go et al. 2005; Shi and Zhang 2006; Shi and Zhang 2009; Wooding 2011), presumably due to environmental pressure such as a changing diet (Leonard 2002; Shi et al. 2003; Li and Zhang 2013). As a consequence of this diversification, there are many non-synonymous substitutions in the coding regions of these genes (Conte et al. 2002; Shi et al. 2003; Fischer et al. 2005; Behrens and Meyerhof 2013), which in some cases result in functional changes in the receptor (e.g. (Kim et al. 2003)).

The most widely studied bitter taste receptor gene, *TAS2R38* (located on chromosome 7), has three functional single nucleotide polymorphisms (SNPs). These SNPs (A49P, V262A and I296V) are inherited together (i.e. they form a haploblock), which results in two common haplotypes named for the respective amino acid substitutions: PAV and AVI (Kim et al. 2003; Kim et al. 2005). The *TAS2R38* diplotype is associated with the ability to detect the bitterants phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP), with PAV homozygotes perceiving greater bitterness than heterozygotes, and AVI homozygotes perceiving little to no bitterness (Kim et al. 2003; Duffy et al. 2004; Bufe et al. 2005; Kim et al. 2005; Wooding et al. 2006; Hayes et al. 2008; Calo et al. 2011; Mennella et al. 2011; Allen et al. 2013; Garneau et al. 2014; Boxer and Garneau 2015).

Polymorphisms in other *TAS2Rs* have also been shown to associate with differential bitterness perception of other foods and ingredients. To date, variability in bitterness perception for various bitter foods and beverages have been associated with polymorphisms in *TAS2R3* (Hayes et al. 2011), *TAS2R4* (Hayes et al. 2011; Risso et al. 2014), *TAS2R5* (Hayes et al. 2011), *TAS2R9* (Allen et al. 2013), *TAS2R14* (Risso et al. 2014), *TAS2R16* (Bufe et al. 2002), *TAS2R19* (Reed et al. 2010; Hayes et al. 2011; Hayes et al. 2015), *TAS2R31* (Roudnitzky et al. 2011; Allen et al. 2013; Hayes et al. 2015), and *TAS2R43* (Pronin et al. 2007). However, it is critical to note that for many of these putatively functional SNPs identified via gene-phenotype association studies in humans, it is unknown whether these SNPs are truly the causal SNP with respect to mechanism. For instance, in association studies, it is possible that the tested SNP is in linkage disequilibrium with an untested functional SNP (e.g. (Reed et al. 2010; Hayes et al. 2011; Hayes et al. 2015)).

Although additional work has also noted associations between gene variants and other behaviors like liking or intake (e.g. (Duffy et al. 2004; Hinrichs et al. 2006; Wang et al. 2007; Duffy et al. 2010; Feeney 2011; Hayes et al. 2011; Dotson et al. 2012; Pirastu et al. 2014), here we focus exclusively on associations between genetic variability and bitterness perception, rather than broader influences on hedonics, food choice, and ingestive behavior (see (Hayes et al. 2013)).

The majority of research on *TAS2R* polymorphisms to date has understandably focused on compounds that are predominantly or solely bitter. However, many other chemosensory stimuli elicit bitterness as a secondary sensation, thus *TAS2R* variation also has the potential to influence perception of these stimuli. For example, *TAS2R31* variants have been associated with differential responses to sulfonyl amide sweeteners (Roudnitzky et al. 2011; Allen et al. 2013) and grapefruit juice (Hayes et al. 2011; Hayes et al. 2015). Likewise, capsaicin, best known for eliciting burning sensations mediated via TRPV1, also provokes bitter sensations in some individuals but not others (Green and Hayes 2003; Green and Schullery 2003; Green and Hayes 2004), although the genetic basis for this is currently unknown. Here, we address this knowledge gap by exploring whether the variability in bitterness of capsaicin, piperine and ethanol associates with select polymorphisms in *TAS2Rs*.

Recently, we reported the bitterness produced by ethanol on the circumvallate papillae was significantly associated with *TAS2R38* diplotype (Allen et al. 2014). Here we expand previously reported data (Allen et al. 2014), and report on the associations between ethanol bitterness and SNPs within *TAS2R3, TAS2R4*, and *TAS2R5*. In a separate group of participants, Hayes and colleagues previously reported four SNPs on chromosome 7 resulted in a haploblock across *TAS2R3* (rs765007), *TAS2R4* (rs2234001), and *TAS2R5* (rs2234012 and rs2227264) (Hayes et al. 2011). The resulting *TAS2R3/4/5* diplotype was significantly associated with perceived coffee bitterness, with TGAG homozygotes reporting more bitterness from sampled espresso coffee compared to CCGT homozygotes (Hayes et al. 2011). Accordingly, we also explore the associations between haplotypes across *TAS2R38* and *TAS2R3/4*/5 and the reported bitterness of capsaicin and piperine here.

2. Materials and methods

2.1. Study overview

Individuals were recruited from the Pennsylvania State University campus and surrounding community (State College, PA) as part of a larger study on the genetics of oral sensation. The overall study consisted of four one-hour one-on-one visits with a researcher in the Sensory Evaluation Center, a custom build sensory testing facility located within the Erickson Food Science Building on the Penn State campus. All testing occurred face to face in a windowless clinical examination room free of distractions. On the first day of the study, researchers provided participants with an oral explanation of the protocol and goals of the study and obtained written consent. This session included collection of anthropometric data (height, weight, bodyfat %, blood pressure, picture of anterior tongue), collection of salivary DNA, reported liking of foods, beverages, and non-food items and rated intensity of sampled stimuli. These stimuli included potassium chloride (salty/bitter), quinine monohydrochloride

dihydrate (bitter), Acesulfame-K (sweet/bitter), a monosodium glutamate / inosine monophosphate (MSG/IMP) blend (umami/savory), sucrose (sweet), and capsaicin. Also, participants were specifically instructed 'You may receive stimuli causing more than one quality. Please attend to all sensations on all trials.' Before scheduling visits 2, 3 and 4, the researcher determined if the circumvallate papillae were visible and could be touched with a wetted cotton swab without triggering a gag reflex. Other than genetic material collected via the salivary DNA sample, data from session one will not be discussed further here, as current hypothesis are focused on data collected in sessions two, three and four. Results from session one have been reported elsewhere (Allen et al. 2013; Byrnes and Hayes 2013; Hayes et al. 2015).

Briefly, a constant protocol was used across visits two, three and four, with a different chemesthetic stimulus presented on each day (either capsaicin, piperine or ethanol). These chemesthetic stimuli were selected based on other work showing that they were capable of eliciting bitterness as a secondary quality, at least in some individuals (Mattes and Dimeglio 2001; Green and Hayes 2004; Nolden and Hayes 2015). Each visit began with a refresher on how to use a general Labeled Magnitude Scale (gLMS) followed by a short practice session. Participants also rated the perceived intensity of five different prototypical tastants presented regionally to each quadrant of the tongue, as reported elsewhere (see (Feeney and Hayes 2014)). Participants also rated the perceived intensity of a chemesthetic stimulus (either capsaicin, ethanol or piperine) applied to the left and right circumvallate (CV) papillae, which are located on the posterior tongue, forming a rearward pointing chevron of 8 to 12 dome-shaped structures. Detection thresholds were collected for chemesthetic stimuli using a forced choice method based on ASTM method E-679. As the final stimulus within a session, participants rated the overall intensity of a whole mouth swish-and-spit chemesthetic stimulus.

2.2. Participants

All four sessions were completed by 106 participants (40 men) with a mean age of 25.2± 0.63 (SEM). Participants reported ethnicity using the recommended wording from the 1997 OMB Directive 15 guidelines. The majority of participants reported Caucasian ancestry (n=69), followed by Asian (n=15) and African American (n=1); 11 participants chose not to disclose their ancestry. Participant characteristics are reported in Table 1.

Prior to study enrollment, individuals interested in participating completed an online screening questionnaire. Eligibility criteria included: between 18–45 years old, not pregnant nor breastfeeding, had not smoked in the last 30 days, no known defects of smell nor taste, no oral piercings (lip, cheek or tongue), no history of chronic pain, not currently taking any prescription pain medication, no history of choking or difficulty swallowing and no history of thyroid disease. Participants also indicated they were willing to provide a saliva sample to obtain DNA. Written informed consent was obtained from all participants. All procedures were approved by the Pennsylvania State University Institutional Review Board (protocol number #33176). The procedure in this study complies with stipulations outlined in the Declaration of Helsinki.

2.3. Stimuli

2.3.1. Regional application of prototypical tastants—Prototypical tastants were applied regionally, one at a time, to each of the four quadrants of the tongue using a single cotton swab. Swabs were submerged into the tastant solution prior to application, and rolled on the tongue for three seconds. Stimuli included sucrose, sodium chloride, citric acid, quinine, and a monosodium glutamate and inosinate monophosphate (MSG/IMP) mixture. Data for these stimuli were not analyzed here, as they have been described previously (Feeney and Hayes 2014).

2.3.2. Irritant swabs—Piperine and capsaicin impregnated swabs were prepared prior to the test session. Solutions of either 10,070 ppm piperine and 30.5 ppm capsaicin were prepared using 95% food grade ethanol, resulting in nominal concentrations of approximately 35 mM piperine and 100 uM capsaicin. The swabs were prepared by submerging a cotton swab in the appropriate solution and drawing it across the lip of a plastic medicine cup before placing it on a strip of parafilm and allowing it to air dry. In the test session, capsaicin and piperine impregnated swabs were rewetted with reverse osmosis (RO) water just before the swab was applied participant's tongue (e.g. (Green and Hayes 2004)). Ethanol swabs were prepared immediately prior to application by dipping the swab in a 50% (v/v) ethanol solution made with food grade ethanol and RO water, and drawing it across the lip of a plastic medicine cup in the same manner as the other swabs. The concentrations given here should be considered nominal, as it is impossible to know the specific amount of stimulus delivered to the tongue from the swabs. We also assume all of the ethanol evaporated during the drying process. To select the concentrations used here, we consulted prior literature (Green and Hayes 2004) and refined them via informal piloting by our team to identify concentrations that would produce roughly similar burn intensities.

2.3.3. Detection threshold—Detection threshold estimates were collected using a 3-alternative forced choice (3-AFC) method based on ASTM method E-679. Six concentrations of piperine, capsaicin and ethanol were each prepared from an appropriate stock solution for that stimulus. The same stock solutions of capsaicin and piperine were used for the detection threshold stimuli described here, and the whole mouth sip-and-spit suprathreshold stimuli (next section). The stock solutions consisted of 120 ppm capsaicin and 9120 ppm piperine in ethanol (95%), respectively. Following an initial 100-fold dilution with RO water, followed by additional 2 fold serial dilutions, the resulting detection threshold stimuli for capsaicin were: 0.6 ppm, 0.3 ppm, 0.15 ppm, 0.075 ppm, 0.037 ppm and 0.018 ppm. For piperine, the concentrations were: 4.56 ppm, 2.28 ppm, 1.14 ppm, 0.57 ppm, 0.28 ppm, and 0.14 ppm. Ethanol stimuli were 8%, 4%, 2%, 1%, 0.5%, and 0.25%, which were prepared using 95% food grade ethanol diluted with RO water. These concentrations were chosen to bracket threshold estimates reported previously for capsaicin (Sizer and Harris 1985; Lawless et al. 2000) and ethanol (Mattes and Dimeglio 2001) we were unable to locate published detection thresholds for piperine, so these levels were selected on the basis of suprathreshold data that suggest piperine is less potent than capsaicin (Stevens and Lawless 1986; Green 1996).

2.3.4. Whole mouth sip-and-spit suprathreshold stimuli—Whole mouth sip-and-spit samples were presented in 15mL aliquots at room temperature. Piperine and capsaicin whole mouth stimuli were prepared via a 100-fold dilution of the same stock solution used to make the detection threshold stimuli (see above). Final concentrations of 9.12 ppm piperine and 1.2 ppm capsaicin, each containing 1% ethanol, were presented to participants (approximately 32uM piperine and 3.9uM capsaicin). Here, we assume the amount of ethanol present (1%) in the capsaicin and piperine samples should be meaningfully alter the irritancy of these stimuli, as this amount falls below the reported detection threshold of 1.43% (v/v) for ethanol (Mattes and Dimeglio 2001). The whole mouth ethanol stimulus consisted of 16% (v/v) ethanol in RO water. Whole mouth dose-response functions for capsaicin and piperine using contemporary scaling methods were not available in extant literature when the experiment was designed, so these concentrations were selected for logistical convenience: they are twice the highest concentration used in the detection threshold concentration series.

2.4. Psychophysical scaling

A general labeled magnitude scale (gLMS) was used to collect psychophysical ratings for all stimuli (Snyder and Fast 2004; Hayes et al. 2013). This scale ranges from ‘no sensation’ at 0 to ‘strongest imaginable sensation of any kind’ at 100, with intermediate descriptors located at 1.4, 6, 17, 35 and 51, which are labeled as ‘barely detectable’, ‘weak’, ‘moderate’, ‘strong’, and ‘very strong’, respectively. During each session, the researcher provided an introduction to the scale and the participant practiced rating 15 items, which included food and non-food items. For each sampled stimulus, participants rated sweetness, bitterness, sourness, saltiness, umami and burning/stinging, unless otherwise noted. Participants were asked if they had any questions regarding the sensations and were given verbal examples to ensure their familiarity with each sensation. All psychophysical data were collected using Compusense five, version 5.2 (Guelph ONT, Canada).

2.5. Protocol: Sessions 2, 3 and 4

2.5.1. Regional ratings for tastants—Data for regionally applied tastants are not analyzed here, as they have been reported elsewhere (Feeney and Hayes 2014); we briefly describe the procedure here to better characterize the context within which the participants made their ratings, as this speaks to their ability to perform the task. A timeline depicting the testing sequence for sessions 2, 3 and 4 is provided in Figure 1. Participants were presented with five different prototypical tastants, one at a time, in a randomized order on each of the four quadrants of the tongue (e.g., front right, front left, back right, back left). Participants retracted their tongue into their mouth and were asked to refrain from touching their tongue to the roof of their mouth. Participants rated perceived intensity of sweetness, bitterness, sourness, saltiness, umami and burning/stinging on a gLMS. Mouth temperature RO water, which was maintained in glass bottles in a thermostatically controlled water bath, was provided as rinse water. Participants were instructed to rinse between stimuli, and they waited a minimum of 30 seconds, or longer if a sensation was still present, before the next stimulus was presented. Twenty tastants were presented in total (5 stimuli in 4 regions) with a multi-attribute time intensity (MATI) trial for the irritant presented halfway through the series. Thus, in every visit, participants tasted and rated 10 different prototypical tastants

before an irritant was applied to the CV papillae. The participant then tasted and rated 10 more prototypical tastants before the same irritant was applied to the CV papillae on the other side of the tongue. As noted elsewhere (Allen et al. 2014), ratings for these regionally applied prototypical tastants act as a negative control; in a superset of present data (from Feeney and Hayes, 2014), group means for the side tastes for each tastant were extremely low (i.e., mean bitterness for sucrose, citric acid, and sodium chloride were 0.3, 3.0, 2.2, respectively, and mean burning/stinging ratings were 0.35, 1.08, and 0.80). Conversely, means for the expected qualities of each tastant (e.g., sourness for citric acid) were close to "moderate" on a gLMS). Although single time point ratings for regionally applied tastants are somewhat different than the multi-attribute time intensity task described below with regard to the demand characteristics placed on participants, they nonetheless suggest participants were able to successfully distinguish between various qualities during the rating task.

2.5.2. Multi-attribute time intensity (MATI) for chemesthetic stimuli—Participants were assigned to receive a different chemesthetic stimulus (capsaicin, piperine or ethanol) during each session; presentation order was counterbalanced across participants, so that all participants received all stimuli upon completion of the study. Following the completion of the first 10 regional tastant stimuli, participants received a chemesthetic stimulus on the circumvallate papillae (CV). A researcher applied two cotton swabs taped in tandem onto the left (CV) for 10 seconds in a circular motion. Participants were asked to keep their tongue in their mouth, and lips closed without touching their tongue to the roof of their mouth. Participants rated sweetness, bitterness, sourness, saltiness, umami and burning/stinging response every thirty seconds for three minutes. Participants were not allowed to rinse with water but were allowed to spit if necessary. After the three minutes of rating, participants were given a four minute break to rinse with mouth temperature RO water. The participants continued with remainding 10 regional tastant stimuli and then the MATI protocol was replicated for the right CV. Another four-minute break was enforced before continuing on the detection threshold task for the same chemesthetic stimulus. To maintain motivation and attention across the testing session, puzzle books and magazines were provided during the longer 4 minute breaks, and participants were allowed access to their smartphones during these breaks.

2.5.3. Detection thresholds for the chemesthetic stimuli—Participants were presented with three plastic medicine cups, and asked to identify the different sample. Each set of samples contained one spiked sample and two blank samples (see above for sample preparation). All triads were presented in ascending order of concentration. To sample each stimuli, participants were asked to swish the solution for 3 seconds, and spit it out, and wait 5 seconds before sampling the next stimuli, in order from left to right. Participants were not allowed to rinse in between stimuli, but were required to rinse in between sets of stimuli (triads) with mouth temperature RO water (from a glass bottle kept in a temperature controlled water bath). In total, 6 sets of stimuli (triads) were presented; with a 30 second break in between each. All stimuli were presented at mouth temperature in 10mL aliquots. A mandatory break of at least 4 minutes was enforced before moving on to whole mouth sip-and-spit ratings for the same chemesthetic stimulus.

2.5.4. Whole Mouth sip-and-spit—The visit ended with participants rating the intensity of a whole mouth sample of that session's chemesthetic stimulus. The stimulus (either capsaicin, piperine or ethanol) was presented in a plastic cup at mouth temperature. Participants rinsed the entire sample (15mLs) in their mouth for 10 seconds before rating the perceived 'overall intensity' of the stimulus on a gLMS.

2.6. Genetic analysis

DNA was collected using Oragene saliva collection kits following manufacturer instructions (Genotek Inc, Ontario, Canada). SNPs (single nucleotide polymorphisms) analyzed here include: rs713598, rs1726866, rs10246939 in *TAS2R38*, as well as SNPs located in *TAS2R3* (rs765007), *TAS2R4* (rs2233998, rs2234001, rs2234002), *TAS2R5* (rs2234012), *TAS2R9* (rs3741845), *TAS2R13* (ra1015443), and *TAS2R31* (rs10772423). Primers were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Genotypes were determined by either MassARRAY technology (Sequenom) or by Taqman assays and independently inspected by two technicians. As a standard procedure, 15% of samples are rerun to ensure reliability. Genotype frequencies of SNPs did not vary from Hardy Weinberg equilibrium (see Table 2). Haploview was used to determine associations between haplotypes and Solid Spline criterion was used to determine haplotype blocks (Figure 2) (Barrett et al. 2005). Diplotypes were determined for three SNPs in *TAS2R38* (rs713598, rs1726866, rs10246939) and also for five SNPs located in *TAS2R3* (rs765007), *TAS2R4* (rs2233998, rs2234001, rs2234002) and *TAS2R5* (rs2234012) using PHASE. Individuals with haplotype probabilities less than 0.8 were reported as missing.

2.7. Statistical analysis

Data were analyzed using SAS 9.2 (Cary, NC). MATI ratings for the left and right CV were combined and averaged for each participant a priori. Repeated measures analysis of variance (ANOVA) and ANOVA were performed via *proc mixed* to determine the association between genotype and time, and significant effects of gene were determined using the slice option in SAS. Individual detection thresholds were calculated as the geometric mean between the first correct answer followed by all correct answers, and the next lowest concentration. Group detection thresholds were calculated as the geometric mean of all individual detection thresholds (Lawless and Heymann 2010).

3. Results

3.1. Psychophysical response to chemesthetic stimuli

MATI ratings for capsaicin, piperine and ethanol collected separately on the left and right CV were averaged for each participant prior to any analysis. Across all three stimuli, burning/stinging was rated as the dominant sensation during the trial, followed by bitterness (Figure 3). The remaining sensations are not shown in Figure 3 to reduce visual clutter: the sourness, sweetness, umami and salty ratings varied in intensity across the stimuli, and for capsaicin and piperine these sensations fell close to or below 'barely detectable'. Ethanol was perceived to have some sourness and sweetness (above 'barely detectable') at time 0 and 30, and these sensations fell below 'barely detectable' by 60 seconds.

3.2. TAS2R38 explains variability in bitterness of chemesthetic stimuli presented on the CV

Due to linkage disequilibrium for the three SNPs in *TAS2R38* (see Figure 2), diplotypes were determined prior to further analysis. Of the 106 participants who completed all four sessions, 105 were successfully genotyped (diplotype probability > 0.8) for the three SNPs in *TAS2R38* (A49P, V262A, and I296V). There were 25 PAV homozygotes, 46 heterozygotes (PAV/AVI) and 20 AVI homozygotes. The remaining 14 individuals expressed rare diplotypes (6 AVI/AAV, 3 PAV/AAV, 1 PAV/PVI, 1 AVI/PVV, 1 AVI/PVI, 1 PVI/PVV, 1 PVI/PVI).

Repeated measures ANOVA were performed to determine the relationship between common *TAS2R38* diplotypes (PAV/PAV, PAVI/AVI, AVI/AVI) and perceived bitterness over 3 minutes for capsaicin, ethanol and piperine presented on the CV papillae (Figure 4). For capsaicin, there was a significant interaction effect between *TAS2R38* diplotype and time for bitterness ([$F(12,528)=2.0$, $p=0.02$]; Figure 4a). As would be expected, the main effect of time was also a significant predictor of capsaicin bitterness, [$F(6,528)=10.76$, $p<0.0001$] which decayed over time. The main effect of diplotype was not significant [$F(2,88)=1.69$, $p=0.19$]. Post-hoc analysis of the significant time by diplotype interaction revealed significant differences in the bitterness of capsaicin at 0 and 30 seconds ($p=0.0025$ and $p=0.0344$, respectively).

As reported previously (Allen et al. 2014), perceived bitterness of ethanol was significantly associated with *TAS2R38* polymorphisms. Present ethanol data do not represent an independent replication of our prior report, but are reanalyzed here as haplotypes to allow direct comparison with the other two chemesthetic stimuli. It is important to point out that the previous analysis was conducted in only participants of European ancestry due to ethnic diversity of a different gene of interest (*TRVP1*) (Allen et al. 2014), and that analysis was done SNP by SNP, rather than on the basis of haplotype. For the current analysis, data from all participants was used with haplotypes generated via PHASE. Here, the bitterness of ethanol differed significantly by the *TAS2R38* diplotype [$F(2,88)=3.82$, $p=0.0256$], time [$F(6,528)=36.36$, $p<0.0001$], and the time by diplotype interaction was significant [$F(12,528)=2.25$, $p=0.009$] (Figure 4b). Post-hoc analysis via the slice option in SAS revealed ethanol bitterness differed significantly across the diplotypes at 0, 30 and 60 seconds (p's=0.0002, 0.002, 0.01, respectively).

For piperine, bitterness ratings were significantly different across time [$F(6,528)=11.5$, $p<0.0001$] while the main effect of *TAS2R38* diplotype was not significant [$F(2,88)=0.84$, $p=0.43$]; we did observe a significant time by diplotype interaction [$F(12,528)=2.36$, $p=0.0059$]. Despite of this significant interaction, none of the individual time points met the criteria for significance in post-hoc analysis (all p's > 0.08); nonetheless, the pattern in Figure 4c is not inconsistent with the results for capsaicin and ethanol (i.e., bitterness appeared to be higher in PAV homozygotes).

3.3. TAS2R3/4/5 diplotype explains variability in capsaicin, piperine and ethanol bitterness

As expected from prior work, SNPs in *TAS2R3* (rs765007), *TAS2R4* (rs2233998, rs2234001 and rs2234002), and *TAS2R5* (rs2234012 and rs2227264) were in linkage disequilibrium

(Figure 2), forming a haploblock, which was independent from the *TAS2R38* haploblock. Of 106 participants, 102 had a diplotype probability greater than 0.8. In these individuals, we observed two common haplotypes – CCCAGT (frequency = 0.518) and TTGGAG (freq=0.413) – which resulted in 3 common diplotypes. There were 28 CCCAGT homozygotes, 46 heterozygotes (CCCAGT/TTGGAG), and 20 TTGGAG homozygotes (see Table 3). Other diplotypes included three individuals expressing CTCAAG/CCCAGT, and one participant for each of the following: CCCAGT/TCCAGT, CCAGT/TCGGAG, CCCAGT/TTGGGT, CTCAGT/CCCAGT, CTGGGG, TTGGAG. Due to low frequencies of the less common diplotypes, analysis was restricted to the three common diplotypes in our cohort.

Repeated measures ANOVA revealed significant associations between common *TAS2R3/4/5* diplotypes and the bitterness from capsaicin and piperine but not ethanol applied to the CV. For capsaicin, the diplotype by time interaction effect was significant [$F(12,540) = 2.09$, $p = 0.0007$], as was the main effect of time [$F(6,540)=9.86$, $p < 0.0001$]; however, the main effect of diplotype was not significant [$F(2,90) = 1.81$, $p = 0.17$]. Post-hoc analysis via the slice option in SAS revealed significant differences in capsaicin bitterness between the diplotypes occurred at 0 and 30 seconds ($p = 0.0001$ and $p = 0.025$, respectively; Figure 5a).

As shown in Figure 5b, ethanol bitterness decayed significantly over time [$F(6,540) = 34.23$, $p <0.0001$]. The main effect of *TAS2R3/4/5* diplotype on ethanol bitterness was marginal [$F(2,90) = 2.67$, $p = 0.07$], and there was no evidence of a time by diplotype interaction [$F(12,540) = 1.34$, $p = 0.19$]). Even though the main effect of the *TAS2R3/4/5* diplotype was not significant, a post-hoc analysis was conducted as we might expect differences to occur when bitterness ratings are the highest before bitterness falls below barely detectable as time goes on. In this additional analysis, participants reported significant difference between diplotypes for the bitterness of ethanol at 0 and 30 seconds ($p = 0.017$ and $p = 0.01$, respectively; Figure 5b).

For piperine, bitterness decayed over time, as expected [$F(6,540) = 9.61$, $p < 0.0001$], and the *TAS2R3/4/5* diplotype by time interaction was significant [$F(12,540) = 2.14$, $p = 0.01$]; there was no evidence of a main effect of diplotype [$F(2,90) = 0.59$, $p = 0.5$] (Figure 5c). As with the *TAS2R38* diplotype data for piperine, there appeared to be a floor effect, as post-hoc analysis of the interaction revealed no significant differences at the individual time points in spite of the significant interaction.

3.4 Reportedly functional SNPs in TAS2R9, TAS2R13, and TAS2R31 did not associate with variability bitterness of capsaicin, piperine nor ethanol on the CV

In parallel analyses to those described in the two proceeding sections, we also tested putatively functional SNPs in *TAS2R9* (rs3741845), *TAS2R13* (rs1015443) and *TAS2R31* (rs10772423). For capsaicin applied to the CV, there was no evidence of any association with bitterness for either the SNP main effect (all p's > .1) or the SNP by time interactions (all p's >.8). Likewise, we failed to observe any effects for piperine (main effect p's > .4; interaction p's > .4) or ethanol (main effect p's > .3; interaction p's > .1).

3.4. Overall intensity of sip-and-spit whole mouth stimuli

Overall intensity ratings for whole mouth (sip-and-spit) chemesthetic stimuli are shown in Figure 6. The average overall intensity rating for piperine was significantly different from capsaicin and ethanol (p's<0.0001). Capsaicin and ethanol did not significantly differ in overall intensity ratings (p=0.13), suggesting our efforts to match their intensity in pilot testing were generally successful.

Individual differences in overall intensity ratings of whole mouth sip-and-spit stimuli were not significantly associated with *TAS2R38 nor TAS2R3/4/5* diplotype (all p's > 0.3). Given the absence of any evidence for an effect with the CV swab, we did not test the SNPs in *TAS2R9*, *TAS2R13*, and *TAS2R31* for the capsaicin and piperine whole mouth stimuli. Also, we did not test for *TAS2R9* and *TAS2R31* effects with whole mouth ethanol; an association between whole mouth ethanol and the rs1015443 SNP in TAS2R13 was reported previously (Allen et al. 2014).

3.5. Detection thresholds for capsaicin, piperine and ethanol

The group geometric mean of individual best estimate thresholds was: 0.52±0.04 ppm for capsaicin, 0.58±0.25 ppm for piperine, and 0.87±0.16% for ethanol (geometric mean± standard error).

TAS2R38 diplotype did not explain variability in individual detection thresholds for any stimuli (capsaicin, piperine or ethanol). However, we did note capsaicin and piperine detection thresholds appeared to trend in the same direction as the suprathreshold data. For capsaicin, AVI homozygotes and heterozygotes had mean detection thresholds of 0.74±0.1 ppm and 0.72±0.06 ppm, compared to PAV homozygotes 0.52±0.09 ppm, however this difference was not significant (p=0.16). Similarly, piperine detection thresholds for AVI homozgyotes were appeared to be slightly higher (3.12±0.5), compared to heterozygotes (2.45±0.39) and PAV homozygotes (1.53±0.54); however these apparent differences were not significant across the diplotypes (p=0.13). Ethanol detection thresholds did not follow this same trend, with thresholds of 0.94±0.38, 1.06±0.24, and 1.67±0.35, respectively for AVI homozygotes, heterozygotes and PAV homozygotes (p=0.28). TAS2R3/4/5 diplotype failed to explain variation in individual detection thresholds for capsaicin, piperine nor ethanol (p's > 0.6). The 3 SNPs in *TAS2R9*, *TAS2R13*, and *TAS2R31* were not tested against the detection threshold data.

4. Discussion

4.1. Bitterness of capsaicin, ethanol and piperine

Chemesthetic stimuli are known to elicit both burning and bitter sensations. Present data confirm previous findings that capsaicin, piperine and ethanol presented on the posterior tongue are perceived as mainly burning/stinging, along with bitterness at least in some individuals (i.e., Green and Hayes 2004). Notably, the burning/stinging ratings from capsaicin and ethanol on the CV were similar across time. At time zero, ratings were reported between weak and moderate, and decayed to below weak after 60 seconds and fell to barely detectable by 180 seconds. Conversely, burn ratings for piperine across time

decayed more slowly than for capsaicin or ethanol, with means between weak and barely detectable even after 180 seconds. This is consistent with other reports that piperine burn decays more slowly than capsaicin burn (Green and Hayes 2004; Mcdonald et al. 2010). Perceived bitterness for ethanol followed a similar time course as burning/stinging, but did not reach the same maximal intensity, suggesting ethanol at this concentration is predominantly burning rather than bitter (Allen et al. 2014; Nolden and Hayes 2015).

When participants were presented with the chemesthetic swabs, they were asked to rate six qualities, including bitterness and burning/stinging. It is possible that these sensations may have been difficult for untrained participants to discriminate between, as there was no training session with exemplars to familiarize participants with these sensations. However, as mentioned in the methods, separate analysis of the 5 prototypical tastants used in the regional taste test (sucrose, citric acid, NaCl, quinine, and a MSG/IMP blend) showed little to no evidence that participants made inappropriate ratings for qualities that should not be present (e.g., bitterness for sucrose) (Allen et al. 2014). Also, the data described here were collected after participants already had substantial practice in rating diverse, perceptually complex stimuli during their first visit to the laboratory (see Byrnes & Hayes 2013; Allen et al. 2013). Finally, while it remains possible that this task may have been potentially difficult for some participants, the overall burning/stinging and bitterness ratings were not confounded as bitter and burning response curves were distinct for these chemesthetic agents (see Figure 3), consistent with other work (Green and Schullery 2003; Green and Hayes 2004). Collectively, this suggests present results were not contaminated by some sort of dumping process or other demand characteristics of the task, in spite of the use of naïve participants who were not previously oriented to these sensations in a training session with specific exemplars.

The capsaicin, piperine and ethanol concentrations applied via a cotton swab to the CV papillae were intended to be intensity matched, to facilitate direct comparisons across the stimuli. This was generally successful, as the burning/stinging ratings were similar at time 0 (as shown in Figure 3). However, it should also be noted that bitterness ratings from ethanol were higher than the bitterness ratings for capsaicin and piperine at these concentrations. It is unknown how bitterness perception may potentially differ when presented at concentrations that are intensity matched for bitterness, rather than burning/stinging. This should be revisited in future work to facilitate comparisons for both bitterness decay rates and associations with bitter taste receptor genetics.

4.2. Reported bitterness on CV is explained by TAS2R38 and TAS2R3/4/5 diplotypes

Here, we explored whether *TAS2R38* diplotype can explain variability in bitterness perception of capsaicin and piperine for stimuli presented on the CV and as whole mouth sip-and-spit stimuli. Genetic variability in *TAS2R38* has been previously associated with the bitterness of numerous stimuli, with the most widely reported association occurring with bitterness from 6-n-propythiouracil and phenylthiocarbamide (Kim et al. 2003; Duffy et al. 2004; Allen et al. 2013), but also ethanol (Allen et al. 2014), vegetables (Sandell and Breslin 2006) and goitrin (Wooding et al. 2010). Present data suggests the bitterness of capsaicin, ethanol and possibly piperine, differs by *TAS2R38* diplotype. Here, PAV homozygotes

perceived greater bitterness from capsaicin presented on the CV papillae compared to heterozygotes and AVI homozygotes, consistent with the idea that the PAV haplotype codes for the more functional form of the receptor. As expected, the significant differences across diplotype occurred within the first few time points following application, as this is where the greatest intensity was reported. As the burn decays over time, we would not expect the significant differences across diplotype to persist as ratings approach barely detectable on the gLMS; indeed, such a floor effect is apparent in Figure 4a. A similar pattern of heightened bitterness among the PAV homozygotes is seen for piperine, although the use of a concentration that never exceeded weak bitterness (on average) likely obscures the ability to cleanly test for differences across *TAS2R38* diplotype in post-hoc analyses.

Present data also extend upon our prior report that ethanol bitterness on the posterior tongue varies by *TAS2R38* diplotype (Allen et al. 2014). These data are not a true replication of our prior report, and should not be treated as such; however, the analyses shown here are novel in two ways. The present analysis a) is based on diplotype rather than individual SNPs in isolation, and b) uses a mixed race cohort that is a superset of the prior European ancestry only sample. As expected, the PAV homozygotes reported greater bitterness from ethanol, and this difference persisted over the first 90s following application.

SNPs in *TAS2R3*, *TAS2R4*, and *TAS2R5* have been previously reported to be in linkage disequilibrium (Hayes et al. 2013). Here we report that six SNPs (1 from *TAS2R3*, 3 from *TAS2R4* and 2 from *TAS2R5*) are in linkage disequilibrium with each other, but are independent from the well-known haplotype for *TAS2R38*. The resulting *TAS2R3/4/5* diplotype significantly explained variability in bitterness perception of both capsaicin and ethanol that had been applied to the CV papillae. CCCAGT homozygotes rated the greatest bitterness compared to heterozygotes and TTGGAG homozygotes for both ethanol and capsaicin. Unexpectedly, these results are not in agreement with previous findings, which suggested CCGT homozygotes individuals (which are comparable to **C**C**C**A**GT** here) reported lower bitterness response to sampled coffee compared to heterozygotes and TGAG homozygotes (comparable to **T**T**G**G**AG**) individuals (Hayes et al. 2011). The reason for this discrepancy is unclear, and requires an additional study.

4.3. Whole mouth ratings and detection threshold

Overall intensity ratings are reported for capsaicin, piperine and ethanol in a whole mouth sip and spit paradigm. In terms of overall intensity, the 1.2 ppm capsaicin and 16% ethanol were not significantly different, while the 9.12 ppm piperine had significantly lower mean ratings. While this suggests the 1.2ppm capsaicin and 16% ethanol were well matched for overall intensity, it is important to note that we do not know the relative contribution of burning/stinging and bitterness to overall intensity, as a whole mouth stimulus. In a separate group of participants, we recently reported that ethanol is more bitter than burning at lower concentrations, with the reverse being true as concentration continues to increase (Nolden and Hayes 2015). Here, our decision to have participants rate overall intensity rather than individual ratings of multiple attributes is, in hindsight, a clear limitation of the present work. More work is needed to understand how the quality specific profile of capsaicin and piperine may shift with concentration.

The group geometric means of individual best estimate thresholds for capsaicin, piperine and ethanol are 0.52±0.04 ppm, 0.58±0.25 ppm and 0.87±0.16% (±S.E.), respectively. An ascending 3-AFC method was chosen in order to keep test session brief and only included six concentrations; however, it remains possible a more sensitive albeit labor intensive method (e.g. up-down staircase) may result in different associations with *TAS2R38* diplotype. Nonetheless, the present cohort had a capsaicin detection threshold similar to previously results (0.31 ppm) (Lawless et al. 2000), suggesting the method used here was valid. For ethanol, the current cohort reported lower detection threshold 0.87±0.16% (S.E.) than the one reported previously: 1.43±0.11% (S.E.) (Mattes and Dimeglio 2001). This difference may be due to the different methods used as Mattes and DiMeglio (2001) used a forced choice staircase method in 25 individuals. While their method should to be more sensitive that the rapid method used here, it is also important to note the difference in sample size. To the best of our knowledge, detection thresholds for piperine have not been previously reported, so we are unable to compare our data to prior values. Finally, the very similar detection thresholds for capsaicin and piperine, in spite of substantial differences in their burn at higher concentrations, serves to recapitulate the observation that threshold values are often a poor predictor of suprathreshold response due to differing slopes of the psychophysical function.

Diplotypes for *TAS2R3/4/5* or *TAS2R38* did not significantly associate with the reported overall intensity of the sip-and-spit stimuli, nor detection thresholds for capsaicin, piperine or ethanol. However, there was a trend for PAV homozygotes to have a lower (more sensitive) detection threshold for capsaicin and piperine compared to AVI homozygotes. This trend was not observed for ethanol. Initially, one might expect that with a more sensitive detection threshold method or with additional participants, that there might be a significant association between *TAS2R38* diplotype and individual detection thresholds for capsaicin and piperine, given the pattern seen in the suprathreshold data. Alternatively however, the primary percept experienced at detection threshold for chemesthetic stimuli like capsaicin and piperine is presumably burning, not bitterness, so accordingly, we should not expect detection threshold to be explained by bitter taste receptor genetics. Thus, determining individual recognition thresholds (rather than detection thresholds), and attempting to associate them with *TAS2R38* diplotype could potentially be more successful in future studies.

4.4. Future direction

In vitro methods have been fundamental in identifying bitter compounds that are ligands for TAS2R receptors (Meyerhof et al. 2010). Furthermore, it can be determined which amino acid substitutions (either naturally occurring in the population as genetic polymorphisms, or via site directed mutations) influence receptor activation. Changes in receptor activation often explain differences in psychophysical response. However, differences in psychophysical response are frequently associated with genetic polymorphisms in gene association studies in advance of functional expression studies (e.g., Reed et al. 2010, Hayes et al. 2011, Allen et al. 2013). Thus, putatively functional SNPs in such studies may be merely associated with a change in perceptual response without being mechanistically involved if functional expression assays have not be conducted to determined whether the

SNP is causal or merely in LD with another nearby SNP that is the functional variant. Presently, it is unknown whether capsaicin, piperine and ethanol activate TAS2R3, −4, −5 or −38. Thus, additional work is needed to determine if these (and other) bitter taste receptors respond to capsaicin, piperine, and ethanol *in vitro*.

5. Conclusions

Here we explore differential bitterness from capsaicin, ethanol and piperine as a function of *TAS2R* polymorphisms. Measures included suprathreshold ratings from stimuli applied to the circumvallate papillae on the posterior tongue, and as whole-mouth sip-and-spit stimuli; detection thresholds were also collected using a rapid method. Traditionally, chemesthetic compounds are characterized and defined by their ability to elicit irritation: yet, little is known about the perception of secondary sensations (e.g. 'side tastes') from these stimuli. Here we replicate prior reports that capsaicin, piperine and ethanol elicit bitterness in addition to burning/stinging sensations, at least at the concentrations tested. Furthermore, differences in the bitterness from these stimuli associated with genetic polymorphisms in bitter taste receptor genes, specifically in *TAS2R3, −4, −5* as well as *TAS2R38*. Additional work is needed *in vitro* to confirm that these compounds are able to activate bitter taste receptors in functional expression systems.

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Highlights

- Capsaicin, piperine and ethanol elicit bitterness in addition to burning/stinging
- Bitterness of capsaicin and ethanol associate with *TAS2R38* diplotypes
- *TAS2R3/4/5* diplotype significantly associates with capsaicin and ethanol bitterness

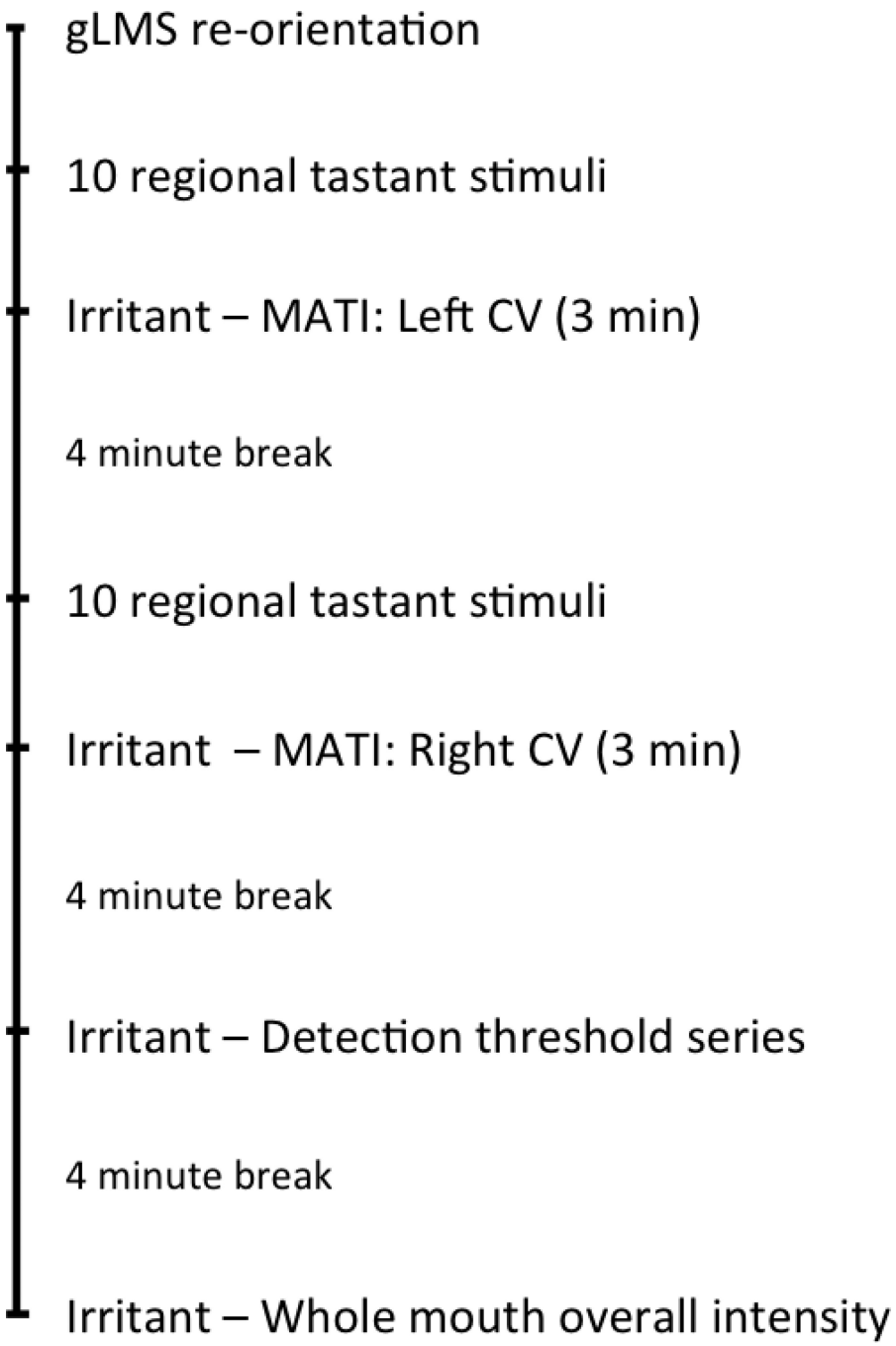

Figure 1.
Timeline for laboratory visits held on days 2, 3 and 4 of the study. A single irritant (capsaicin, piperine, or ethanol) was presented during each visit, with the order of presentation counterbalanced across participants. Abbreviations in the figure are: gLMS, general Labeled Magnitude Scale, and MATI, multiple attribute time intensity.

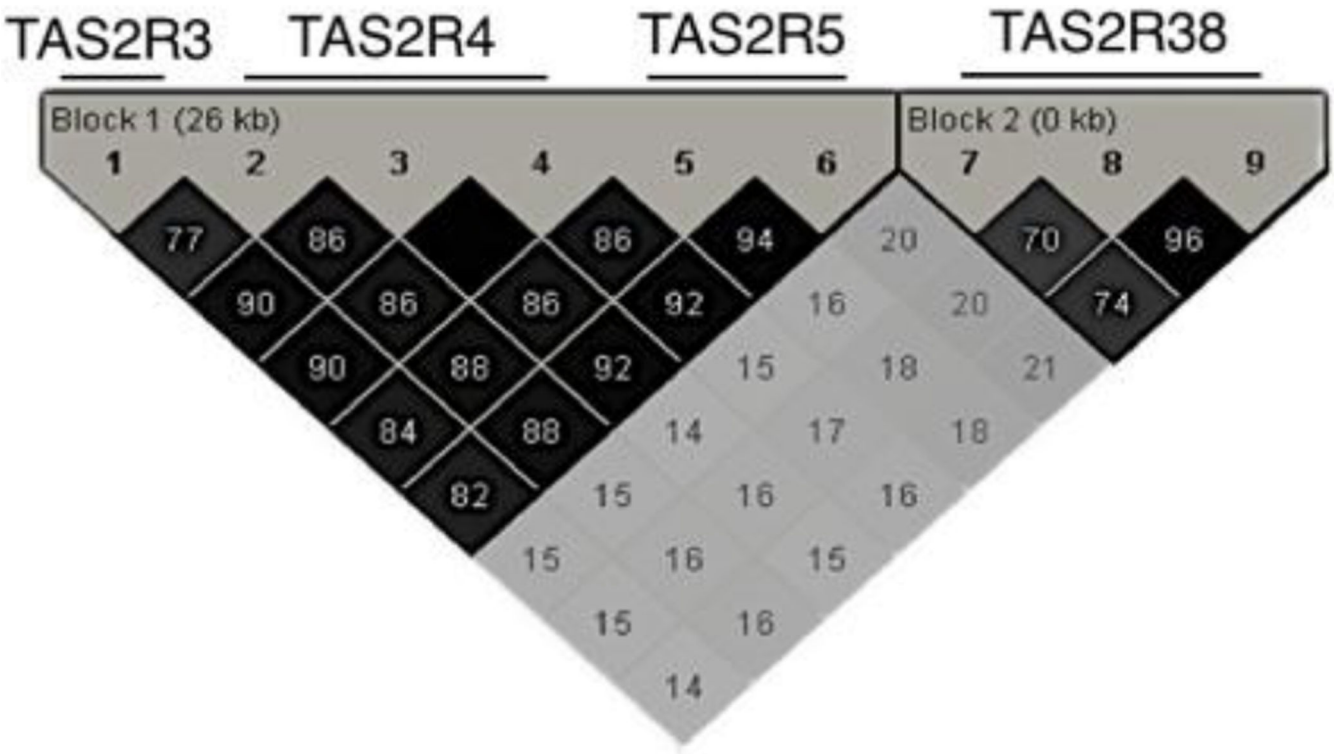

Figure 2.

Linkage disequilibrium plot for *TAS2R* SNPs on chromosome 7. Numbers in LD plot indicate rounded R^2 values and darker shading indicates higher R^2 values generated via Haploview. For SNP identification, see LD Plot # listed in Table 2.

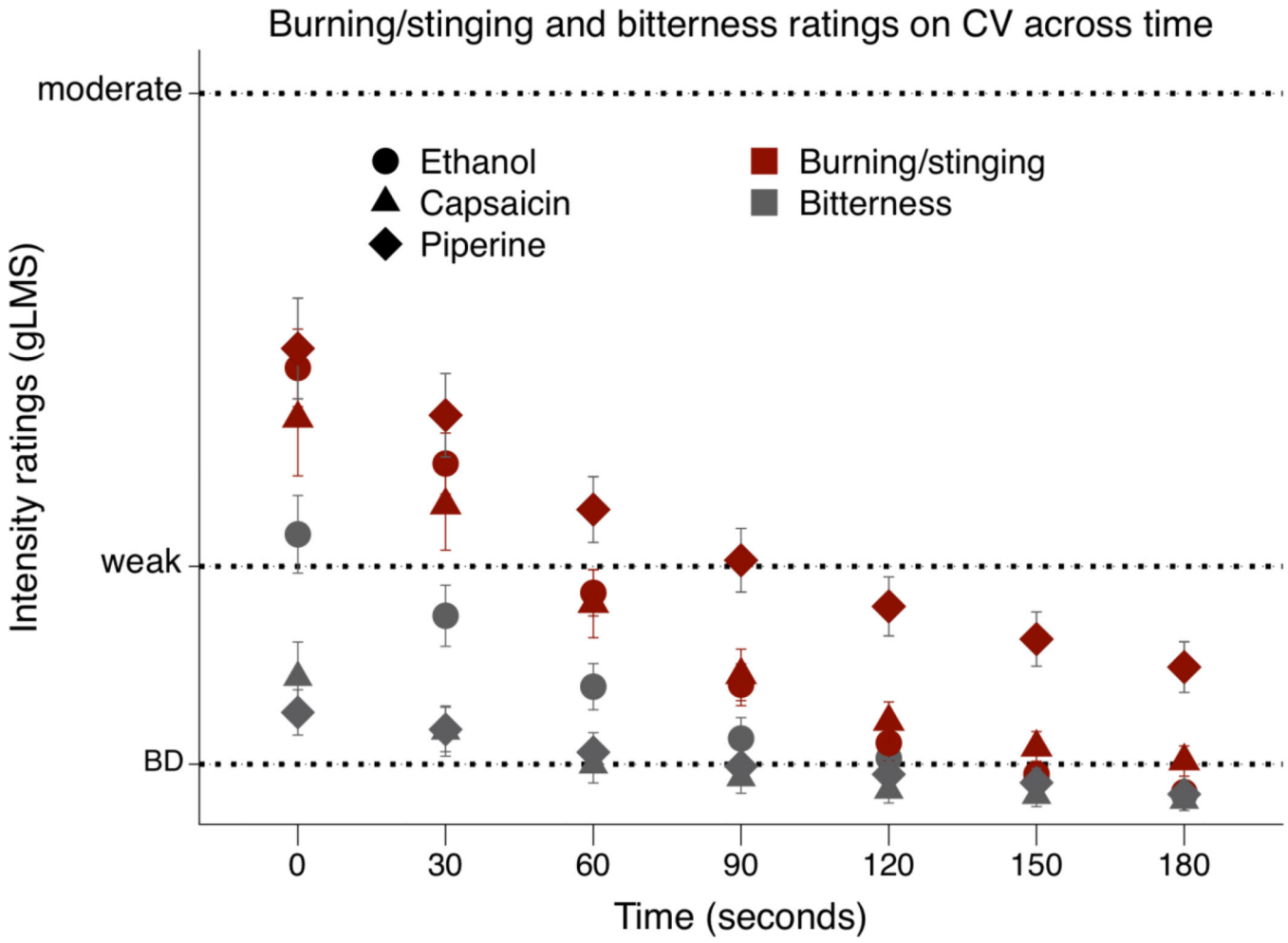

Figure 3.

Means (±S.E.M) for burning/stinging and bitterness for 30.5 ppm capsaicin, 50% (v/v) ethanol, and 10,070 ppm piperine presented to the CV via cotton swabs. Ratings were made on a gLMS every 30 seconds over 3 minutes.

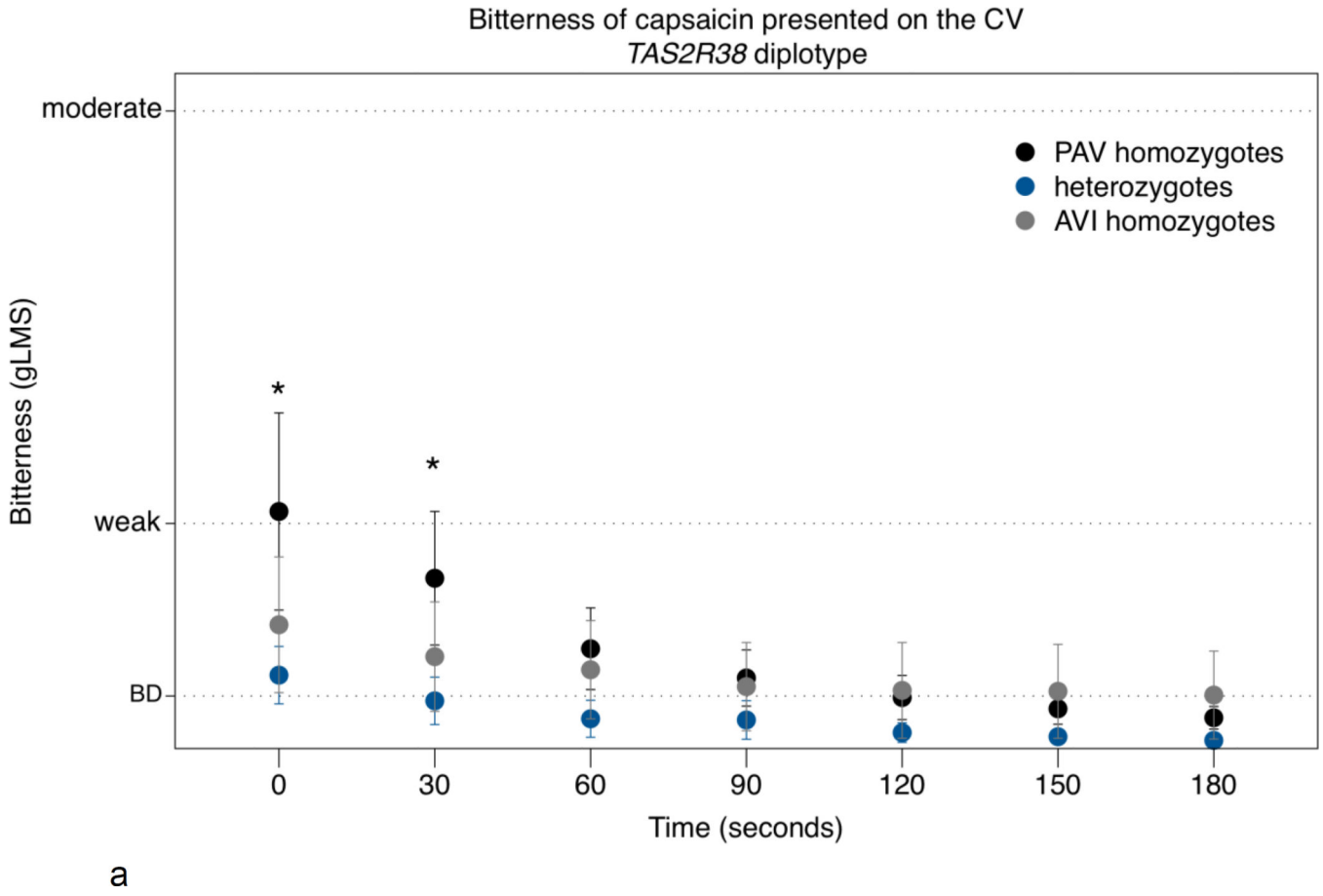

Figure 4a

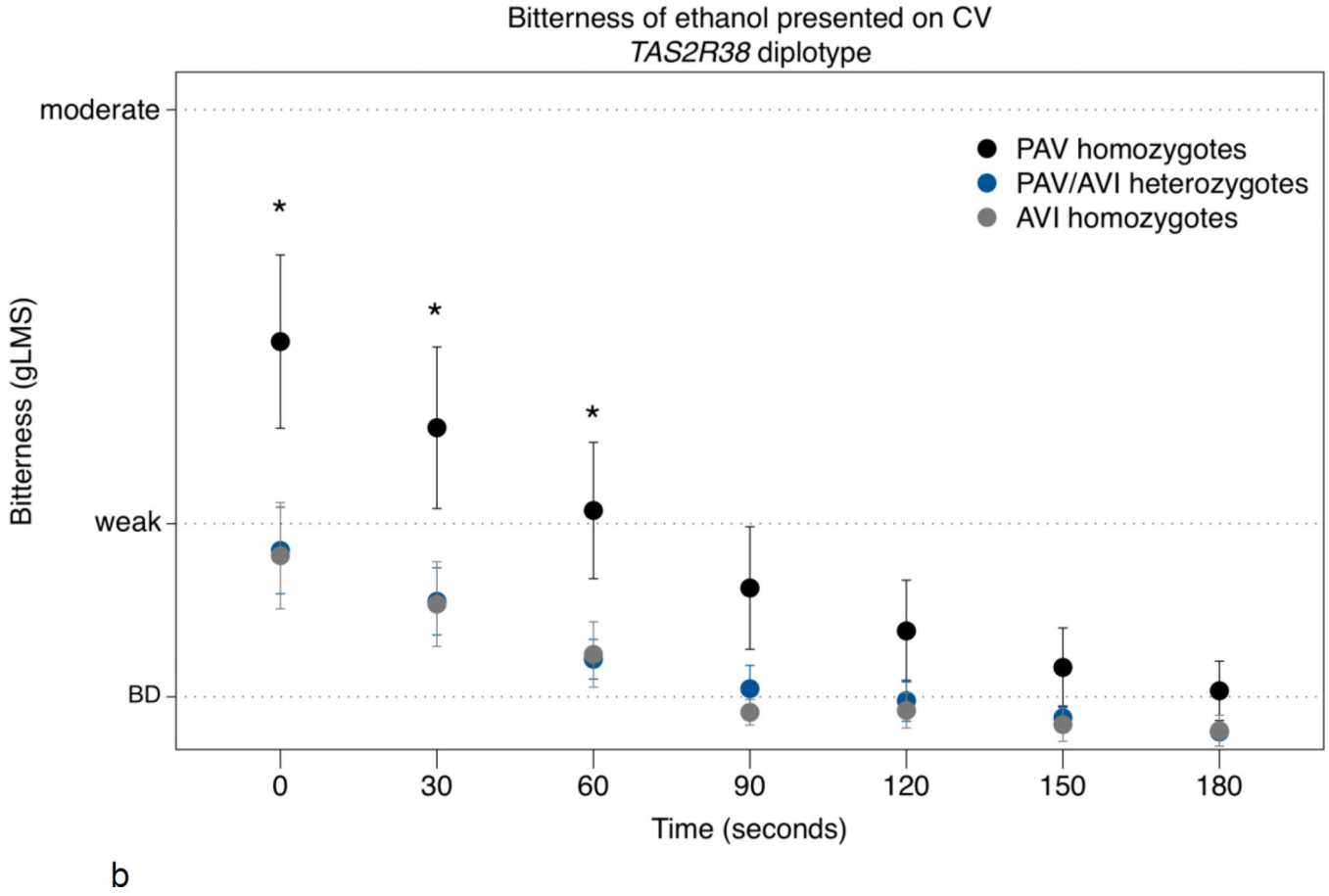

Figure 4b

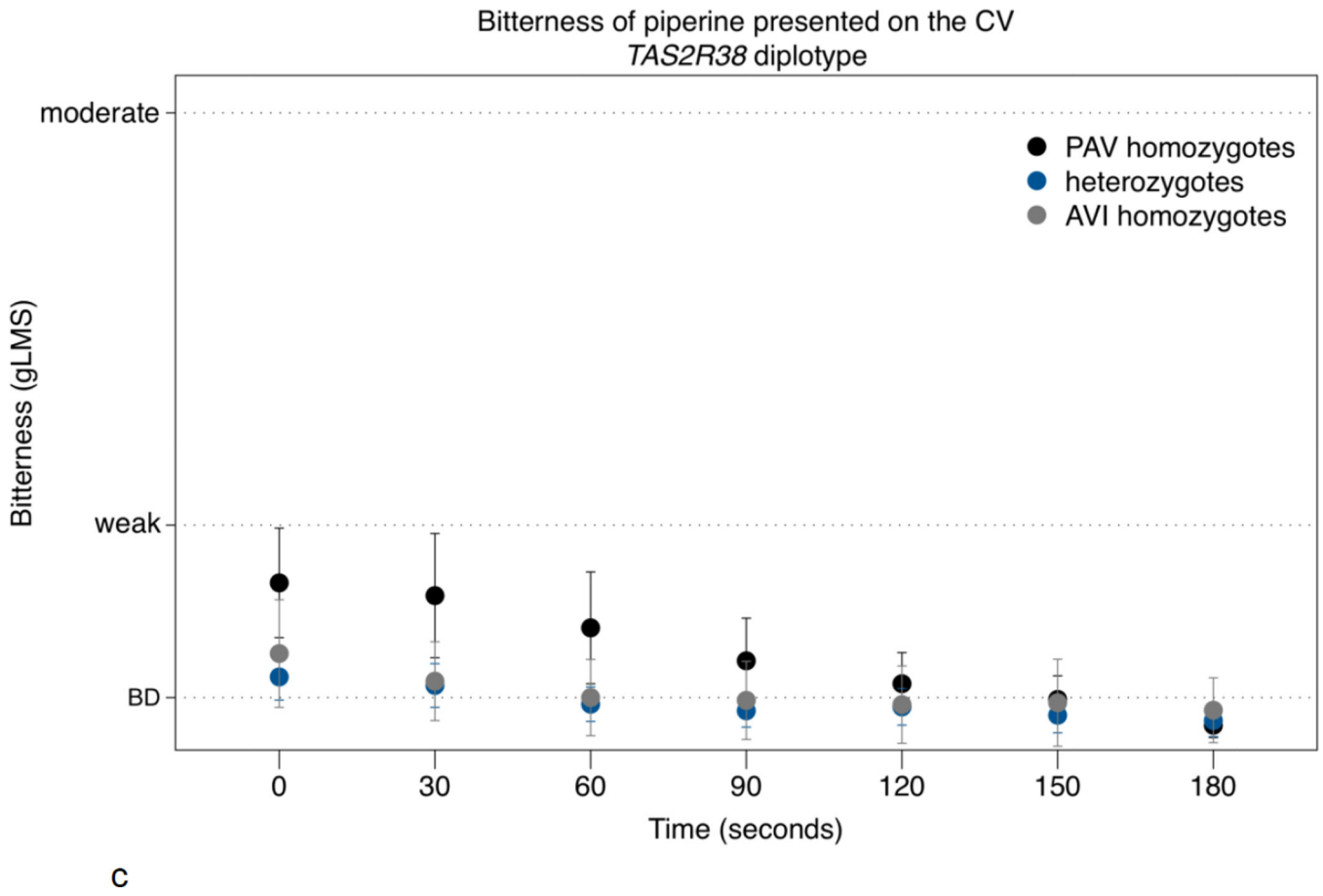

Figure 4c

Figure 4.

Mean (±S.E.M) bitterness ratings for a) 30.5 ppm capsaicin b) 50% (v/v) ethanol and c) 10,070 ppm piperine presented on the CV, stratified by common *TAS2R38* diplotypes. Individuals with rare diplotypes were excluded from the analysis.

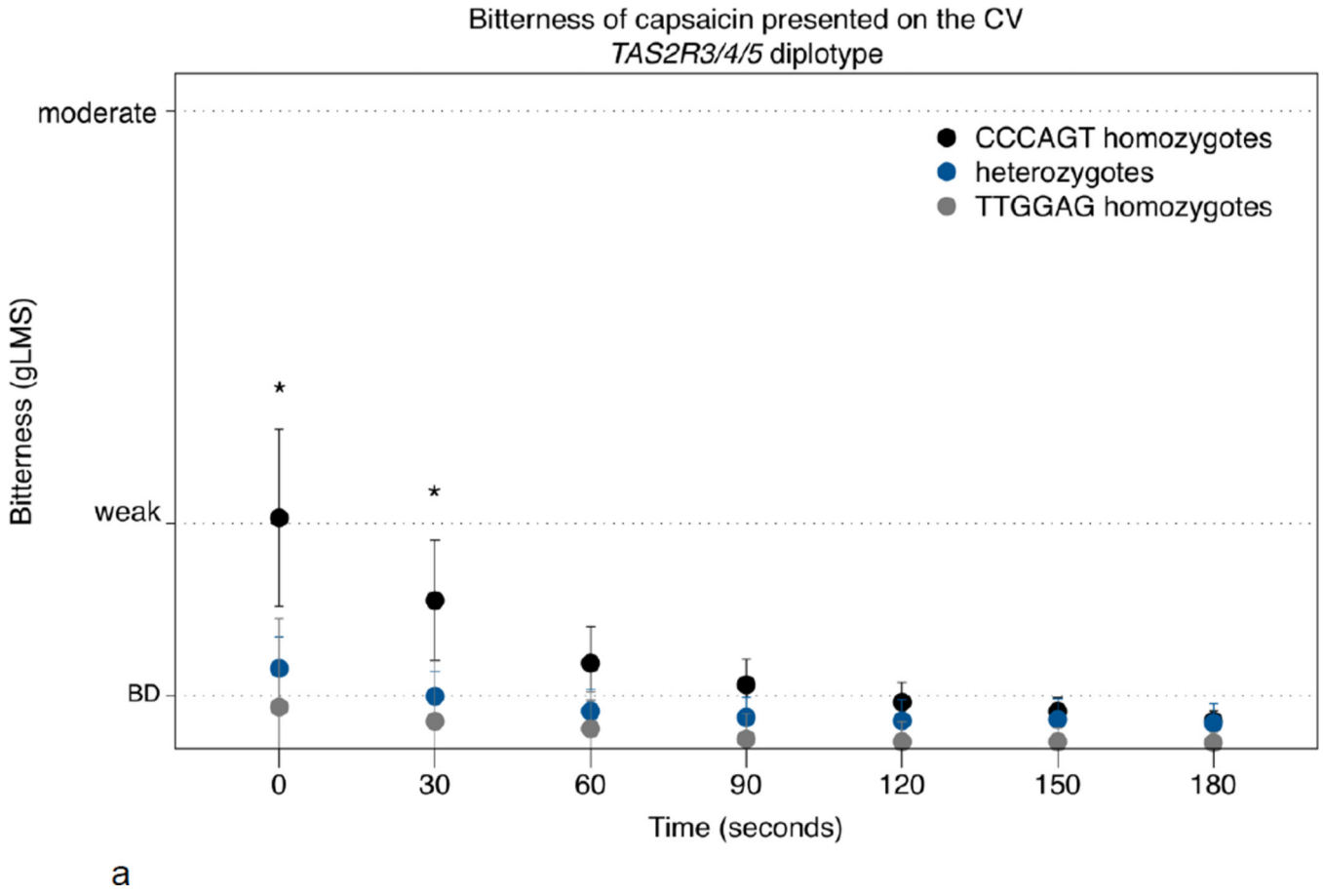

Figure 5a

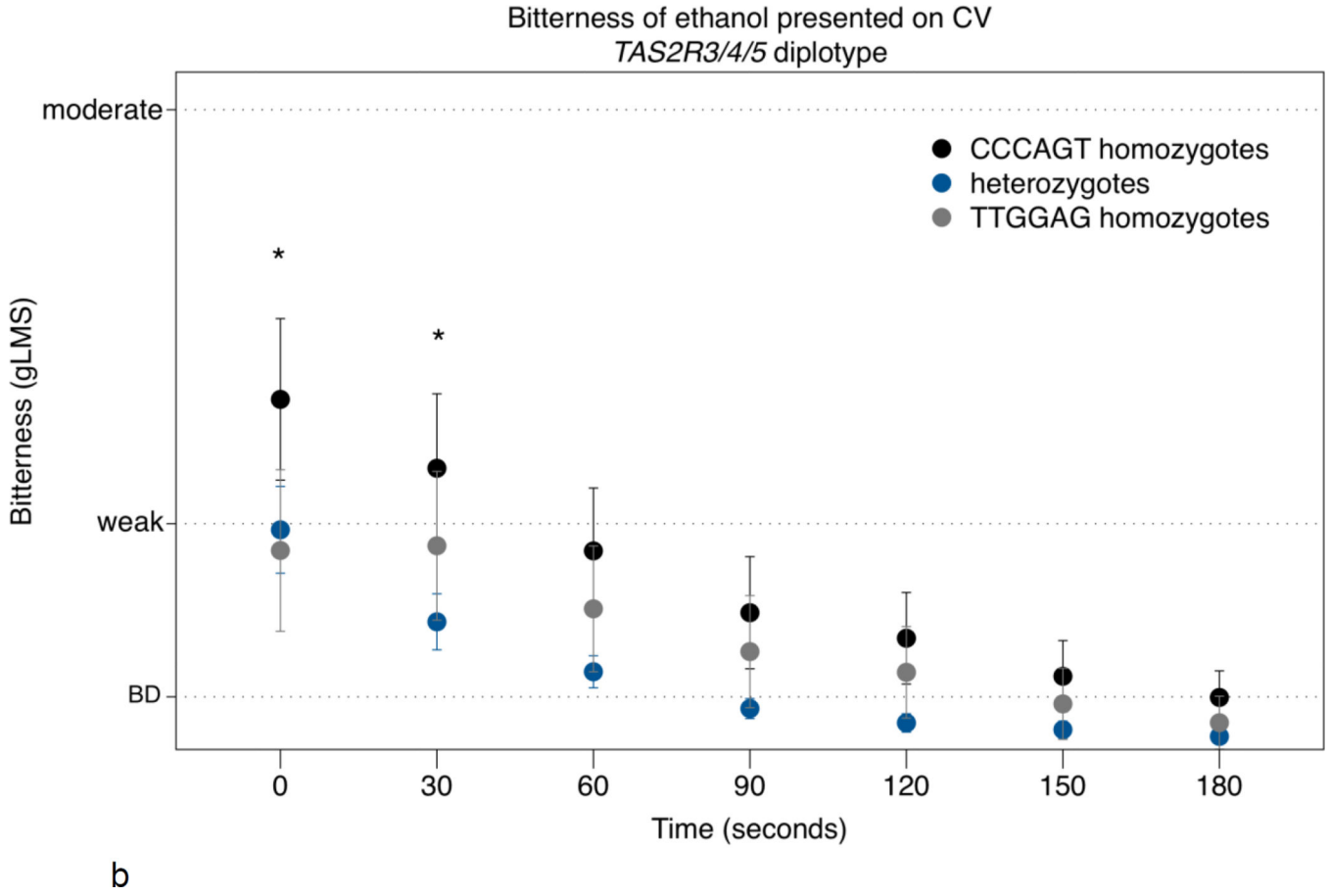

Figure 5b

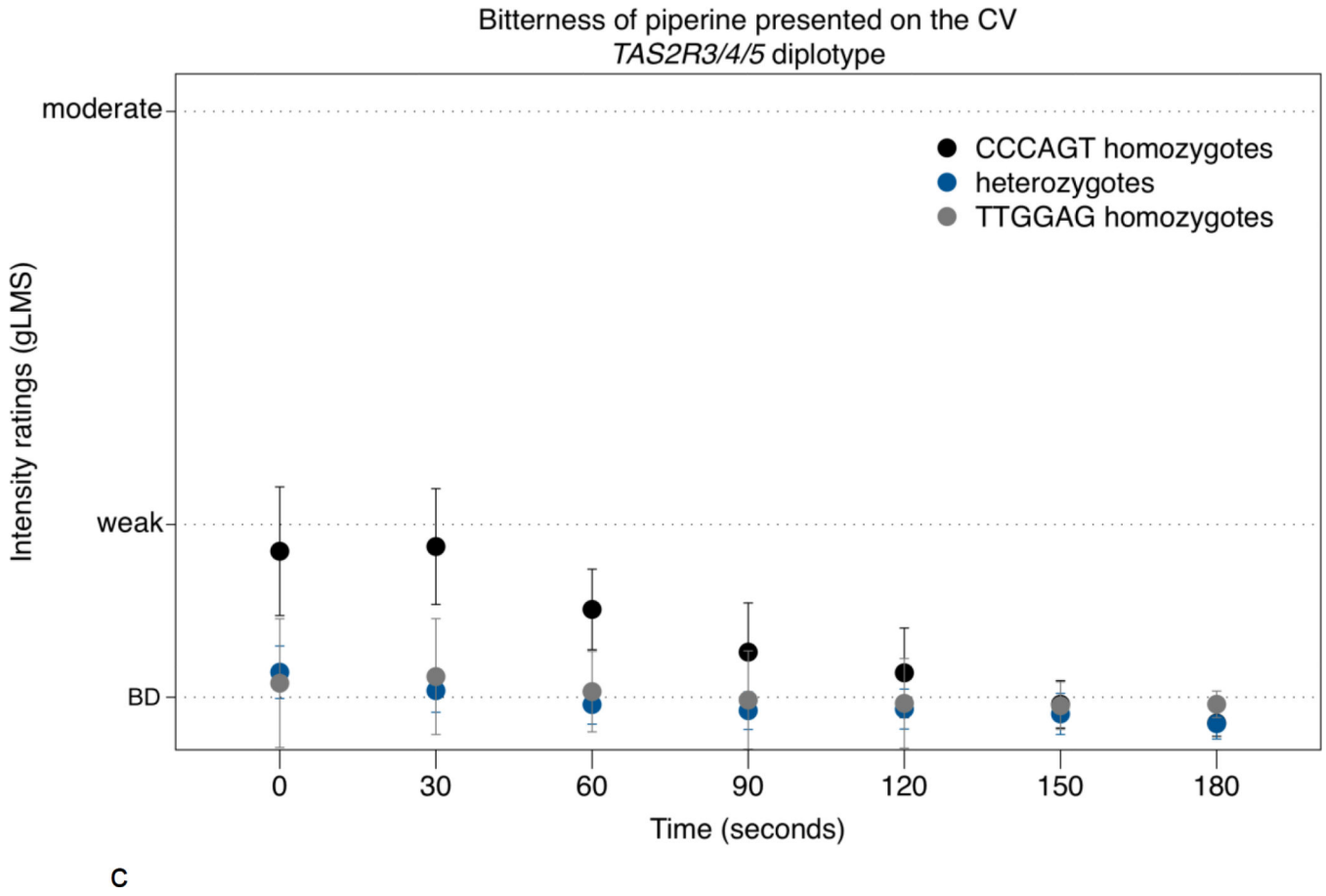

Figure 5c

Figure 5.

Mean (±S.E.M) bitterness ratings for a) 30.5 ppm capsaicin b) 50% (v/v) ethanol and c) 10,070 ppm piperine presented on the CV, stratified by common *TAS2R3/4/5* diplotypes. Individuals with rare diplotypes were excluded from the analysis.

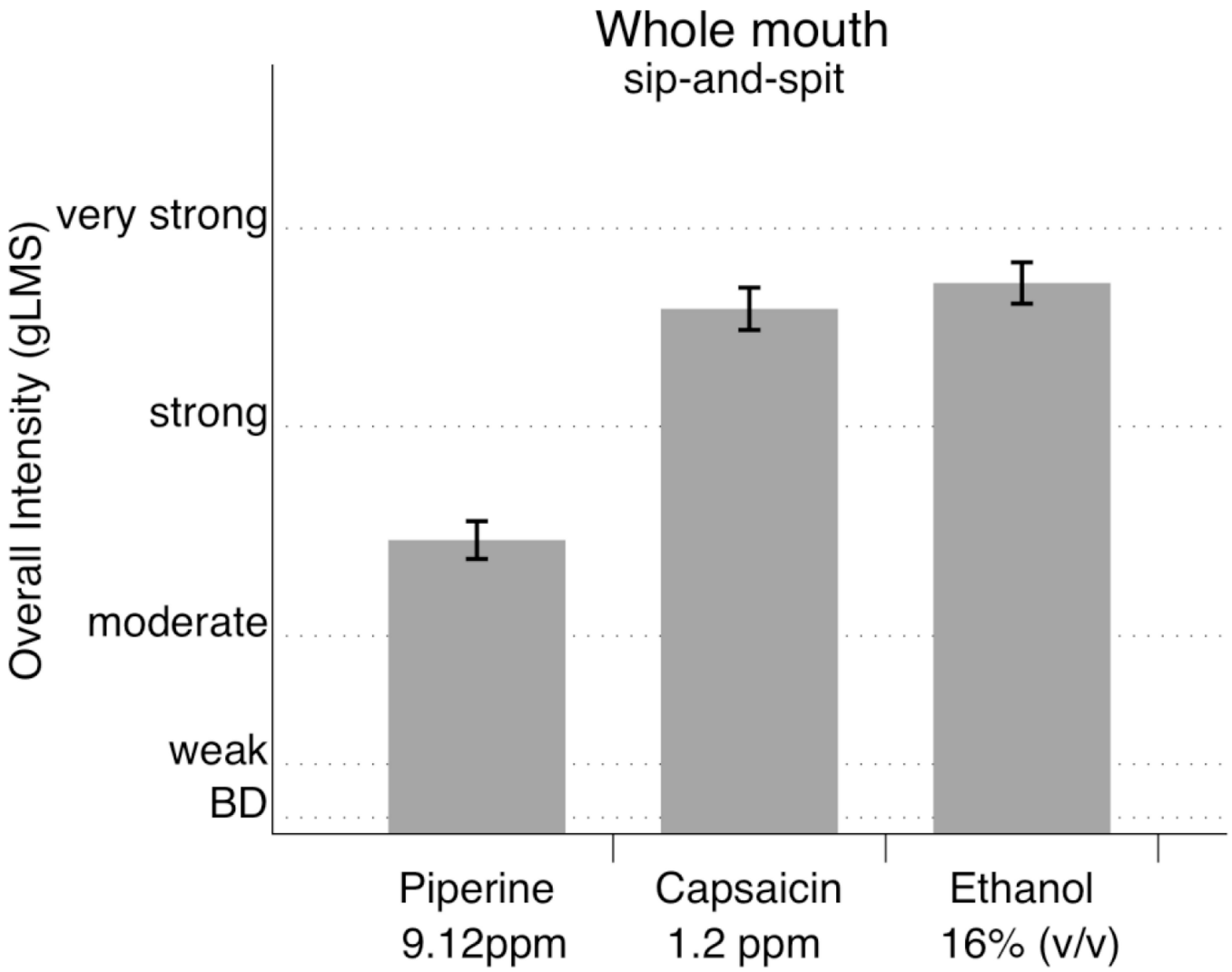

Figure 6.
Overall intensity ratings (mean±S.E.M) for whole mouth sip-and-spit stimuli (15 mL).

Table 1

Summary of participant characteristics

	Freq.	Mean age (±SEM)	Self-reported ethnicity Freq. (row %)			
			Caucasian	Asian	African American	Unreported
Total	106	25.2 ± 0.63	79 (74%)	15 (14%)	1 (1%)	11 (10%)
Female	66	24.7 ± 0.75	50 (83%)	11 (16%)	1 (1%)	4 (6%)
Male	40	25.8 ± 1.14	29 (72%)	4 (10%)	0 (-)	7 (17%)

Table 2

Summery for SNPs analyzed

Receptor	LD Plot #	SNP ID	Location	HWE *p*-value
TAS2R3	1	rs765007	5'UTR	0.97
TAS2R4	2	rs2233998	Phe7Ser	0.90
	3	rs2234001	Val96Leu	1.00
	4	rs2234002	Ser171Asn	0.97
TAS2R5	5	rs2234012	5'UTR	1.00
	6	rs2227264	Ser87Asn	1.00
TAS2R38	7	rs713598	Ala49Pro	0.78
	8	rs1726866	Val262Ala	1.00
	9	rs102466939	Ile296Val	0.88

LD Plot #: corresponds to SNP numbers presented in Figure 2

HWE: Hardy-Weinberg equilibrium

Table 3

Summary of common *TAS2R3/4/5* diplotypes

TAS2R3 rs765007	TAS2R4 rs2233998	rs2234001	rs2234002	TAS2R5 rs2234012	rs2227264	n (%)
C	C	C	A	G	T	28 (27%)
Y	Y	S	R	R	D	46 (45%)
T	T	G	G	A	G	20 (20%)

Y = C or T S = G or C R = A or G D = A or G or T

Percentages were based on 102 individuals that had a diplotype probability >0.8. See text for frequency of uncommon diplotypes.