Bitter–Sweet Processing in Larval *Drosophila*

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Abstract

“Sweet-” and “bitter-” tasting substances distinctively support attractive and aversive choice behavior, respectively, and therefore are thought to be processed by distinct pathways. Interestingly, electrophysiological recordings in adult *Drosophila* suggest that bitter and salty tastants, in addition to activating bitter, salt, or bitter/salt sensory neurons, can also inhibit sweet-sensory neurons. However, the behavioral significance of such a potential for combinatorial coding is little understood. Using larval *Drosophila* as a study case, we find that the preference towards fructose is inhibited when assayed in the background of the bitter tastant quinine. When testing the influence of quinine on the preference to other, equally preferred sweet tastants, we find that these sweet tastants differ in their susceptibility to be inhibited by quinine. Such stimulus specificity argues that the inhibitory effect of quinine is not due to general effects on locomotion or nausea. In turn, not all bitter tastants have the same potency to inhibit sweet preference; notably, their inhibitory potency is not determined by the strength of the avoidance of them. Likewise, equally avoided concentrations of sodium chloride differ in their potency to inhibit sugar preference. Furthermore, *Gr33a-Gal4*-positive neurons, while being necessary for bitter avoidance, are dispensable for inhibition of the sweet pathway. Thus, interactions across taste modalities are behaviorally significant and, as we discuss, arguably diverse in mechanism. These results suggest that the coding of tastants and the organization of gustatory behavior may be more combinatorial than is generally acknowledged.

Key words: bitter, combinatorial coding, *Drosophila*, inhibition, sweet, taste

Introduction

Investigating the gustatory systems of insects is of interest not only because of the roles of gustation in complex social behaviors, learning, and decision-making (Itskov and Ribeiro 2013; Schleyer et al. 2013) but also from an applied point of view: Various insects and/or their larvae cause substantial damage to crops, sustain pollination, feature as disease vectors or pests, not to mention their role for the stability of ecosystems in general. To understand insect appetite, taste, and gustatory choice, therefore, is similarly interesting as it is important. Furthermore, the relatively simple central brain organization of taste as compared with olfactory systems (for classical reviews of *Drosophila* chemosensation, see Stocker 1994; Cobb 1999) makes it an attractive study case for how brains achieve adaptive behavioral control (more reviews featuring in-depth discussions of *Drosophila* gustation: Ishimoto and Tanimura 2004; Amrein and Thorne 2005; Dahanukar et al. 2005; Scott 2005; Hallem et al. 2006; Ebbs and Amrein 2007; Gerber and Stocker 2007; Cobb et al. 2009; Gerber et al. 2009; Montell 2009; Tanimura et al. 2009; Yarmolinsky et al. 2009).
The fruit fly *Drosophila melanogaster* is widely used as a model organism to elucidate gene-brain-behavior relationships (Benzer 1971; Sokolowski 2001; Heisenberg 2003; Greenspan and van Swinderen 2004), and in the last decade also larval *Drosophila* received increasing experimental attention (Heimbeck et al. 1999; early reviews include Cobb 1999 as well as Stocker 2001). As compared with adults, they combine a numerically yet simpler brain (roughly 10000 neurons) with *Drosophila’s* general potential for transgenic manipulation (Brand and Perrimon 1993; reviewed in Phelps and Brand 1998; Elliott and Brand 2008; Venken et al. 2011). However, larvae still possess fundamental mnemonic and cognitive faculties (Scherer et al. 2003; recently reviewed in Diegelmann et al. 2013; Schleyer et al. 2013), arguing that they are simple enough to be tractable, yet complex enough to remain interesting.

Our knowledge about the gustatory system of the larva is fragmentary as compared with the olfactory system (reviews featuring in-depth discussions of chemosensation in larval *Drosophila*: Gerber and Stocker 2007; Melcher et al. 2007; Vosshall and Stocker 2007; Stocker 2008; Cobb et al. 2009; Gerber et al. 2009). Taste information is sensed via approximately 90 gustatory sensory neurons per body side, distributed across 3 external (terminal organ, ventral organ, and the bulge of the dorsal organ) and 3 internal (ventral, dorsal, and posterior pharyngeal) taste sensilla; these gustatory sensory neurons project, via multiple nerve tracts, to defined regions of the subesophageal ganglion (recently renamed as subesophageal zone; Ito et al. 2014), depending on both the sense organ they originate from and the receptor genes they express (Singh and Singh 1984; Heimbeck et al. 1999; Python and Stocker 2002; Gendre et al. 2004; Colomb et al. 2007). The connections from the subesophageal ganglion towards the brain (Melcher and Pankratz 2005; Colomb et al. 2007; Selcho et al. 2009) as well as towards (pre-) motor neurons presumably in the ventral nerve cord remain largely clouded, however.

On the molecular level, sweet and bitter tastants (please note that throughout this study we refer to tastants as “sweet” or “bitter,” respectively, based on whether humans would classify them as such) likely are detected by non-overlapping sets of G-protein coupled gustatory receptors (GRs) of the *Gr* gene family (Clyne et al. 2000; Dahanukar et al. 2001; Thorne et al. 2004; for reviews, see Montell 2009; Yarmolinsky et al. 2009). In adult *Drosophila*, putative sugar receptors and putative bitter receptors are expressed in distinct sets of sensory neurons (Wang et al. 2004; Marella et al. 2006; Weiss et al. 2011), with a single sensory neuron typically expressing several *Gr* genes of either kind (Thorne et al. 2004; Weiss et al. 2011; this holds true for larvae, too: Kwon et al. 2011). Additionally, it has been reported that bitter-activated sensory neurons expressing different combinations of GRs respond to different subsets of bitter substances (Meunier et al. 2003; Weiss et al. 2011). Based on *Gr-Gal4* transgene analyses, 39 *Gr* genes (out of 68) were suggested to be expressed in the taste organs of the larval head (Colomb et al. 2007; Kwon et al. 2011). Of the known bitter-sensitive GRs expressed in adults (*Gr33a*, *Gr66a*, *Gr93a*: Scott et al. 2001; Thorne et al. 2004; Lee et al. 2009; Moon et al. 2009), *Gr33a* and *Gr66a* are expressed also in the larva and can be coexpressed with up to 15 other *Gr*s within a given sensory neuron (Kwon et al. 2011).

Regarding bitter processing, Apostolopoulou et al. (2014) recently reported that ablating the 12 sensory neurons covered by *Gr33a-Gal4* as well as by *Gr66a-Gal4* reduced feeding suppression by quinine to about half. In a binary choice assay, such ablation furthermore abolished avoidance of quinine (within these cells, as the authors argue, it is likely the *Gr33a* receptor rather than the *Gr66a* receptor that is responsible for quinine detection); in turn, transgenically expressing the VRI receptor in this set of sensory neurons was sufficient to confer sensitivity to, and ensuring avoidance of, capsaicin, a substance towards which larvae otherwise are indifferent. Using more specific *Gal4*-driver lines covering subsets of *Gr33a-Gal4*, *Gr66a-Gal4*-positive sensory neurons, it turned out that ablating the C3 neuron (as part of the *Gr97a-Gal4* and *Gr57a-Gal4* expression pattern) and apparently also the C2 neuron (covered by *Gr47b-Gal4*, *Gr94a-Gal4*, *Gr57a-Gal4*, and *Gr59d-Gal4*) entailed partial defects in avoidance. In turn, expressing VRI in the C3 neuron by *Gr97a-Gal4* was sufficient to confer capsaicin avoidance (the C2 neuron has not been tested in this respect). As the Discussion will show, it is particularly important in the present context that the *Gr33a-Gal4*/*Gr66a-Gal4*-positive sensory neurons, strikingly, were found to be dispensable for odor-quinine associative memory formation and retrieval. This implies that already at the sensory periphery there are at least 2 cellularly distinct quinine-sensing pathways that are differentially hooked up to avoidance behavior (for an earlier suggestion of such type of organization based on behavior analyses, see Schipskani et al. 2008; El-Keredy et al. 2012): a first pathway that is *Gr33a-Gal4*/*Gr66a-Gal4*-dependent and that is mediating both aversion and feeding suppression, and a second pathway that is *Gr33a-Gal4*/*Gr66a-Gal4*-independent and that is processing the mnemonic impact of quinine.

For most of the known adult-expressed sugar-sensitive *Gr*s (*Gr5a*, *Gr64a*, *Gr64f*) (Thorne et al 2004; Dahanukar et al. 2007; Jiao et al. 2007, 2008), in turn, no expression has been found in the larva, such that the molecular basis of sugar detection in larvae remains largely unknown. Only recently, Mishra et al. (2013) reported that replacing the coding region of the *Gr43a* receptor gene by *Gal4* reduces attraction to 500 mM fructose to about half and appears to turn attraction of 100 and 20 mM fructose (loc cit Figure S3A) as well as to 100 mM melezitose (loc cit Figure 2B), 100 mM glucose and sorbitol (loc cit Figure 3B) into aversion; this may tentatively suggest a role of *Gr43a*, which is also expressed in adults, in larval sugar processing. In any event, besides the *Gr* gene family, receptors of the
TrpA family (Kim et al. 2010) and/or the Ir family (Benton et al. 2009) may participate in aspects of gustation in the larva. Thus, the function of most GRs in larvae and the behavior(s) they support is only partially clear. What is clear, however, is that larvae are able to sense different substances that to humans are tasting sweet (Schipanski et al. 2008; Rohwedder et al. 2012), salty (Miyakawa 1981; Liu et al. 2003; Niewalda et al. 2008; Russell et al. 2011), or bitter (Hendel et al. 2005; El-Keredy et al. 2012; Apostolopoulou et al. 2014), as these tastants influence choice and feeding behavior, and support reinforcement in Pavlovian conditioning experiments.

In comparison to olfactory behavior, gustatory behavior appears to be relatively simple, rigid, and reflexive, largely because gustatory processing features much less obvious connections towards the brain “proper” as compared with olfaction, and because the evolutionary “meaning” of tastants seems relatively more straightforward: Sweet taste hints at nutritional, energy-rich food, whereas bitter tastants are themselves toxic or hint at toxic food in many cases. Therefore, sweet tastants are usually preferred, enhance feeding, and act as rewards in associative learning. Bitter tastants, in turn, usually are avoided, suppress feeding, and act as punishment. In particular, tastant categories such as sweet and bitter (or high concentrations of salt) apparently can be funneled rather directly into appetitive or aversive behavior: For example in larvae, as in adults (Marella et al. 2006), experimentally driving sensory neurons covered by Gr33a/Gr66a-Gal4 neurons is sufficient to induce aversive behavior (Colomb et al. 2007; Apostolopoulou et al. 2014). Given these striking cases of sufficiency, the electrophysiological observations by the Tanimura group (e.g., Meunier et al. 2003; Gruber et al. 2013; see also the recent paper by Jeong et al. 2013) suggesting a more combinatorial organization of taste have received less attention (discussion in Gerber et al. 2009). That is, both bitter and salty tastants can inhibit sweet receptor neurons, such that the presence of bitter (or of highly concentrated salt) could be coded in a combinatorial way by increased activity in bitter (or salt) sensory neurons plus decreased activity in sweet-sensory neurons (for early reviews on this topic in other insects, see Dethier 1978; Dethier 1987; for corresponding studies on locusts, see Haskell and Schoonhoven 1969; Chapman et al. 1991; Simpson et al. 1991; reviewed in Schuppe and Newland 2009). To examine whether these electrophysiological observations of inhibition, in adult labellar sensilla at least partially mediated by so-called odorant-binding proteins (Jeong et al. 2013), also entail inhibition at the behavioral level, we test whether larval attraction towards sugars is sensitive to modifications by gustatory context. Specifically, we introduce a paradigm that distinguishes effects of inhibition from mere summation and use that paradigm to ask whether the presence versus the absence of bitter or salty tastants influences the animals’ attraction towards sweet tastants.

Materials and methods

Larvae

Third-instar feeding-stage Drosophila melanogaster larvae were used throughout the study. Every day, approximately 200 adult flies were transferred to fresh standard food vials and allowed to lay eggs for 24 h at 25 °C, 60–70% relative humidity, under a 14:10-h light:dark cycle. Then, adult flies were removed, and vials were kept at the mentioned culture conditions for 4 additional days until larvae were collected for experiments. A spoonful of medium from a food vial was taken, and larvae were briefly rinsed twice in tap water to remove residual food. Afterwards, experiments were started, using cohorts of 15 larvae each.

Throughout this study, we used the wild-type strain Canton-S, with a single exception. For the experiments displayed in Figure 9, the following crosses were performed:

- Flies of the w1118, Sp/CyO, Gr33a-Gal4-17/TM3 driver strain (kindly provided by L. Weiss) were crossed to w1118 flies, yielding the driver control genotype heterozygous for Gr33a-Gal4;
- the driver strain was crossed to the w1118, UAS-hid,rpr effector strain (kindly provided by A. Thum) to yield the experimental genotype heterozygous for both Gr33a-Gal4 and UAS-hid,rpr and thus expressing the cell death genes hid and rpr in the Gr33a-Gal4-positive neurons to ablate these cells;
- the effector strain was crossed to w1118 flies yielding the effector control genotype heterozygous for UAS-hid-rpr.

Petri dishes and substrates

We used Petri dishes of 55 mm inner diameter (Sarstedt), covered one-half with a plastic stamp (Figure 1A), and filled the other side with freshly boiled agarose solution that, for example, in addition contained a sweet tastant. Once solidified, we removed the stamp and filled the second-half with another substrate, for example, one with agarose but without an added sweet tastant (to prevent spurious effects of the handling sequence, we alternately poured one or the other substrate). After the second-half had solidified, we covered the dishes with their lids and left them at room temperature until the experiment started 1–4 h later; using the Petri dishes so relatively soon after their preparation ensures that diffusion is minimal. Indeed, for some of the bitter tastants, we observed that using the Petri dishes the following day degrades avoidance scores (data not shown; no such effects were seen for the sweet tastants). Fittingly, the bitter avoidance scores our group has reported previously by using Petri dishes the day following their preparation (El-Keredy et al. 2012) were lower than those we report here; the concentration to induce about a half-maximal avoidance, however, are apparently unaltered (in the case of quinine between 0.05 and 0.5 mM).
As substrates we used either pure 1% aqueous agarose solution (pure; electrophoresis grade) or agarose in addition containing different sweet and/or salty or bitter tastants at the concentrations mentioned in the Results. We used fructose (CAS: 57-48-7, purity > 99.5%), glucose (CAS: 50-99-7, purity > 99.5%), sucrose (CAS: 57-50-1, purity > 99.5%), or sorbitol (CAS: 50-70-4, purity > 98%; all obtained from Roth) as potential sweet tastants, and quinine hemisulfate (CAS: 6119-70-6, purity 92%; Sigma-Aldrich), berberine sulfate trihydrate (CAS: 60352-97-2, purity > 98%, Wako Chemicals), denatonium benzoate (CAS: 3734-33-6, purity > 98%; Sigma-Aldrich), salicin (CAS: 138-52-3, purity > 90%; Wako Chemicals), or L-canavanine (CAS: 543-38-4, purity > 98%; Sigma-Aldrich) as bitter tastants. Additionally, we used sodium chloride (CAS: 543-38-4, purity > 99.5%; Roth) as salty tastant.

Choice

For the choice experiments, one-half of the Petri dishes contained pure agarose, whereas the other half contained either a sweetened, or a bitter, or a salty substrate. In half of the cases, the tastant side was to the left, and in the other half of the cases, the tastant side was to the right, to average-out spurious effects of the experimental surround. We placed 15 larvae in the middle of these Petri dishes and closed the lid. At the time point(s) mentioned in the Results, we scored the number of larvae located at either the pure side, the tastant side, a 1 cm wide “neutral” middle stripe (Figure 1B), or the lid. To calculate a gustatory preference index (PREF), we subtracted the number of larvae on the pure side (#pure) from the number of larvae on the tastant side (#tastant) and divided this difference by the total number of larvae on the dish (thus excluding the larvae on the lid):  

\[
PREF = \frac{#_{\text{tastant}} - #_{\text{pure}}}{#_{\text{total}}} 
\]

Thus, PREF values were constrained between 1 and −1, positive values indicating preference for and negative values indicating aversion of the tastant.

In Figure 1C, we show the time dependency of innate (i.e., experimentally naïve) larvae towards fructose. Larvae were confronted with a choice between pure agarose and agarose containing 2M fructose. In accordance with previous studies (Schipanski et al. 2008; Schleyer et al. 2011; Rohwedder et al. 2012), we found strong attraction towards fructose: Preference scores were positive for all tested time points and increased with time up to preference = 0.8 after 8 min. For all following experiments, we therefore restricted data analyses to this 8-min time point (time-resolved data are presented as supplementary material).
Interaction

Interaction experiments were performed in principle as the choice experiments. However, the interaction test always involved 2 experimental groups.

For the first group, Petri dishes contained a sweet tastant on 1 side and pure agarose on the other side (sweet vs. pure), and the preference for the sweet tastant was determined as follows:

$$\text{PREF} = \frac{\#_{\text{sweet}} - \#_{\text{pure}}}{\#_{\text{total}}}. \quad (1b)$$

For the second group, Petri dishes also contained a sweet tastant on only 1 side, but a bitter tastant was added to both sides (sweet + bitter vs. bitter). We then calculated the preference for the sweet tastant side as follows:

$$\text{PREF} = \frac{\#_{\text{sweet+bitter}} - \#_{\text{bitter}}}{\#_{\text{total}}}. \quad (1c)$$

Thus, also in this case, preference for the sweet-containing side is indicated by positive PREF scores.

We reasoned that if the bitter substance had no influence on the way the sweet substance is processed, that is, if there were no interaction between them, the behavioral tendencies for bitter and sweet should merely add up (such summation-only can explain the behavioral [yet not the electrophysiological] effects as reported in for example Jeong et al. 2013).

To illustrate the underlying rational, consider that when you climb a chair your height and the height of the chair do add up, yet they do not interact—your height remains the same no matter how high the chair is (or the heels for that matter). Thus, based on such summation-only, larvae facing a choice of sweet versus pure and larvae confronted with a choice sweet + bitter versus bitter should behave the same. This is because as a result of summation in both cases, it is the presence of the sweet tastant that remains as the difference between both halves of the Petri dish (Figure S1A and B). An inhibitory interaction, however, would be indicated by the preference for the sweet-containing side being lessened by the bitter tastant (Figure S1C). In other words, such a result would indicate that the bitter tastant acts on the way the sweet stimulus is processed—such that the bitter tastant “disables” the sweet sensory-motor loop.

We note that although this experimental design does allow testing whether there is an inhibitory interaction, it is not intended to draw conclusions about the level of processing at which such interaction takes place (see Discussion).

In the experiment presented in Figure 8, we used sodium chloride instead of a bitter tastant for an interaction experiment.

Statistical analyses

All statistical analyses were performed with Statistica on a PC. Preference values were compared across multiple groups with Kruskal–Wallis tests (KW). For subsequent pair-wise comparisons, Mann–Whitney U tests (MWU) were used. To test whether values of a given group differ from 0, we used 1-sample sign tests (OSS). When multiple tests were performed within a given experiment, we adjusted significance levels by a Bonferroni correction to keep the experiment-wide error rate at 5%. This was done by dividing the critical P-value of 0.05 by the number of tests. Data are presented as box plots, which represent the median as the bold middle line, and 25%/75% and 10%/90% quantiles as box boundaries and whiskers, respectively.

Results

Quinine inhibits fructose processing

We tested for an interaction between bitter and sweet processing by comparing 2 groups of larvae (Figure 2A): One group was confronted with a choice of fructose versus pure agarose, whereas for the second group, fructose preference was tested in the background of quinine (fructose + quinine vs. quinine). Quinine tastes bitter to humans and is reported to be aversive to adult (e.g., Meunier et al. 2003; Weiss et al. 2011) and larval Drosophila (Hendel et al. 2005; El-Keredy et al. 2012; Apostolopoulou et al. 2014). If quinine processing would not do anything to fructose processing, that is if there were no interaction and the behavioral tendencies regarding fructose and quinine would merely add up, both groups should behave the same. This is because for both groups the difference between the sides of the Petri dish is the same: The presence versus the absence of fructose (see Methods and Figure S1 for more detail concerning this rational). However, the preference for the fructose-containing side was less in the background of quinine (Figure 2A), suggesting that quinine is acting onto fructose processing in an inhibitory way.

The inhibition of quinine onto fructose processing was partial for a relatively high concentration of 2 M fructose (Figure 2A); expectedly, as fructose concentration was reduced, fructose preferences were less strong and these less strong preference scores could be inhibited more fully (Figure 2B and C). More importantly, inhibition was getting stronger with increasing quinine concentration (Figure 3A and B): The differential presence of fructose mattered the less the more quinine was present.

We conclude that quinine and fructose processing interact, such that quinine inhibits fructose processing in a concentration-dependent manner. Obviously, the level of processing at which this inhibition takes place as well as its cellular and molecular mechanisms remain unspecified at this point.

Which sweet tastants are inhibited by quinine?

As a next step, we examined the stimulus specificity of this inhibitory effect of quinine. Specifically, we asked upon which sweet tastants quinine exerts inhibition. To allow
“fair” comparisons of quinine inhibition onto the processing of different sweet tastants, however, we first needed to determine the dose-response functions for preference towards 4 sweet tastants (Figure 4A–D), namely for the 3 sugars fructose, glucose, and sucrose, as well as for sorbitol, a substance used as an artificial sweetener in humans. We found strong and dose-dependent attraction towards all tested sweet tastants (Figure 4E). Despite some differences in method, we qualitatively replicated earlier studies (Schipanski et al. 2008; Rohwedder et al. 2012) in that preference scores for sorbitol, although showing from rather early on (Figure S4), are over-all relatively low and in that larvae are more sensitive to fructose and sucrose (Figure 4E: detection threshold for fructose and sucrose is lower than 0.02 M) than to glucose (Figure 4E: detection threshold for glucose is higher than 0.06 M); this latter result fits with what had been found in humans (Pangborn 1963). Also in line with previous reports (Schipanski et al. 2008; Rohwedder et al. 2012), the dose-response functions for fructose and sucrose are strikingly similar. Furthermore, in this as well as in the previous studies, the attraction to sucrose follows an optimum function; reduced attraction to very high sugar concentrations may be due to non-gustatory effects such as for example the stickiness of the substrate. Conceivably also for the other sweet tastants, similar optimum functions would be observed if yet higher concentrations were used. Indeed, for 4 M concentrations of both sucrose and fructose, previous studies found reduced attraction (Schipanski et al. 2008; Rohwedder et al. 2012) and suppression of feeding (Schipanski et al. 2008). We note that the “falling phase” in preference at high concentrations of sweet tastants quantitatively appears less reproducible across studies than the “rising phase.” In this study, attraction decreased already for 2 M sucrose, whereas in both reports cited previously, this was the case only for a 4 M concentration.

In any event, based on the current dose-response functions of attraction, we chose concentrations of the sweet tastants such that they supported equal scores of approximately preference = 0.5 in all 4 cases (dashed line in Figure 4E). This allowed us to ask how strongly quinine could inhibit these about equally-preferred sweet tastants. It turned out that for glucose, inhibition was almost complete; for fructose and sorbitol, inhibition was partial, whereas for sucrose, no inhibition could be detected (Figure 5A). Notably, although preferences for glucose and sorbitol were equally strong (Figure 5B), the extent to which quinine could inhibit glucose processing as measured by an inhibition index (see legend of Figure 5 for details) was higher than was the case for sorbitol (Figure 5B); a similar result was found for fructose and sucrose (Figure 5C and C’).

We conclude that some but not all sweet substances are inhibited by quinine, and that the susceptibility of sweet tastants to be inhibited is not determined by the level of preference for them. This stimulus specificity argues that the inhibitory effect of quinine does not come about by generally adverse effects on behavior (general sluggishness due to “nausea”).

Figure 2  Quinine inhibits fructose processing. (A) In both groups, the presence of fructose (F, at a 2 M concentration) is the sole difference between the halves of the Petri dish. If there was no interaction between quinine (Q, at 5 mM) and fructose processing, the larvae should distribute themselves according to this differential, leading to equal levels of preference for the fructose-containing half in both groups (see Methods and Figure S1 for more detail about the underlying rational). However, fructose preference is lessened in the background of quinine (P < 0.05, U = 1522, MWU) suggesting that quinine inhibits fructose processing. Using lower concentrations of fructose, the same inhibition effect is seen, at respectively lower over-all levels of preference (B) 0.2 M, P < 0.05, U = 993; (C) 0.02 M, P < 0.05, U = 1536.5; MWU). Time-resolved data of this experiment are displayed in Figure S2. Open boxes indicate choice in a bitter background. Significant differences between groups are indicated by asterisks (P < 0.05; MWU). Note that the data of (B) and (C) are also included in Figure 3A and B, respectively. For further details, see legend of Figure 1.
Which bitter tastants inhibit fructose?

We next asked whether in turn bitter tastants other than quinine would inhibit fructose preferences. Again, we first determined dose-response curves for the aversion towards 5 bitter tastants (Figure 6A–E): The alkaloids quinine and berberine, the quaternary ammonium cation denatonium, the β-glucoside salicin and the non-proteinegic amino acid L-canavanine. We found quinine, denatonium, and L-canavanine to evoke very similar levels of avoidance across 3 orders of magnitude (Figure 6A, C, and E), whereas berberine and salicin induce neither preference nor aversion (Figure 6B and D; in the case of berberine, we would like to note that in independent sets of experiments, trends for either avoidance or attraction were observed [not shown]; these variations may be due to variations in berberine-associated olfactory cues as they were not seen in anosmic Orco-null mutant animals). Except for quinine, none of the used bitter tastants were previously used in larval choice behavior to our knowledge. Regarding adult Drosophila, it is interesting that berberine was shown to induce aversion and to activate bitter detecting neurons (e.g., Meunier et al. 2003; Weiss et al. 2011). Thus, there might be a difference in the spectrum of perceptible bitter compounds between adult and larval Drosophila (and also between larvae and man, as all used substances taste bitter to humans). Quinine, denatonium, and L-canavanine are avoided by adult Drosophila (Meunier et al. 2003; Mitri et al. 2009; Weiss et al. 2011; Lee et al. 2012), whereas the lack of behavioral responses towards salicin is consistent between fly and larva (Meunier et al. 2003).

Testing the inhibitory potency of these various bitter substances, we found that all bitter substances except for salicin inhibit fructose attraction (Figure 7) (a trend for L-canavanine having a somewhat weak inhibitory effect was not substantiated in a partial replication of this experiment; Figure S6). Strikingly, berberine, which is inducing no aversion (see Figure 6B), nevertheless strongly inhibits fructose preference.

We conclude that some but not all bitter substances inhibit fructose preference, and that the potency of bitter substances to do so does not depend on the level of aversion for them. Again, this stimulus specificity argues that the inhibition by bitter substances does not come about by generally adverse effects.

Does salt inhibit sugar preference?

After probing various bitter substances for their inhibitory capacity, we wondered whether other, non-bitter tastants have similar effects. Specifically, we tested whether salt inhibits
Figure 4  (A–D) Dose-response functions of the preference for (A) fructose (F), (B) glucose (G), (C) sucrose (Su), and (D) sorbitol (So). (E) Overview, using the median preference score for the respective tastant and concentration. The dashed line represents a level of comparable preference (preference = 0.5), such that “fair” concentrations of these tastants (colored arrows) can be used for the experiment in Figure 5. Time-resolved data of this experiment are displayed in Figure S4. For further details, see legend of Figure 1.
Figure 5  Inhibition of various but not all sweet tastants by quinine. (A) Gustatory preferences in choice between pure agarose versus agarose containing 0.2 M fructose (F), 1.24 M glucose (G), 0.06 M sucrose (Su), or 2 M sorbitol (So), as well as between 5 mM quinine (Q) versus quinine plus the respective sweet tastant (F + Q, G + Q, Su + Q, So + Q). A comparison across groups reveals significant differences (P < 0.05, H = 245.2, df = 7, KW). The presence of quinine results in statistically different levels of sweet preference in the cases of fructose, glucose, and sorbitol (P < 0.05/8, from left to right: U = 3662, 1253, 5259, MWU), but not in the case of sucrose (P > 0.05/8, U = 6076, MWU). (B and B') Comparing glucose and sorbitol in terms of the levels of preference they support (B) and their susceptibility to inhibition (B'). The inhibition index quantifies the differences as displayed in (A); for convenience, it is defined within the boundaries of |−1; 1| as: \( \frac{\text{PREF}_{\text{sweet vs. pure}} - \text{PREF}_{\text{sweet + Q vs. Q}}}{2} \). Despite equal preference (P > 0.05/8, U = 6785, MWU), glucose is more susceptible to quinine inhibition than sorbitol (P < 0.05/8, U = 3032; MWU). (C and C') Same as in (B and B') but for fructose and sucrose ([C] P > 0.05/8, U = 5957; [C'] P < 0.05/8, U = 5189; MWU). In case of sucrose, the inhibition index is not significantly different from 0 (P > 0.05/2; OSS). Open boxes indicate choice in a bitter background. Significant differences between groups are indicated by asterisks (P < 0.05/8; MWU). No time-resolved data were gathered for this experiment (i.e., larvae were scored only after 8 min). For further details, see legends of Figure 1.
Figure 6  (A–E) Dose-response functions of the preference for (A) quinine (Q), (B) berberine (B), (C) denatonium (D), (D) salicin (Sa), and (E) L-canavanine (C). For berberine and salicin, none of the concentrations result in significant attraction or avoidance ($P < 0.05/4$, OSS). (F) Overview, using the median preference score for the respective tastant and concentration. The dashed line represents a level of comparable preference (preference $= -0.7$), such that for quinine, denatonium, and L-canavanine, “fair” concentrations of these tastants can be used for the experiment in Figure 7; for berberine and salicin, we decided to use the very same concentration. Time-resolved data of this experiment are displayed in Figure S5. For further details, see legend of Figure 1.
preference towards glucose (using glucose in this experiment is based on the finding that from all the sweet tastants tested, glucose is most prone to inhibition; Figure 5A, B’, C’).

First, we tested animals’ direct preference towards 2 concentrations of salt and found that both of them are highly and equally aversive (Figure 8A). Both concentrations of salt inhibit glucose preference (Figure 8B), yet the lower concentration of salt evokes milder inhibition. These findings, again, speak for a dissociation between levels of aversion and inhibition and thus against aversiveness as the sole cause of inhibition.

**Are bitter-activated neurons necessary for sweet inhibition?**

From the GRs in the gustatory system, GR33a and GR66a have been repeatedly shown to be necessary for bitter sensation in adults (Scott et al. 2001; Thorne et al. 2004; Lee et al. 2009; Moon et al. 2009) and to be expressed also in the larva (Kwon et al. 2011; Apostolopoulou et al. 2014). It has been suggested that GR33a acts as a co-receptor for many bitter substances and therefore is expected to be expressed in many bitter-activated neurons (Moon et al. 2009; Weiss et al. 2011). Interestingly, GR33a and GR66a are expressed in a broad range of cells and their expression patterns seem to completely overlap, in both adults and larvae (Kwon et al. 2011; Weiss et al. 2011; Apostolopoulou et al. 2014). In larvae, Gr33a-Gal4/Gr66a-Gal4-positive neurons have been shown to be necessary for aversion of quinine and sufficient mediate aversion (Colomb et al. 2007; Apostolopoulou et al. 2014).

To investigate whether signaling of Gr33a-expressing neurons is necessary for inhibition of the sweet pathway, we genetically ablated these gustatory receptor neurons (GRNs) by co-expression of 2 apoptosis genes, hid and reaper (White et al. 1996; Kurada and White 1998; Selcho et al. 2009, 2012). First, we tested for quinine choice and found that killing Gr33a-expressing cells abolished quinine avoidance (Figure 9A), replicating the recent findings of Apostolopoulou et al. (2014). We would like to note that our results feature some data points indicating slight attraction in the experimental group. This may hint at an attractive (maybe non-gustatory) component of quinine being revealed only when the Gr33a-Gal4-dependent aversive component is switched off.

In any case, when probing for inhibition of glucose preference by quinine, it was striking to see strong inhibition in all genotypes (Figure 9B). Thus, we conclude that those cells that are required for quinine avoidance are not necessary for quinine-mediated inhibition of the sweet pathway.

**Discussion**

We find that many—but not all—bitter tastants inhibit fructose processing, and that many—but not all—sweet tastants are susceptible to inhibition by quinine. Before entering into

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**Figure 7** Inhibition of fructose by various but not all bitter tastants. Gustatory preferences for the choice between pure agarose versus agarose containing 0.2 M fructose (F), as well as between 5 mM quinine (Q), berberine (B), denatonium (D), salicin (Sa), or L-canavanine (C) versus the respective bitter tastant plus 0.2 M fructose (F + Q, F + B, F + D, F + Sa, F + C). A comparison across groups reveals significant differences between groups ($P < 0.05$, $H = 81.9$, $df = 5$; KW). The presence of a bitter tastant results in statistically different levels of fructose preference in case of quinine, berberine, denatonium, and L-canavanine ($P < 0.05/5$, from left to right: $U = 3662, 3334, 3450.5, 5207.5$; MWU), but not in case of salicin ($P > 0.05/5$, $U = 6817$; MWU). A partial repetition of this experiment is shown in Figure S6. No time-resolved data were gathered for this experiment (i.e., larvae were scored only after 8 min). Open boxes indicate choice in a bitter background. Significant differences between groups are indicated by asterisks ($P < 0.05/5$; MWU). For further details, see legend of Figure 1.
the discussion about how this pattern of results can be understood, we would like to stress that the inhibition effects observed here cannot be accounted for by a summation of behavioral tendencies (see Methods and Figure S1 for more detail about this rational). As the concentration of the bitter tastant is the same on both sides of the Petri dish, bitter-induced aversion should cancel out, such that what differs between both sides is the sweet tastant. Thus, if behavioral tendencies towards sweet and bitter tastants would not interact but would merely sum up, we would observe the same level of preference in the presence and in the absence of the bitter component. An inhibitory interaction, however, would decrease the preference for the sweet-containing side in the background of the bitter tastant—and this is what we observe throughout this study.

Bitter → sweet inhibition and levels of attraction/avoidance

Can the levels of attraction to the sweet tastants and/or the levels of avoidance of the bitter tastants account for the result that many—but not all—bitter tastants inhibit fructose processing, and that many—but not all—sweet tastants are inhibited by quinine?

When probing the inhibitory effect of quinine towards a number of different sweet tastants, we chose their concentrations such that they induce a similar level of attraction (Figures 4 and 5). Nevertheless, they strongly differ in their susceptibility to be inhibited by quinine (Figure 5): For example, glucose and sorbitol are preferred equally, but glucose is inhibited much more strongly than sorbitol (Figure 5B and Bʹ), and a similar result is found for fructose and sucrose (Figure 5C and Cʹ). Testing, in turn, the inhibitory power of various bitter substances towards fructose reveals that berberine induces inhibition to the same extent as quinine, although in contrast to quinine it is not significantly aversive (Figures 6 and 7). Furthermore, 2 concentrations of salt, being equally aversive, differ in their ability to inhibit glucose preference (Figure 8). These findings suggest that inhibition is determined neither by the level of attraction to sweet tastants nor by the level of aversion of bitter/salty tastants. Regarding quinine, this conclusion is further supported by the finding that aversion and inhibition employ distinct pathways already from the sensory periphery on: Gr33a-Gal4-positive neurons are necessary for quinine avoidance (and neurons positive for Gr66a-Gal4, which is thought to completely overlap with Gr33a-Gal4, are sufficient for aversion [Colomb et al. 2007; Apostolopoulou et al. 2014]) but not for quinine-mediated inhibition of the sweet pathway (Figure 9). What, then, are the mechanisms of this inhibition?

A view towards the physiology of taste processing in adult Drosophila

For a discussion of the mechanisms of inhibitory interactions between bitter and sweet processing, a view towards adult Drosophila might be elucidating. It is known that the
For adult *Drosophila*, thus both basic scenarios of how bitter substances inhibit sugar processing, namely operating either via bitter-sensitive neurons (Figure 10A) or not (Figure 10B and B’), remain conceivable and may indeed

![Figure 9](http://chemse.oxfordjournals.org/)  
**Figure 9**  
Gr33a-Gal4-positive neurons are necessary for quinine avoidance (A) but not for sweet inhibition (B). (A) Gustatory choice between 5 mM quinine (Q) and pure agarose in the driver control (left), the experimental group in which the Gr33a-Gal4-positive neurons are ablated by expression of the cell death genes hid and rpr (middle), and in the effector control (right). A comparison across these genotypes reveals significant differences between them ($P < 0.05$, $H = 29.48$, $df = 2$; KW). Upon ablation of the Gr33a-Gal4-positive neurons, avoidance of the bitter substrate is reduced ($P < 0.05/2$, from left to right: $U = 465.5$, 379.5; MWU) to 0 ($P > 0.05/3$, OSS). (B) Gustatory choice between pure agarose versus glucose (G: 1.24 M) as well as between quinine (Q: 5 mM) on 1 side versus glucose plus quinine on the other side (G + Q) of the same genotypes as in (A). A comparison across genotypes reveals significant differences ($P < 0.05$, $H = 352.9$, $df = 5$; KW). Notably, in all genotypes, and thus also in the larvae that lack the Gr33a-Gal4-positive neurons, the presence of quinine results in a reduced level of glucose preference ($P < 0.05/3$, from left to right: $U = 2742.5$, 2849.5, 1387.5; MWU). No time-resolved data were gathered for this experiment (i.e., larvae were scored only after 8 min). Open boxes indicate choice in a bitter background. Significant differences between groups are indicated by asterisks ([A] $P < 0.05/2$, [B] $P < 0.05/3$; MWU). For further details, see legend of Figure 1.
differentially apply to different sensillae. Might this be the case for larvae as well?

Possible modes of bitter $\rightarrow$ sweet inhibition in *Drosophila* larvae

Regarding larvae, it is clear that the inhibitory effect of bitter tastants onto sweet processing does not solely rely on those neurons that command avoidance behavior and that are covered by *Gr33a-Gal4*. The scenario would thus be that inhibition comes about by bitter sensory neurons that are not part of the *Gr33a-Gal4* pattern (Figure 10A), that are not necessary for avoidance behavior, and that differ from the *Gr33a-Gal4* neurons in their ligand profile: Berberine, for example, can activate gustatory sensory neurons (at least in adults: Meunier et al. 2003; Weiss et al. 2011) and does initiate the inhibitory effect onto sugar processing but no aversion, whereas quinine, denatonium, and L-canavanine (as well as salt) activate both (Figures 6 and 7). Is there reason to remain sceptical that this is an exhaustive explanation?

We note that inhibition acts differentially depending on the identity of the sweet tastant. In adults, glucose is sensed by the GR5a receptor molecule, whereas fructose and sucrose are sensed by GR64a (Dahanukar et al. 2007). However, both these sugar receptor molecules in adults are usually expressed in the same cells (Weiss et al. 2011). This implies that although the identity of the sweet tastant could be discerned using 2 differentially tuned receptor molecules, such discriminative power is “abandoned” at the level of the sensory neuron. If for a given sweet tastant a similar organization would apply in the larva, too (see Introduction), the found differences between sweet tastants in their sensitivity to inhibition may hint at a relatively peripheral site of inhibition along the sweet pathway, that is, at a point of processing before the signals carried by the respectively expressed GRs converge (see Discussion). Quinine is reportedly able to enter artificial liposomes and rat taste cells (Peri et al. 2000) and to directly activate G-proteins in liposomes (Naim et al. 1994). We note that direct interactions between BTs and STs seem to be unlikely on stoichiometric grounds (BTs are effective inhibitors in concentrations up to 1000-fold lower than the inhibited ST).

![Figure 10](http://chemse.oxfordjournals.org/) Working hypotheses of bitter $\rightarrow$ sweet inhibition. A, and B, B’ show 2 scenarios to account for the inhibitory effect of quinine on sweet processing. (A) The sweet pathway may be inhibited by the bitter pathway. Specifically, bitter tastants (BTs) may activate 2 types of bitter sensory neurons (BN; BTs may differ in their ability to activate one or the other BN), 1 steering aversion (*Gr33a-Gal4* positive), and the other (not *Gr33a-Gal4* positive) inhibition of the sweet pathway. A sweet tastant (ST) activates a sweet-sensory neuron (SN), but its downstream effect is then inhibited by the inhibitory bitter pathway. (B) BTs may compromise the function of some of several sugar receptor (SR) molecules. Indeed, in adults, CO$_2$-induced avoidance behavior can be inhibited by a particular class of odors, directly acting on the CO$_2$ receptor (coded by *Gr21a* and *Gr63a*: Turner and Ray 2009). (B’) A BT or an intracellular cascade triggered by it may act within sweet-sensitive gustatory receptor neurons (SN), that is, at an intracellular domain of the SR or downstream of the SR. In case of several SRs in 1 cell that are activated by different STs, inhibition may take place before those tastant-specific pathways converge (see Discussion).
its peripheral, sensory features, the metabolic feedback it induces, and its nutritional value factor in these scenarios.

Outlook
Compared with the olfactory system, the taste system is often described as relatively simple, with primary taste categories like sweet and bitter rather strictly commanding behavior (approach and avoidance, respectively). However, the complexity of the taste system, also relative to the olfactory system, becomes obvious when contemplating the number of involved genes, cells, nerve tracts and organs, the dichotomy of internal–external gustatory organs, and the entanglement of taste and touch. This study additionally suggests that there are unexpected complexities in the way sweet and bitter processing interact. This hints at a coding strategy for gustation that is more combinatorial than previously thought (see also Charlu et al. 2013; for a review and discussion about combinatorial elements in the taste system of fly and mouse, see Yarmolinsky et al. 2009).

Supplementary material
Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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