Temporal responses of bumblebee gustatory neurons encode sugar identity

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The sense of taste permits the recognition of valuable nutrients and the avoidance of potential toxins. Models of gustatory coding propose that within modalities (e.g. sweet, bitter, etc.), taste ligands are not distinct stimuli. However, these models are based on data from mice or fruitflies that have omnivorous, non-specialist diets. A specialist feeder might, however, be expected to have acuity within modality if stimulus resolution was critical to survival. Here, we show that the gustatory system of the bumblebee is capable of performing within-modality discrimination of sugar compound identity. The temporal firing patterns of two sugar-sensing gustatory neurons (GRNs) within the galeal sensilla of the bees' mouthparts separate sugars into four distinct groups that correlate with sugar nutritional value. Representative sugars from each of these groups could be discriminated by bumblebees in a two-choice assay. Furthermore, the bees could discriminate among high nutritional value sugars such as fructose, sucrose, and maltose, implying that GRNs from other locations on the mouthparts improve the resolution of sugar identity in the bee brain. The bumblebee's refined sense of sweet taste is likely to be an adaptation that enables precise calculations of the nature and nutritional value of floral nectar.

gustation | sensory neurons | temporal population coding | taste discrimination

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Introduction

Sensory acuity evolves in animals when information resolution improves fitness. Visual acuity for colour, for example, arises when the ability to discriminate among colours improves the location

of food or the choice among mates (Osorio and Vorobyev, 2008). Where high acuity is necessary to perform optimal stimulus identification, sensory systems become more specialized. While this has been observed in vision, audition, and olfaction, it has relatively rarely been studied in the sense of taste.

The canonical model of the organization of the gustatory system proposes that individual cells or neurons express receptors that detect compounds from specific modalities (Breslin and Spector, 2008; Chen and Dahanukar, 2020). This arrangement permits a form of coding specificity whereby tastants in particular modalities (e.g., sweet, bitter, salty) are represented through the activity of individual neurons. In insects, contact chemosensilla house several (i.e. 2-6) gustatory neurons, each of which responds to compounds from a specific modality (Dethier, 1976). For example, in a fly taste sensillum housing four neurons, one GRN would detect sugars, another 'low' salt concentrations, another would detect water, and the last would detect 'high' concentrations of salt and/or bitter compounds (Amrein and Thorne, 2005; Chapman and Chapman, 1998; Dethier, 1976). The sensitivity of each neuron type is determined by the receptor proteins (Grs) it expresses.

Sugar compounds are important nutrients for insects. In Drosophila, the GRN in each sensillum that responds to sugars expresses Gr proteins from the 5a, 61a, and the 64a-f family that form complex and diverse heteromeric receptors that enable flies to detect many sugars using the same neuron in each sensillum (Hiroi et al., 2002; Fujii et al., 2015; Dahanukar et al., 2007). These GRNs also express another Gr protein, 43a, which forms functional homomeric receptors tuned towards the detection of fructose (Miyamoto et al., 2012). To date, most studies of sugar detection in insects focus on differences in the tuning of GRNs towards sugars of different structure in order to relate structure to receptor specificity (Dahanukar et al., 2007; Fujii et al., 2015; Miyamoto et al., 2012).

Bees are unique among insects because they possess few genes for gustatory receptors (Robertson and Wanner, 2006; Sadd et al., 2015). Adult bees have specialized mouthparts for collecting floral nectar, a sugary reward offered by plants to pollinators. Sugars have a large impact on bee survival; for example, adult worker honeybees require the greatest amount of ATP of any animal recorded (Suarez et al., 1996). For this reason, one might predict *a priori* that bees would have proportionally greater acuity for the detection and discrimination of sugars in food than other insects, but this has rarely been investigated.

Floral nectar is mainly composed of sucrose, glucose and fructose. However, many

bee species collect honeydew produced by Hemipteran insects as a carbohydrate-rich food source and it often contains sugars such as maltose and melezitose (Shaaban et al., 2020). Previously, we found that the bumblebee's gustatory system exhibits a specialized mechanism for encoding sugar concentration (Miriyala et al., 2018). We observed that two GRNs per sensillum coordinated their activity to produce bursts of spikes at concentrations of sucrose >25 mM. Here, we performed a series of experiments to test the acuity of the gustatory system towards sugars relevant to bumblebees. Specifically, we tested the canonical model of taste organization that argues that compounds classified within a modality (i.e. sweet) cannot be discriminated. To this end, we measured how compound detection, such as the spiking pattern produced by the sugar-sensing GRNs, was influenced by compound identity. We also tested whether bees could discriminate among sugars and how each sugar influenced feeding reflexes and bout duration.

Results & Discussion

Sugar type affects the responses of three of four neurons per galeal sensillum

Bumblebees have a unique mechanism for sensing sucrose in nectar; sucrose evokes a bursting pattern of spikes derived from the activity of two GRNs (GRN1, GRN2) that communicate via a gap junction (Miriyala et al., 2018). This contrasts with the strategy of taste coding described in *Drosophila* where sucrose evokes phasic activity in a single GRN in each sensillum (Hiroi et al., 2004). Temporal codes from peripheral neurons relay important qualitative information about stimuli in other senses including olfaction (Raman et al., 2010). We therefore tested whether temporal coding occurred in bee gustatory neurons. We stimulated bumblebee type A sensilla with seven sugars found in floral nectar and aphid honeydew (fructose, glucose, sucrose, maltose, melezitose, trehalose, sorbitol). We also tested four sugars less commonly encountered by bees (sorbose, xylose, lactose, mannose). Sugars evoked a diverse range of GRN responses that were unrelated to similarity in their compound structure (Figure 1A).

Sucrose, fructose, and maltose elicited the highest rates of bursting; GRN firing rate also increased as a function of concentration for every sugar tested except lactose, xylose, and mannose (Figure 1B and D-F). We used a spike sorting algorithm that differentiates individual GRNs based on their interspike intervals (ISIs, Figure S1). As expected (Miriyala et al., 2018), we observed coherent bursts of spikes from GRN1 punctuated by a single GRN2 spike. We also detected a third sugar-sensitive GRN in each sensillum (Figure 1C). GRN3 spikes occurred sparsely over the course of sugar stimulation and were not tied to the burst structure of GRN1

and GRN2 (Figure 1C, Figure S1).

Sucrose, maltose and melezitose activated GRN1 and GRN2 at the lowest threshold concentration (10 mM, Figure 1G). Another group of sugars (glucose, sorbitol, and sorbose) activated GRN1 but required sugar concentrations >100 mM to activate GRN2 (e.g., glucose, 1000 mM, Figure 1D-F). The firing rate of GRN3 was low: at the highest concentrations GRN3 fired at 2-6 spikes/s, whereas GRN1 fired at 25-75 spikes/s and GRN2 fired at 4-20 spikes/s (Figure 1D-F). GRN3 was most strongly tuned towards fructose but also responded to sucrose, maltose, and glucose (Figure 1G, Figure S1). This could indicate that GRN3 expresses gustatory receptors (GRs) specific for fructose detection (e.g., AmGr3, (Simcock et al., 2017; Takada et al., 2018)). None of the GRNs responded to lactose, xylose or mannose.

Sugar identity and concentration are encoded by a temporal pattern created by bursting neurons

At least three parameters of spike trains encode information: relative spike timing, changes in spike rate (i.e., adaptation) and instantaneous firing rate. GRN responses are typically compared by averaging firing rates over relatively long intervals for neurons (e.g, 1 s, Figure 1D-F). Bumblebee GRNs, however, clearly have a temporal pattern of firing which could contribute to coding over shorter time scales (Figure 1A). Sugar-induced changes to the temporal structure of GRN firing is readily visualized in the traces in Figure 1A.

Analysis of the relationship between the firing of GRN1 and GRN2 over the time course of sugar exposure revealed that concentration and sugar identity affect the bursting pattern of activity (Figure 1H-J). GRN1 spike rate increased as a function of GRN2 for all 10 mM stimuli (except maltose) with an average slope of 2 (Figure 1H and Table S1). At >100 mM (Figure 1I and 1J), sugars which elicited regular and relatively high rates of GRN2 spikes (fructose, sucrose, maltose, melezitose) became distinguishable from all other sugars because the rate of GRN1 depends less on the rate of GRN2 firing. Finding that the dynamic response between GRN1 and GRN2 depends on sugar identity (as in Figure 1J) and not entirely on the rate of GRN1 firing indicates that GRN2 also plays an active role in sensing sugars.

The temporal activity of GRNs could potentially reveal information about a sugar's chemical identity (Reiter et al., 2015) (Figure S1). To test this, we employed a consensus clustering analysis (CCA) of output from a principal components analysis of the binned spike trains of GRNs 1 and 2 (as in Stopfer et al (Stopfer et al., 2003), Figure S2 and Table S2). Sugars that did not evoke spikes such as xylose, mannose, and lactose were not included in the analyses. The

CCA predicted how the pattern of spiking, rates of spiking, and involvement of all three neurons encoded information about sugar identity (Figure 1K). At 10 mM, the CCA was only able to identify two groups: one for maltose and sucrose and one that contained all the other sugars (Figure 1K).

At concentrations >10 mM, the CCA identified four distinct groups. Cluster 1 was composed of fructose, sucrose, and maltose. Responses to these sugars exhibited high rates of burst firing in GRN1 and GRN2. Cluster 2 represented melezitose, which evoked high rates of burst firing, but fewer GRN1 spikes per burst (lower GRN1 rate). (Note: sugars in Clusters 1 and 2 were also more likely to evoke GRN1 spikes at 10-100 mM, Figure 1G). Cluster 3 represented glucose. Glucose evoked relatively high firing activity in GRN1, but less activity in GRN2. Furthermore, glucose responses also exhibited a strong rate of adaptation, as indicated by the slope of the relationship between GRN1 and GRN2 in Figure 1D-F (Table S1). Cluster 4 represented trehalose, sorbitol, and sorbose. Although sorbitol and sorbose elicited high GRN2 firing rates at 1000 mM, this did not correspond with high rates in GRN1 (Figure 1J). The clustering of sugar-evoked responses revealed that the pattern of spiking was an important facet of the code for identity.

Sugars can be discriminated by identity

The CCA in Figure 1K indicates that the temporal pattern of firing of the three GRNs contains information that makes it possible to organize sugars into groups. Based on this, we predicted that bumblebees should be able to differentiate between sugars in different groups. We therefore tested whether bumblebees could distinguish between sucrose or fructose and a representative sugar from each of the other clusters using a two-choice assay (Kessler et al., 2015). We adjusted the concentrations of the sugars by caloric value (see methods). Bumblebees discriminated sucrose and fructose from all the sugars we tested (Figure 2A-B). Furthermore, the bees performed better than the CCA: bees also discriminated sugars within the same CCA group (sucrose from maltose or fructose, Figure 2A-B).

Taste instructs feeding behaviour. To identify how input from the mouthparts influenced the relative phagostimulatory power of each sugar, we used two additional behavioural assays. Bees reflexively extend the proboscis to feed when the sensilla on the mouthparts are stimulated (Miriyala et al., 2018). All of the 500/1000 mM sugar stimuli that elicited GRN spikes (Figure 1) also provoked proboscis extension when the mouthpart sensilla were directly stimulated, with the exception of sorbitol (Figure 2C). Furthermore, the phagostimulatory properties of the sugars also affects the duration of feeding once it commences (Ma et al., 2016). We used a 2 min

158 feeding assay to assess how the structure of feeding was affected by the sugars. At 100 mM,
159 the only sugars that bees consumed in significant volumes were fructose, sucrose, maltose
160 and melezitose, however bees only maintained contact with sucrose and maltose solutions
161 significantly longer than water (Figure 2D). Bees consumed >10 µl of all of the 500/1000 mM
162 sugar stimuli except lactose, xylose, sorbitol and mannose (Figure 2E). They also spent more
163 time in contact with the solution and had longer first bouts on the sugars that provoked the highest
164 spike rates in the GRNs (Figure 2E).

165 Whether or not a bee feeds on a solution is likely to be related to the metabolic or nutritional
166 value of a sugar. We fed bees with 500/1000 mM solutions of each of the sugars and measured
167 their lifespan over a 10-day period. At least 60-80% of the bees fed with fructose, sucrose,
168 melezitose, or sorbitol, survived for 10 days, whereas nearly 90% of the bees fed with maltose
169 survived (Figure 2F). Unexpectedly, only 50% of the bees fed with glucose or trehalose survived
170 over the 10 day period. In contrast, most bees fed with sorbose, lactose, xylose or mannose died
171 within 3 days of the start of the experiment (Figure 2F). These bees did not consume much of the
172 solution and died of starvation or of toxicity (Figure 2G).

173 **GRN3 is not activated by ligands from other modalities**

174 Our data indicate that three of the four GRNs in bumblebee galeal sensilla are sugar sensing
175 neurons. This finding contrasts the classical model in which insects possess one sugar-sensing
176 GRN per sensillum (Thorne et al., 2004). To our knowledge, the only other example of an
177 insect with multiple within-sensillum GRNs sensitive to sugars is in hawkmoth larvae (*Manduca*
178 *sexta*). The taste sensilla of these caterpillars contain at least two GRNs that are responsive to
179 sugars. However, these GRNs do not burst, and dual activation of GRNs occurs primarily with
180 sugar mixtures rather than single sugars (Glendinning et al., 2007). GRN1 and GRN2 clearly
181 demonstrate modulation of firing activity consistent with sugar-sensing. However, the low rate
182 of GRN3 firing to sugars might indicate that it responds more strongly to other specific ligands.
183 The tip-recording technique does not always obtain/retrieve traces that permit the identification
184 of GRNs 1-3 responses by the shape of their spikes. For this reason, we could not determine
185 whether GRN3 responded to other ligands presented as monomolecular stimuli. We instead
186 tested the responses of the GRNs to fructose mixed with salts (NaCl, KCl), bitter compounds
187 (caffeine, quinine), or amino acids (glutamic acid, proline, phenylalanine) to identify if GRN3 could
188 be activated by ligands from other taste modalities (Figure 3A). As shown in other studies in bees,
189 quinine completely silenced all GRN responses to fructose (Wright et al., 2010; Kessler et al.,

2015). None of the other compounds influenced GRN3 firing activity (Figure 3B). The pattern of activity of GRN1 and 2 over time was moderately affected by proline (Figure S3), which reduced GRN2 firing rates and affected bursting structure by reducing the number of GRN1 spikes per burst and increasing its rate of adaptation (Figure S3). However, when the patterns of activity of all three GRNs were integrated into a CCA of the binned spikes, none of the stimuli were significantly grouped into a distinct cluster except for the fructose-quinine mixture (Figure 3C-D, Figure S3).

Non-nutritive sugars reduce GRN responses to sucrose

In the previous experiments, we established that sugars with nutritional value activate GRNs 1-3. Some of the sugars we tested did not activate any GRNs. To test whether activity evoked in GRNs 1-3 relates to the nutritional value or toxicity of the sugars, we fed bees with sorbose, xylose, lactose, or mannose in equimolar mixtures of the sugars with sucrose. Xylose had some nutritional value, as bees fed with sucrose mixed with xylose lived longer than those fed with sucrose alone. Sorbose provided no additional nutritional value, and it was not toxic. However, bees fed with sucrose laced with mannose and lactose died at a much faster rate, indicating that these sugars were toxic (Figure 4A). In a 2 min assay of feeding, the addition of mannose, lactose and xylose to sucrose did not significantly alter the quantity of food a bee would consume (Figure 4B). However, bees fed sucrose containing mannose over a 24 h period ate <30% of the total volume consumed by bees fed with the other sugar mixtures (Figure 4C) indicating that post-ingestive feedback from the toxicity of mannose inhibited ingestion. Unlike sorbose, the sugars mannose, xylose, and lactose did not evoke feeding or spikes, but might still be detectable in sugar solutions. To test this, we stimulated the sensilla with mixtures of sucrose and each individual sugar. As a positive control, we also tested a fructose-sucrose mixture. The firing rates of GRN1 and GRN2 to sucrose were attenuated by the addition of mannose, lactose, and xylose. Sorbose reduced the rate of GRN1 firing but not the rate of GRN2 firing. In contrast, the addition of fructose increased the rate of firing of both GRN1 and GRN2 (Figure 4D). Although addition of the sugars to sucrose affected GRN1 and GRN2 average firing rates, the pattern of activation of these GRNs was similar across stimuli (Figure 4E, Figure S4). The CCA did not distinguish responses of sensilla to the various sugar mixtures (Figure 4F, Figure S4). This clustering predicts that, although the mixtures have effects on absolute GRN firing rates, bees cannot distinguish between these mixtures based on their taste alone.

Conclusions

Our data here indicate that the temporal pattern of firing of GRNs in the bumblebee's galeal sensilla has the capacity to partition sugars into at least 5 categories according to their nutritive value: 'very valuable' (fructose, sucrose, maltose), valuable (melezitose), slightly less valuable (glucose), or not very valuable (trehalose, sorbose, sorbitol). In addition, the galeal GRNs do not respond to toxic or low value sugars (xylose, mannose and lactose). The bee's mouthparts have hundreds of taste sensilla in other locations, such as the labial palps and glossa. It is therefore remarkable that information from only a subset of galeal sensilla is sufficient to predict the behaviour of bees. In fact, the bees performed better in the behavioral assays than the CCA grouping according to the temporal pattern of the galeal sugar-sensing GRNs predicted, because they could discriminate sucrose, fructose and maltose. These data indicate that the sensilla in other locations on the mouthparts likely contribute to the resolution of molecular identity of sugars in the brain, consistent with the analysis of taste input from the maxillary nerve of adult hawkmoths by Reiter et al (Reiter et al., 2015).

Fine tuning of the gustatory system towards high-value sugars is likely to provide bees the ability to select the optimally formulated nectar to fuel flight. This is particularly important for a small flying animal to derive the maximum energy from the least weight that it can manage. Interestingly, although fructose and glucose have the same molecular weight and are both found in nectar, bumblebees prefer fructose and fructose elicits higher GRN responses. Unlike fructose, glucose crystallizes at low temperatures, making it harder to drink from cold nectar solutions (Silva et al., 2010). Fructose is also particularly valuable to bumblebees because they use it as the substrate for activation of flight muscles to heat up during 'shivering' (Beenakkers et al., 1984). The only sugar for which we did not see a relationship between GRN activation and survival was sorbose. Sorbose elicited PER and drinking behaviour in the first bout of feeding but it was not consumed in large volumes over 24 h (Figure 2G). For this reason, bees fed sorbose did not live longer than 4 days (Figure 2E). Likewise, bees that consumed mannose mixed with sucrose stopped feeding only after they had consumed the solution. These data indicate that post-ingestive feedback plays an important role in modulating feeding towards sugars over periods as long as 24 h. Integration of taste information with post-ingestive cues, therefore, is likely to improve the accuracy of the brain to identify the nutritional value of foods which are often mixtures of substances.

Methods

Resource availability

Lead contact. Further information and requests regarding the methods used should be directed to the lead contact, Geraldine A. Wright (geraldine.wright@zoo.ox.ac.uk).

Materials availability. This study did not generate new unique reagents.

Data and code availability. All (.csv) data files have been deposited at *[to be confirmed]* and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. The original code for consensus clustering has been deposited at *[to be confirmed]* and is publicly available as of the date of publication. DOIs are listed in the key resources table. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Bumblebee colonies. Bumblebees Experiments were performed at Newcastle University and the University of Oxford with commercial colonies of *Bombus terrestris audax* (Koppert Biological Systems, Haverill, UK and Biobest, Westerlo, Belgium). Bumblebees were kept inside their colonies and maintained at laboratory conditions (22-27℃ and 25-40% RH) until use for experiments. They were fed ad libitum with honeybee-collected pollen (Koppert Biological Systems, Haverill, UK and Agralan, Swindon, UK) and the proprietary sugar syrup provided with the colonies (Biogluc, Biobest) or 1 M sucrose. Workers were collected directly from the colony. To minimize the likelihood of nurse bee inclusion, only bees with a thorax width >4.5 mm were used in experimentation (Goulson et al., 2002). Bees used were of unknown age and were experimentally naïve. Treatments were counterbalanced across colonies and bees from within a colony were randomly allocated to a treatment group for all experiments. Health status of the colonies was unknown but they are presumed to be disease-free due to the strict quality controls conducted by commercial bumblebee suppliers (Huang et al., 2015).

Method Details

Sugar Solutions. Solutions of sugars were prepared in demineralised water for both electrophysiological and behavioural experiments. Sugars were purchased from Sigma-Aldrich (Dorset, England) or Alfa Aeasar (Heysham, UK) at a 98% minimum purity. One trisaccharide [D-(+)-melezitose monohydrate], four disaccharides [D-(+)-sucrose, D-(+)-maltose monohydrate,

D-(+)-trehalose, β-lactose], five monosaccharides [D-(-)-fructose, D-(+)-glucose, D-(+)-mannose, L-(-)-sorbose, D-(+)-xylose] and one sugar alcohol [D-sorbitol] were tested.

24-hour preference assay. To test whether bees could distinguish between sugars we employed a 24-hour feeding assay where bees were given *ad libitum* access to two sugars. Bees were collected directly from colonies and placed into clear acrylic cages (145 × 130 × 60 mm) with 1 mm ventilation holes. Two Additional 10 mm holes were positioned on either side of the cage where feeding tubes could be inserted horizontally. Feeding tubes were modified 2 ml Eppendorf tubes with four 2 mm holes drilled equidistantly on one side so the bumblebees could alight on the tubes and feed from the openings. Each solution was presented in two tubes on one side of the cage; 4 tubes per cage total. Tube positions were counterbalanced to exclude positional bias. Following a 1 h period of acclimation to the caged environment, feeding tubes were inserted into the test cages and the cages were placed in an incubator for the duration of the feeding assays. Test cages were maintained in complete darkness at a constant temperature (28◦C) and relative humidity (60%) throughout the experiment. Experimenters were not blind to the treatments. Molarity of the sugar solutions were adjusted for approximately equal caloric value to reduce the effect of preferences derived from the caloric value of the sugars. We performed two assays, the first with sucrose (500 mM) versus fructose (1000 mM), maltose (500 mM), melezitose (333 mM), trehalose (500 mM), or glucose (1000 mM). In the second assay we tested preferences for fructose (1000 mM) versus sucrose (500 mM), melezitose (333 mM), glucose (1000 mM), or trehalose (500 mM). Preference between sugar pairs were tested with bees housed in groups of 5, 15 cages per sugar pair. An additional 5 cages containing ten tubes per sugar were also included in cages without bees to quantify the amount of evaporation over the experiment. The change in weight of feeding tubes was assessed after 24 h, with the average evaporation weight removed. The density of each solution was measured at room temperature (21◦C). For the 24 h preference assays (Figure 2A-B) we used the volumes of sugar solution consumed to calculate a preference index (PI) for sucrose or fructose for each cage of bees in each of the nine sugar pairings. This was calculated as:

$$\mathrm{PI} = \frac{(\text{volume of A consumed} - \text{volume of B consumed})}{(\text{volume of A consumed} + \text{volume of B consumed})},$$

where solution A was always sucrose (first assay) or fructose (second assay). Values on this index vary between -1 to 1, with 0 being no preference, positive values indicating a preference for sucrose or fructose, and negative values indicating a preference for the alternative sugar (i.e.,

solution B).

Proboscis extension response. To test the phagostimulatory effect of sugars, proboscis extension response (PER) experiments were performed on bumblebees. Individual bees were collected directly from the colony in small glass vials and cold anaesthetized on ice for approximately 3 min, or until movement slowed sufficiently, before being harnessed. Harnesses were modified 1000 µl pipette tips with a portion of the tip removed to allow placement of the bee. A single duct tape strip (2 mm in diameter) was placed across the bees thorax to hold the animal in place. A small piece of wire was positioned directly below the bees mouthparts and was affixed to the harness with melted beeswax. The wire functioned to stop the bee from folding the mouthparts below their mandibles. Following harnessing, bees were starved for 3-8 h at room temperature in a dark environment to habituate them to the harnesses and to motivate them to feed during the assay. A 1.0 ml syringe with a female adapter was used to deliver a small drop (3.5 µl) of solution directly to the bees mouthparts for a period of 4 s and the PER was monitored visually by the same experimenter. The test solution was tested first, followed by a water control and a 1000 mM sucrose control. Five bees were tested for each sugar tastant. A PER was counted if the bee extended its proboscis to at least 90° with the glossa passing the point between the mandibles. Bees that displayed extensive licking behavior of the glossa immediately before or during the assay were excluded as this behavior can confound accurate PER quantification. Animals that did not display the PER to 1000 mM sucrose were excluded from analyses, as were those that displayed the PER to water. Experimenters were not blind to the treatments.

Capillary taste assay. The feeding behaviour of free-moving bumblebees was examined in more detail using a capillary taste assay previously described in Ma et al (Ma et al., 2016). Individual bees were collected directly from colonies using small plastic vials (7 cm long, 2.8 cm inner diameter, with a perforated plastic stopper) and placed in a dark box maintained at laboratory conditions (24 ℃ and 38% RH) for 3-6 h to starve them sufficiently to ensure their motivation to feed during the assay. Following starvation, bees were transferred into a modified 15 ml centrifuge tube (119 mm length, 17 mm diameter), with a 3 mm hole drilled at the tip and a steel mesh (10×30 mm, 1 mm mesh diameter) inserted at the bottom of the tube to allow the bumblebees to grip to the wall of the tube. Bumblebees were free to move within the holding tubes throughout the experiment. The tube was then placed into a polystyrene holder and held in place using dental wax. White cardboard affixed to either side of the holder shielded the bees from visual stimuli.

Bees were allowed a 3 min habituation period to the test environment before being presented with a droplet of 500 mM sucrose (3.5 µl) using a 1.0 ml syringe connected to a female adapter. The droplet served to bait the bees to extend their proboscis. If the droplet was not consumed within 5 min, the bees were excluded from the experiment. Immediately after consuming the sucrose droplet the bees were offered a 100 µl microcapillary tube 60 mm in length (Blaubrand intraEnd, Ref 7091 44) filled with the test solution.

Solutions were blinded for this assay. The microcapillary tube was held in place by feeding the tube through a modified 1.0 ml syringe with the tip removed. The syringe was in turn affixed to a micromanipulator using dental wax. The proximity of the solutions to the bees' mouthparts could be controlled by adjusting the micromanipulator and by squeezing a silicone tube (60 mm long, 1 mm inside diameter) which allowed the microcapillary tube to function as a pipette bulb. The microcapillary was adjusted such that the solution was always readily accessible to the bee. The feeding period lasted for 2 min after the bumblebee tasted the solution within the microcapillary with its mouthparts. Bumblebees failing to taste the test solution with their mouthparts within 10 min of being presented the test solution were discarded from the experiment. The movement of the mouthparts was recorded using a digital microscope (Dino-Lite AM4815ZT) which was positioned 20 cm above the end of the microcapillary tube. Video recordings were made using DinoLite Digital Microscope software at resolution of 640×480 pixels, 26 Hz frame rate, and 25x magnification. Videos were manually scored at 0.5x speed for contact times of the mouthparts with the feeding solution using the Noldus Observer software (The Observer® XT 5.0.25, Noldus, Wagenigen, The Netherlands). A feeding bout was defined as a contact between the extended proboscis and the solution not interrupted by an absence of contacts of 5 s or more. For each sugar and concentration n=9 to 12 bees were tested.

Each microcapillary tube was scanned at 600 d.p.i. before and after the experiment, and fluid levels were measured using ImageJ (version 1.48) (Schneider et al., 2012). The reference scale was set to 60 mm. Image files were zoomed to approximately 400% and the length of the solution was measured meniscus to meniscus. The length of test solution consumed by each bee was calculated as the difference between the length of the liquid before and after the test phase. These lengths were converted to volumes using the formula:

$$\frac{\text{capillary volume}}{\text{capillary length}}\,(\text{liquid before} - \text{liquid after}). \qquad \textbf{(1)}$$

Evaporation was monitored by measuring liquid levels in 34 capillaries filled with water fixed on the experimental set up for a 12 min period (the maximum duration of an experimental trial) in

375 the absence of a bee. Evaporation value appeared to be marginal (0.9±0.3 µl, mean ± s.e.m.).
376 Water consumption was consequently marginal, but slightly higher than evaporation (1.7±0.4 µl).
377

378 **Longevity assay.** The ability of sugars to support bumblebees' survival was tested. Bees were
379 collected and caged in an identical fashion to that of the 24-hour preference assay, with the
380 exception that bees were constrained to a single diet for the duration of the experiment. The
381 experiments ran for a total of 10 days. Mortality was assessed every 24±2 h from the beginning of
382 the experiment and dead bees were removed from the cage using forceps. A piece of paper was
383 placed in the bottom of each cage and replaced when needed to maintain hygienic conditions.
384 Feeding tubes were weighed and replaced every 24 h to monitor the total volume of solutions
385 consumed over the course of the experiment, as detailed earlier. Longevity of bumblebees with
386 access to 500 mM melezitose or lactose, or 1000 mM sucrose, trehalose, maltose, fructose,
387 glucose, xylose, mannose, sorbose, sorbitol, or water were measured, and n=33 to 42 bees
388 were tested for each sugar. In the toxicity experiment, longevity of bumblebees having access
389 to equimolar mixtures (500 mM) of sucrose and lactose, sucrose and mannose, sucrose and
390 sorbose, sucrose and xylose and 500 mM sucrose alone was assessed during 10 consecutive
391 days (n=20 bees per sugar mixture). Experimenters were not blind to the treatments.

392 **Electrophysiology.** To investigate how sugar perception is encoded by GRNs on the mouthparts,
393 tip recordings Hodgson et al. (1955) were performed on individual galeal sensilla chaetica A-type
394 contact chemoreceptors (Supplemental Figure 1A). Electrophysiological preparations were made
395 as already described (Miriyala et al., 2018; Kessler et al., 2015). For tip recordings, sensilla were
396 stimulated using a motorized micro-manipulator (MPC-200, Sutter Instruments, USA) for 2 s
397 with a borosilicate (Clark capillary glass 30-066, GC150TF-10) recording electrode (15 µm tip
398 diameter, made with a Narishige PC-10 electrode puller) containing the test solution. Signals
399 were acquired using a non-blocking pre-amplifier (TasteProbe; Syntech, Germany), amplified
400 (AC amplifier 1800, A-M Systems, USA), digitized at 10 or 30 kHz (DT9803 Data Translation)
401 and stored using dbWave (version 4.2014.3.22) or DataView (version 11.5). A minimum
402 latency period of 3 minutes was allowed between simultaneous recordings to avoid adaptation.
403 Recordings were made from among the first 6 most distal sensilla chaetica on the galea of
404 bumblebees. For each sugar, n = 6 to 8 bumblebees were tested.

405 **Spike detection.** Both spikes and bursts were detected using custom routines written in Matlab
406 (The MathWorks) following a modified analysis described in Miriyala et al (Miriyala et al., 2018).

The first 2 s following the contact artefact (automatically detected with findpeaks [fixed thresholds: minpeakheight = 1.5, npeaks = 1]) were first band-pass filtered at 300-2500 Hz (second order butterworth filter) with the butter function. Assuming a normal distribution of noise frequencies, the standard deviation of the background noise was estimated using:

$$\hat{\sigma} = 1.4826\,\text{MAD}(R(t)).$$

Each recording was then normalised using:

$$\tilde{R}(t) = \frac{R(t)}{\sigma},$$

where $R(t)$ is the filtered recording and MAD the median absolute deviation. Spikes were then detected using the peakfinder function with adequate thresholds. A logarithmic equation of the form:

$$\text{ISI} = a\log(bt + c),$$

where ISI are the interspike-intervals and t are the spike time indices, was used to detect the end of burst positions (EOB). A spike was considered as EOB if its ISI exceeded two times its fitted value. The number of spike doublets (2 spikes events separated by an ISI <2.5 ms) were quantified within each burst (i.e., after removing the last spike event of each burst). The total number of spikes, bursts and doublets within each burst were quantified during a 0.1 to 1.1 s recording window following the peak of the contact artefact. GRN1 spikes were identified as any low-frequency responses to sugars, and as the high frequency component of the bursts, while GRN2 spikes were identified exclusively at the EOB position, as described in Miriyala et al (Miriyala et al., 2018). The presence of within burst doublets and end of burst triplets were identified in the responses to some sugars, representing the spikes from GRN3.

Spike train analyses. We compared the average firing rates of GRNs housed in sensilla 1-6 in response to water and found that sensilla 4-6 responded with higher firing rates than sensilla 1-3 (Supplemental Figure 1B). Sugars that did not elicit strong GRN1 responses (lactose, xylose, mannose) shut down the water cell at concentrations above 10 mM (Supplemental Figure 1C). Spike sorting methods could not differentiate the GRNs responding to water versus GRN1 spikes, so the water response resulted either from activity in GRN1, or another GRN with a similar amplitude, and this presented a confound in measuring sugar responses. Sensilla 4-6 were

removed from analyses to define the "activation threshold" (lowest concentration that elicited a significant GRN response compared to water). Responses of GRNs 1-3 from sensilla 1-3 were compared using the number of spikes from 0.1 to 1.1 s from stimulus onset, i.e., the average firing rate. To assess the importance of time-dependent GRN responses, we additionally compared the spike trains of GRN1 and GRN2, by binning spikes for each 100 ms of recording from 0.1 to 1.1 s after stimulus onset. Time series of GRN spikes in 100 ms bins were aligned so that a single sensillum response was contained in a single vector (i.e., 20 features per vector containing binned normalized spike counts for GRN1 and GRN2).

Quantification and Statistical Analysis

Consensus clustering. For subsequent analyses, we focused on the responses to sugars that elicited significant GRN responses only (trehalose, sorbitol, sorbose, glucose, melezitose, sucrose, maltose and fructose at concentrations $\geq$10 mM). Dimensionality of the GRN time series responses (normalized to peak GRN firing rates) was reduced with a principal component analysis. We used a consensus clustering analysis (CCA) to assess whether GRN responses to sugars were differentiable based on sugar identity or intensity. CCA is an unsupervised class discovery algorithm for determining cluster count and membership by aggregating the results from many clustering iterations to detect possible groupings of items based on intrinsic features. We used the `ConsensusClusterPlus` package in R (Wilkerson and Hayes, 2010), to implement consensus clustering. We used the first eight principal components from the GRN time series data for clustering analyses. We clustered the GRN responses to the sugars that elicited significant GRN responses. We selected a k-means clustering algorithm and ran the consensus clustering algorithm with 2000 iterations, k=2 to 8 clusters, and 90% of the samples and features per iteration (i.e., 10% of the responses and 10% of the bins were omitted from each clustering run). Optimal cluster count (k) was determined using the consensus matrix, cumulative distribution function, as well as cluster and item consensus. The stimuli were grouped by assessing the individual cluster assignments for the responses of individual sensilla: variability in responses between sensilla resulted in some scattering of the responses across different clusters. The stimulus was assigned to the cluster that represented the greatest proportion of the responses across sensilla. We performed a sensitivity analysis by adjusting the hyperparameters of the clustering analysis (i.e., by varying the proportion of features and samples included in each iteration between 0.8 and 1) and additionally by running the clustering analysis on the normalized binned spikes (rather than the principal components), with similar results. Consensus clustering

was also used to compare the time series responses of GRNs stimulated with fructose and fructose mixtures, as well as sucrose and sucrose mixtures. These analyses were performed as described above.

Other statistical analyses. Statistical analyses were performed in R (version 4.0.3). All data were tested for normality and equal variance using the Shapiro-Wilks and Levene's tests. Parametric repeated measures data, including average firing rates of GRNs 1-3, maximum number of spikes per burst, and GRN adaptation rates, were modeled using linear mixed effects models (`lmerTest` package, (Kuznetsova et al., 2017)), with animal ID as a random effect. Estimated marginal means (`emmeans` package, (Lenth et al., 2018)) was used for *post hoc* multiple comparisons of linear mixed models. The aligned rank transform (`ARTool` package, (Wobbrock et al., 2011)) was used to transform non-parametric data, including the water response of sensilla 4-8, and fructose mixtures electrophysiological responses, for modeling in a linear mixed model with animal ID as a random effect. Dose response curves for (Figure 1D-E) were modeled with and without sugar as a variable, and these models were compared to quantify differences in the average GRN responses for each sugar (`drc` package, (Ritz et al., 2015)). The Kruskal-Wallis test (with Wilcoxon Rank Sum *post hoc* multiple comparisons) was used to compare responses of sensilla 1-6 to water, and compare 24 h feeding preferences and consumption of sugars over 2 min. The Kaplan Meier Log Ranks test was used to assess differences in survival of bees fed different sugars or sucrose mixtures. Wilcoxon signed-rank tests were used to test if the median preference index for each sugar pair was significantly different from zero. Where appropriate, p-values were adjusted for multiple testing using Bonferroni corrections within each assay. Significance was assessed at $\alpha<0.05$. The results of *post hoc* analyses are denoted either by letters to show significant differences between groups, or with asterisks: '*' <0.05; '**' <0.001; '***' <0.0001.

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Author contributions

RH Parkinson, SC Kessler, A Miriyala, and GA Wright conceptualized and designed the experiments. RH Parkinson, SC Kessler, J Scott, J Bu, and A Simpson collected data. JG Pattrick analyzed the data for the preference assay. A Mahdi assisted in implementing the consensus clustering method. RH Parkinson performed all other analyses and prepared all visualizations. RH Parkinson and GA Wright wrote the manuscript. All authors contributed to revising the manuscript.

Declaration of Interests

The authors declare no competing interests.

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585 ## Figure Legends

586 **Figure 1: Stimulation with sugars elicits responses from three gustatory receptor neurons**
587 **per galeal sensillum.**

588 **(A)** Sugar structural relationships and representative extracellular tip-recordings of galeal GRNs
589 stimulated with sugars at 100 mM. **(B)** Rasters of GRN spikes over 1 s stimulations with sugars of
590 increasing concentrations from 0 to 1000 mM (Blue lettering is used to indicate sugar stimuli
591 that were 500 mM at their maximum concentration in this experiment due to solubility). **(C)**
592 Spike sorting exploited inter-spike intervals (ISI) to differentiate GRNs: bursts of spikes with
593 5-10 ms ISIs (GRN1 spikes) were terminated by a single GRN2 spike marked by an end of
594 burst doublet (EBD) with a very short ISI (<2.5 ms) (Miriyala et al., 2018). A third neuron
595 (GRN3) fired occasionally within bursts as a within burst doublet (WBD) or end of burst triplet
596 (EBT) with ISIs <2.5 ms. Bursts were followed by longer (>15 ms) inter-burst intervals. **(D-F)**
597 The average firing rates of GRNs 1-3 over 1 s stimulations varied by sugar type and increased
598 as a function of concentration (n=18 to 24 sensilla from n=6 to 8 bees per sugar). Points
599 represent mean ($\pm$ s.e.m) responses across animals. Concentration response curves for
600 GRNs 1 and 2 varied significantly by sugar treatment (GRN1: $F_{30,807}$=45.55, p<0.0001; GRN2:
601 $F_{30,837}$=23.86, p<0.0001). **(G)** Activation thresholds for GRNs 1-3 for all sugars. Box colours
602 denote significant activation of a single sugar at a given concentration versus water (linear mixed
603 effects, concentration: GRN1: $F_{4,257}$=213.21, p<0.0001; GRN2: $F_{4,257}$=77.98, p<0.0001; GRN3:
604 $F_{4,257}$=20.37, p<0.0001, sugar: GRN1: $F_{10,66}$=23.33, p<0.0001; GRN2: $F_{10,66}$=11.57, p<0.0001;
605 GRN3: $F_{10,66}$=7.58 p<0.0001; conc:sug: GRN1: $F_{40,257}$=13.22, p<0.0001; GRN2: $F_{40,257}$=7.43,
606 p<0.0001; GRN3: $F_{40,257}$=5.48, p<0.0001) with estimated marginal means (EMM) *post hoc*
607 multiple comparisons across concentrations for each sugar (coloured box denotes p<0.05 for
608 a sugar versus water at a given concentration). EMM *post hoc* comparisons between sugars in
609 Figure S1. **(H-J)** Firing rates (spikes per 100 ms bin) of GRN1 versus GRN2 over 1 s stimulations
610 with sugars that significantly activated GRN2 at 10 **(H)**, 100 **(I)**, and 1000 mM (**J**, 500 mM for
611 maltose and melezitose). Points represent mean spikes/s in each bin across all trials for a
612 given sugar, connected with lines over time (over 10 bins). The symbol for the first bin for each
613 sugar (i.e., time = 100 ms) is an open marker with a black outline. GRN2 significantly predicts
614 the trajectory of GRN1 (GLM, GRN2: $F_{1,194}$=1723.0, p<0.0001; sugar: $F_{6,194}$=67.8, p<0.0001;
615 concentration $F_{2,194}$= 138.6, p<0.0001). **(K)** Cluster assignment for each sugar and concentration
616 using the PCs of GRN1 and GRN2 responses in 100 ms bins over 1 s of stimulation. Stimuli were

assigned to the cluster to which the largest proportion of individual sensilla responses matched (mean cluster consensus across stimuli: 0.79, CI 0.07). Boxes surround stimuli assigned to a single cluster. Sugars are abbreviated throughout the manuscript as follows: sucr = sucrose, fruc = fructose, malt = maltose, mele = melezitose, gluc = glucose, sbtl = sorbitol, srbs = sorbose, treh = trehalose, lact = lactose, xylo = xylose, mann = mannose.

Figure 2: Bumblebees discriminate sugars of equivalent nutritional value.

(A-B) 24-hour preference assay between sucrose (**A**, 500 mM) or fructose (**B**, 1000 mM) and other sugars (500 mM maltose, 333 mM melezitose, 1000 mM glucose, and 500 mM trehalose). Bars show mean $\pm$ s.e.m. preference index (positive is preference for sucrose **A**, or fructose **B**). Bees displayed a significant preference in all cases (Wilcoxon rank sum for each preference pair p<0.001 with Bonferroni corrections). **(C)** The probability of eliciting the proboscis extension reflex depended on sugar type (Kruskal-Wallis, χ^2_{10}= 36, p<0.0001, n=5 per sugar). **(D-E)** Feeding behaviour over 2 min varies by sugar type. Bees consumed more of the sugar solutions at the highest concentrations (top, 100 mM, **D**, Kruskal-Wallis, χ^2_{11}=48.5, p<0.0001, n=10 to 13; 1000 mM, **E** χ^2_{11}=69.4, p< 0.0001, n=8 to 13 per sugar), and spent more time in contact with the most phagostimulatory sugars over the first bout (middle, 100 mM, χ^2_{11}=25.8, p<0.05, n=10 to 13; 1000 mM, χ^2_{11}=42.7, p<0.0001, n=8 to 13 per sugar) and entire 2 min period (bottom, 100 mM, χ^2_{11}=38.4, p<0.0001, n=10 to 13; 1000 mM, χ^2_{11}=69.5, p<0.0001, n=8 to 13 per sugar). Blue letters denote 500 mM concentrations. Asterisks represent significant differences in sugar consumption versus water (Wilcoxon rank-sum multiple comparisons). **(F)** Survival of bumblebees fed with 1000 mM (or 500 mM for lactose, maltose, and melezitose) solutions depended on sugar type (Kaplan-Meier Log-Rank statistic =1576, df=11, p<0.0001). Holm-Sidak *post hoc* analysis showed significant differences in survival between the following groups of sugars (from highest to lowest survival): 1) maltose; 2) fructose, sucrose, melezitose; 3) glucose, sorbitol, trehalose; and 4) sorbose, lactose, xylose, mannose and water. (n=33 to 42 bees per treatment). **(G)** Total consumption of sugar solutions per 24 h period over the 10-day longevity assay in F.

Figure 3: GRN3 responses to fructose are not affected by the addition of secondary compounds.

(A) Representative tip-recordings from galeal A-type sensilla stimulated with 100 mM fructose, or 100 mM fructose plus 1 mM bitter compound (quinine, caffeine), salt (KCl, NaCl), or amino acid

(glutamate, proline, phenylalanine). Individual sensilla were tested with all compounds (in total 24 sensilla, n=8 bees). **(B)** Average GRN3 spike rates (over 1 s stimulation) to fructose are not affected by the addition of secondary compounds (linear mixed effects, $F_{6,267}$=0.452, p>0.05). Responses to quinine not included; quinine shuts down all GRN responses. **(C)** Firing rates (spikes per 100 ms bin) of GRN1 versus GRN2 over 1 s of stimulation with fructose and fructose mixtures. Points represent mean spikes/s in each bin across all trials for a given sugar, connected with lines over time (over 10 bins). GRN2 significantly predicts the trajectory of GRN1 over time (GLM, GRN2: $F_{1,65}$=786.1, p<0.0001; sugar $F_{6,65}$=36.0, p<0.0001). **(D)** Responses to fructose and fructose mixtures cluster in two groups (with or without quinine) using a consensus clustering analysis of the binned GRN responses (Figure S3).

Figure 4: GRN responses to sucrose are attenuated by the addition of non-nutritive secondary compounds.

(A) Bees fed with sucrose mixed with a non-nutritive sugar (500 mM equimolar solutions) over 10 days did not survive if the mixture included mannose and lactose, but did not have a different rate of mortality if mixed with sorbose. Those fed with sucrose mixed wtih xylose were more likely to survive than the control. Letters denote significant differences in survival (Kaplan-Meier log-rank statistic =370.9, df=4, p<0.001, n=20 to 23 bees per sugar). **(B)** Feeding behaviour over 2 min did not vary in the total volume consumed with the addition of a non-nutritive sugar (ANOVA: $F_{5,88}$=1.66, p=0.15, n=15 to 17 per sugar solution). **(C)** Over 24 h, the quantity of food consumed per bumblebee was significantly less if the bees had been fed with the sucrose-mannose mixture (ANOVA with Tukey multiple comparisons, $F_{5,18}$=10.53, p<0.001, n=4 cages, 20 bees per treatment). Asterisks represent significance versus sucrose alone. **(D)** Average GRN1 (left) and GRN2 (right) firing rates following stimulation with sucrose (500 mM) and equimolar (500 mM) sugar mixtures, including a sucrose-fructose mixture (Aligned Rank Transform, GRN1: $F_{5,263}$=26.8, p<0.0001; GRN2: $F_{5,258}$=19.7, p<0.0001). Violin plots show the distribution of responses of individual sensilla, with boxplots overlaid. Asterisks denote significant differences of GRN responses to the mixture versus sucrose (red boxes). **(E)** Firing rates (spikes per 100 ms bin) of GRN1 versus GRN2 over 1s of stimulation with sucrose and sucrose mixtures. Points represent mean spikes/s in each bin across all trials for a given sugar, connected with lines over time (over 10 bins). GRN2 significantly predicts the trajectory of GRN1 over time, slopes do not vary significantly between sugars (GLM, GRN2: $F_{1,48}$=195.7, p<0.0001; sugar $F_{5,48}$=36.8, p<0.0001, GRN2*sugar $F_{5,48}$=214.9, p>0.05). **(F)** The consensus clustering analysis

of the binned GRN responses to sucrose and sucrose mixtures did not partition the stimuli into separate groups.

Figures

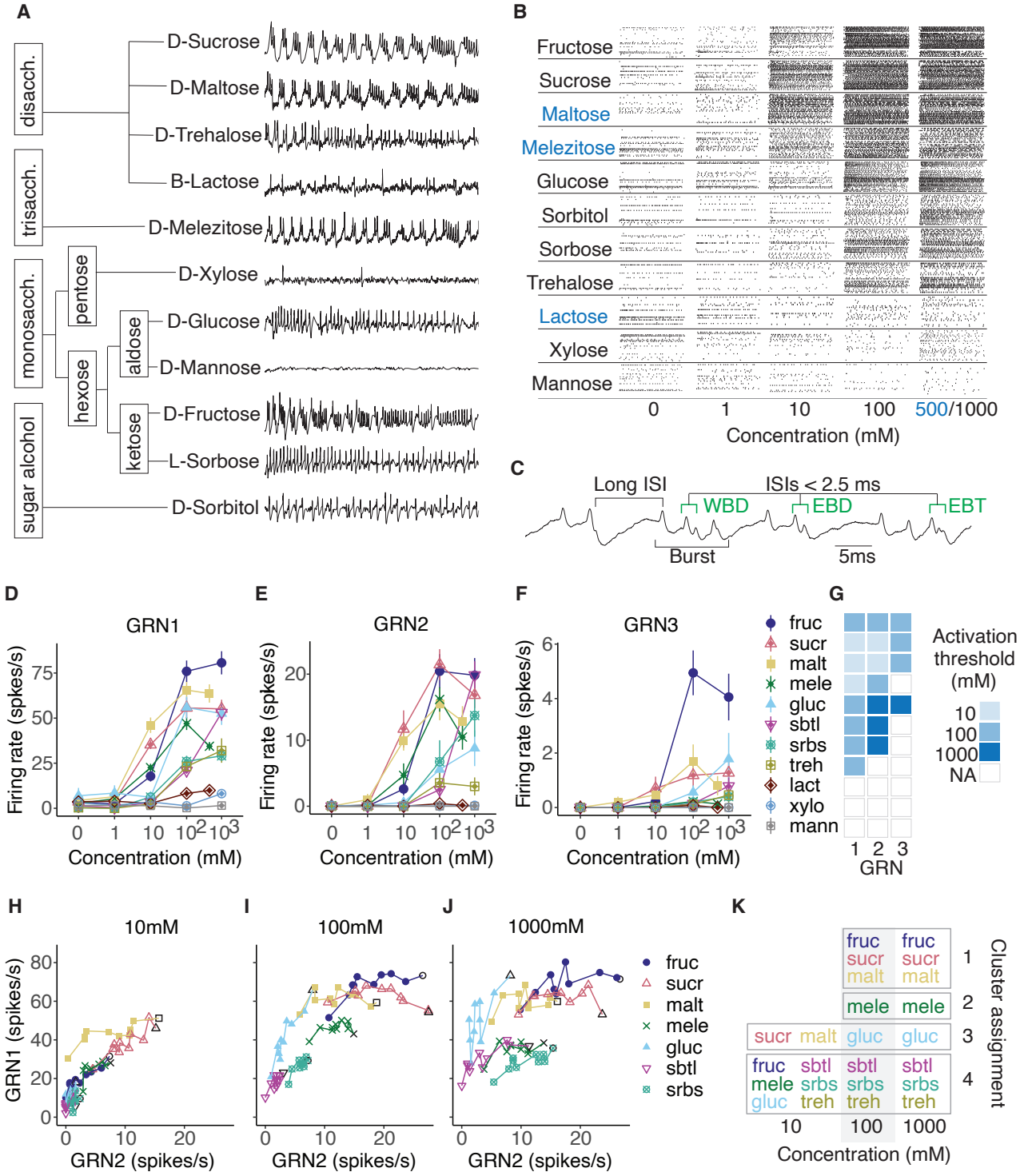


Figure 1. Stimulation with sugars elicits responses from three gustatory receptor neurons per galeal sensillum.

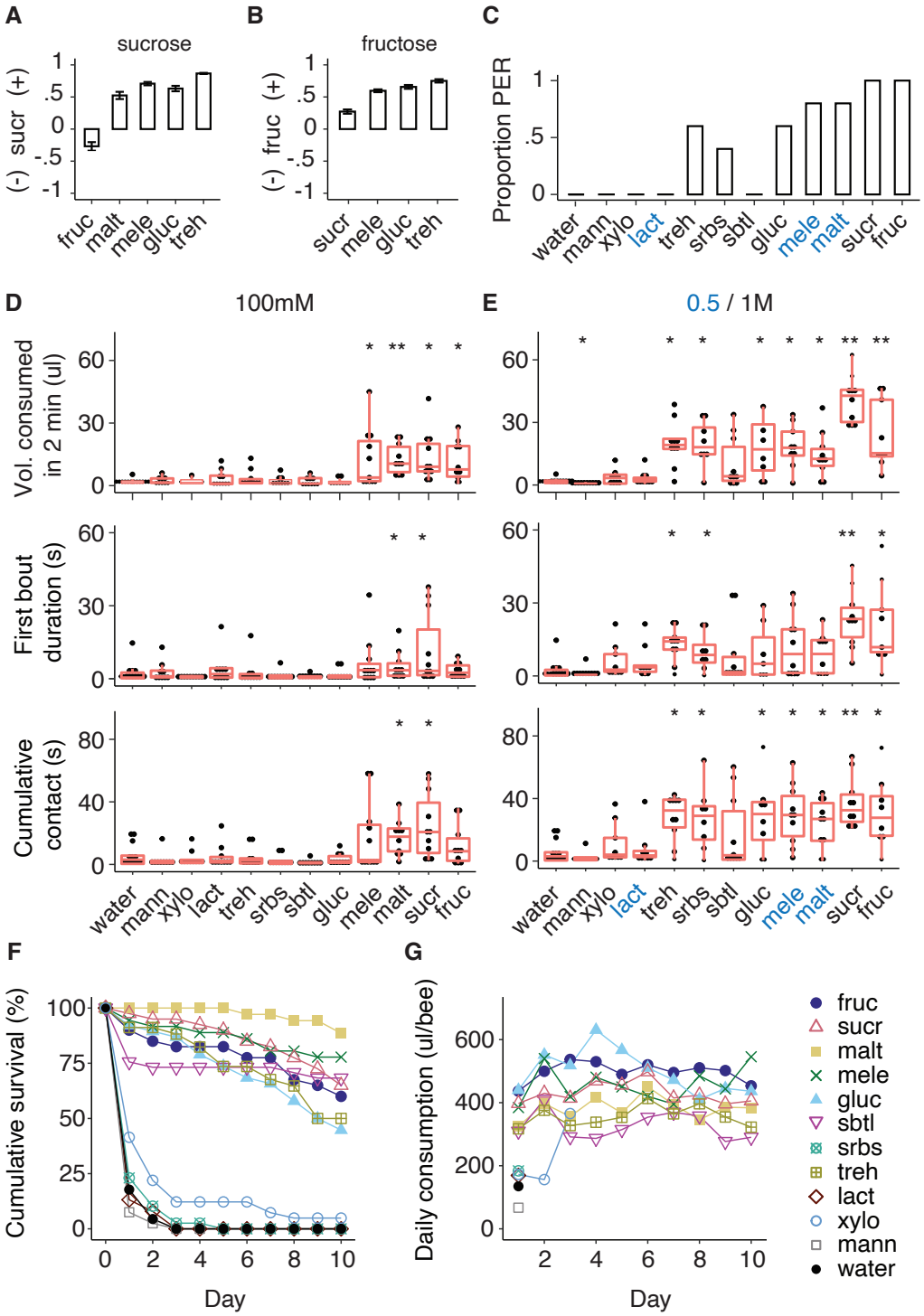


Figure 2. Bumblebees discriminate sugars of equivalent nutritional value.

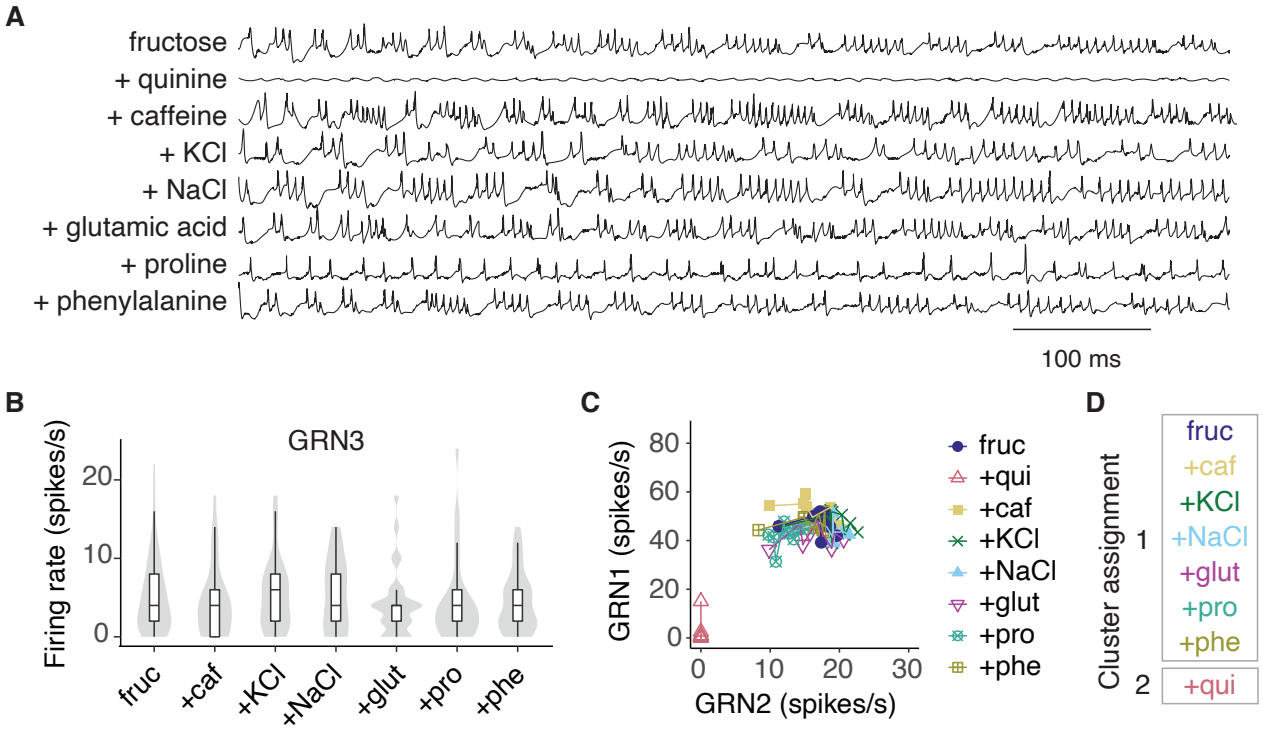


Figure 3. GRN3 responses to fructose are not affected by the addition of secondary compounds.

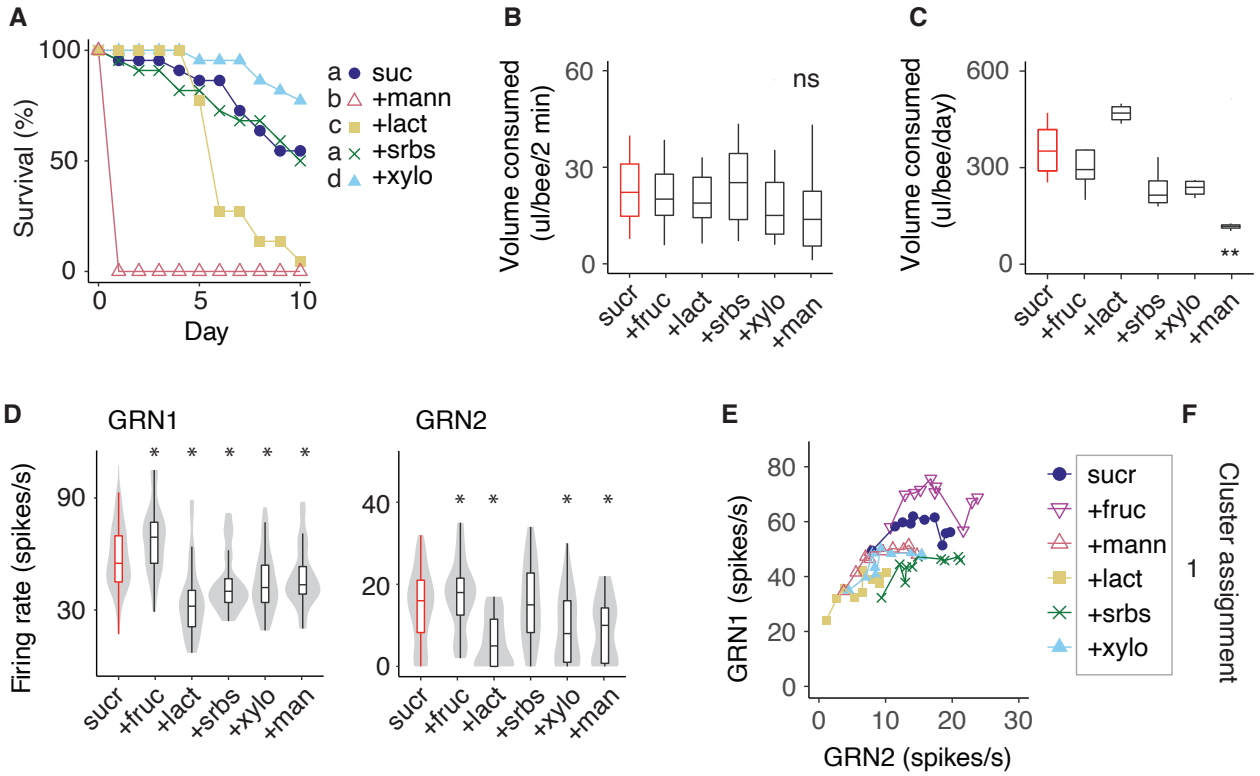
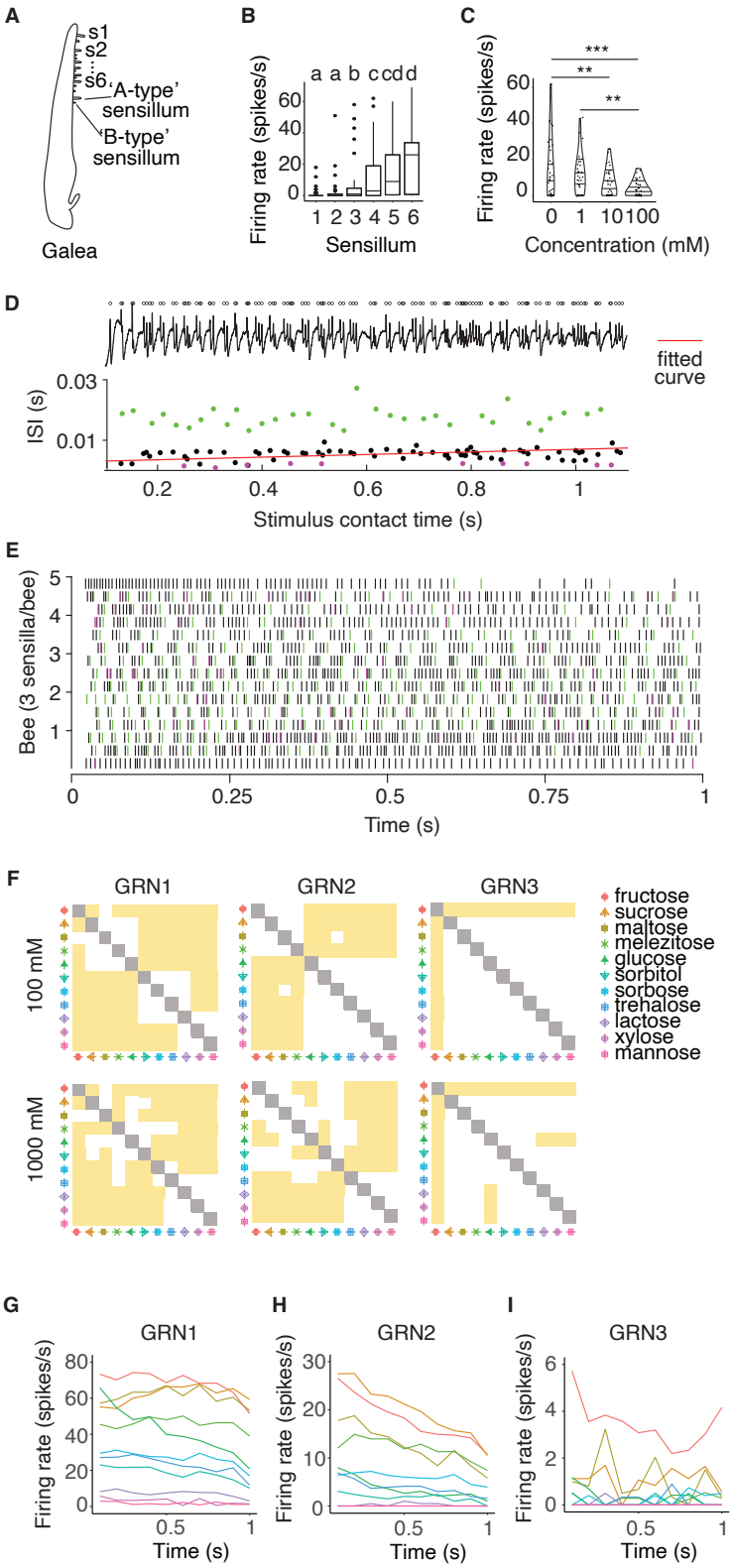
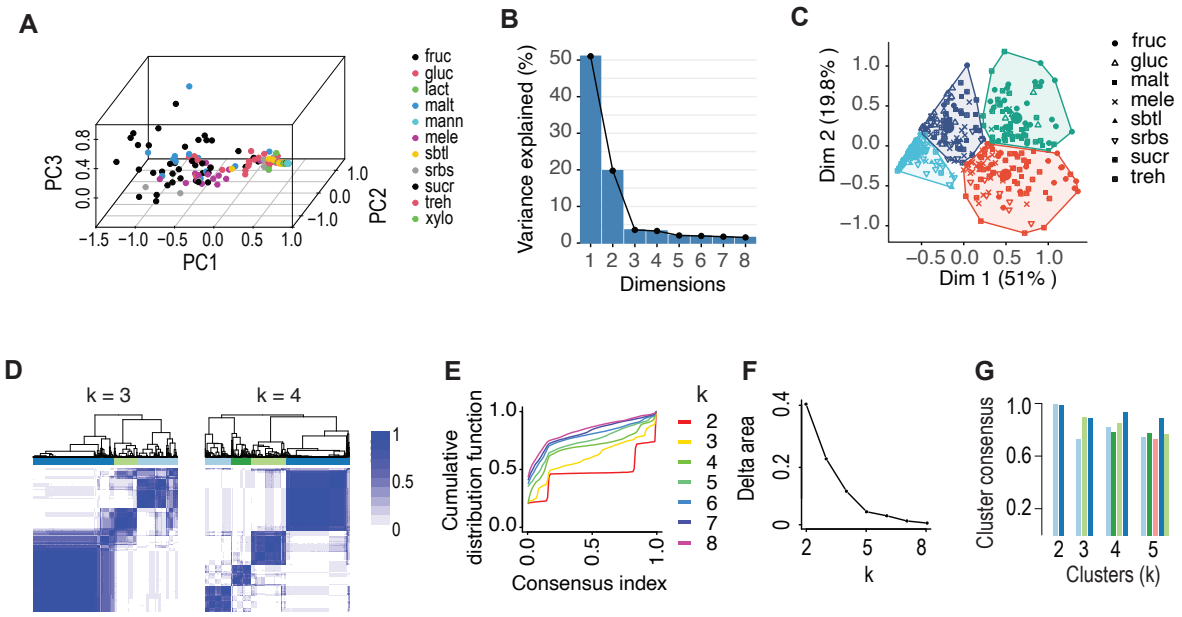


Figure 4. GRN responses to sucrose are attenuated by the addition of non-nutritive secondary compounds.

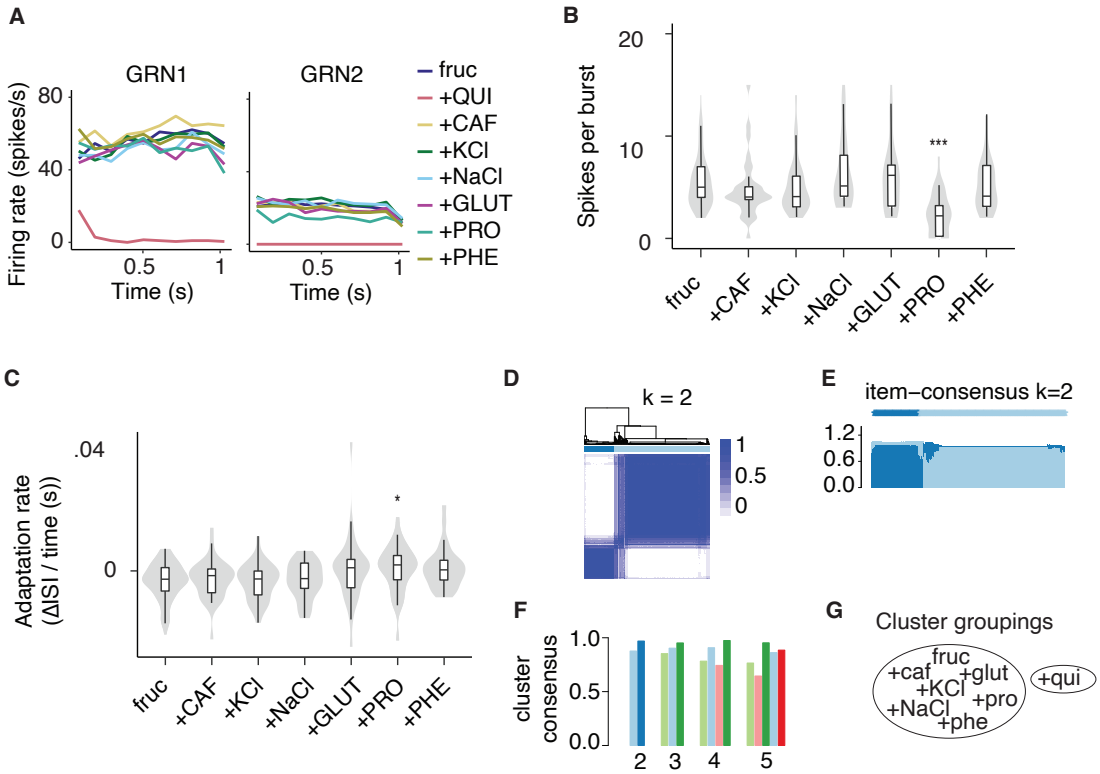
Supplemental Figures



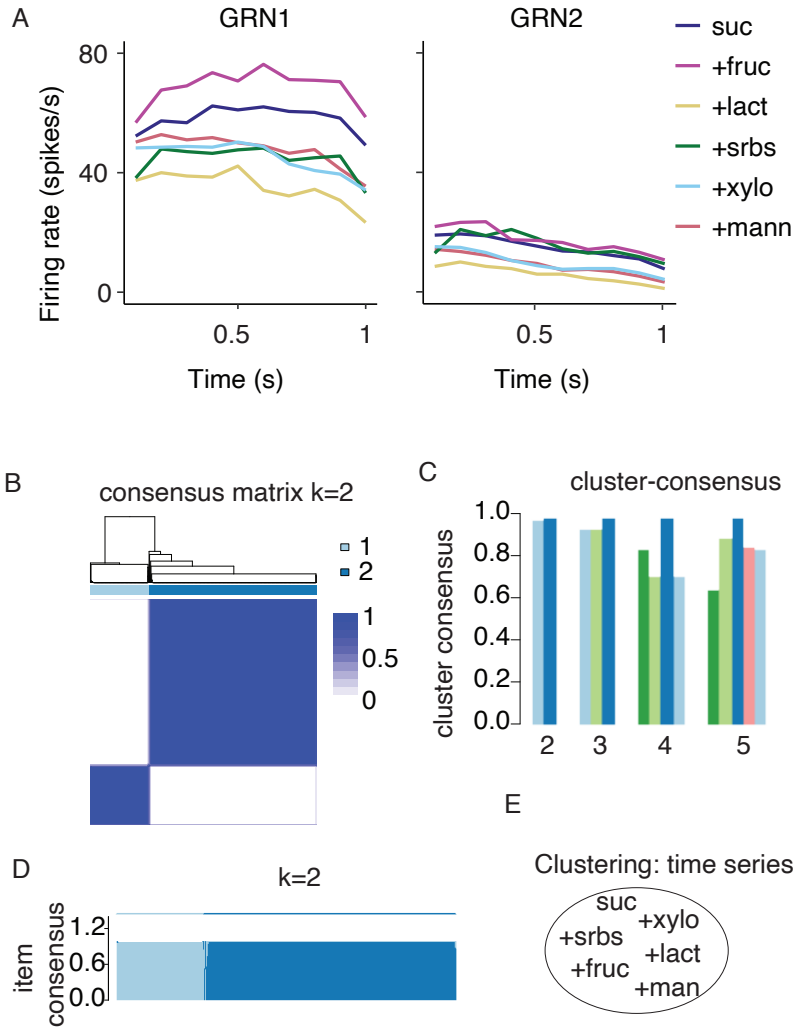
Supplemental Figure 1. **A**, Diagram of a bumblebee galea displaying 'A type' and 'B type' sensilla. A-type sensilla are labeled from s1 to s6 from the most distal sensillum. **B**, Average firing rates of the 6 most distal galeal A-type sensilla to water varied by sensillum position (Kruskal-Wallis, χ^2_5 =65.94, p<0.0001). Letters denote pairwise comparisons using Wilcoxon rank sum with continuity correction. **C**, Average firing rate of GRN1 in sensilla 4-6 decreased with increasing concentration for sugars that displayed low valence for GRNs (lactose, xylose and mannose, aligned rank transform ANOVA, $F_{3,217}$ =11.4, p<0.0001). Violin plots show median, 1st and 3rd quartile lines, and individual data points. Asterixis denote post hoc multiple comparisons. **D**, The ISIs versus time were fit to a logarithmic model (red line) to differentiate GRN1 (black points) and GRN2 spikes (green points). GRN3 spikes (magenta) were identified by their <2.5 ms ISIs (and not preceding a 10-20 ms ISI, i.e. a GRN2 spike). **E**: Raster plots of GRN1 (black), GRN2 (green) and GRN3 (magenta) spikes over 1 s stimulation with 100 mM fructose. Data represents single sensillum responses (sensilla 1-3) from 5 bees. **F**, Results of post hoc multiple comparisons (estimated marginal means) between sugars at 100 mM (top row) and 1000 mM (bottom row) for GRNs 1-3. Yellow squares represent significant differences between sugars with a 0.95 confidence level, and the Sidak correction for multiple comparisons. **G-I**, Firing rate histograms over 1 second of stimulation with 100 mM concentrations of sugars for GRN1 (B), GRN2 (C), and GRN3 (D). Mean firing rates were calculated for spikes in 100 ms bins. Note the changing y-axis limits between panels.



Supplemental Figure 2. **A**, Visualization of the first three principal components (PCs) from a principal component analysis of the binned spike times (100 ms bins) of GRNs 1-3 to sugars at 100 mM. **B**, Scree plot showing the percent of explained variances by PCs 1-8 using binned spike time data for responses of GRNs 1-3 to sugars at $10 - 1000$ mM concentrations. **C**, Kmeans clustering of the first 3 principal components (PCs) for all sugars and concentrations from $10 - 1000$ mM. **D**, Consensus matrices constructed from iterative consensus clustering algorithms using k-means clustering of the 8 PCs derived from the time series GRN responses. The matrices quantify how consistently certain data points were clustered together over clustering iterations, and the consensus matrix itself is then used to cluster unique GRN responses together and incorporate the information across clustering runs. Matrices show that k=4 clusters results in increased cluster consensus across runs. Color bar indicates matrix consensus from 0 (no consensus) to 1 (consensus on every clustering iteration). **E-G**, Consensus clustering validation: E: Cumulative distribution function (CDF) for the consensus matrix values with k varied from 2 to 8. The CDF displays only modest increases with increasing cluster count after 4 clusters. F: Delta area plot shows the relative change in area under the curve from the CDF plot at k versus k-1, showing there is no appreciable increase in consensus with k¿4. G: Cluster consensus with clusters varying from k=2 to 5 showing the mean pairwise consensus values between a cluster's members, with high values indicating a cluster with high stability. Using these metrics we determined that the greatest cluster consensus is obtained with k=4 clusters.



Supplemental Figure 3. GRN responses to fructose mixtures. **A**, Firing rate histograms (spikes in 100ms bins) over 1 s of stimulation for GRN1 and GRN2. Lines represent mean measurements across all sensilla for a given stimulus. **B-C**: Proline affected burst structure and GRN1 adaptation: the maximum number of GRN1 spikes per burst was reduced (B, aligned rank transform ANOVA, $F_{6,261}$ =9.61, $p<0.0001$) and the adaptation rate for GRN1 was increased (C, $F_{7,262}$ =3.61, $p<0.05$). Violin plots show density of response with overlaid boxplots displaying 1st, 2nd and 3rd quartiles with 95% confidence interval whiskers. Asterisk denotes significant effects versus fructose alone. **D-G**, Consensus clustering of GRN responses to fructose mixtures. **D**, Consensus matrix, **E**, item consensus, and **F**, cluster consensus show high levels of consensus with k=2 clusters. **G**, Clustering analysis resulted in the separation of responses in two clusters, with quinine clustering separately from all other responses.



Supplemental Figure 4. Consensus clustering of GRN responses to sucrose mixtures. **A**, Firing rate histograms (spikes in 100ms bins) over 1 s of stimulation for GRN1 and GRN2 in response to sucrose and sucrose mixtures. Lines represent mean measurements across all sensilla for a given stimulus. **B-E**, Consensus clustering of time-series GRN responses with 500 mM sucrose or 500 mM equimolar sucrose mixtures results in k=2 clusters. Consensus matrix (**B**), cluster consensus (**C**) and item consensus (**D**) show high levels of consensus with k=2 clusters. **E**, Responses that clustered together in the two groups were equally distributed amongst stimuli (i.e. clustering represented variation in sensilla responses to a single stimulus) and the responses in the second cluster represented a small proportion of the responses to any stimulus (average of 0.24 across stimuli). Thus, the greatest proportion (0.76) of sensilla responses clustered together in a single group.

Supplemental Tables.

Table 1. Adaptation of GRN1 vs GRN2 over 1 s. Values are the slopes of fitted models corresponding to Figure 1H-J

Sugar	10 mM	100 mM	1000 mM
fructose	2.264	0.995	0.750
sucrose	2.010	-0.453	0.118
maltose	0.936	0.056	0.398
melezitose	1.683	0.700	0.768
glucose	3.384	5.706	4.747
sorbitol	2.095	3.776	1.613
sorbose	2.095	3.234	1.211

Table 2. PCA factor loadings Loadings shown for PC1 to PC8 for each 0.1 s bin of GRNs 1-3 between 0.1 s and 1 s.

GRN	Time (s)	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
GRN1	0.1	0.1268	0.3356	-0.2356	0.2763	-0.0042	0.4793	-0.1599	0.1950
	0.2	0.1652	0.3174	-0.1029	0.1741	0.0107	0.2339	0.0340	0.3205
	0.3	0.1859	0.2844	-0.0320	-0.0018	-0.0666	0.1166	0.1059	-0.0317
	0.4	0.2081	0.2578	-0.0770	0.0828	-0.0266	-0.0402	-0.3113	-0.0664
	0.5	0.2025	0.2543	-0.0860	-0.0622	-0.0162	-0.0388	0.1357	-0.2124
	0.6	0.2194	0.2253	-0.0132	-0.0705	0.0401	-0.0596	0.1775	-0.1014
	0.7	0.2140	0.2056	0.0272	-0.1102	0.1374	-0.3526	0.1094	0.2359
	0.8	0.2129	0.1700	-0.0336	-0.0311	-0.2842	-0.1729	-0.0332	-0.2183
	0.9	0.2166	0.1712	-0.0064	-0.1439	0.0004	-0.3293	-0.0509	-0.0703
	1	0.2059	0.1128	-0.0565	-0.0789	0.0395	-0.2501	0.2080	-0.1283
GRN2	0.1	0.3246	-0.0778	0.3700	-0.5031	-0.0071	0.3068	-0.1902	0.1661
	0.2	0.3264	-0.1897	0.1178	-0.2159	0.0739	0.2940	-0.1362	-0.3011
	0.3	0.2875	-0.2078	-0.0105	0.0659	0.2145	-0.0826	-0.2942	0.0076
	0.4	0.2483	-0.2209	-0.1047	-0.1083	0.0155	0.1058	0.4999	0.1439
	0.5	0.2454	-0.2376	-0.0458	0.0984	-0.0056	-0.1168	-0.1478	0.3046
	0.6	0.2196	-0.2451	-0.1558	0.1521	-0.1245	-0.1270	-0.2217	0.0808
	0.7	0.2008	-0.2240	-0.1549	0.2194	-0.2021	0.1633	0.0970	-0.4310
	0.8	0.1893	-0.2045	-0.1600	0.1052	0.3272	-0.0808	0.1975	0.2259
	0.9	0.1685	-0.2005	-0.1705	0.2394	-0.1198	0.0408	0.2460	-0.0001
	1	0.1299	-0.1409	-0.0612	0.1719	-0.1160	-0.0824	-0.1274	0.0527
GRN3	0.1	0.0756	0.0719	0.3748	0.4245	0.6168	0.0089	-0.0263	-0.2976
	0.2	0.0504	0.0268	0.2325	0.2123	-0.1404	-0.0904	-0.0353	0.1117
	0.3	0.0629	0.0093	0.4800	0.1725	-0.2205	0.1105	0.2890	0.1704
	0.4	0.0409	0.0136	0.1894	0.0963	-0.1659	-0.1734	-0.1917	0.1951
	0.5	0.0367	0.0082	0.2336	0.0329	-0.0822	0.0508	0.1543	-0.0329
	0.6	0.0238	0.0200	0.1235	0.1010	-0.0368	-0.2037	-0.0319	0.0251
	0.7	0.0369	0.0132	0.1842	0.1226	0.0760	0.0169	0.1020	0.0305
	0.8	0.0400	-0.0152	0.0893	0.1511	-0.2989	-0.0270	-0.0912	-0.1571
	0.9	0.0462	-0.0313	0.1476	0.0704	-0.1069	-0.0407	0.0188	-0.0168
	1	0.0483	-0.0064	0.2223	0.1566	-0.2402	-0.0522	0.0534	0.0107