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Nutritional Value–Dependent and Nutritional Value–Independent Effects on *Drosophila melanogaster* Larval Behavior

Astrid Rohwedder[1,2,*], Johanna E. Pfitzenmaier[1,2,*], Noel Ramsperger[2], Anthi A. Apostolopoulou[1,2], Annekathrin Widmann[1,2] and Andreas S. Thum[1,2]

[1]Department of Biology, University of Fribourg, 1700 Fribourg, Switzerland and [2]Department of Biology, University of Konstanz, 78464 Konstanz, Germany

Correspondence to be sent to: Andreas S. Thum, Department of Biology, University of Konstanz, Universitätsstraße 10, 78464 Konstanz, Germany. e-mail: andreas.thum@uni-konstanz.de

*These authors contributed equally to this work.

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Abstract

Gustatory stimuli allow an organism not only to orient in its environment toward energy-rich food sources to maintain nutrition but also to avoid unpleasant or even poisonous substrates. For both mammals and insects, sugars—perceived as "sweet"—potentially predict nutritional benefit. Interestingly, even *Drosophila* adult flies are attracted to most high-potency sweeteners preferred by humans. However, the gustatory information of a sugar may be misleading as some sugars, although perceived as "sweet," cannot be metabolized. Accordingly, in adult *Drosophila*, a postingestive system that additionally evaluates the nutritional benefit of an ingested sugar has been shown to exist. By using a set of seven different sugars, which either offer (fructose, sucrose, glucose, maltodextrin, and sorbitol) or lack (xylose and arabinose) nutritional benefit, we show that *Drosophila*, at the larval stage, can perceive and evaluate sugars based on both nutrition-dependent and -independent qualities. In detail, we find that larval survival and feeding mainly depend on the nutritional value of a particular sugar. In contrast, larval choice behavior and learning are regulated in a more complex way by nutrition value–dependent and nutrition value–independent information. The simplicity of the larval neuronal circuits and their accessibility to genetic manipulation may ultimately allow one to identify the neuronal and molecular basis of the larval sugar perception systems described here behaviorally.

Key words: *Drosophila* larvae, feeding, gustation, learning, nutritional benefit, sugar, survival

Introduction

In the last decade, *Drosophila* larvae have turned into a suitable model organism to study the neuronal, molecular, and behavioral basis of chemosensation and chemosensory learning due to their numerical simplicity on the neuronal level and genetic tractability (Gerber and Stocker 2007; Gerber et al. 2009). A comprehensive set of studies has described the anatomy of the olfactory and gustatory sensory systems even partially including their organization within higher brain centers (Fishilevich et al. 2005; Kreher et al. 2005; Colomb et al. 2007; Kwon et al. 2011).

The larval gustatory system consists of three external and three internal organs. The three external organs are located at the head region: dorsal organ (DO), terminal organ (TO), and ventral organ (VO). The three internal organs are closely attached to the pharynx: dorsal pharyngeal sense organ (DPS), ventral pharyngeal sense organ (VPS), and posterior pharyngeal sense organ (PPS) (Gendre et al. 2004; Gerber and Stocker 2007; Vosshall and Stocker 2007). Gustatory receptor neurons (GRNs) project from the peripheral sensory organs toward the brain via distinct nerves: GRNs from the DO via the antennal nerve, GRNs from the TO and VO via the maxillary nerve, GRNs from the DPS and PPS via the labral nerve, and GRNs from the VPS via the labial nerve (Gendre et al. 2004; Colomb et al. 2007; Kwon et al. 2011). In the central nervous system, the terminal endings are organized in a specific pattern within the suboesophageal ganglion (SOG), the major taste center (Colomb et al. 2007; Kwon et al. 2011). It was suggested that the terminal sites of individual GRNs deriving from external and internal

sensory organs significantly differ. Although little is known about the potential target neurons of these terminals, several candidates were recently described. About 20 neurons of the SOG express the *hugin* gene and connect the SOG with the protocerebrum, the ventral nerve cord, the ring gland, and the pharynx (Melcher and Pankratz 2005; Bader et al. 2007). Interestingly, neuronal signaling of these neurons is involved in larval feeding (Melcher and Pankratz 2005), in addition to another set of neurons that express the neuropeptide F (NPF). Only about six NPF-positive neurons are located within the SOG or the protocerebrum and connect the SOG with higher brain centers (Garczynski et al. 2002; Wu et al. 2003; Xu et al. 2008). Octopaminergic and dopaminergic neurons are supposed to signal the sugar-dependent reward information onto higher brain centers like the mushroom body, a brain structure necessary for establishing odor–sugar associations (Hammer and Menzel 1998; Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009; Pauls et al. 2010b).

Kwon and colleagues (2011) analyzed the relation between cellular identities and the expression of putative gustatory receptor genes of 60 members containing Gr gene family. They identified 39 Gr genes that were expressed in 10 different GRNs of larvae, which fall into three classes: the DO class (cell body located in the DO ganglion and projecting toward the DO), the TO distal class (cell body located in the TO ganglion and projecting toward the TO), and the TO dorsolateral class (cell body located in the DO ganglion and projecting toward the TO). Furthermore, they showed that single GRNs express at least two Gr genes, for example, C6 cell (*Gr21a* and *Gr63a*) that detect CO_2 (Badre et al. 2005; Kwon et al. 2007; 2011). Other types of GRNs express many more Gr genes, up to 17 for C1 neuron (Kwon et al. 2011). The 10 neurons identified are likely to respond to bitter food compounds as most of them express the "bitter co-receptor" GR33a. Interestingly, salt reception, however, was shown to be mediated by another set of GRNs (located within the TO) that express DEG/ENaC channel genes *Pickpocket* (ppk). Genetic interference with *ppk11* and *ppk19* disrupted the ability of larval to detect low salt concentrations (Liu et al. 2003). On the contrary, larval sugar perception is still mysterious. None of the Gr genes that are involved in sugar sensation in adults (*Gr5a, Gr61a, Gr64a-f, and Gr43a*) were detected in the larval system (Colomb et al. 2007; Kwon et al. 2011). Thus, although larvae can sense sugars and different sugar concentrations (Schipanski et al. 2008), it is not known how this chemosensory stimulus is detected at the receptor level.

Compared with *Drosophila* olfactory system, gustatory system has a lower dimensionality and seems to be designed to classify substances in a handful of hedonic values, for example, "nonedible" versus "edible" (Colomb et al. 2007; Colomb and Stocker 2007; Gerber and Stocker 2007). However, it was shown that the edible category can trigger sugar-dependent responses differently in larvae (Schipanski et al. 2008). Thus, it seems reasonable to expand our current view by including more dimensions like nutritional value of a substance.

Three studies have recently convincingly demonstrated at the behavioral level and partially by using electrophysiological and blood sugar concentration measurements that adult *Drosophila* can detect and even remember the caloric content of different sugars (Burke and Waddell 2011; Dus et al. 2011; Fujita and Tanimura 2011). Three different groups of sugars were investigated for their diet-dependent survival, feeding, choice, and olfactory appetitive learning. The first group of sugars is perceived by the fly as "sweet" and has a nutritional value (e.g., fructose or sucrose), the second one is also perceived as "sweet" but cannot be metabolized (zero calorie; e.g., xylose or arabinose), and the third group is not perceived as "sweet" but has a nutritional value (e.g., sorbitol). Taken together, the data suggest that adult flies may use two distinct systems in order to discriminate and learn different sugar identities: one that is Gr gene dependent and evaluates the "sweetness" and second postingestive system of unknown identity that recognizes the nutritional value.

By investigating how different sugars affect larval survival, choice, feeding, and appetitive olfactory learning, we establish whether or not such a nutrition-based system exists in *Drosophila* larvae. We use a set of seven different sugars that offer nutritional benefit to fruitflies (fructose, sucrose, glucose, sorbitol, and maltodextrin) or lack nutritional benefit (arabinose and xylose). Taken together, our behavioral data show first that a nutrition-dependent system in larvae indeed exists. Second, survival and feeding mainly depend on the nutritional value of sugars. Third, larval choice behavior and learning are regulated in a more complex way by nutrition value–dependent and nutrition value–independent information of sugars. Therefore, we argue that sugar-dependent behavioral changes are based on a more complex multistimulus signal. Given the lack of information on the basic organization of the larval external and internal sensory neurons that detect sugar, our data provide further understanding of how sugar information is processed in *Drosophila* larvae.

Materials and methods

Fly strains

For all experiments, we used wild-type Canton-S larvae. Fly strains were kept on standardized cornmeal medium at 25 °C under a 14:10 h light:dark cycle. Adult flies were transferred to fresh food vials every second day and were allowed to lay eggs for 48 h. Third instar feeding-stage larvae aged 96–144 h were removed from vials and divided into groups of 30 animals that were briefly washed in tap water to remove food residues.

Survival

To measure sugar-dependent larval survival, 12 wild-type larvae were placed in a vial that contained either 1% agarose (Sigma Aldrich cat. no.: A5093) only, or 1% agarose plus 2 M sugar at 25 °C . We used the following seven sugars: D-fructose (Sigma Aldrich cat. no.: 47740), D-sucrose (Sigma Aldrich cat. no.: 84100), D-glucose (Sigma Aldrich cat. no.: G8270), maltodextrin (Sigma Aldrich cat. no.: 419699), D-sorbitol (Sigma Aldrich cat. no.: W302902), D-xylose (Sigma Aldrich cat. no.: X3877), and D-arabinose (Sigma Aldrich cat. no.: A3131). The number of larvae that were alive was counted from day 1 to day 8. Some drops of tap water were occasionally added to prevent larvae from dehydrating. The percentage of surviving larvae was calculated as follows:

$$\text{Percentage survival} = (\text{number of living larvae} / \#\text{total number of larvae}) \times 100$$

For each group (control and respective sugar), 15 independent experimental groups were analyzed (n = 15). The data shown in Figure 1 depict the mean and the standard deviation of them daily for each sugar.

Gustatory preference

For gustatory preference tests, 2.5% agarose solution (Sigma Aldrich) was boiled in a microwave oven and filled as a thin layer into Petri dishes (85-mm diameter; Greiner). After cooling, the agarose was removed from half of the plate. The empty half was filled with 2.5% agarose solution containing fructose, sucrose, glucose, maltodextrin, sorbitol, xylose, or arabinose (all sugars: 0.1, 1, 2, or 4 M). Assay plates were used on the same day or stored at 4 °C until the day of experiments. Groups of 30 larvae were placed in the middle of the plate, allowed to crawl for 5 min, and then counted on the sugar side, the sugar-free agarose side, and the neutral zone (about 1 cm between both sides). By subtracting the number of larvae on the pure agarose side (#nS) from the number of larvae on the sugar side (#S) divided by the total number of counted larvae (#TOTAL), a preference index for the respective sugar and its concentration was calculated:

$$\text{PREFsugar} = (\#\text{S} - \#\text{nS}) / \#\text{TOTAL}$$

Negative PREFsugar values indicate sugar avoidance, whereas positive PREFsugar values represent sugar attractiveness.

Feeding

To measure feeding behavior, 30 feeding third instar larvae were placed on a Petri dish containing one of the seven different sugars at a concentration of 2 M, dissolved in 1% agarose and 2% indigocarmin (Sigma Aldrich cat. no.: 73436). An additional control group was put on plates containing only 1% agarose and 2% indigocarmin. Larvae from all these groups were allowed to feed on this substrate for 30 min, washed in tap water, and, as a group, homogenized in 500 µl of a 1 M ascorbic acid solution (Sigma Aldrich cat. no.:

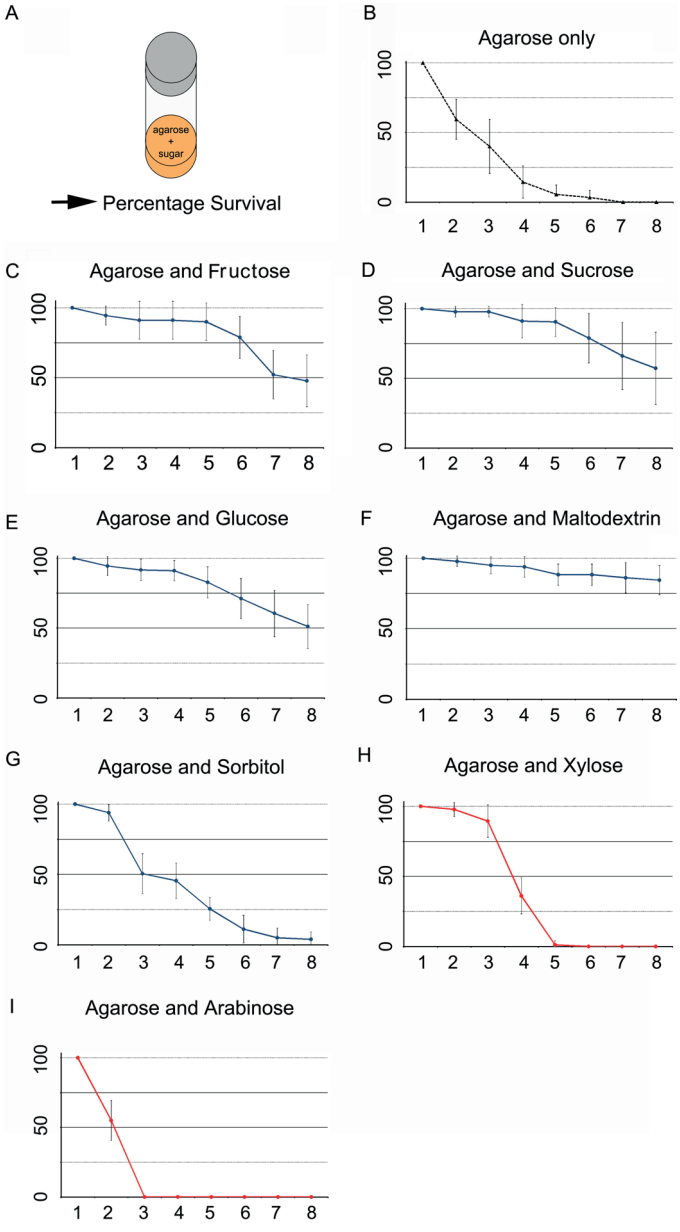

Figure 1 Larval survival on different sugar diets. A) A scheme of the experimental setup. B-I) Percentage survival of wild-type Canton-S larvae that were raised on agarose only (B) or agarose containing fructose (C), sucrose (D), glucose (E), maltodextrin (F), sorbitol (G), xylose (H), or arabinose (I). In each panel, larval survival is presented as the mean survival and its standard deviation. The curve in B indicates baseline survival on pure agarose. Curves in C–F show prolonged larval survival defined by surviving larvae up to day 8. Curves in G and H show no prolongation of lifespan compared with baseline survival as all larvae are dead on day 8. Sample size for each data point is n = 15.

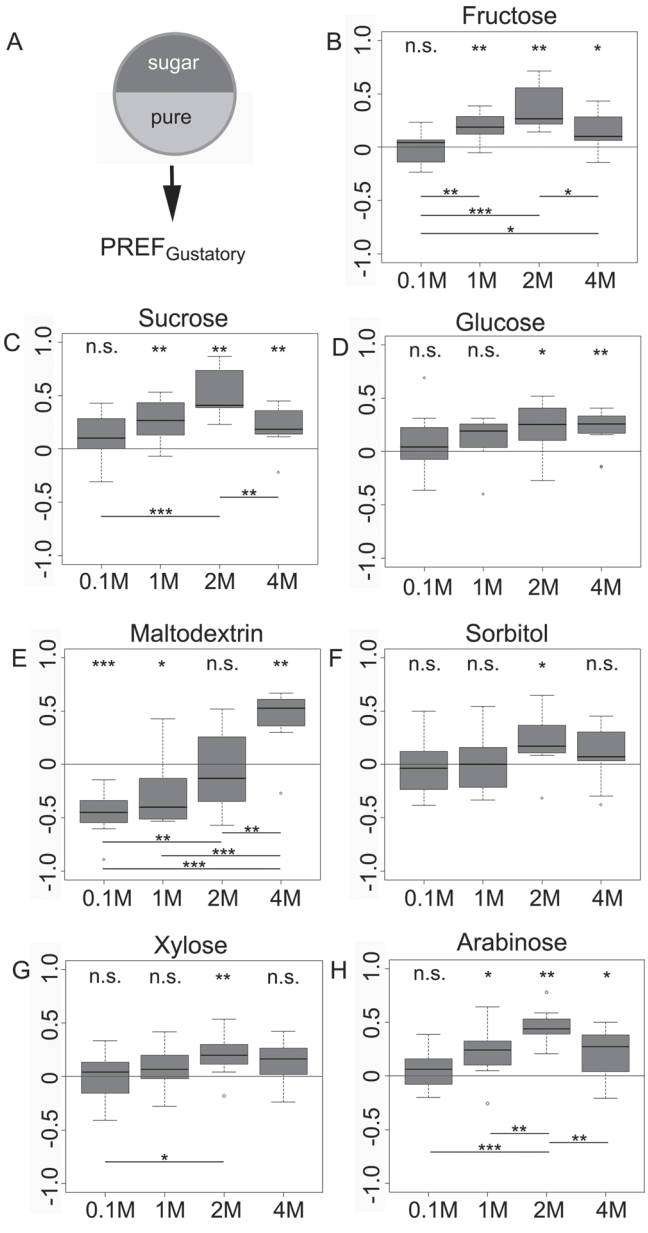

Figure 2 Larval choice responses for seven different sugars. A) A scheme of the experimental setup. B-I) Preference indices are depicted for fructose, sucrose, glucose, maltodextrin, sorbitol, xylose, and arabinose, respectively. Sample size for each box plot is $n = 12$. Significant differences of two groups are indicated at the bottom of each panel. Differences against zero are given at the top of each panel. n.s. (nonsignificant $P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

A7506). The homogenate was centrifuged for 5 min at 13'400 rpm. The supernatant was then filtered using a syringe filter (millipore, 5-µm pores) into a fresh Eppendorf cup and centrifuged again for 5 min at 13'400 rpm. In all, 100 µl of the supernatant was loaded into single wells of a 96-well plate (Hartenstein, Würzburg, Germany). Then, using a Sunrise spectrophotometer (Tecan AG, Männedorf, Switzerland) or an Epoch spectrophotometer (BioTek, Bad Friedrichshall, Germany), absorbance at 610 nm was measured.

Relative feeding was calculated by dividing each photometrically measured value minus the blank control by the mean score obtained for larvae feeding on a pure agarose minus the blank control:

$$\text{Relative feeding} = (\#\ \text{absorption on sugar plate} - \#\ \text{absorption for blank control}) / (\#\ \text{mean absorption on pure agarose} - \#\ \text{mean absorpiton for blank control})$$

For each sugar, 15 independent experimental groups were analyzed ($n = 15$). The data shown in Figure 3 are presented as relative feeding in box plots for each type of sugar.

Odor-sugar learning

All learning experiments were conducted on assay plates filled by a thin layer of agarose solution (as described above) containing either pure 2.5% agarose or 2.5% agarose plus fructose, sucrose, glucose, maltodextrin, sorbitol, xylose, or arabinose at concentrations of 1, 2, and 4 M. As olfactory stimuli, we used 10 µl amyl acetate (AM, Fluka cat. no.: 46022; diluted 1:250 in paraffin oil, Fluka cat. no.: 76235) and benzaldehyde (BA, undiluted; Fluka cat. no.: 12010). Odorants were loaded into custom-made Teflon containers (4.5-mm diameter) with perforated lids as described in Gerber and Stocker (2007).

Learning ability was tested by exposing a first group of 30 animals to BA while crawling on agarose medium containing sugar as a positive reinforcer. After 5 min, larvae were transferred to a fresh Petri dish in which they were allowed to crawl on pure agarose medium for 5 min while being exposed to AM. A second group of larvae received the reciprocal training. Immediately, after three training cycles, larvae were transferred onto test plates on which AM and BA were presented on opposite sides. After 5 min, individuals were counted on the AM side (#AM), the BA side (#BA), and in a neutral zone. By subtracting the number of larvae on the BA side from the number of larvae on the AM side divided by the total number of counted individuals (#TOTAL), we calculated an preference index for each training group:

$$\text{PREF}_{\text{AM+/BA}} = (\#\ \text{AM} - \#\ \text{BA}) / \#\text{TOTAL}$$

$$\text{PREF}_{\text{AM/BA+}} = (\#\ \text{AM} - \#\ \text{BA}) / \#\text{TOTAL}$$

We then compiled a performance index (PI):

$$\text{PI} = (\text{PREF}_{\text{AM+/BA}} - \text{PREF}_{\text{AM/BA+}}) / 2$$

Negative PIs represent aversive learning, whereas positive PIs indicate appetitive learning.

Statistical methods

Comparison between two experimental groups was done by using Wilcoxon rank sum test. To compare experimental

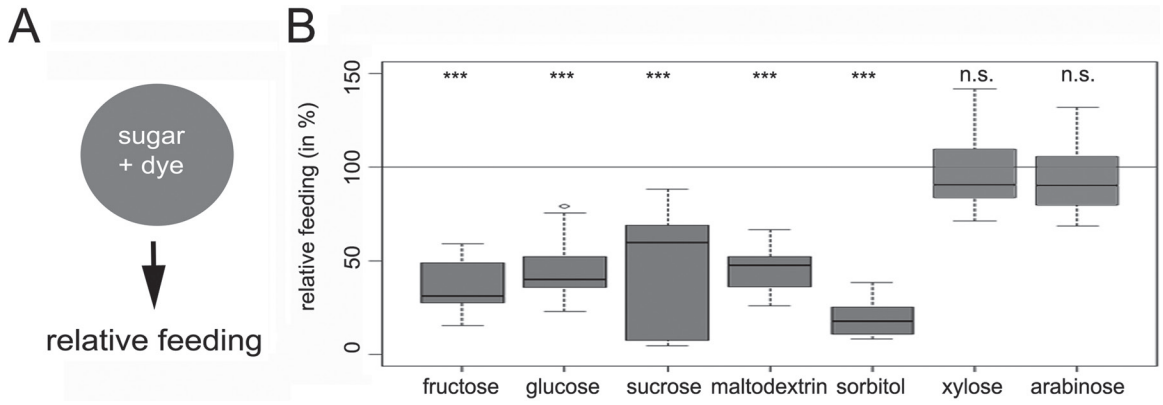

Figure 3 Larval feeding behavior for seven different sugars in relation to baseline feeding on pure agarose. A) A scheme of the experimental setup. B) Relative feeding on fructose, sucrose, glucose, maltodextrin, sorbitol, xylose, and arabinose. All values were normalized for baseline feeding on pure agarose (100 %). Fructose, sucrose, glucose, maltodextrin, and sorbitol significantly reduce larval feeding. However, xylose and arabinose do not change baseline feeding. Sample size for each box plot is $n = 15$. Differences against zero are given at the top of each panel. n.s. (nonsignificant $P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

groups against chance level, we applied Wilcoxon signed ranked test (Selcho et al. 2009). All statistical analyses and visualizations were done with R version 2.8.0 and Windows Excel, respectively. Behavioral data are presented as box plots, including all values of a given genotype, 50% of the values being located within the box. The median performance index is indicated as a bold line within the box plot. Outliers are depicted as small circles. The data in Figure 1 are presented as line plots. Significance levels shown in the figures refer to the P value obtained in the statistical tests: n.s. for $P > 0.05$, * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$.

Results

Experiment 1: Larval survival on specific sugar diet

Led by prior studies on adult *Drosophila* (Hassett 1949, Burke and Waddell 2011), we first analyzed how a specific sugar diet affects larval survival. Therefore, we put first instar wild-type Canton-S larvae into food vials that either contained 1% agarose or 1% agarose plus 2 M fructose, sucrose, glucose, maltodextrin, sorbitol, xylose, or arabinose as their sole food source. Daily for 1 week, the percentage of surviving larvae was calculated for each vial (Figure 1A). The experiment was completed on day 8 although in several cases, larvae were still alive. For all different sugar diets, larvae did not pupate.

Larvae kept on an agarose-only diet were used as control. About 50% of them had died after 2 days; none survived until the end of the experiment (Figure 1B). This result is in line with data found for adult flies (Hassett 1949; Burke and Waddell 2011). Larvae feeding on agarose that contained in addition fructose, sucrose, or glucose survived much longer. Even at the end of the experiment, after 8 days, about 50% survived (Figure 1C–E). Raising larvae on agarose containing maltodextrin had a similar effect on the survival rate: after 8 days about 85% of them were alive, suggesting a higher nutritional benefit for maltodextrin (Figure 1F). Sorbitol diet also improved the survival of the larvae compared with pure agarose (Figure 1G). However, the number of surviving larvae was lower than for the previously mentioned sugars, only about 4% of the larvae were still alive at the end of the experiment. Regarding xylose and arabinose, we did not detect any extended lifespan compared with agarose only (Figure 1H,I). All larvae died in about 3 days.

Taken together, the data suggest that the seven tested sugars differ with respect to their nutritional values. Fructose, sucrose, glucose, and maltodextrin and on a lower level also sorbitol offer carbohydrate sources that prolong larval survival. However, under these experimental conditions, xylose and arabinose can apparently not be metabolized.

Experiment 2: Sugar choice

We next tested if naive feeding third instar wild-type Canton-S larvae prefer one of the seven different sugars dissolved in 2.5% agarose compared with pure 2.5% agarose (Figure 2A). Preferences were studied at four concentrations: 0.1, 1, 2, and 4 M. Specifically, we counted the number of larvae after 3 min on a Petri dish that was split into a sugar-containing agarose side and a pure agarose side (Figure 2A). Schipanski and colleagues (2008) used a similar approach to study the sugar-dependent choice behavior of naive feeding third instar wild-type larvae for fructose, sucrose, glucose, and trehalose. Although some of their parameters were slightly different (e.g., diameter of Petri dish, concentration of agarose and sugar, test duration), we obtained essentially similar results.

Larvae preferred intermediate concentrations of about 2 M fructose (Figure 2B) and 2 M sucrose (Figure 2C), given a significant difference of the preference indices of 0.1 and 2 M ($P = 0.0002$ for fructose and $P = 0.0006$ for

sucrose) and the significantly lower scores for 4 M compared with 2 M ($P = 0.015$ for fructose and $P = 0.0014$ for sucrose). Notably, larvae did not show any significant preference for 0.1 M fructose ($P = 0.97$) and 0.1 M sucrose ($P = 0.24$) when tested against pure agarose. Schipanski and colleagues (2008) further reported that the preferences for glucose differed from those for fructose and sucrose. Under their test conditions, glucose did not induce larval choice behavior. Similarly, we also found low preference indices for glucose, which were significantly different from zero only for 2 M and 4 M (Figure 2D; $P = 0.42$ for 0.1 M; $P = 0.083$ for 1 M; $P = 0.034$ for 2 M and $P = 0.0024$ for 4 M).

Next we tested larval choice behavior for maltodextrin and sorbitol that had nutritional benefit in the first experiment while being reported to be less palatable for adult flies. Maltodextrin induced a unique appetitive response in larvae (Figure 2E), while being highly aversive at 0.1 M ($P = 0.00048$). No preference was detectable for 2 M ($P = 0.42$), whereas 4 M was highly appetitive ($P = 0.0033$). For sorbitol the preference was much weaker. Whereas 0.1 M ($P = 0.79$), 1 M ($P = 0.81$), and 4 M ($P = 0.09$) did not elicite any choice behavior, there was a slight preference for 2 M when tested against pure agarose ($P = 0.029$).

Finally, we used two sugars that were reported to be palatable for adult flies but were not nutritionally beneficial in the survival assay (Figure 1). For xylose, we only found weak attraction (Figure 2G). While 0.1 M ($P = 0.91$), 1 M ($P = 0.155$) and 4 M ($P = 0.056$) were not preferred compared with pure agarose, a moderate concentration of 2 M was significantly attractive ($P = 0.0068$). The larval response for arabinose was comparable to fructose and sucrose responses; 0.1 M arabinose dissolved in agarose was not preferred over pure agarose (Figure 2H; $P = 0.46$). However, 1, 2, and 4 M arabinose were preferred compared with agarose only ($P = 0.014$; $P = 0.0025$; $P = 0.012$, respectively). Here again we observed a concentration optimum at 2 M as this response was significantly higher than at 1 M ($P = 0.0042$) and 4 M ($P = 0.0073$).

Taken together, all seven sugars were preferred at an intermediate concentration of 2 M (Figure 2). Only for maltodextrin, larvae showed appetitive and aversive choice responses (Figure 2E). Furthermore, some sugars induced significant responses at three different concentrations from 1 to 4 M (fructose, sucrose, or arabinose), whereas three others were only preferred at 2 M, or at 2 and 4 M (glucose, sorbitol, and xylose).

Experiment 3: Sugar-dependent feeding

We photometrically measured the amount of ingested food in third instar wild-type Canton-S larvae by placing 30 of them for 30 min on a Petri dish that contained 1% agarose and 2% of a blue dye (indigocarmin). To analyze in which way the seven different sugars affect feeding, we added each of them individually at 2 M to the agarose. Relative feeding was calculated by dividing each photometrically measured sugar-dependent value by the mean agarose feeding scores (Figure 3A). Thus, a value of 100% for relative feeding represents baseline agarose feeding.

Similar to Schipanksi et al. (2008), we found that baseline feeding is significantly reduced by adding either fructose or sucrose (Figure 3B; $P = 1.2 \times 10^{-6}$ and $P = 2.6 \times 10^{-5}$, respectively). Also glucose, maltodextrin, and sorbitol significantly reduced feeding of third instar larvae with respect to food ingestion on pure agarose (Figure 3B; $P = 4.1 \times 10^{-5}$, $P = 2.9 \times 10^{-5}$ and $P = 1.0 \times 10^{-8}$, respectively). On the contrary, xylose and arabinose did not significantly change feeding when compared with pure agarose feeding (Figure 3B; $P = 0.77$ and $P = 0.68$, respectively). Given the results in Figure 1 that among the seven sugars, only xylose and arabinose do not offer nutritional benefit, we suggest that larval feeding is mainly controlled by a postingestive system that evaluates the nutritional value of a sugar resource at a concentration of 2 M.

Experiment 4: Larval olfactory learning reinforced by seven different sugars

In a final experiment, we analyzed the reinforcing potency with respect to odor-sugar learning of our set of seven sugars at concentrations of 1, 2, and 4 M. We used a standard assay for analyzing sugar-reinforced associative olfactory learning that is schematically shown in Figure 4A (Gerber et al. 2004; Michels et al. 2005; Pauls et al. 2010a; Schleyer et al. 2011; von Essen et al. 2011). Larvae were trained with two odors one of which was presented together with the respective sugar. Training was repeated three times. Immediately after training, in the test, the distribution of larvae between the sugar-paired odor and the unrewarded odor was measured. By comparing two groups that were trained by either rewarding odor A or odor B, a performance index reflecting associative olfactory learning was calculated.

Similar to the report by Schipanski et al. (2008), fructose and sucrose had at all concentrations a similar reinforcing potential to induce appetitive olfactory memory (Figure 4B, $P = 0.0020$ compared with zero for all three fructose concentrations; Figure 4C, $P = 0.0.008$ for 1 M, $P = 0.0019$ for 2 M, and $P = 0.0020$ for 4 M of sucrose). Also, larvae trained with glucose as a reinforcer showed learning for all three concentrations (Figure 4D). There was no significant difference between the three groups if compared pairwise ($P > 0.05$), and each group significantly differed from zero ($P = 0.014$ for 1 M, $P = 0.004$ for 2 M, and $P = 0.002$ for 4 M). Remarkably, the sugar-induced behavioral change for maltodextrin differed from that for all other sugars (Figure 4E). Only 4 M maltodextrin-trained larvae showed appetitive learning when compared with zero ($P = 0.002$). Learning at 4 M was significantly different from learning at 2 M ($P = 0.0025$) and 1 M ($P = 0.014$). Sorbitol, on the other hand, was able to reinforce appetitive learning at all

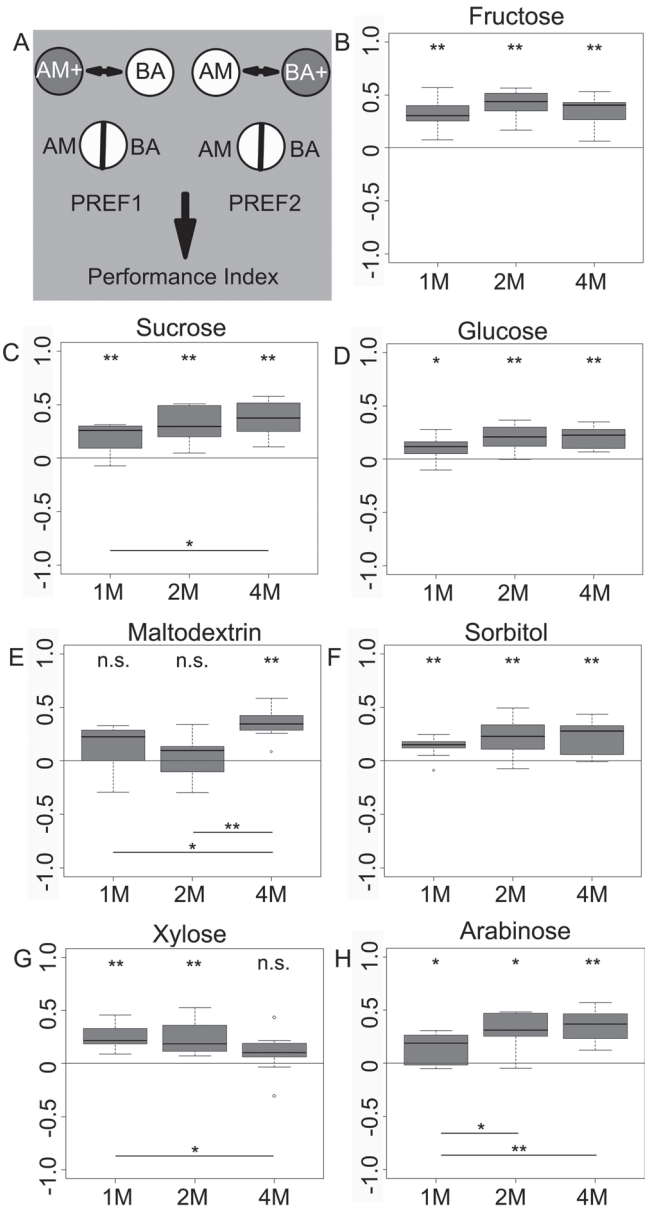

Figure 4 Larval appetitive olfactory learning reinforced by seven different sugars. A) A scheme of the experimental procedure. B–I) Performance indices are depicted for fructose, sucrose, glucose, maltodextrin, sorbitol, xylose, and arabinose, respectively. Sample size for each box plot is $n = 10$. Differences against zero are given at the top of each panel. Differences between two groups are shown if present at the bottom of the panel. n.s. (nonsignificant $P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

three concentrations at similar levels (Figure 4F; $P > 0.05$ for all pairwise comparisons), which differed significantly from zero ($P = 0.006$ for 1 M, $P = 0.006$ for 2 M, and $P = 0.004$ for 4 M). Xylose at lower concentrations significantly reinforced appetitive olfactory learning (Figure 4G), as values for 1 M ($P = 0.0019$) and 2 M ($P = 0.0019$) were significantly different from zero; however, this was not the case for 4 M ($P = 0.084$). Finally, larvae were also significantly attracted to an odor paired with arabinose at all tested concentrations

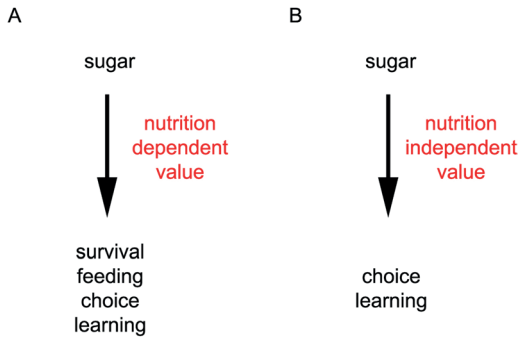

Figure 5 The effect of sugar-related information on larval survival, choice, feeding and learning. A) Nutrition-dependent information affects all tested behaviors: survival, feeding, choice, and learning. B) Nutrition-independent information of sugars is necessary for choice behavior and learning but does affect neither the survival of larvae nor their feeding behavior.

(Figure 4H; $P = 0.027$ for 1 M, $P = 0.011$ for 2 M, and $P = 0.002$ for 4 M). Higher concentrations of arabinose tended to increase performance, as a pairwise comparison of 1 M and 2 M ($P = 0.045$) and 1 M and 4 M ($P = 0.007$) showed significantly different results.

In conclusion, we were able to detect significant appetitive olfactory learning for all seven sugars. Notably, maltodextrin and arabinose seem to reinforce learning more efficiently at concentrations above 2 or 1 M, respectively, whereas xylose showed the opposite effect, being more efficient at concentrations below 4 M (Figure 4).

Discussion

We systematically analyzed seven different sugars for their effects on survival (Figure 1), choice (Figure 2), feeding (Figure 3), and learning (Figure 4). Our analysis includes pentoses (D-xylose and D-arabinose), hexoses (D-fructose and D-glucose), a disaccharide (D-sucrose), a polysaccharide (maltodextrin), and a polyhydric alcohol (D-sorbitol) at different concentrations. In the following, we discuss each of these behaviors separately and outline how these results can be integrated into our current understanding regarding the neuronal circuits and mechanisms of larval chemosensation (Figure 5).

Survival

In our first experiment, we tested how specific sugar diets affect larval survival. We used wild-type Canton-S first instar larvae 24 h after egg laying, that is, shortly after hatching. Constantly feeding with fructose, sucrose, glucose, maltodextrin, or sorbitol supported larval survival for up to 8 days (although for sorbitol this is only true for a small number of individuals) (Figure 1). Thus, all of these sugars offered a nutritional benefit to the larvae compared with pure agarose, although larval development was retarded in all cases,

likely due to the lack of protein containing food resources (data not shown). We found that xylose and arabinose did not offer a nutritional benefit for the larvae because none survived till day 8 (Figure 1). However, additional factors may complicate the direct correlation of survival and sugar-dependent nutritional benefit. For instance, our data indicate that larvae ingest less sorbitol compared with all other sugars (Figure 3). Thus, it is possible that the reduced survival on sorbitol is based on a reduced feeding rate. In this case, we would underestimate the nutritional benefit of sorbitol. In addition, larvae die even faster on arabinose compared with agarose only (Figure 1). Therefore, we cannot exclude a poisonous effect of arabinose (e.g by changing the metabolism of the larvae or even more directly). Interestingly, a similar potential detrimental effect for arabinose was also shown for adult *Drosophila* (Hassett 1949; Burke and Waddell 2011). Also in this case, we underestimate the nutritional benefit of the sugar resource.

However, the effects we observe are similar to those reported by Hassett (1949), describing that adult flies exclusively fed with fructose, sucrose, glucose, maltodextrin, or sorbitol showed increased survival compared with controls fed with agarose only. In his studies, sorbitol was least efficient as about 50% of the adult flies were dead after 5 days. In contrast to our data, maltodextrin was less efficient in his assay, as after 8 days, 50% of the adult flies were dead. Hassett's results were recently confirmed by two independent studies. Fujita and Tanimura (2011) showed that either glucose or to a lesser extent sorbitol offer a nutritional benefit, which allowed adult flies to survive for up to 120 h. Burke and Waddell (2011) fed sucrose, fructose, maltodextrin, and sorbitol to adult flies and demonstrated its nutritional benefit allowing survival up to 96 h. Taken together, all studies convincingly demonstrated that fructose, sucrose, glucose, maltodextrin, and sorbitol each offers a nutritional benefit for the fly. The same seems to be true for larvae.

Consistent with our data that neither xylose nor arabinose offers any nutritional benefit to larvae (Figure 1), Hassett (1949) showed that neither arabinose nor xylose feeding allows half of the tested adult flies to survive for more than 3 days. These results were recently reproduced by Burke and Waddell (2011) as they also found no extension in lifespan for adult *Drosophila* fed on arabinose or xylose compared with adult flies raised on pure agarose. Thus, for the two tested sugars that did not extend larval survival (Figure 1), similar results were obtained with adult flies, suggesting that independent of the different developmental stages the same metabolic pathways are used to exploit sugar-dependent energy sources.

Interestingly, it seems that the ability to exploit sugar as energy source is partially conserved between insects and humans. Fructose and glucose are metabolized anywhere in the body (Stryer 1999). The same is true for the disaccharide sucrose, which is composed of fructose and glucose (Stryer 1999). The polysaccharide maltodextrin consists of glucose units linked as chains of variable length, which are as rapidly digested and absorbed as glucose (Chao et al. 1969; Chao and Graves 1970; Chao and Weathersbee 1974). Sorbitol can be converted in humans to fructose and is therefore also metabolized; it is also known as the food additive E420 (Wick et al. 1951; Olmsted 1953; Stryer 1999). On the contrary, xylose is not metabolized in humans and is excreted by the kidneys (Chasis et al. 1933; Hemingway 1935; Shannon and Smith 1935). Arabinose is a naturally occurring monosaccharide that is not used as sweetener in human food, so the metabolic value is unknown.

Taken together, the seven different sugars used in our study can likely be classified in two groups, similar as in humans: A group that offers nutritional benefit (fructose, sucrose, glucose, maltodextrin, and sorbitol) and a group that does not (xylose and arabinose).

Choice behavior

Sugar-induced choice behavior of naive larvae between pure agarose and agarose mixed with different sugars at various concentrations was tested in an established Petri dish assay (Gerber and Stocker 2007). All seven sugars significantly induced appetitive larval choice behavior (Figure 2). However, some of them showed a stronger response than others. Similar to Schipanski et al. (2008), fructose and sucrose supported a strong and fast response for concentrations of 1 M and higher (Figure 2). The same was also true for arabinose. Moreover, in line with the initial study was the result that glucose induces weaker attraction at concentrations of 2 M and higher (Schipanski et al. 2008). Sorbitol and xylose only showed a significant response at 2 M (Figure 2). Interestingly, larval maltodextrin-induced choice behavior was different, with respect to not only its strong attraction at 4 M but also its avoidance at lower concentrations (Figure 2). The reason for the opposing effect is unknown. Taken together, our data support the idea that larval preference responses toward sugars follow an optimum function (Schipanski et al. 2008).

In adult flies, sugar-dependent choice behavior was often measured as the ability of a sugar to induce a proboscis extension response (PER) (DeJianne et al. 1985; Vaysse et al. 1988; Fujishiro et al. 1990; Wang et al. 2004; Chabaud et al. 2006; Inoshita and Tanimura 2006; Shiraiwa and Carlson 2007; Gordesky-Gold et al. 2008; Wong et al. 2009; Masek and Scott 2010; Marella et al. 2012). Similar to our findings, sucrose, fructose, and arabinose induce PER very efficiently in adult flies, whereas sorbitol and xylose have only a limited potential (Gordesky-Gold et al. 2008; Burke and Waddell 2011). Even the response to glucose was reduced compared with fructose or sucrose (Gordesky-Gold et al. 2008). Thus, the similar response profiles for larvae and adults triggered by each of these sugars might support a conservation of the underlying neuronal mechanism. In addition, it is remarkable that neither larval nor adult choice behavior depends on

the nutritional value of the sugar, as arabinose triggers these responses similar to fructose or sucrose (Gordesky-Gold et al. 2008; Burke and Waddell 2011). However, the similar response profiles for larval choice behavior regarding sorbitol and xylose might suggest that nutritional value–dependent and nutritional value–independent information of a sugar can induce the behavior. Yet, these conclusions have to be handled with care, given the limited information on larval sugar sensation (Colomb et al. 2007; Kwon et al. 2011). In addition, we cannot exclude other stimuli that might significantly affect larval choice behavior, such as differences in the viscosity, osmolarity, and surface structure of the two halves of the Petri dish that either contain sugar or did not contain sugar.

Feeding behavior

In our third experiment, we photometrically measured the amount of ingested food, either on agarose plates containing only the dye indigocarmine or on plates onto which one of the seven sugars was added (Figure 3). Similar to Schipanski et al. (2008), we found reduced food intake in larvae when 2 M fructose or 2 M glucose was present. The same was true for glucose, maltodextrin, and sorbitol, sugars that all offer a nutritional benefit for the larvae (although potentially limited in the case for sorbitol). On the contrary, xylose and arabinose did not reduce larval food intake (Figure 3), two sugars that do not have a nutritional benefit for the larvae. Thus, we suggest that at this concentration, a postingestive evaluation system exists that recognizes the nutritional value of a sugar and downregulates larval food intake. Interestingly, for even lower sucrose and fructose concentrations, Schipanski et al. (2008) did not only report a lack of repression of food intake but even a modest upregulation. Thus, the nutrition-dependent downregulation of feeding seems to be restricted to high sugar concentrations allowing an appropriate exploitation of energy rich-food sources.

Learning

The reinforcing potency of sugar-mediated olfactory associative learning was analyzed using a well-established assay, in which larvae were trained with two odors: one paired with sugar and the other one presented alone (Gerber and Stocker 2007). After training, in the test situation, larvae were allowed to distribute between the two odors. The associative character of the calculated performance index is given by the comparison of two groups that received reciprocal training (either first odor or second odor paired with sugar). Appetitive olfactory learning can efficiently be reinforced by all of the seven tested sugars when tested directly after training (Figure 4). For five of them, this is even true for all tested concentrations. In detail, fructose, sucrose, glucose, sorbitol, and arabinose at concentrations from 1 to 4 M—when paired with an odor—induce a positive association when tested afterward. Furthermore, for three of these (fructose, glucose, and sorbitol) there was no concentration dependence for its reinforcing function. However, for sucrose and arabinose, learning scores significantly increased at higher sugar concentrations (Figure 4). Only maltodextrin and xylose did not efficiently induce an appetitive olfactory memory at all tested concentrations. Whereas larvae trained with maltodextrin did not show any performance at concentrations of 2 M and below, the opposite was true for xylose-trained animals. Here only concentrations of 2 M and below induced significant performance scores (Figure 4). Interestingly, for maltodextrin, we found no aversive odor-sugar learning at a concentration of 1 M, although larvae avoid maltodextrin at this concentration in the choice assay (Figure 2E). Thus, aversive choice behavior is not directly correlated with aversive odor-sugar learning. This result is in line with data published for larval low-salt learning, as larvae usually avoid a concentration of 0.375 M sodium chloride but form an appetitive association if the same concentration of sodium chloride is paired with an odor (Niewalda et al. 2008).

Similar to Schipanski et al. (2008), we also found that high concentrations of sucrose and fructose act as potent reinforcers while only being little effective in governing choice behavior. They both might reach the asymptote at around 1 to 2 M without any decrement at higher concentrations. The same effect was also reported for an assay that used individual larvae instead of groups (Neuser et al. 2005). Interestingly, we also found that sugars irrespective of their nutritional benefit can induce appetitive olfactory learning at all tested concentrations. This suggests that the reinforcing function of a particular sugar is not exclusively based on its nutritional value. Given the fact that we found learning for all tested sugars, we argue that either nutritional value–dependent or nutritional value–independent information alone can reinforce appetitive olfactory learning. Thus, reinforcement in larvae may not be based on a single appetitive input, but rather on a complex function or at least on two different but parallel reinforcing stimuli that potentially establish independent types of appetitive memory.

Interestingly, in adult flies, too, it was recently suggested that the nutritional value of a sugar can be learned (Burke and Waddell 2011; Fujita and Tanimura 2011). However, also arabinose that induces a PER but does not support survival can act as an appetitive reinforcer (Burke and Waddell 2011). Interestingly, arabinose was only able to efficiently induce adult short-term memory but not long-term memory. Thus, short-term memory in larvae and adult flies can be reinforced by different types of sugars depending on nutritional value and also additional stimuli apart from the nutritional benefit of the sugar. This might not be the case for long-term memory, as only sugars offering a nutritional benefit were able to induce a stable high performance level in adult flies when tested after 24 h (Burke and Waddell 2011).

Outlook

We have analyzed how different sugars affect four different behaviors in *Drosophila* larvae. Similar to a previous study, we have uncovered discrepancies between the dose-effect functions of sugars with regard to choice compared with their reinforcing potency (Niewalda et al. 2008; Schipanski et al. 2008; Schleyer et al. 2011). We did not find any correlation between choice and learning behavior on the one hand and the nutritional benefit of the tested sugars on the other. Thus, both the nutritional value of a sugar and nutrition-independent signaling are sufficient for the execution of these behaviors. This may be different for larval feeding, which seems to depend mainly on the nutritional value of a sugar. Given the importance for the constantly feeding larvae to initiate an appropriate behavioral response that maximally exploits a sugar source, by extracting and learning all kinds of possible parameters, sugar perception by a multidimensional system that includes peripheral and even postingestive mechanisms appears most reasonable (Figure 5). However, sugar sensation is likely even more complex, as larval feeding was suggested to be under neuropeptide control. It was reported that wandering third instar larvae switch from food attraction to food aversion regulated via neuropeptide F signaling. Thus, sugars may also induce aversive behaviors within a specific developmental time frame (Xu et al. 2008).

Our data clearly demonstrate that different types of sugars are not only vital for *Drosophila* larvae, but can also be perceived by them. The lack of information about the basic organization (and even the existence) of external and internal sugar-sensing neurons or about sugar processing in the larval brain is therefore very puzzling. Our set of experiments provide first insights into how sugars may be perceived and evaluated at a behavioral level. In a next step, it should now be possible to uncouple the nutrition-dependent and -independent sugar-sensing systems that so far complicated a neuronal and molecular analysis.

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