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Immediate and delayed effects of nutrient-sensing in fruit fly *Drosophila melanogaster*☆

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ABSTRACT

Starting in late 1980's, Bill Timberlake and associates conducted a series of experiments on anticipatory contrast which showed that rats' feeding decisions were regulated by the nutritive value of currently ingested and anticipated food. The effects of nutrient sensing on feeding regulation have been studied intensively in rodents, and recently, in the fruit fly *Drosophila melanogaster*. In this study, we developed a new behavioral test to study rapid feeding decisions of tethered flies within 6–8 s of ingestion. Using a two-phase experimental design, we presented individual flies one of four serial combinations of a non-nutritive sugar, arabinose, or a nutritive sugar, sucrose. Feeding decisions of wildtype (Canton-S) flies are altered both by immediate effects of nutrient sensing and 1-hour delayed effects of nutrient-feeding, and the two effects act additively to yield a signature pattern of behavioral contrast based on nutritive contrast. Feeding phenotype of flies that carry a mutation of the *dSLC5A11* (*cupcake*) gene varied with the mutant allele and genetic background. Fasted *dSLC5A11* mutants showed an overeating phenotype and a defect in short-term feeding regulation irrespective of the nutritive value of sugar. Flies that carried the $dSLC5A11^{1}$ allele showed differential feeding for arabinose and sucrose. However, $dSLC5A11^{2}$ allele yielded a conspicuous deficit in delayed effects of nutrient ingestion, but only when it was expressed on a Canton-S background. Our results suggest that *dSLC5A11* might function to integrate external stimulus properties and internal state for feeding regulation and action selection.

1. Introduction

Bill Timberlake was a versatile scientist. Although he is best known for his contributions to the theories of reinforcement (Timberlake and Allison, 1974) and Pavlovian conditioning (Timberlake, 1983, 1988) which was highly inspired by Tinbergen's model of instinct (1951), his experimental work on the feeding system of rats spanned a wider range of topics that included how internal state variables like circadian rhythms (White and Timberlake, 1994, 1995), or stimulus properties like the nutritive or hedonic value of food (Lucas et al., 1988,1990, Timberlake and Engle, 1995) regulated feeding. For example, in a series of experiments that started in late 1980's, Timberlake and associates showed that rats' ingestion of a non-nutritive sugar was regulated in anticipation of the opportunity to feed on substances with higher nutritive or hedonic value (Lucas et al., 1988,1990).

Regulation of feeding by nutritive cues has been studied intensively in rodents due to its relevance for important human health problems including obesity (Sclafani, 2013). In recent years, the fruit fly *Drosophila melanogaster* has become a popular model of feeding regulation due to the remarkable similarity of its neural mechanisms of food intake to those of vertebrates (Bhumika, 2018; Lin et al., 2019). Flies exhibit a complex feeding system whereby the initiation, maintenance and termination of food-driven responses depend on both external stimulus factors and internal state (Pool and Scott, 2014). Initiation of feeding is a probabilistic process triggered by taste input conveyed by the peripheral receptors on the tarsi and proboscis (fly mouthparts). In general, taste and nutrient quality are correlated, i.e., nutritive value of foods increase with their concentration. However, highly palatable foods might also have low nutrient quality, as in the case of non-metabolizable sugars that are capable of activating peripheral taste receptors to give sweet sensation. Flies use post-ingestive nutrient-sensing mechanisms to terminate feeding on non-nutritive compounds (Miyamoto et al., 2012; Dus et al., 2011). For fruit flies that have been fasted for 36 h, the first feeding bout lasts approximately 30–40 s and comprises

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more than 60% of total consumption (Qi et al., 2015). Therefore, nutrient-sensing should be used to suppress feeding rapidly in the order of seconds to prevent satiation with non-nutritive compounds. High-throughput feeding tests have been developed for screening for internal nutrient-sensors that can be used for rapid feeding decisions (Yapici et al., 2016), yet none of the feeding tests reported so far provide a temporal resolution below tens of seconds or minutes (Qi et al., 2015).

Nutritive value of sugars has been shown to affect food consumption via the action of different neural circuits (Dus et al., 2013, 2015; LeDue et al., 2015; Miyomato et al, 2012; Stafford et al., 2012; Qi et al., 2015; Yapici et al., 2016), including those that are involved in the formation of long-term appetitive memories in fruit flies (Burke and Waddell, 2011; Fujita and Tanimura, 2011). Independent studies associated internal nutrient-sensing with small groups of central neurons that express the receptor Gr43a that detects circulating hemolymph fructose levels (Miyamoto et al., 2012, LeDue et al., 2015), as well as their downstream targets located in the subesophageal ganglion (Yapici et al., 2016), the primary neuropil for taste processing and feeding control in insects. Neurosecretory cells located in the *pars intercerebralis*, a neuroendocrine center in the insect brain, have also been shown to affect post-prandial nutrient sensing (Dus et al., 2015). A fairly surprising finding was the involvement of SLC5A11 (*cupcake*) gene that encodes a Na^+/solute co-transporter, whose expression is largely confined to a group of ring neurons (R4) that innervate the ellipsoid body (Dus et al., 2013), a structure that has been suggested to be involved in action selection in homology with the vertebrate basal ganglia (Strausfeld and Hirth, 2013). How the activity of these circuits converge on a common path to control feeding decisions is currently not known (Miroschnikow et al., 2018).

In this study, we present a simple feeding test that we used to measure rapid feeding decisions of tethered flies within 6–8 s of feeding initiation. The test has the advantage of being readily useable in any fly lab as it does not require any special equipment or software, along with the disadvantage of being low-throughput as it is conducted manually on individual flies.

Flies tethered on their dorsal thorax can display a number of behaviors on a Styrofoam ball, including grooming, walking, and feeding if the ball is dipped in an appetitive tastant. We conducted a two-phase experiment to test the immediate effects of nutrient sensing and delayed effects of nutrient-ingestion on feeding decisions of individual flies. The experiment involved two feeding tests: In the first test, flies that have been food deprived for 17 h were presented a Styrofoam ball that was dipped in either a non-nutritive (arabinose, A) or a nutritive (sucrose, S) sugar, and their feeding activity was recorded every 2 s for a total of 10 trials (20 s). The pattern of feeding during the first test reflects the effects of immediate nutrient-sensing and short-term feeding regulation for fasted flies. The second feeding test started exactly 1 h later where each fly was tested with either the same (arabinose-arabinose, AA, or sucrose-sucrose, SS) or the other sugar (arabinose-sucrose, AS, or sucrose-arabinose, SA). Notice that each condition represents a different combination for testing immediate feeding regulation driven by the nutritive value of currently ingested sugar, and the delayed metabolic regulation driven by nutritive properties of the previously ingested sugar. For example, the pattern of feeding of group SA shows both the delayed effects of ingesting a nutritive sugar and the immediate effects of ingesting a non-nutritive sugar on the decision to maintain or terminate feeding.

In anticipation of our results, feeding decisions of wildtype flies are altered both by immediate effects of nutrient sensing and 1-hour delayed effects of nutrient-feeding, and the two effects act additively to yield a signature pattern of behavioral contrast based on nutritive contrast. We tested flies that carry a mutation of the *dSLC5A11* (*cupcake*) gene, and found that their feeding phenotype varied with the mutant allele and the genetic background. Fasted *dSLC5A11* mutants were able to feed differentially on A or S during the first test, yet they showed an overeating phenotype and a defect in short-term feeding regulation irrespective of the nutritive value of sugar. Mutant flies that carried the $dSLC5A11^1$ allele showed evidence for feeding differentiation based on both immediate effects of nutrient-sensing and delayed effects of nutrient-feeding. However, $dSLC5A11^2$ allele yielded a conspicuous deficit in delayed effects of nutrient ingestion, but only when the mutant allele was expressed on a Canton-S background. Our results suggest that *dSLC5A11* functions to integrate external stimulus properties and internal state for feeding regulation and response termination.

2. General method

2.1. Fly maintenance

Fly stocks were kept in a Memmert ICH 260 L incubator under a 12 h light: 12 h dark cycle. The lights were on between 6 a.m. – 6 pm. The temperature and relative humidity in the incubator were set to 25 °C and 50%, respectively. Flies were raised on standard medium (Bloomington Stock Center formula), collected on the day of eclosion and transferred to fresh food vials. 4–5 day old male flies were used in the experiments.

2.2. Fly stocks

Canton-S flies were used as the wildtype strain. $dSLC5A11^1$ (Bloomington stock #22498: $y^1 w^{67c23}$; P{w[+mC] y[+mDint2] =EPgy2}$CG8451^{EY21708}$) and $dSLC5A11^2$ (Bloomington stock #6768: $y^1 w^*$; P{y[+t7.7]=Mae-UAS.6.11}$CG8451^{UY1824}$) were obtained from the Bloomington Stock Center. They were crossed to Bloomington stock #5907 (w^{1118}/Dp(1;Y) y^+; sna^{Sco}/SM6a) for at least 6 generations to express the mutant alleles on the Canton-S genetic background.

2.3. Experimental protocol

2.3.1. Feeding tests

Flies were removed from food between 15:30 − 18:30 PM, transferred to 150 ml plastic vials covered with wet tissue paper, and placed in the incubator for the next 16 h. They were removed from the vial between 7:30 − 10:30 AM and tethered to a steel pin on their dorsal thorax using melted wax under cold anesthesia. They were then transferred to a water-containing 25 × 15 × 10 cm^3 Styrofoam box where they recovered in a closed, humid environment for ~45-50 min before the experiment.

The first feeding test started at exactly 17 h of food deprivation. The tethered fly was stabilized to a 2 × 2 × 2 cm^3 foam cube and placed under a Zeiss Stemi 2000-C stereomicroscope (Fig. 1). The fly was positioned vertically so the experimenter had a full view of the anterior surface of the head and the proboscis. At the beginning of the session, the fly was presented a ~1.5 mm^3 Styrofoam ball that was dipped in daily prepared sucrose (Merck 1.07651.1000) or D-Arabinose (Sigma A3131) solutions. Flies were observed immediately to extend their proboscis and start feeding upon getting in contact with the sugar dipped ball. Behavioral coding commenced within 2 s of ball presentation, and feeding activity was recorded every 2 s at the tick of a metronome for the next 20 s. Flies were then transferred back to the humidified box at the end of the first feeding session, and tested again exactly 1 h later with either sucrose or D-arabinose. Therefore, 2 × 10 feeding codes were obtained for each fly in two sessions.

Flies that have been tethered as described can walk, groom, fly, or feed without showing behavioral signs of discomfort. Behavior was recorded as feeding if the labellum was in contact with the ball surface and pumping movements of the proboscis were visible. Proboscis extensions without labellar contact were not recorded as feeding.

2.3.2. Habituation tests

Habituation tests were used in Experiment 2 to dissociate the effects

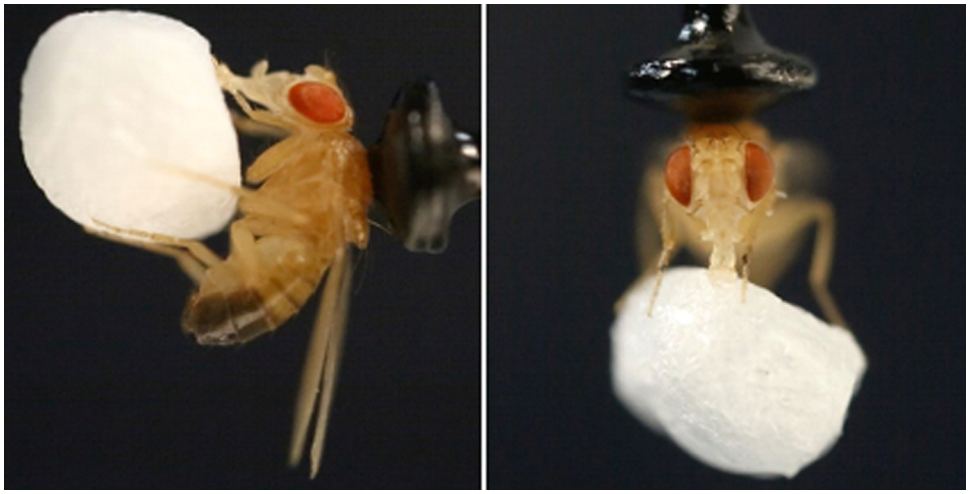

Fig. 1. A tethered fly feeding on a sucrose-dipped Styrofoam ball.

of immediate and delayed nutrient sensing on behavioral contrast. To test habituation, flies' prothoracic tarsi were continuously kept in contact with the sugar solution presented by a hypodermic needle for 20 s, and proboscis extensions were recorded every 2 s. The contact of meso- or metathoracic tarsi with sugar was allowed but flies were discarded if the proboscis touched the sugar solution.

2.3.3. Measuring sugar responsiveness

Flies' responsiveness was assessed by recording their proboscis extensions upon tarsal stimulation with a concentration series of 0.01, 0.03, 0.1, 0.3, 1 M sucrose or arabinose at 17 h of food deprivation.

2.3.4. Statistics

SPSS IBM 20 were used for statistical analyses. A chi-square analysis with two-tailed asymptotic significance criteria was used to compare probability of feeding on pairs of trials. Total number of bins with feeding was calculated for each fly in each session, and analyzed using ANOVA. Effect size (η^2) was reported along with statistical significance (p) in reporting ANOVA results. Scheffe analysis was used for post-hoc tests and Bonferroni corrections were applied to control for Type I error rates in sequential comparisons.

3. Experiment 1

In the first group of experiments, we assayed the immediate effects of nutrient-sensing and delayed effects of nutrient-ingestion on feeding decisions of wildtype (Canton-S) flies for 1 M A and variable concentrations (0.1–1 M) of S.

3.1. Results and discussion

We first determined a pair of sucrose (S) and arabinose (A) concentrations that yield equivalent and high palatability for fasted wildtype (Canton-S) flies. Stimulation of flies' tarsal receptors with sugar triggers the proboscis extension reflex (PER), and the proportion of flies that respond with a PER can be taken as a behavioral measure of sugar pallatability. Following 17 h of food deprivation, approximately 80% of flies responded with a PER to 1 M A and 0.5 M S (Supplementary Figure S1), which provided a reliable starting point for the assessment of feeding facilitation or suppression.

Fig. 2a shows the proportion of Canton-S flies feeding when they were tested using 1 M A and 0.5 M S following 17 h of food deprivation. Proportion of flies feeding on A or S was similar on the first bin ($\chi^2(1) = .34$, $p < .56$), confirming that the two sugars were equally effective in initiating feeding. However, maintenance of feeding on A or S diverged rapidly, such that the proportion of flies feeding on A was lower than that on S as early as the 3rd 2-second bin ($\chi^2(1) = 8.6$, $p < .003$). Although more than 80% of the flies started feeding on A on the first bin, roughly 40% continued to feed after the third, suggesting that nutrient ingestion is necessary for maintenance of feeding in hungry flies. By the end of the first test, flies spent significantly fewer number of bins feeding on A relative to S ($F(1, 105) = 33.9$, $p < .001$, $\eta^2 = .24$).

When we conducted the second feeding test 1 h later, consumption of A (groups AA and SA) was again lower than S (groups SS and AS), yielding a significant effect for the nutritive value of currently ingested sugar ($F(1, 105) = 51.0$, $p < .001$, $\eta^2 = .33$, Fig. 2b). Further, flies that fed on nutritive S 1 h earlier (groups SS and SA) spent fewer bins feeding than the flies that were still fasted after feeding on A (groups AA and AS), yielding a significant effect for the delayed metabolic effects of previously ingested sugar ($F(1, 105) = 23.4$, $p < .001$, $\eta^2 = .18$).

Clearly, the combination of immediate nutrient-sensing and delayed effects of nutrient-ingestion, rather than either alone, determined the amount of feeding for the four groups (Fig. 2g). We re-analyzed the group differences using a one-way ANOVA to follow up the results with pairwise comparisons. Average number of bins with feeding was significantly different across four groups, AA, AS, SA and SS ($F(3, 103) = 32.1$, $p < .001$, $\eta^2 = .48$). Scheffe analysis confirmed that total feeding for group SA was lower than that of group AA that was feeding on the same non-nutritive sugar ($p < .02$) and group SS that was equally sated for having fed on to the same nutritive sugar 1 h ago ($p < .001$). In fact, proportion of flies feeding in group SA was already lower than that in group SS ($\chi^2(1) = 8.5$, $p < .004$), or group AA ($\chi^2(1) = 6.6$, $p < .01$) as early as the 3rd bin. The converse was true for group AS whose feeding was facilitated to yield total feeding scores higher than those of both AA ($p < .001$), and SS ($p < .001$). Proportion of flies feeding on the 3rd bin in group AS was higher than that in group AA that was equally hungry ($\chi^2(1) = 13.1$, $p < .001$), or that in group SS that was exposed to the same nutritive sugar ($\chi^2(1) = 10.2$, $p < .001$). These results show that both suppression of feeding for group SA and facilitation of feeding for group AS were in effect by the end of the 3rd bin, i.e., between 6–8 seconds into the session.

Interestingly, groups AA and SS showed neither facilitation nor suppression as the proportion of flies feeding in both groups declined at similar rates, yielding similar total feeding scores ($F(1, 48) = 1.6$, $p < .21$). The pattern of *de novo* feeding on A or S during the first test was different for the fasted flies, so we reasoned that the similarity of feeding behavior between groups AA and SS during the second test could have stemmed from reduced food-driven activity of group SS due

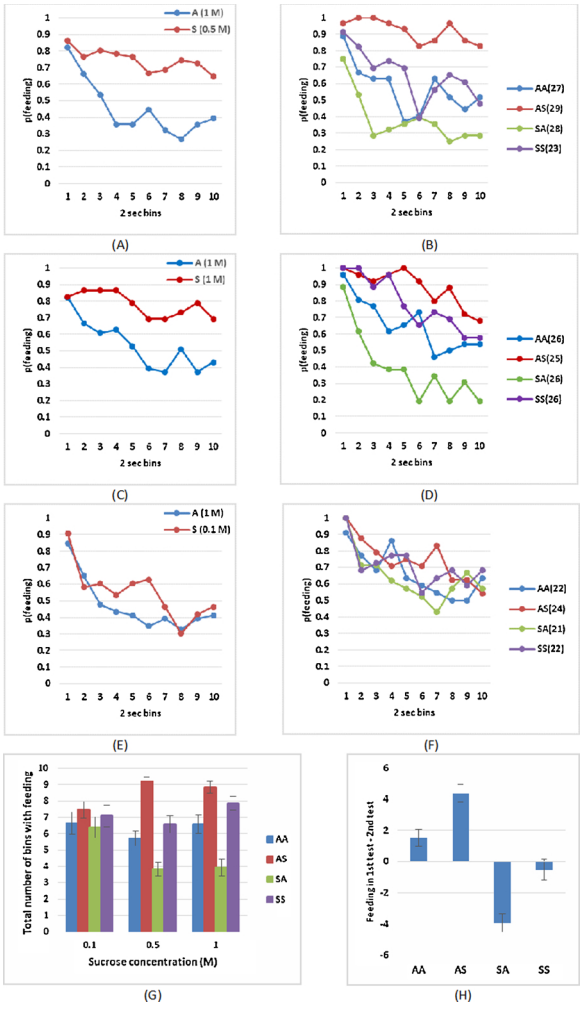

Fig. 2. The effects of sucrose concentration on nutritive contrast in Canton-S flies. A. Proportion of Canton-S flies feeding on 1 M A or 0.5 M S during the first test. **B.** Proportion of Canton-S flies feeding on 1 M A (groups AA and SA), or 0.5 M S (groups SS and AS) during the second test. **C.** Proportion of Canton-S flies feeding on 1 M A or 1 M S during the first test. **D.** Proportion of Canton-S flies feeding on 1 M A (groups AA and SA), or 1 M S (groups SS and AS) during the second test. **E.** Proportion of Canton-S flies feeding on 1 M A or 0.1 M S during the first test. **F.** Proportion of Canton-S flies feeding on 1 M A (groups AA and SA), or 0.1 M S (groups SS and AS) during the second test. **G.** Total feeding scores during the second test. **H.** Mean difference in the total feeding scores of the first and second tests for each group in the data set depicted in A–B. Error bars show SEM. Number of flies in each group is indicated in parantheses.

to relative satiety. In order to test for this explanation, we calculated the difference in feeding scores between the second and the first tests for each fly in groups AA and SS. A negative difference would indicate that the fly spent fewer bins feeding during the second test, and vice versa. Fig. 2h shows that the pattern of difference scores mirrored that of total feeding scores for the four groups ($F(3, 103) = 39.0$, $p < .001$, $\eta^2 = .53$). Difference scores were positive for group AA but negative for group SS, suggesting that the proportion of flies feeding in groups AA and SS converged as the former increased and the latter decreased during the second test.

Next, we manipulated the concentration of sucrose to test how changing the nutritive contrast between A and S affects feeding. Increasing the concentration of sucrose from 0.5 M to 1 M did not change the overall pattern of behavioral contrast during either the first or the second test (Fig. 2c-d). Flies consumed significantly more S than A during the first test ($F(1, 101) = 21.9$, $p < .001$, $\eta^2 = .18$, Fig. 2c), and the total number of bins with feeding was significantly different across groups during the second test ($F(3, 99) = 19.9$, $p < .001$, $\eta^2 = .38$, Fig. 2d, g). A Scheffe analysis showed that the feeding scores of group SS was no longer lower than those of AS ($p < .54$), suggesting that the likelihood of feeding increased with sucrose concentration when flies were sated, although the difference was not high enough to distinguish the total feeding scores of groups SS and AA ($p < .32$).

We then repeated the experiment using a lower concentration (0.1 M) of S and 1 M arabinose. Decreasing the concentration of S prevented the augmentation of feeding on S relative to A during the first test (Fig. 2e), causing the proportion of flies feeding to overlap extensively at each bin to yield similar total feeding scores for 0.1 M S and 1 M A ($F(1, 87) = 1.8$, $p < .19$). Presenting the flies low concentration S obscured group differences during the second test as well ($F(3, 85) = .61$, $p < .61$), selectively diminishing the facilitation or suppression of feeding for groups AS and SA, respectively, while leaving AA and SS unchanged. Indeed, comparing the data sets in Fig. 2b and f, we found that changing S concentration did not change the overall level of feeding ($F(1, 188) = 2.5$, $p < .12$). However, the interaction between S concentration and group was significant ($F(3, 188) = 6.4$, $p < .001$, $\eta^2 = .09$, Fig. 2g) because the nulling effect of sucrose concentration reduction was selective for cases where nutrient-sensing differentiated appetitive responses, i.e., feeding on a nutritive sugar when hungry (facilitation of feeding for S on the first test and AS on the second), or feeding on a non-nutritive sugar when sated (suppression of feeding for SA). These results confirm that both the facilitation of feeding for group AS and the suppression of feeding for group SA depend on the nutritional contrast between S and A, and reducing the concentration of sucrose diminished both effects, sparing the overall level of feeding for groups AA and SS.

4. Experiment 2

The first group of experiments suggested that ingestion of high concentration (0.5–1 M) S was necessary for nutrient-sensing based differentiation of feeding on A and S. However, we were not able to dissociate immediate and delayed effects of nutrient feeding on the behavioral contrast obtained during the second test of the previous set of experiments by manipulating S concentration. In two experiments that follow, we only allowed the flies to taste 0.5 M S without ingestion during either the first or the second test to separate the effects of immediate and delayed effects of nutrient feeding on behavioral contrast.

First, we replaced the first feeding test with a habituation test where flies' tarsal receptors were stimulated with either 0.5 M S or 1 M A without allowing them to feed. The second test was conducted as usual. This manipulation disallowed the use of immediate post-ingestive cues during the first test and delayed post-ingestive cues during the second test. In effect, flies that were habituated to S or A without feeding were restricted to using pre-ingestive taste cues during the first test, and were equally hungry and devoid of delayed post-ingestive effects of nutrient sensing during the second test. Next, we conducted the converse experiment where the flies were allowed to feed during the first, but not the second test, which prevented the flies from using immediate nutrient-cues for feeding decisions.

4.1. Results and discussion

Fig. 3a shows that the proboscis extension reflex habituatated at a similar rate to 0.5 M sucrose and 1 M arabinose in the absence of feeding ($F(1, 118) = .12$, $p < .73$), suggesting that 0.5 M sucrose and 1 M arabinose had equivalent palatability, and that the differentiation of feeding on A and S during the first test requires immediate post-ingestive assessment of nutritive value. We then compared the data sets on Figs. 2a and 3 a to understand how immediate nutritive cues affected feeding in fasted flies. When the flies were presented A, they spent effectively the same number of bins with feeding (4.5 ± .35) or with PER

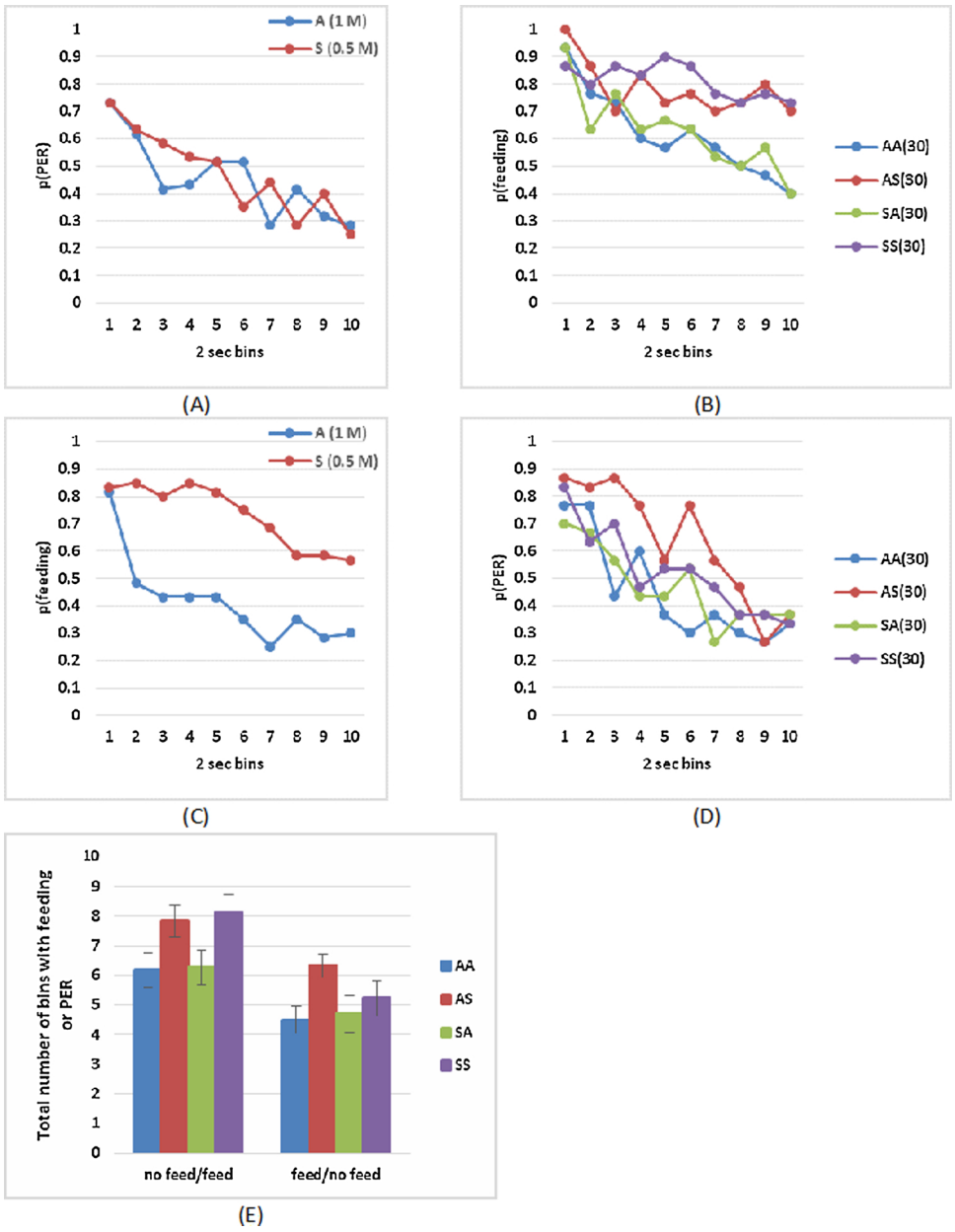

Fig. 3. Dissociation of the effects of immediate and delayed nutrient sensing on behavioral contrast. A. Proportion of Canton-S flies that made a PER upon stimulation with 1 M A or 0.5 M S during the first test. **B.** Proportion of Canton-S flies feeding on 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **C.** Proportion of Canton-S flies feeding on 1 M A or 0.5 M S during the first test. **D.** Proportion of Canton-S flies that made a PER upon stimulation with 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **E.** Total feeding or total PER scores during the second test. Error bars show SEM. Number of flies in each group is indicated in parantheses.

(4.5 ± .4) in the presence and absence of ingestion, respectively, indicating that the appetitive responses towards this non-nutritive sugar is based solely on peripheral taste, i.e., not affected by post-ingestive cues. In contrast, when the flies were presented S, they spent significantly higher number of bins feeding (7.5 ± .36) than with PER (4.7 ± .37) in the presence and absence of ingestion, respectively ($F(1, 109) = 27.6$, $p < .001$, $\eta^2 = .20$). Given also that appetitive responses for S were similar to those for A in the absence of feeding, but higher when feeding was allowed, these results suggest that immediate-nutrient sensing facilitates feeding on S when the flies were fasted.

Fig. 3b shows that when ingestion was not allowed during the first test, feeding was distinguished only with respect to immediate nutrient-sensing in the second test. The pattern of results resembled those that were obtained during the first test when fasted flies were allowed to feed on A or S *de novo* (compare Figs. 2a and 3 b). Accordingly, the group effect was significant ($F(3, 116) = 3.3$, $p < .02$, $\eta^2 = .08$, Fig. 3e), as flies that fed on sucrose (AS and SS) spent higher number of bins feeding than those that fed on arabinose (SA and AA). A visual comparison of Figs. 2b and 3 b shows that the suppression of feeding for group SA, facilitation of feeding for group AS, and the convergence of feeding for groups SS and AA were all lacking when the flies did not feed on A or S during the first test. In particular, for group SA, the proportion of flies feeding failed to show a rapid decline by trial 3 ($\chi^2(1) = 3.3$, $p < .07$), and the total number of bins with feeding was now higher for this group compared to the experiment where the flies were allowed to feed during the first test ($F(1, 56) = 12$, $p < .001$, $\eta^2 = .18$), suggesting that delayed satiating effects of S ingestion are necessary for subsequent termination of feeding on A. Interestingly, number of trials with feeding was lower for group AS ($F(1, 57) = 5.3$, $p < .03$, $\eta^2 = .09$), for which proportion of flies feeding now showed a steady decline up to the 3rd trial ($\chi^2(1) = 10.6$, $p < .001$) instead of a facilitated continuous feeding pattern, suggesting that ingestion of A during the first test had a delayed sensitizing effect on subsequent ingestion of S during the second test of the experiment depicted in Fig. 2b. These results suggest that ingestion of nutritive and non-nutritive sugars yields delayed bi-directional effects that are required for the emergence of both facilitatory and suppressive behavioral contrast in Fig. 2b.

In a converse experiment, we allowed the flies to feed during the first test, but used a habituation protocol in the second. Fig. 3c shows that the first test replicated the finding that flies spent significantly higher number of bins feeding on S than A ($F(1,118) = 51.4$, $p < .001$, $\eta^2 = .30$). During the second test where the flies were not allowed to ingest, the proportion of flies making a PER in all four groups showed a similar fast-decreasing trend (Fig. 3d). Although the proportion of flies making a PER in group AS was higher throughout the session, the difference in the total number of proboscis extensions of four groups failed to reach significance ($F(3,116) = 2.4$, $p < .07$, Fig. 3e). Taken together, these results suggest that both immediate and delayed metabolic effects of post-ingestive nutritive sensing are necessary for the behavioral contrast observed in Fig. 2b.

5. Experiment 3

Under two choice assays where a high concentration non-nutritive sugar and a lower-concentration nutritive sugar are simultaneously available, wiltype flies prefer the less palatable nutritive sugar if they are fasted (Dus et al., 2013). The *Drosophila* mutant *cupcake* prefers sweeter non-nutritive L-glucose to lower-concentration nutritive D-glucose after fasting, suggesting that they fail to discriminate D- and L-glucose on the basis of their nutritive content using post-ingestive cues (Dus et al., 2013, 2015; Qi et al., 2015). In this section, we tested flies that carry two different mutant alleles of the *cupcake* gene, previously termed *dSLC5A11*^{1} (Bloomington Stock #22498) and *dSLC5A11*^{2} (Bloomington Stock #6768) to assay their feeding phenotype under the current behavioral paradigm.

5.1. Results and discussion

Each data set reported in this section was merged across two replications. Fig. 4a-b shows results that were obtained by merging the data set shown in Fig. 2a-b with a second replication where the wildtype flies were tested with 0.5 M S and 1 M A. Fig. 4c shows the proportion of *dSLC5A11*^{1} (Bloomington Stock #22498) flies feeding on 0.5 M S or 1 M A following 17 h of food deprivation. The proportion of flies feeding on A or S was not different on the first trial ($\chi^2(1) = 3.7$, $p < .06$), suggesting that the two sugars had similar palatability for *dSLC5A11*^{1} flies. Proportion of flies feeding on S remained close to a maximum throughout the session whereas that on A decreased roughly by 10% to yield a small but significant difference in total feeding for A and S ($F(1,217) = 20.0$, $p < .001$, $\eta^2 = .08$), indicating that *dSLC5A11*^{1} mutants were able to feed differentially on the two sugars after 17 h of food deprivation. Unlike the wildtype flies, however, the proportion of *dSLC5A11*^{1} mutants feeding on A failed to show a fast reduction by the end of the 3rd trial ($p < 1$). Mutant flies spent significantly higher number of bins feeding on both A and S relative to Canton-S flies ($F(1,435) = 244.7$, $p < .001$, $\eta^2 = .36$), yielding a distinctly hyperorexic phenotype.

Fig. 4d shows that in spite of their hyperorexic tendency, *dSLC5A11*^{1} mutants showed the wildtype-pattern of behavioral contrast during the second feeding test when the mutation was expressed in the BL#22498 genetic background. On average, *dSLC5A11*^{1} mutants spent higher number of bins feeding relative to the wildtype flies ($F(1, 431) = 48.3$, $p < .001$, $\eta^2 = .10$, Fig. 4g). Nevertheless, total number of bins with feeding differentiated significantly for the 4 groups ($F(3, 215) = 46.6$, $p < .001$, $\eta^2 = .39$), and feeding was suppressed selectively for group SA. A Scheffe analysis showed that feeding was facilitated for group AS relative to groups AA ($p < .02$) and SS ($p < .01$), suppressed for group SA relative to groups AA ($p < .001$) and SS ($p < .001$), and indistinguished between groups AA and SS ($p < 1$).

Fly strains show considerable variation with respect to sensory thresholds. In order to make sure that hyperorexia of *dSLC5A11*^{1} mutants was not the result of variation in the genetic background, we repeated the experiment after crossing *dSLC5A11*^{1} to the Canton-S background (using BL#5907). Fig. 4e shows that although being expressed on the Canton-S background meliorated the hyperorexia of *dSLC5A11*^{1} mutants, as the proportion of flies feeding still ran higher for the *dSLC5A11*^{1} mutants relative to the wildtype controls during the first test. An ANOVA showed that total feeding scores were different across wildtype and two strains of *dSLC5A11*^{1} flies during the first test ($F(2,658) = 94.0$, $p < .001$, $\eta^2 = .22$, compare Fig. 4a, c, and e), and a Scheffe analysis confirmed that hyperorexia of *dSLC5A11*^{1} mutants was moderated on the Canton-S (BL#5907) background relative to BL#22498 ($p < .001$), although they still spent higher number of bins with feeding relative to the wildtype flies ($p < .001$). Fasted *dSLC5A11*^{1} mutants spent fewer trials feeding on A than S when the mutation was expressed on the Canton-S background ($F(1,223) = 10.6$, $p < .001$, $\eta^2 = .045$), although this was a small effect that explained 4.5% of variance in total feeding. The proportion of flies feeding failed to show a rapid reduction within the first three trials when fasted *dSLC5A11*^{1} flies were tested on A ($\chi^2(1) = 1.2$, $p < .28$), such that the curves in Fig. 4e decreased in parallel when the flies were feeding on A or S, indicating that *dSLC5A11*^{1} flies failed to show differential rates of feeding suppression based on nutrient-sensing.

Expression of *dSLC5A11*^{1} on the Canton-S background ameliorated hyperorexia during the second test which enabled the differentiation of feeding in a wider dynamical range for the four groups ($F(3, 221) = 22.5$, $p < .001$, $\eta^2 = .23$, Fig. 4f). A Scheffe analysis showed that feeding scores of group AS was higher than those of group SS ($p < .002$) and marginally higher than those of group AA ($p < .047$). Total feeding scores of group SA were lower relative to those of groups AA ($p < .001$) and SS ($p < .001$). Importantly, rapid suppression of feeding recovered for group SA during the second test irrespective of genetic background: The proportion of flies feeding in group SA decreased significantly by the third bin when *dSLC5A11*^{1} was expressed on either BL#22498 ($\chi^2(1) = 9.9$, $p < .002$) or Canton-S backgrounds ($\chi^2(1) = 12.0$, $p < .001$). Taken together, these results show that *dSLC5A11*^{1} mutants do express both the immediate effects of nutrient sensing and delayed effects of nutrient feeding, although they also show a deficit in rapid termination of feeding on both nutritive and non-nutritive sugars when fasted.

Next, we tested the effects of another mutant allele, *dSLC5A11*^{2}, expressed both on its original BL#6768, and on Canton-S backgrounds. Fig. 5a shows that a higher proportion of *dSLC5A11*^{2} mutants (BL#6768) were feeding on S relative to A following 17 h of food deprivation ($F(1, 216) = 13.1$, $p < .001$, $\eta^2 = .06$), although this was a small effect that accounted for only 6% of the variance in total feeding. Interestingly, *dSLC5A11*^{2} mutants showed a slightly increasing trend in feeding on S, which is a pattern that we had not observed in other flies, and the proportion of flies feeding on A failed to show a significant change within the first three bins ($\chi^2(1) = 1.2$, $p < .28$). The hyperorexic tendency of *dSLC5A11*^{2} yielded significantly higher feeding scores relative to wildtype controls ($F(1, 434) = 86.9$, $p < .001$, $\eta^2 = .17$). During the second test, *dSLC5A11*^{2} (BL#6768) flies showed higher feeding scores ($F(1, 430) = 28.5$, $p < .001$, $\eta^2 = .06$) along with diminished behavioral contrast compared to the wildtype flies (Fig. 5b). The flat, slow-decreasing trend was evident for all groups whereby the proportion of flies feeding failed to change rapidly during the early trials in accordance with nutritive value. Although the group effect was significant ($F(3, 214) = 6.0$, $p < .001$, $\eta^2 = .08$, Fig. 5e), Scheffe analyses only confirmed the difference between groups SA and AS ($p < .001$). Notably, the proportion of flies feeding in group SA did not show any reduction at all within the first three bins ($\chi^2(1) = 0.5$, $p < .82$), showing that *dSLC5A11*^{2} flies showed defects in early feeding regulation during the second test as well.

Fig. 5c shows that when *dSLC5A11*^{2} mutation was expressed on a Canton-S background, flies spent fewer bins feeding on A than S after 17 h of food deprivation ($F(1, 224) = 17.0$, $p < .001$, $\eta^2 = .07$). Once again however, *dSLC5A11*^{2} flies failed to show a rapid suppression of

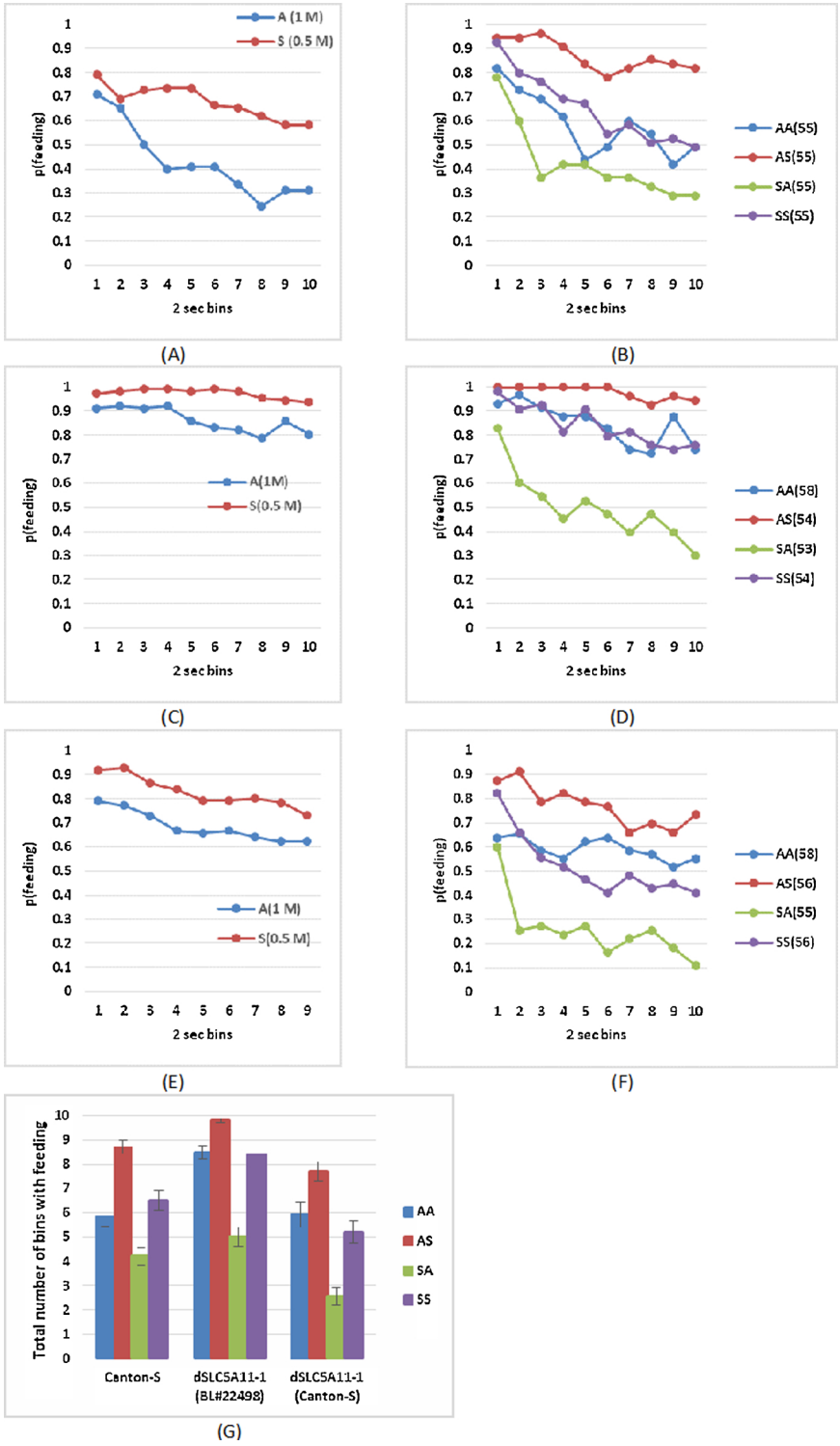

Fig. 4. Nutritive contrast in *dSLC5A11*^{1} flies. A. Proportion of Canton-S flies feeding on 1 M A or 0.5 M S during the first test. **B.** Proportion of Canton-S flies feeding on 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **C.** Proportion of *dSLC5A11*^{1} flies (BL#22498 background) feeding on 1 M A or 0.5 M S during the first test. **D.** Proportion of *dSLC5A11*^{1} flies (BL#22498 background) feeding on 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **E.** Proportion of *dSLC5A11*^{1} flies (BL#5907 background) feeding on 1 M A or 0.5 M S during the first test. **B.** Proportion of *dSLC5A11*^{1} flies (BL#5907 background) feeding on 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **G.** Total number of bins with feeding during the second test. Error bars show SEM. Number of flies in each group is indicated in parantheses.

feeding by the third trial ($\chi^2(1) = 2.23$, $p < .14$). During the second test, *dSLC5A11*^{2} mutants showed a surprising result where the proportion of flies feeding differentiated only with respect to the nutrient value of the currently ingested sugar, in a pattern that resembled the data set when the wildtype flies were not allowed to feed during the first test (compare Figs. 5d and 3 b). A two-way ANOVA showed an effect of feeding on A or S during the second test ($F(1, 222) = 19.2$, $p < .001$, $\eta^2 = .08$, Fig. 5d). There was, however, no effect of the nutrient value of the sugar used during the first test ($F(1, 222) = .09$, $p < .8$), i.e., *dSLC5A11*^{2} mutants failed to show the delayed effects of feeding on A or S 1 h earlier. In particular, both the facilitation of feeding for AS, and the suppression of feeding for SA were completely missing, and proportion of flies feeding in groups AA and SS failed to converge, showing that when expressed on a Canton-S background, *dSLC5A11*^{2} mutation prevented the delayed metabolic effects of nutrient ingestion on subsequent feeding decisions.

6. General discussion

In this study, we used a new feeding test under an experimental

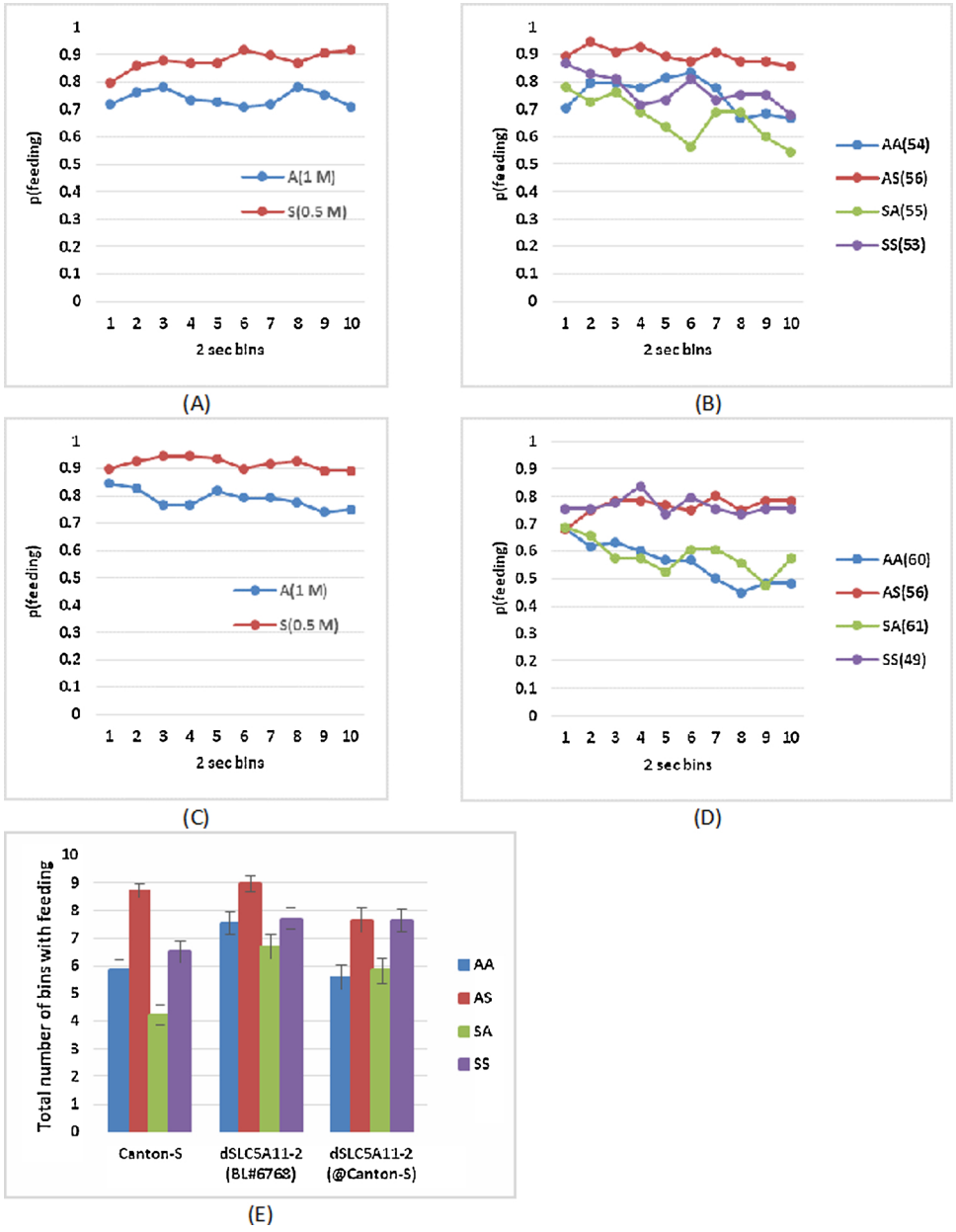

Fig. 5. Nutritive contrast in *dSLC5A11*^{2} flies. A. Proportion of *dSLC5A11*^{2} (BL#6768 background) flies feeding on 1 M A or 0.5 M S during the first test. **B.** Proportion of *dSLC5A11*^{2} (BL#6768 background) flies feeding on 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **C.** Proportion of *dSLC5A11*^{2} (BL#5907 background) flies feeding on 1 M A or 0.5 M S during the first test. **D.** Proportion of *dSLC5A11*^{2} (BL#5907 background) flies feeding on 1 M A (groups AA and SA) or 0.5 M S (groups SS and AS) during the second test. **E.** Total number of bins with feeding during the second test. Error bars show SEM. Number of flies in each group is indicated in parantheses.

design that allowed us to dissociate the immediate and 1-hour delayed effects of nutrient-feeding. We carried out two feeding tests spaced 1-hr apart and presented flies with one of four serial combinations of nutritive sucrose (S) and non-nutritive D-arabinose (A), namely AA, AS, SA and SS. The first test allowed us to measure the immediate effects of nutrient-sensing on feeding for fasted flies. The second test allowed us to measure how 1-hour delayed effects of ingesting nutritive or non-nutritive sugars interacted with the immediate effects of nutrient-sensing to change flies' decisions to terminate or sustain feeding.

6.1. *Fasted wildtype flies show rapid bi-directional feeding regulation within few seconds of ingestion*

Our results showed first and foremost that nutrient-sensing affects feeding decisions within a few seconds of ingestion. For example, this was observable in the first feeding tests of the fasted wildtype flies, where the proportion of flies feeding on A was significantly lower than that on S by the end of the third 2-second bin. Given that the behavioral code for the first bin was entered 2 s after the presentation of the sugar-dipped ball, it is safe to conclude that immediate effects of nutrient sensing made a significant impact on feeding decisions as early as 8 s of ingestion.

6.2. *Immediate and delayed effects of nutrient feeding act additively to change feeding decisions*

We tested each fly twice with 1 h interspaced between the two tests, which enabled us to dissociate the immediate and delayed effects of ingesting nutrient and non-nutrient sugars during the second test. Immediate effects of nutrient-sensing rapidly produced either a suppression or facilitation of feeding for A and S, respectively. Delayed effects of former nutrient ingestion, on the other hand, changed average feeding activity whereby flies that had formerly ingested S 1 h ago fed less than those that had ingested A, which can readily be explained by relative satiety.

An analysis of the difference in the feeding patterns of groups AA, AS, SA and SS showed that rapid nutrient-sensing for the currently ingested sugar and the delayed nutritive effects of the previously ingested

sugar had additive effects on feeding decisions during the second test, suggesting that they can be dissociated. For example, group SA that was presented non-nutritive A 1 h after feeding on nutritive S showed suppression of feeding on A relative to group SS that was equally sated, or group AA that was presented the same non-nutritive sugar. Conversely, group AS that was presented nutritive S 1 h after feeding on non-nutritive A showed facilitation of feeding on S relative to group SS that was feeding on the same nutrient-sugar, or group AA that was equally hungry.

6.3. Fasted dSLC5A11 (cupcake) mutants show hyperorexia and a failure of feeding termination

In this study, we found that the feeding phenotype of *dSLC5A11* mutants varies with the allele and genetic background (Chandler et al., 2013; Yoshihara and Yoshihara, 2018). Nevertheless, the following three properties were invariably observed when fasted *dSLC5A11* flies received the first feeding test, irrespective of the mutant allele or the genetic background: First and foremost, both $dSLC5A11^{1}$ and $dSLC5A11^{2}$ mutants showed marked hyperorexia relative to the wild-type controls, irrespective of the nutritive value of sugar. Second, when the flies were fasted, the rate of decline in the proportion of flies feeding was noticeably slower for *dSLC5A11* mutants relative to the wildtype controls. In particular, rapid suppression of feeding on A was conspicuously absent for both *dSLC5A11* mutants. Further yet, proportion of $dSLC5A11^{2}$ flies feeding on S increased over the 20-second period in spite of the potential down regulating effects of gut distention. Finally, within the 20 s test period, proportion of flies feeding on A was always lower than that on S, showing that fasted *dSLC5A11* mutants can feed differentially on nutrient and non-nutrient sugars. Taken together, these results suggest that fasted *dSLC5A11* mutants have a deficit in short-term regulation of feeding for both nutritive and non-nutritive compounds. In particular, their temporal pattern of feeding is suggestive of a defect in feeding termination after fasting.

The deficit of *dSLC5A11* mutants in rapid feeding suppression is likely to have contributed to their previously reported failure to prefer nutritive sugars under two-choice paradigms (Dus et al., 2013). Hyperorexia that results from a deficit in short-term regulation of feeding can bias preference scores in a two-choice test even if the flies are capable of discriminating sugars based on their nutritive value using post-ingestive cues. If flies fail to rapidly terminate feeding on non-nutritive sugars, they will be physically full and incapable of further ingestion by the time they resume foraging to locate the nutritive-sugar. It follows that the rapid termination of feeding based on nutrient-sensing is necessary for differential consumption of nutritive sugars, and a failure of this behavioral switch will bias preference in favor of higher concentration sugars, as may be the case for fasted *dSLC5A11*.

6.4. Sated $dSLC5A11^{2}$ flies **show** a genetic-background dependent deficit in delayed effects of nutrient feeding

During the second feeding test, *dSLC5A11* mutants showed variable feeding phenotypes that changed with both the mutant allele and the genetic background (Chandler et al., 2013; Yoshihara and Yoshihara, 2018).

When the $dSLC5A11^{1}$ allele was expressed on the original BL#22498 background, we observed the wildtype pattern of feeding differentiation for groups AA, AS, SA and SS in spite of their overeating tendency. Expression of the same mutant allele, $dSLC5A11^{1}$, on the Canton-S background ameliorated hyperorexia during the second test which enabled the differentiation of feeding in a wider dynamical range for the four groups. Our results suggested that $dSLC5A11^{1}$ flies were capable of expressing both immediate effects of nutrient-sensing and delayed effects of nutrient-feeding.

Flies that carry the $dSLC5A11^{2}$ allelle, on the other hand, showed a more complex feeding phenotype in a genetic-background dependent manner. When we tested the original BL#6768 strain, all four groups exhibited high levels of feeding which allowed limited differentiation with respect to the nutritive value of the sugar ingested during either the first or the second feeding test. We obtained a very surprising result when $dSLC5A11^{2}$ was expressed on the Canton-S background whereby feeding was differentiated only with respect to the nutritive value of the currently ingested sugar. The curves that showed the proportion of flies feeding segregated as if sated flies were feeding de novo with virtually no evidence for a delayed metabolic effect of the previously ingested sugar. Although feeding curves for A and S showed a steady segregating trend throughout the session, there was no evidence for rapid feeding termination on A whether or not the flies were sated (SA) or fasted (AA). Finally, both groups AS and SS showed a slightly increasing, rather than a satiating trend in feeding. These results suggest that the $dSLC5A11^{2}$ allele yielded both a failure of short term feeding regulation and a deficit in using delayed metabolic effects of nutrient ingestion for feeding decisions when it was expressed on the Canton-S background.

Our results are compatible with the previously reported feeding phenotype of *dSLC5A11* mutants, as four differences in experimental protocol might account for the discrepancies in results: First of all, we used sucrose as a nutritive sugar, whereas D-glucose had been used as the nutritive agent in the original experiments that had identified *dSLC5A11* as a nutrient sensor (Dus et al., 2013). In a follow up study, $dSLC5A11^{1}$ mutants were shown to be able to discriminate D-fructose and L-fructose via the activity of internal Gr43a receptors (Qi et al., 2016). Given that sucrose can rapidly be hydrolyzed to glucose and fructose, it is possible that testing the flies with sucrose failed to disclose the glucose-specific deficit in nutrient-sensing. Second, nutrient ingestion might affect feeding behavior in different time scales, through direct activation by nutrients (e.g., binding of fructose to internal Gr43a receptors), or delayed activity of metabolites. Hence the duration of feeding tests potentially changes the involvement of multiple nutrient-sensing mechanisms that have dissociable effects on feeding decisions. *dSLC5A11* has been identified as a nutrient sensor using a two-choice feeding assay that lasted 2 h, which is long enough to disclose the inability of $dSLC5A11^{2}$ mutants to use delayed metabolic nutrient cues for feeding regulation (Dus et al., 2013). Third, the results of two-choice assays are reported as preference indices which do not reflect the absolute amount of ingestion. When short-duration manual feeding assays are used to measure the amount of ingested sugar, $dSLC5A11^{1}$ mutants have been shown to consume equally high amounts of nutritive and non-nutritive glucose enantiomers, suggesting that they displayed a deficit in terminating feeding for the non-nutritive sugar (Qi et al., 2015). Finally, we calibrated the concentration of nutritive S and non-nutritive A to yield equivalent palatability, whereas higher concentrations of non-nutritive sugars were used in earlier studies. Given that feeding initiation is triggered by palatability, this difference in experimental protocols might also have contributed to the higher preference for non-nutritive compounds in earlier studies.

dSLC5A11 gene encodes a Na^{+}/solute co-transporter that is expressed in the ellipsoid body R4 ring neurons. The ellipsoid body of the insect brain is suggested to be a higher-order sensorimotor integration center where input from different modalities converge with those from the internal milieu to control behavioral transitions and action selection (Strausfeld and Hirth, 2013). Higher order evaluative processing prevents animals from inflexible and repetitive responding vis a vis external stimuli (Mesulam, 2000). We suggest that *dSLC5A11* gene might function like a gate in R4 neurons to integrate the internal state with external stimulus value to control action selection, or to trigger a behavioral transition. Given the multi-modal integrative nature of feeding decisions, and the complex distributed processes involved in action selection, it is not surprising that *dSLC5A11* mutants exhibit genetic background dependent variations in behavioral phenotype (Chandler et al., 2013). Further studies are necessary for a more detailed assessment of the behavioral space of *dSLC5A11* mutants to understand how R4 neurons mediate selection, maintenance or termination of appetitive responses.

7. Epilogue

Give me a fish and I eat for a day. Teach me to fish and I eat for a lifetime. Bill Timberlake was a great mentor who taught his students to fish. His lab was a thing to remember as it was full of one of a kind experimental boxes that he himself designed to study species-typical behavior of rats. Bill did not work on fruit flies, but being a polymath he followed the work, foresaw the importance of the fly as a model organism for behavioral analysis, and invited fruit fly researchers as speakers to introduce them to his students. That's how one of us (M.Ö. Çevik) got to know about fly neurogenetics when she was Bill's graduate student. We are grateful for having the opportunity to join the Timberlake festschrift with a new behavioral test, hoping that the skills we inherited from Bill will be of good use for research on the new lab rat, the venerable fruit fly.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2019.04.016.

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