Video Article

The CApillary FEeder Assay Measures Food Intake in *Drosophila melanogaster*

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Abstract

For most animals, feeding is an essential behavior for securing survival, and it influences development, locomotion, health and reproduction. Ingestion of the right type and quantity of food therefore has a major influence on quality of life. Research on feeding behavior focuses on the underlying processes that ensure actual feeding and unravels the role of factors regulating internal energy homeostasis and the neuronal bases of decision-making. The model organism *Drosophila melanogaster*, with its great variety of genetically traceable tools for labeling and manipulating single neurons, allows mapping of neuronal networks and identification of molecular signaling cascades involved in the regulation of food intake. This report demonstrates the CApillary FEeder assay (CAFE) and shows how to measure food intake in a group of flies for time spans ranging from hours to days. This easy-to-use assay consists of glass capillaries filled with liquid food that flies can freely access and feed on. Food consumption in the assay is accurately determined using simple measurement tools. Herein we describe step-by-step the method from setup to successful execution of the CAFE assay, and provide practical examples to analyze the food intake of a group of flies under controlled conditions. The reader is guided through possible limitations of the assay, and advantages and disadvantages of the method compared to other feeding assays in *D. melanogaster* are evaluated.

Video Link

The video component of this article can be found at https://www.jove.com/video/55024/

Introduction

Eating is essential; however, deregulation of food intake resulting in eating disorders such as bulimia, anorexia or the general tendency to overeat imposes costs on individuals and society[1,2,3]. The goal of the present research is to uncover regulatory mechanisms of food intake and to provide a strategy for circumventing disorder formation. Numerous studies using mammalian model organisms have provided new insights of the circuitry and the role of signaling systems in eating disorders[4,5,6]. Nevertheless, our knowledge of the neuronal and molecular bases underlying these disorders remains far from complete. In recent years, the fruit fly *Drosophila melanogaster* has become a valuable model system for unraveling basic mechanistic insights into the regulation of metabolism[7,8,9]. The CApillary FEeder (CAFE) assay for *Drosophila melanogaster* was established in the lab of Seymour Benzer in 2007 inspired by an earlier work by Dethier in blowfly[10, 11]. The CAFE assay made it possible to directly measure food intake in *Drosophila melanogaster*. In this behavioral test system, flies feed on liquid food provided in graded glass capillaries placed inside a vial. The decline of the capillary meniscus indicates loss of food solution *via* evaporation and food consumption. Determining the evaporation rate by vials without flies allows the accurate quantification of food intake.

The CAFE assay is one of several behavioral paradigms used to measure feeding in *Drosophila melanogaster* and researchers have to choose the most appropriate one for their specific question. The decision to use a certain assay should consider the following points: the nature of the food provided; the feeding condition; the measurement of intake or uptake of nutrients and investigation food consumption or response to food.

The CAFE assay as described in this report is ideal for following food intake of a liquid food source under an upright feeding condition. Alternatively the food intake can be measured for a fly group on a colored food source in a vial or on a plate. Flies are normally killed or anesthetized after feeding and the amount of ingested dye is determined by spectrometry or visual inspection of the stained abdomen. Flies start to excrete the ingested food only 30 min after intake, therefore this approach is difficult to use for the analysis of continuous longer feeding behaviors[12, 13].

In contrast flies are kept intact when absorbable dyes with radioactive tracers are used and their consumption of radioisotope is scored in a scintillation counter[14, 15]. Absorption of the radiolabel by the fly digestive system makes long-term food uptake measurement possible, but might lead to underestimation of consumption because of non-absorbed and excreted tracer molecules. Another approach to measure response to food in *Drosophila melanogaster* is the proboscis extension response (PER), which normally occurs for food intake[16]. This elegant method measures the initial response to a food stimulus but does not record the quantity of intake. Food intake is dynamically adjusted during feeding

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using several post-digestive feedback signals that are critical for the regulation of feeding[17, 18]. Several attempts have been made in recent years to semi-automate data collection in the PER assay[19, 20]. The PER is detected by an electric pad or a combination of electrodes and counted via computer. Combining the PER assay with radioisotope uptake revealed that this assay is limited by low sensitivity to detecting quantity feeding differences[18]. The manual feeding assay (MAFE)[21], in which a fly is fed manually with a glass capillary, was recently developed to measure food uptake in a single immobilized fly. The MAFE assay eliminates the interferences of foraging and feeding initiation and has a time resolution of seconds, and initiation of PER and food consumption can be monitored independently in the assay. However, the way in which immobilization of the fly affects certain aspects of feeding behavior (*e.g.* locomotion, motivation) still needs to be investigated. For excellent comparative reviews of different assays for measuring food consumption in *Drosophila melanogaster* and to help researchers finding the most appropriate one, see reports by Deshpande and Marx [13, 22].

The CAFE assay avoids some of the disadvantages of other assays described above and combines simplicity of use with reliable measurement of food intake. Here, a detailed description of the CAFE assay is provided and we show a simple setup modification to reduce evaporation. Representative results including a two food choice assay (short and long term) and the sucrose uptake of flies is demonstrated. In the discussion we compare our described method with alternative ways to perform the CAFE assay, and highlight potential limitations.

Protocol

1. The CAFE Assay

NOTE: The assay consists of three components: an experimental vial, a specific lid and micro- capillaries. A plastic box with cover is used to transport the prepared vials and to control the humidity more efficiently.

1. Use a *Drosophila melanogaster* culture plastic vial (optional 8 cm height, 3.3 cm diameter) as a tube for the assay.
2. Seal the vial with a manufactured Plexiglas lid containing an O-ring (**Figures 1A, 1B**). Load flies by tapping or with a blowpipe through the lid's central opening (0.9 cm diameter), which also allows for air circulation and water supply, and close the hole with a sponge bung. Six smaller conical openings (0.4 cm upper diameter, 0.3 cm inner diameter) surround the central hole and fit the pipette tips of 2 - 20 µL volume to hold the capillaries in place. (see supplementary figures for technical details of the lid.)
NOTE: The use of a sponge stopper with openings for the capillaries instead of the custom-made lid used in our manuscript is possible. Our customized lid allows safe handling of the prepared vials minimizing the risk of capillaries falling down.
3. To present the liquid food, use 5 µL microcapillaries with 1 µL marks. Position the capillaries in the conical openings in the lid by cutting off the top of a 2 - 20 µL pipette tip and inserting the tip into the hole (**Figure 1B**, marked with red edge). To prevent flies from escaping, insert an uncut 2 - 20 µL pipette tip into the same opening.
4. To safely handle multiple prepared vials, place them into a plastic box with a gridded inlay (**Figure 2A**).

2. Preparation of Flies

1. Keep flies on standard food at 25 °C, 60% relative humidity and a 12 h/12 h light-dark cycle.
2. To control breeding conditions, introduce 35 virgin females and 15 males for each experimental group into a plastic culture vial (9.8 cm height, 4.8 cm diameter) containing 50 mL fly food. Allow flies to lay eggs for the first 3 days, then transfer adult flies to fresh food vials and let them lay eggs for two more days. After this repeat the transfer again. Discard adult flies after 2 more days.
3. As food intake is dependent on fly size, determine the weight of a group of 100 flies by anesthetizing 2- to 3-day-old adult flies using a CO_2 fly pad and collect them into a 1.5 mL plastic tube and measuring with a standard laboratory scale. Determine the wet weight of at least four independent fly groups sorted by sex (**Table 1**); use the weight to calculate µL food consumption per mg fly. Use the value to determine the amount of food that a single fly feeds per experiment and adjust the number of food-filled capillaries accordingly to avoid emptying of the capillaries by feeding.
    1. For a 3 h assay, use 20 flies and two filled capillaries. For a long-term experiment (> 3 h and up to 9 days), use a group of eight flies with a supply of four filled capillaries (reliable results cannot be obtained with less than eight flies under the described conditions).
4. Separate flies into groups (8 or 20 flies) after measuring weight under CO_2 exposure. Transfer the group to a new food vial (containing 15 mL standard food) to allow recovery from CO_2 sedation for 48 h prior to the experiment. Use 4- to 6-day-old flies for the CAFE assay.
5. As non-starved wild-type flies feed only marginally[19, 21], pre-starve flies for 3 h feeding experiments. No fasting is required when food consumption is monitored over several days. For fasting, transfer flies 16 to 20 h prior to testing by gently tapping them into a vial containing only a 45-mm diameter folded filter paper moistened with ~0.5 mL ddH_2O (double-distilled water), and close with a plugged CAFE assay lid.

3. Preparation of Liquid Food

1. Prepare a 3 M (10%, w/v) sucrose stock solution by filling 102.6 g sucrose ($C_{12}H_{22}O_{11}$) to 100 mL ddH_2O. Pipette 3 µL, 33 µL, 333 µL, 3.3 mL and 6.6 mL of the stock solution into a 15 mL plastic tube; add 2 mL of food color (for red: Cochineal [E124]; for blue: Indigo carmine [E132]) and fill to 10 mL with ddH_2O. The resulting concentrations are 0.001, 0.01, 0.1, 1, and 2 M sucrose.
NOTE: The food dye allows visualizing the meniscus more easily. However the dye might have an impact on the food intake. To avoid a bias due to the dye dispense the food dye or randomized the usage of dyes to the food samples during the experiment and groups.
2. To test for alcohol preference pipette 333 µL of the 3 M sucrose stock solution in a 15 mL plastic tube. Add 1.5 mL (2.3 mL) of 100% EtOH (ethanol) and add ddH_2O up to 10 mL to result in 15% (0.25 mM) and a 23% (0.39 mM) working solutions.
3. Keep stock solutions at -20 °C and working solutions at 4 °C; use within 1 week.
4. Fill up to 10 capillaries at the same time with a colored food solution, by capillary force. Insert the ends of the capillaries into the sucrose solution (holding the capillaries at a 45° angle to the solution). Stop if the liquid reaches the top (5 µL) mark of the capillary, and remove excess solution on the outside and inside with tissue paper.

4. Assembly and Performing the CApillary FEeder Assay

1. If fasting is not needed, transfer the experimental flies to the assay by tapping or by blow-pipe. Make sure to include three control vials without flies to quantify evaporation.
2. Carefully remove a pipette tip (2 - 20 µL volume) that is closing one of the outer openings, and insert a filled glass capillary, bottom-end first. Secure the capillary by placing the pipette tip back next to the capillary. If several food solutions are being tested, repeat this procedure accordingly.
3. Place the capillary ends inside all vials at the same level to avoid bias that could occur if the food sources were located at different heights (3 - 4 cm from the lid); keep a distance to the filter paper to prevent the capillary from leaking by accidently touching the filter paper or different viscosities of food sources.
4. Label the upper end of the colored liquid using a marker pen ($mark_{beginning}$). To ensure the different capillaries can be identified, label them individually using a color or stripe code.
5. Place multiple prepared CAFE assays inside a plastic box with gridded inlay and transfer the box (**Figure 2A**) to a secure position under laboratory conditions or in a temperature-, light- and humidity-controlled climate chamber (parameters: 25 °C, 60% relative humidity, 12 h/12 h light-dark cycle) for the experimental period (*e.g.* 3 h or days).
6. As bottom filter paper dries out if the assay is performed over several days, apply fresh water every 24 h *via* the sponge bung (100 µL) to keep humidity constant inside the assay. Use four separate vials (8 cm height, 3.3 cm diameter) filled with 30 mL ddH_2O as humidity devices and place them next to the CAFE assays in the plastic box. Use a cover for the plastic box to create humidity controlled environment during the experiment (**Figure 2A**).
   NOTE: Broader variability occurs under laboratory conditions; however, it is feasible to perform the CAFE assay at room temperature (*e.g.*, in a classroom). The use of a humidification device (filter paper, with or without a wet sponge bung, filled water vials and cover for the plastic box) is highly encouraged to decrease evaporation (**Figure 2B**).
7. Replace the capillaries with freshly filled ones for long term experiments every 24 h. Make note of dead flies before each 24 h interval and use the number of live flies to calculate consumption per fly for the following period. Discard the old capillaries after measuring the decline of the meniscus (see 5.1).
   NOTE: During a 3 h experiment we hardly see any dead flies. During a 4 days study we usually find 1 - 3 dead flies.
8. At the end of the assay or before replacing the capillary, mark the lower meniscus of the capillary ($mark_{end}$) with a marker pen while the CAFE assay is still in the upright position. Discard the data if $mark_{end}$ is not below the initial mark ($mark_{beginning}$). Do not remove the lid, as this might change the meniscus.
9. Carefully remove the capillaries from the assay and store them for data collection. Check if the liquid inside the capillary reached the lower end if not discard the data, as food was not accessible to the flies. Collect all capillaries per vial as a group. Insert uncut pipette tips into all openings to prevent flies from escaping. Dismantle the setup and wash the vials, lids and sponge bungs in a soap bath and dry overnight at room temperature for further use.
   NOTE: Flies can be further analyzed after the assay. Confirm food uptake by eye or under a dissection microscope.
10. Repeat experiments with the same genotypes on at least three different days.

5. Data Collection and Analysis

1. Measure the distance between $mark_{beginning}$ and $mark_{end}$ on the capillary using a caliper or a ruler. To transfer data directly to a spreadsheet, use a USB (Universal Serial Bus) connected digital caliper (**Figure 1E**). Discard the capillaries after the measurement.
2. Account for capillary size to calculate food uptake or evaporation. For example, consider a capillary that is 73 mm long and contains 5 µL of food solution. A 14.6 mm decrease in the meniscus reflects the uptake of 1 µL solution. Calculate food uptake using the following formula:
   Food uptake (µL) = measured distance (mm)/ 14.6 mm
3. To exclude the effect of evaporation on food intake, calculate mean evaporation in the three (at minimum) control vials without flies. Subtract this mean value from the value obtained for food consumption by the flies.
4. Use the following formula to determine total consumption per fly:
   Food consumption (µL) = (Food uptake [µL] - Evaporative loss [µL])/total number of flies in the vial. For long-term experiments use the number of flies alive before the start of the 24 h interval.
5. To account for differences in body size, such as between male and female flies, normalize food consumption to body weight (µL food/mg fly).
6. Use statistical software for data analysis. For normally distributed data, use student's *T*-tests to determine differences between two fly groups, and use ANOVA (analysis of variance) with post hoc Tukey Cramer tests for more than two groups. In a choice situation, analyze differences from random choice using a nonparametric one-sample sign test.

Representative Results

Flies of the w^{1118} genotype are used to demonstrate how the assay is performed. The w^{1118} mutants are commonly used to generate transgenic lines and to control the genetic background of transgenes marked with the *white* gene. Normally, for behavioral experiments, all transgenic lines are backcrossed for five generations to the same w^{1118} stock, which is used as an experimental control. We show different experiments: a comparison of evaporation loss for our modified setup, a short-term food choice experiment, a long-term food intake experiment, and an experiment on different sucrose dilutions.

Evaporation plays a critical role in the performance of the CAFE assay. We included additional approaches to our assay to decrease evaporation: i) the central sponge bung is refilled with water every 24 h; ii) additional water filled vials within the transport box and iii) the use of a cover for the box to create a humidity enclosure (see 4.6). Comparing the evaporation between a setup without and with above mentioned devices, a significant reduction in evaporation is seen. Even the effect of higher volatility of an ethanol containing solution is not detectable using the new setup.

In a two-choice food experiment a group of 20 flies can feed for 3 h. In natural environments, fruit flies feed preferentially on fermenting fruits with alcohol[22], and it has been shown, using a similar setup, that flies prefer yeast-sucrose solutions with ethanol over yeast-sucrose solutions without ethanol[23]. Here, two food choices are offered, a 0.1 M sucrose solution labeled with red food color and a 0.1 M sucrose solution with 15% EtOH labeled with blue food color (**Figure 1A, C**). Visual examination of the abdomen indicates that the flies feed on both solutions (**Figure 1D**). Food consumption per fly is significantly greater (nearly 2-fold) for the sucrose solution containing EtOH (**Figure 3A**).

In a following experiment, a long-term study, a group of eight flies has access to similar food sources for 4 days, and flies consume more of the ethanol-containing food on each day (**Figure 3B**). The preference index for ethanol ([Suc + EtOH] - [Suc]/total consumption) remains constant over this period (average = 0.29, **Table 4**). The observed ethanol preference is consistent with several other publications and shows that flies can distinguish between different food sources[24,25,26]. The observed ethanol attraction might be a result of the different caloric contents of the offered solutions and of the rewarding properties of ethanol[24]. The assay can also be used to measure negative effects of food supplements. Ja and colleagues showed in the first publication of this method that application of paraquat (an oxidizing drug) decreases food consumption[10].

In the next experiment, the difference in food intake between the sexes is shown. Metabolic requirements differ between male and female *D. melanogaster*. For example, while male flies prefer carbohydrate-rich food, during egg production, a phase that requires increased protein biosynthesis, females prefer protein-rich diets over carbohydrate-rich diets[27]. Mated male and female flies were used in this experiment. To analyze differences in food intake between 20 male and 20 female flies within a 3 h feeding interval, a CAFE assay is performed using a sucrose concentration series. Five capillaries were provided, with solutions ranging from 10^{-3} to 2 M sucrose, and consumption of each solution was measured (**Figure 4A**). Results showed that both sexes preferred high-concentration sucrose solutions as a food source (**Figure 4A**). However, females consumed significantly more of the two lowest-concentration sucrose solutions compared to males ($P < 0.05$); on the other hand, males consumed significantly more of the higher-concentration solutions ($P < 0.001$). Note that these data did not account for differences in body size. Female *D. melanogaster* are usually larger and heavier than males (**Table 1**). When food consumption is normalized to fly mass, differences between males and females in consumption of low-sucrose solutions are no longer significant. In summary, males consume more sucrose solution than mated females, consistent with previous data, reflecting possible different metabolic demands, nutrient preferences or simple differences in the ability to feed on the capillaries between the two sexes.

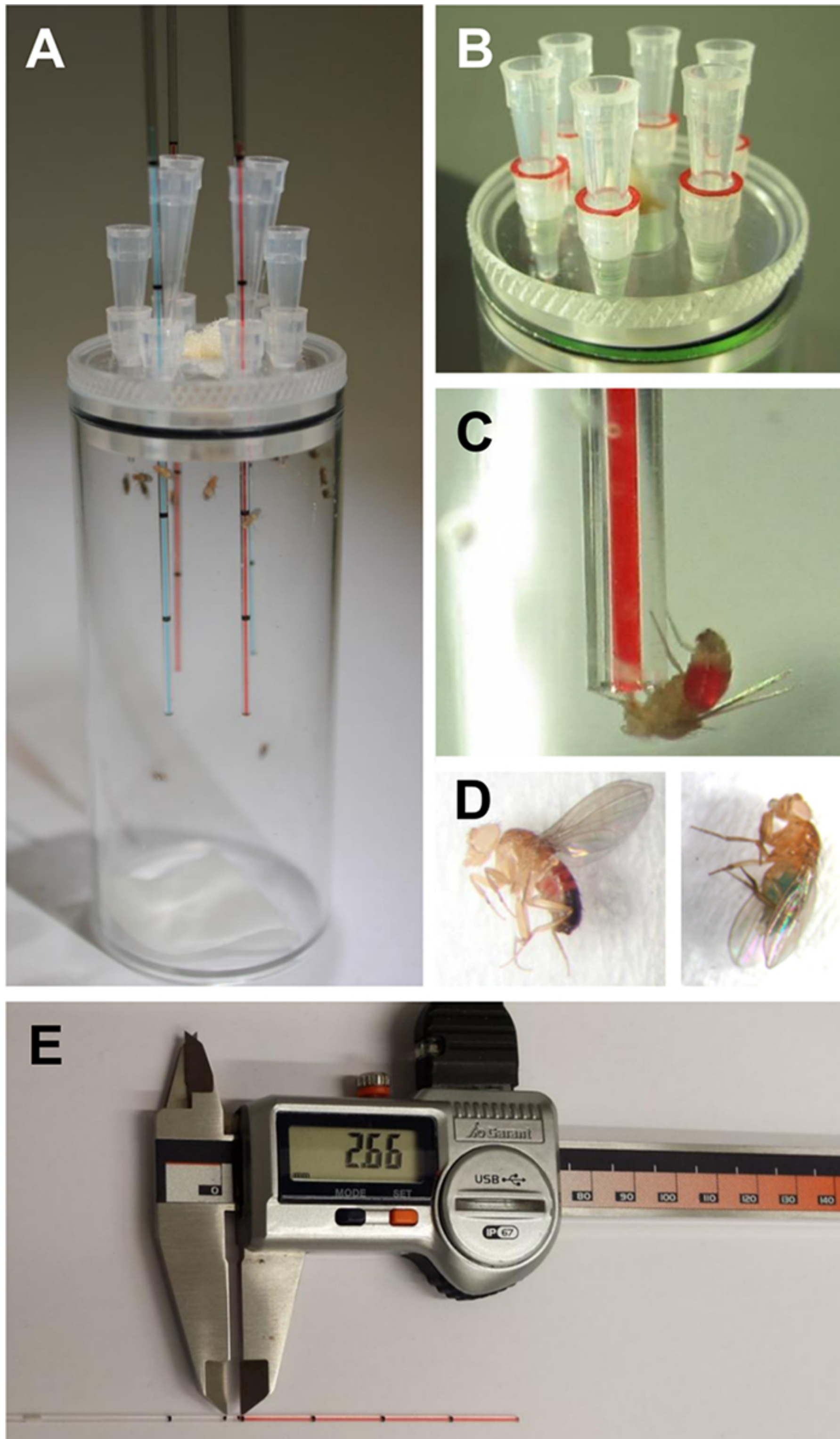

Figure 1: The *Drosophila melanogaster* CApillary FEeder Assay. A) The feeding assay with flies. Moistened filter paper provides water at the bottom of the vial. Four capillaries are provided during the experiment (red- and blue-colored food in opposite capillaries). Note that the capillaries are secured in position by a second pipette tip, and unused positions are closed using pipette tips. A foam plug in the center of the lid allows air exchange. **B)** Detailed view of the lid. Cut pipette tips (2 - 20 µL, red borders) are inserted into the conical openings of unused positions, and a second pipette tip is inserted into the cut tip to close the hole. The cut pipette tips are used to control placement of the microcapillaries, and uncut tips are used to hold the capillaries tight. **C)** A *D. melanogaster* fly feeds on a capillary. **D)** After feeding, food color is clearly visible in the fly abdomen. **E)** A digital caliper is used to measure the distance between $mark_{beginning}$ and $mark_{end}$ of the meniscus. The data are transferred directly to an Excel spreadsheet via USB. Please click here to view a larger version of this figure.

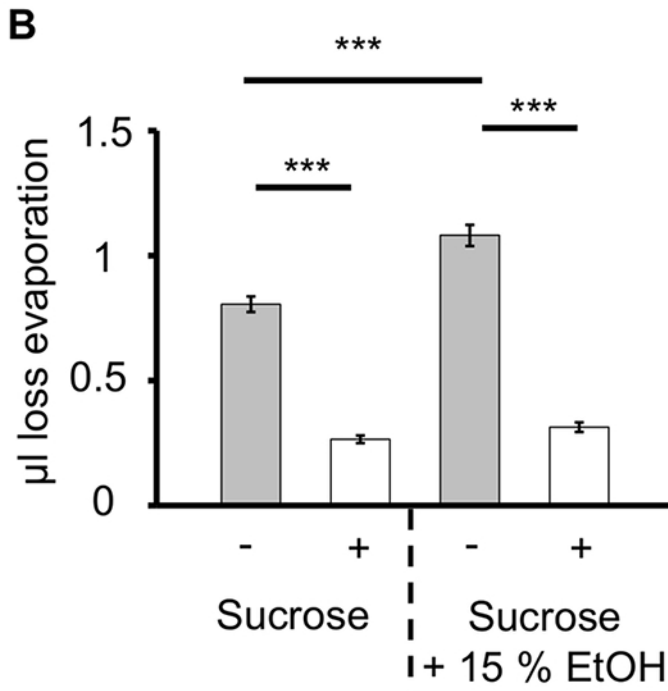

Figure 2: Influence of Evaporation in the Capillary Feeder Assay. A) Multiple CAFE assay placed inside a plastic box with a gridded inlay. For controlling the humidity during the experiment four water filled vials (red rims) are placed inside the grid. The evaporation controls are placed in direct proximity to these vials. A cover for the whole setup is shown in the background. **B)** Comparison of the volume loss through evaporation. The mean value for evaporation over 4 days is shown. Humidity is controlled by (i) applying water to the central sponge bung (24 h interval); (ii) adding four water filled vials into the grid; and (iii) using a plastic cover for the whole setup. The evaporation is significantly lower if humidity is controlled for both solutions tested (***$P \leq 0.001$; N = 48). No differences in volatility between EtOH containing and non-containing sucrose solution is detectable with the humidity devices used. Please click here to view a larger version of this figure.

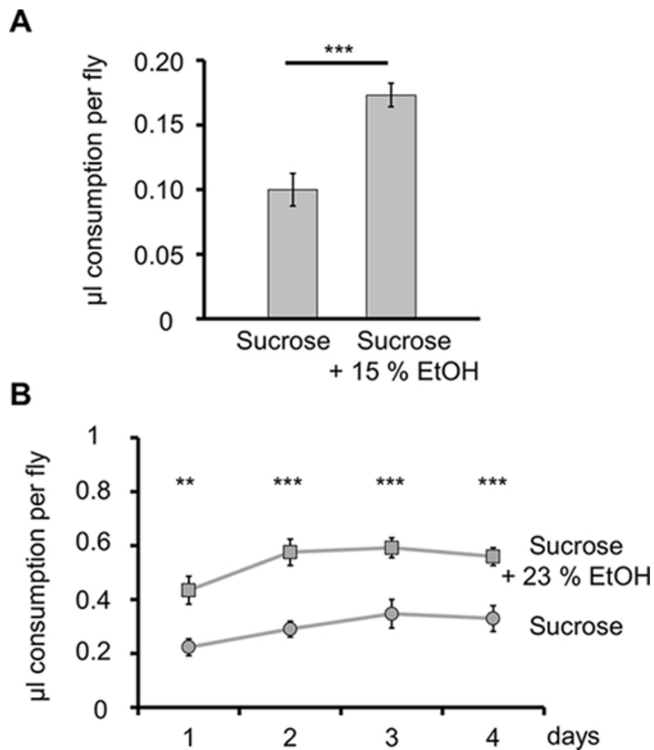

Figure 3: Preference for Ethanol (EtOH) Containing Sucrose over Sucrose Solution. A) Food consumption for male w^{1118} flies is shown. Males consume significantly more of a 15% EtOH containing sucrose solution than of a plain sucrose solution. ***$P \le 0.001$; $N = 27$. **B)** Flies significantly prefer a sucrose solution containing 23% EtOH during a 4-day trial. ***$P \le 0.001$; **$P \le 0.01$; $N = 16$. Please click here to view a larger version of this figure.

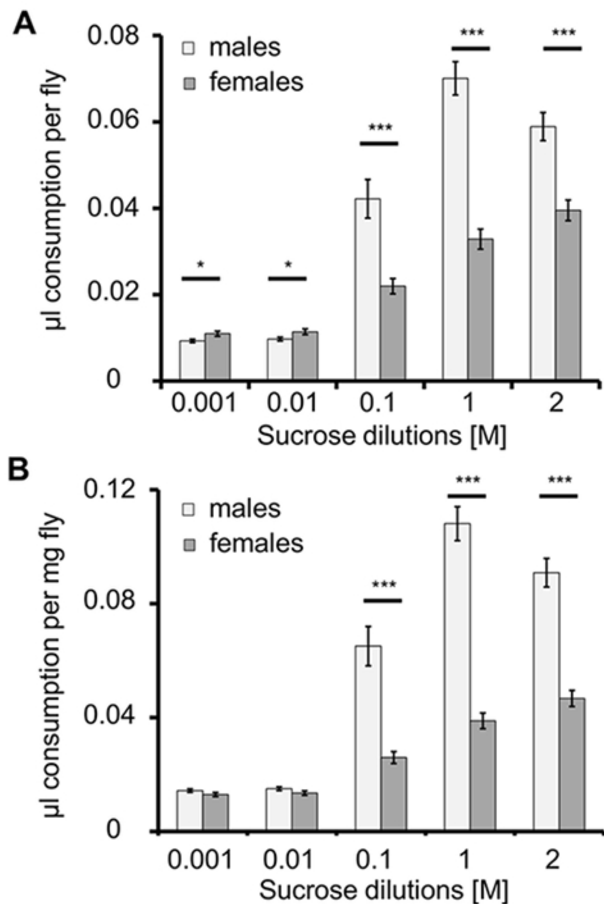

Figure 4: Consumption (µL/fly and µL/mg fly) of Different Sucrose Concentrations by Male and Female w^{1118} Flies. A) The consumption of different concentrations of sucrose solutions differs significantly between males and females. Female flies consume more at lower sucrose concentrations, and male flies consume more at higher concentrations. *$P < 0.05$; ***$P < 0.001$; N = 27 trials with 20 males each, N = 30 trials with 20 females each). **B)** Food uptake on a mass basis. A significant increase in consumption occurs between male and female flies for the 0.1 to 2 M sucrose solutions when normalized to fly mass. ***$P \leq 0.001$; N = 27 males, N = 30 females. Please click here to view a larger version of this figure.

Genotype (sex)	Number of flies	Weight per fly [mg]
w ^{1118} (male)	100	0.645
w ^{1118} (male)	100	0.640
w ^{1118} (male)	100	0.642
w ^{1118} (male)	100	0.702
w ^{1118} (male)	100	0.613
	MEAN	**0.648**
	STDEV	**0.033**
	STERROR	**0.015**
w ^{1118} (female)	100	0.822
w ^{1118} (female)	100	0.870
w ^{1118} (female)	100	0.848
w ^{1118} (female)	100	0.845
	MEAN	**0.846**
	STDEV	**0.020**
	STERROR	**0.010**

Table 1: Body Weight of Male and Female *w*^{1118} Flies. Four to five groups of 100 flies were measured, and body weight (mg/fly) was calculated. Mean values (with STDEV (standard deviation) and STERROR (standard error)) are shown. Mean values are used to normalize food consumption to fly mass (µL/mg fly). Please click here to download this spreadsheet.

Day	5 % Suc / -	5 % Suc / +	5 % Suc + 15 % EtOH / -	5 % Suc + 15 % EtOH/ +
Day 1 [µl loss]	0.768	0.408	1.220	0.399
Day 1 [µl loss]	0.764	0.286	1.049	0.368
Day 1 [µl loss]	0.750	0.210	1.196	0.198
Day 1 [µl loss]	0.761	0.600	1.379	0.200
Day 1 [µl loss]	0.673	0.247	1.266	0.589
Day 1 [µl loss]	1.031	0.500	1.050	0.541
Day 1 [µl loss]	0.778	0.156	1.377	0.140
Day 1 [µl loss]	1.014	0.270	1.118	0.350
Day 1 [µl loss]	1.314	0.073	1.103	0.268
Day 1 [µl loss]	0.780	0.348	1.266	0.193
Day 1 [µl loss]	0.854	0.148	1.050	0.268
Day 1 [µl loss]	0.953	0.117	1.758	0.242
Mean Day 1	0.870	0.280	1.236	0.313
Day 2 [µl loss]	0.708	0.165	1.406	0.288
Day 2 [µl loss]	0.708	0.203	1.634	0.395
Day 2 [µl loss]	0.621	0.412	1.216	0.463
Day 2 [µl loss]	0.847	0.186	1.285	0.434
Day 2 [µl loss]	0.768	0.294	1.168	0.231
Day 2 [µl loss]	1.097	0.230	1.682	0.275
Day 2 [µl loss]	0.659	0.316	0.929	0.421
Day 2 [µl loss]	0.763	0.307	1.029	0.287
Day 2 [µl loss]	0.631	0.311	0.936	0.259
Day 2 [µl loss]	0.671	0.252	1.072	0.353
Day 2 [µl loss]	0.618	0.297	1.148	0.676
Day 2 [µl loss]	1.045	0.216	0.614	0.478
Mean Day 2	0.761	0.266	1.177	0.380
Day 3 [µl loss]	0.855	0.165	1.454	0.273
Day 3 [µl loss]	0.961	0.094	1.151	0.386
Day 3 [µl loss]	0.787	0.248	1.059	0.156
Day 3 [µl loss]	0.999	0.315	0.583	0.397
Day 3 [µl loss]	0.758	0.203	0.983	0.226
Day 3 [µl loss]	0.725	0.229	1.042	0.215
Day 3 [µl loss]	0.748	0.196	1.358	0.145
Day 3 [µl loss]	1.549	0.370	1.043	0.226
Day 3 [µl loss]	0.914	0.258	1.052	0.468
Day 3 [µl loss]	0.707	0.175	1.129	0.203
Day 3 [µl loss]	0.868	0.371	0.648	0.313
Day 3 [µl loss]	0.677	0.236	0.418	0.300
Mean Day 3	0.879	0.238	0.993	0.276
Day 4 [µl loss]	0.810	0.215	1.322	0.225
Day 4 [µl loss]	1.060	0.261	0.845	0.133
Day 4 [µl loss]	0.823	0.298	1.099	0.365
Day 4 [µl loss]	0.824	0.430	1.233	0.172
Day 4 [µl loss]	0.795	0.101	0.768	0.494
Day 4 [µl loss]	0.809	0.365	0.642	0.338
Day 4 [µl loss]	0.708	0.242	0.879	0.333
Day 4 [µl loss]	0.215	0.237	0.680	0.190
Day 4 [µl loss]	0.851	0.221	0.421	0.165
Day 4 [µl loss]	0.827	0.125	0.995	0.177
Day 4 [µl loss]	0.195	0.422	0.821	0.310
Day 4 [µl loss]	0.634	0.403	1.342	0.553
Mean Day 4	0.713	0.277	0.920	0.288
Mean	0.806	0.265	1.082	0.314
STDEV	0.217	0.109	0.298	0.129
STERROR	0.031	0.016	0.043	0.019

Day 1	0.001 M Suc	0.01 M Suc	0.1 M Suc	1 M Suc	2 M Suc
Vial 1 [µl loss]	0.105	0.091	0.100	0.014	0.000
Vial 2 [µl loss]	0.110	0.145	0.118	0.002	0.000
Vial 3 [µl loss]	0.112	0.097	0.045	0.021	0.000
Mean	0.11	0.11	0.09	0.01	0.00
Day 2	0.001 M Suc	0.01 M Suc	0.1 M Suc	1 M Suc	2 M Suc
Vial 1 [µl loss]	0.046	0.086	0.099	0.000	0.000
Vial 2 [µl loss]	0.077	0.065	0.045	0.000	0.000
Vial 3 [µl loss]	0.092	0.069	0.062	0.000	0.000
Mean	0.07	0.07	0.07	0.00	0.00
Day 3	0.001 M Suc	0.01 M Suc	0.1 M Suc	1 M Suc	2 M Suc
Vial 1 [µl loss]	0.120	0.080	0.096	0.002	0.000
Vial 2 [µl loss]	0.061	0.072	0.054	0.000	0.000
Vial 3 [µl loss]	0.038	0.053	0.049	0.000	0.000
Mean	0.07	0.07	0.07	0.00	0.00

Table 2: Evaporation Loss (µL) in the CAFE Assay. The quantity of liquid lost through evaporation is shown for 4 days. Humidity is controlled (+) or not (-) as described in **Figure 2**. Evaporation data for two different solutions (sucrose and sucrose plus EtOH) are shown. Mean values are presented for each day and over the period (with STDEV and STERROR). The evaporation loss of the sucrose dilutions experiment is shown underneath separately (mean values). Please click here to download this spreadsheet.

Day 1	Genotype (sex)	Number of flies	µl [Suc]	µl [Suc+EtOH]	Total (µl)	µl [Suc] per fly	µl [Suc+EtOH] per fly
1	*white* ^{1118} (male)	20	0.14	3.36	3.49	0.01	0.17
1	*white* ^{1118} (male)	20	0.41	3.97	4.38	0.02	0.20
1	*white* ^{1118} (male)	20	0.62	2.53	3.15	0.03	0.13
1	*white* ^{1118} (male)	20	0.96	2.81	3.77	0.05	0.14
1	*white* ^{1118} (male)	20	0.96	3.29	4.25	0.05	0.16
1	*white* ^{1118} (male)	18	0.96	2.33	3.29	0.05	0.13
1	*white* ^{1118} (male)	20	2.05	2.67	4.73	0.10	0.13
1	*white* ^{1118} (male)	20	0.55	3.84	4.38	0.03	0.19
1	*white* ^{1118} (male)	20	0.75	2.74	3.49	0.04	0.14
2	*white* ^{1118} (male)	20	0.82	1.99	2.81	0.04	0.10
2	*white* ^{1118} (male)	19	1.51	3.90	5.41	0.08	0.21
2	*white* ^{1118} (male)	20	2.26	4.79	7.05	0.11	0.24
2	*white* ^{1118} (male)	20	1.71	3.49	5.21	0.09	0.17
2	*white* ^{1118} (male)	20	2.05	3.42	5.48	0.10	0.17
2	*white* ^{1118} (male)	30	1.71	6.58	8.29	0.06	0.22
3	*white* ^{1118} (male)	11	2.74	3.90	6.64	0.25	0.35
3	*white* ^{1118} (male)	20	4.04	3.22	7.26	0.20	0.16
3	*white* ^{1118} (male)	20	4.11	3.42	7.53	0.21	0.17
3	*white* ^{1118} (male)	20	2.05	3.77	5.82	0.10	0.19
3	*white* ^{1118} (male)	22	2.60	3.42	6.03	0.12	0.16
3	*white* ^{1118} (male)	20	4.73	3.22	7.95	0.24	0.16
3	*white* ^{1118} (male)	20	2.60	3.63	6.23	0.13	0.18
3	*white* ^{1118} (male)	20	1.92	3.36	5.27	0.10	0.17
3	*white* ^{1118} (male)	19	3.08	3.36	6.44	0.16	0.18
3	*white* ^{1118} (male)	20	1.58	3.42	5.00	0.08	0.17
3	*white* ^{1118} (male)	20	2.67	2.74	5.41	0.13	0.14
3	*white* ^{1118} (male)	20	2.60	3.01	5.62	0.13	0.15
		Group Mean	1.93	3.41	Individual Mean	0.10	0.17
		STDEV	1.18	0.86	STDEV	0.07	0.05
		STERROR	0.23	0.17	STERROR	0.01	0.01

Table 3: Consumption of 0.1 M Sucrose with/ without 15% EtOH by Male *w*^{1118} Flies Fed for 3 h. Consumption of both solutions by groups of 20 flies was measured for 3 h on 3 days. Consumption values for fly groups are divided by the number of tested flies to estimate microliter uptake per fly after subtracting evaporative loss. Mean values (with STDEV and STERROR) are shown. Please click here to download this spreadsheet.

Table 4: Consumption of 0.1 M Sucrose With and Without 23% EtOH for Four Days by Male *w*^{1118} Flies. Consumption of both solutions by groups of 8 flies was measured for 24 h for 4 days. Preference index for ethanol was calculated by using the following formula ([Suc + EtOH] - [Suc]/total consumption). Consumption values for fly groups are divided by the number of tested flies to estimate µL uptake per fly after subtracting evaporative loss. Mean values (with STDEV and STERROR) are shown for each day. Please click here to download this spreadsheet.

Day	Genotype (sex)	0.001 M Suc [µl]	0.01 M Suc [µl]	0.1 M Suc [µl]	1 M Suc [µl]	2 M Suc [µl]	total uptake [µl]	0.001 M Suc [µl/mg fly]	0.01 M Suc [µl/mg fly]	0.1 M Suc [µl/mg fly]	1 M Suc [µl/mg fly]	2 M Suc [µl/mg fly]
1	w^{1118} (male)	0.009	0.006	0.050	0.065	0.033	0.162	0.014	0.012	0.077	0.100	0.046
1	w^{1118} (male)	0.009	0.013	0.096	0.040	0.087	0.245	0.014	0.020	0.148	0.063	0.134
1	w^{1118} (male)	0.007	0.008	0.042	0.089	0.061	0.207	0.011	0.013	0.064	0.137	0.094
1	w^{1118} (male)	0.008	0.007	0.027	0.063	0.047	0.154	0.013	0.011	0.042	0.097	0.073
1	w^{1118} (male)	0.008	0.010	0.076	0.070	0.059	0.223	0.013	0.015	0.117	0.108	0.092
1	w^{1118} (male)	0.008	0.013	0.084	0.047	0.075	0.226	0.012	0.020	0.130	0.072	0.115
1	w^{1118} (male)	0.011	0.010	0.015	0.089	0.054	0.178	0.017	0.015	0.022	0.137	0.083
1	w^{1118} (male)	0.008	0.006	0.029	0.057	0.053	0.152	0.012	0.010	0.044	0.087	0.081
1	w^{1118} (male)	0.013	0.007	0.031	0.091	0.047	0.190	0.020	0.012	0.048	0.140	0.073
2	w^{1118} (male)	0.008	0.010	0.030	0.071	0.085	0.204	0.013	0.015	0.046	0.109	0.131
2	w^{1118} (male)	0.008	0.008	0.029	0.091	0.052	0.189	0.013	0.013	0.045	0.141	0.080
2	w^{1118} (male)	0.009	0.008	0.083	0.065	0.089	0.255	0.015	0.012	0.128	0.101	0.137
2	w^{1118} (male)	0.012	0.009	0.050	0.115	0.076	0.262	0.019	0.014	0.077	0.177	0.117
2	w^{1118} (male)	0.011	0.014	0.045	0.070	0.050	0.189	0.017	0.021	0.069	0.108	0.078
2	w^{1118} (male)	0.008	0.009	0.043	0.064	0.069	0.193	0.012	0.014	0.066	0.098	0.106
2	w^{1118} (male)	0.013	0.015	0.027	0.062	0.066	0.182	0.019	0.023	0.042	0.095	0.101
2	w^{1118} (male)	0.011	0.011	0.039	0.048	0.060	0.170	0.017	0.016	0.061	0.075	0.093
2	w^{1118} (male)	0.010	0.010	0.041	0.076	0.045	0.182	0.015	0.016	0.063	0.117	0.069
3	w^{1118} (male)	0.010	0.009	0.020	0.044	0.083	0.165	0.015	0.014	0.031	0.068	0.127
3	w^{1118} (male)	0.008	0.008	0.081	0.058	0.075	0.230	0.012	0.012	0.125	0.090	0.116
3	w^{1118} (male)	0.010	0.009	0.039	0.063	0.063	0.184	0.016	0.013	0.060	0.098	0.097
3	w^{1118} (male)	0.005	0.007	0.035	0.123	0.034	0.203	0.008	0.010	0.053	0.189	0.053
3	w^{1118} (male)	0.005	0.012	0.014	0.066	0.044	0.141	0.008	0.018	0.021	0.102	0.068
3	w^{1118} (male)	0.009	0.010	0.015	0.085	0.029	0.147	0.014	0.015	0.023	0.131	0.045
3	w^{1118} (male)	0.011	0.009	0.052	0.052	0.063	0.186	0.016	0.014	0.080	0.080	0.098
3	w^{1118} (male)	0.012	0.010	0.033	0.078	0.045	0.178	0.018	0.015	0.051	0.120	0.070
3	w^{1118} (male)	0.011	0.012	0.014	0.052	0.049	0.138	0.017	0.018	0.022	0.081	0.076
	Mean	0.009	0.010	0.042	0.070	0.059	0.190	0.014	0.015	0.065	0.108	0.091
	STDEV	0.002	0.002	0.023	0.020	0.017	0.033	0.004	0.004	0.036	0.031	0.026
	STERROR	0.000	0.000	0.004	0.004	0.003	0.006	0.001	0.001	0.007	0.006	0.005

Day	Genotype (sex)	0,001 M	0,01 M	0,1 M	1 M	2 M	total uptake	0,001 M	0,01 M	0,1 M	1 M	2 M
1	w^{1118} (female)	0.011	0.016	0.036	0.048	0.045	0.156	0.013	0.019	0.042	0.057	0.053
1	w^{1118} (female)	0.009	0.010	0.021	0.016	0.042	0.096	0.010	0.012	0.024	0.019	0.049
1	w^{1118} (female)	0.018	0.013	0.018	0.036	0.060	0.145	0.021	0.015	0.021	0.043	0.071
1	w^{1118} (female)	0.012	0.013	0.018	0.041	0.039	0.123	0.014	0.015	0.021	0.049	0.046
1	w^{1118} (female)	0.014	0.015	0.022	0.026	0.042	0.119	0.017	0.018	0.026	0.031	0.049
1	w^{1118} (female)	0.010	0.010	0.028	0.015	0.044	0.107	0.011	0.012	0.033	0.018	0.052
1	w^{1118} (female)	0.012	0.013	0.039	0.031	0.023	0.118	0.014	0.015	0.046	0.037	0.028
1	w^{1118} (female)	0.009	0.008	0.021	0.020	0.034	0.091	0.010	0.009	0.024	0.024	0.040
2	w^{1118} (female)	0.012	0.014	0.013	0.018	0.037	0.094	0.014	0.016	0.015	0.022	0.044
2	w^{1118} (female)	0.009	0.016	0.021	0.052	0.050	0.148	0.010	0.019	0.025	0.062	0.060
2	w^{1118} (female)	0.019	0.019	0.019	0.051	0.027	0.135	0.022	0.023	0.023	0.060	0.032
2	w^{1118} (female)	0.013	0.014	0.029	0.053	0.054	0.162	0.015	0.017	0.034	0.062	0.064
2	w^{1118} (female)	0.017	0.012	0.030	0.056	0.052	0.167	0.020	0.014	0.035	0.066	0.062
2	w^{1118} (female)	0.014	0.017	0.021	0.009	0.032	0.092	0.016	0.020	0.024	0.011	0.038
2	w^{1118} (female)	0.017	0.019	0.032	0.034	0.064	0.168	0.020	0.023	0.038	0.040	0.075
3	w^{1118} (female)	0.011	0.011	0.009	0.023	0.043	0.098	0.013	0.013	0.010	0.027	0.050
3	w^{1118} (female)	0.013	0.008	0.007	0.046	0.017	0.091	0.015	0.009	0.008	0.054	0.021
3	w^{1118} (female)	0.009	0.010	0.013	0.035	0.054	0.120	0.011	0.012	0.015	0.041	0.064
3	w^{1118} (female)	0.008	0.007	0.008	0.043	0.040	0.106	0.010	0.008	0.009	0.051	0.048
3	w^{1118} (female)	0.007	0.007	0.008	0.026	0.044	0.092	0.008	0.008	0.010	0.031	0.052
3	w^{1118} (female)	0.011	0.012	0.018	0.046	0.037	0.123	0.013	0.014	0.021	0.054	0.044
3	w^{1118} (female)	0.010	0.011	0.012	0.031	0.054	0.118	0.012	0.013	0.014	0.037	0.064
4	w^{1118} (female)	0.009	0.010	0.018	0.019	0.016	0.072	0.011	0.012	0.021	0.022	0.019
4	w^{1118} (female)	0.008	0.012	0.029	0.040	0.034	0.124	0.010	0.014	0.034	0.047	0.041
4	w^{1118} (female)	0.009	0.008	0.036	0.026	0.055	0.133	0.010	0.009	0.042	0.031	0.065
4	w^{1118} (female)	0.008	0.007	0.032	0.030	0.032	0.109	0.010	0.008	0.038	0.035	0.038
4	w^{1118} (female)	0.008	0.009	0.020	0.019	0.048	0.104	0.009	0.010	0.024	0.023	0.057
4	w^{1118} (female)	0.009	0.009	0.013	0.033	0.021	0.085	0.010	0.011	0.015	0.039	0.025
4	w^{1118} (female)	0.008	0.006	0.041	0.025	0.018	0.097	0.010	0.007	0.048	0.030	0.021
4	w^{1118} (female)	0.009	0.009	0.030	0.035	0.026	0.109	0.010	0.010	0.035	0.041	0.031
	Mean	0.01	0.01	0.02	0.03	0.04	0.12	0.01	0.01	0.03	0.04	0.05
	STDEV	0.00	0.00	0.01	0.01	0.01	0.03	0.00	0.00	0.01	0.02	0.02
	STERROR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Genotype (sex)	quantity	total weight [gr]	weight per fly [mg]
w^{1118} (male)	100	0.065	0.645
w^{1118} (male)	100	0.064	0.640
w^{1118} (male)	100	0.064	0.642
w^{1118} (male)	100	0.070	0.702
w^{1118} (male)	100	0.061	0.613
	Mean		0.648
	STDEV		0.033
	STERROR		0.015

Genotype (sex)	quantity	total weight [gr]	weight per fly [mg]
w^{1118} (female)	100	0.082	0.822
w^{1118} (female)	100	0.087	0.870
w^{1118} (female)	100	0.085	0.848
w^{1118} (female)	100	0.085	0.845
	Mean		0.846
	STDEV		0.020
	STERROR		0.010

Table 5: Consumption of Five Concentrations of Sucrose by Male and Female w^{1118} Flies. Intake of each solution, and the value for the sum of sucrose intakes, is shown. Mean values for each concentration are given below each column (with STDEV and STERROR). To calculate intake based on fly mass (microliter uptake per milligram of fly), food consumption is divided by the average weight of male or female flies (from **Table 1**, shown to the right). Please click here to download this spreadsheet.

Discussion

The report describes the CAFE assay in a step-by-step fashion, focusing on the technical setup and its successful performance in the laboratory. Due to its simplicity, this assay could also be used educationally as a school experiment. The examples show that the assay allows investigation of food sensing, preference and consumption in *Drosophila melanogaster* over short and longer time periods (hours to days). The CAFE assay has been used widely in the field to investigate subjects including food and drug consumption, addictions, energy homeostasis and neuronal control of feeding[16, 18, 24, 25].

In the CAFE assay, experimental flies must successfully perform several tasks to obtain food, such as foraging, sensing and locomotion; inability to perform these tasks might result in reduced consumption. Foraging behavior depends mainly on the hunger state of the flies and can be increased by fasting[19, 21]. Sensing, and thereby localization, of the food source can be influenced by the ability of the fly to smell or taste and might indirectly result in a lower consumption rate[28]. The display of the food at the end of a capillary forces the fly to climb down and actively hold itself in an upside-down position to feed. To hold the drinking position on the capillary, the fly must coordinate its muscle contraction. Impairment or hyperactivity of locomotion clearly would affect food uptake, as do locomotion deficiencies due to ageing. In addition, interference by other flies during this maneuver leads to premature termination of food ingestion. Therefore, the number of flies to be used should be determined prior to the experiment. This number should ensure that all flies can feed properly and should control for fly density in the vial (from 8 up to a maximum of 20 flies in our *D. melanogaster* CAFE assay vial). Feeding is influenced by the nutritional value of the meal, and flies dynamically adjust their ingestion accordingly[24, 29]. It was shown that mutants missing the neurotransmitter octopamine have normal PER response scores but at the

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same time show a significant decrease in food intake[14]. Furthermore, during feeding, the motivation to continue eating decreases and leads to termination of the behavior.

The above-mentioned considerations apply not only to the CAFE assay, they influence feeding behavior measured in other test systems as well. Therefore, the ability of flies to perform the assay must be taken into account when measuring food intake. Although it is not technically challenging, the CAFE assay has some potential practical drawbacks. The decline of the meniscus inside the capillary depends on evaporation loss and food intake by the flies. High evaporation is problematic regarding the signal to noise ratio and should therefore be minimized. We applied several additional approaches and devices to control the humidity during the experimental period (see 4.6). These accessories helped us to reduce the evaporation significantly and even eliminated effects of different volatility of the food sources we used. Nevertheless, if no climate chamber is available the assay can be performed at room temperature (*e.g.* in a classroom) with higher evaporation values as a drawback.

As mentioned in the protocol, the ends of the capillaries need to be placed at the same level inside the vial to avoid bias in the fly's choice due to different distances to the food source. To achieve this, the capillary position is fixed with a second pipette tip. The length of the capillary appears not to be a criterion for feeding in wild-type flies[10]. Any spillage of the liquid can undermine accurate readout of food consumption (see 4.3 and 4.9); a vibration-free environment prevents spills. Particles in the solution block capillary flow and prevent food consumption. The food solution, especially if it contains yeast, needs to be completely dissolved to avoid such a blockage. The use of water soluble yeast extract can overcome this problem but as an incomplete source of nutrition it may cause additional fitness costs. Food accessibility needs to be evaluated before and after the experiment. The only fly data that should be included in the analysis is that obtained where access to food was present during the entire experiment (see 4.9). The upside-down feeding position is a critical feature of the experiment. Under natural conditions, this feeding position is not unfamiliar to the fly, as fruits hang down from trees and they might climb down a rotten fruit. This is supported by experiments comparing the meal sizes of flies feeding in an upside-down position in the CAFE assay to (i) a horizontal eating position of immobilized flies in the MAFE assay and (ii) a right-side-up feeding position using radiolabeled food[13, 21]. Although the upside-down food display does not seem to be an issue for the flies, it could affect the composition of food inside the capillary. Suspended supplements such as yeast cells could sink via gravity to the bottom of the capillary and therefore might be more concentrated at the bottom or might plug the capillary. This would influence fly behavior and thus the results. Ensuring that the components of the feeding solution are completely dissolved, and frequently introducing fresh capillaries during long-term experiments, minimizes this influence on food intake.

The use of the CAFE assay described here allows measurement of food intake in a fly group over time spans of hours or days. If more detailed analysis is required (*e.g.*, the behavior of a single fly or behavior in the range of minutes), other feeding assays, such as the MAFE assay, are more appropriate. It might be possible for the number of flies to be further reduced by using a 1.5 mL microcentrifuge tube and a single capillary[30].

The number of experiments used to obtain the representative results varies from 15 to 27, consistent with experiments described in the literature[17, 24]. The assay can be performed in a classic blind fashion that rules out potential bias from the experimenter, and it is normally repeated at least four to five times on each of several days. Data obtained with the CAFE assay can be normalized to body weight to account for differences in feeding behavior related to body size. The results obtained with this assay are robust and reproducible, so that it has been introduced successfully in practical courses for graduate students.

The CAFE assay is widely used in the field of metabolic and taste research in *Drosophila melanogaster*; it has multiple applications in testing the role of food supplements and/or drugs on feeding behavior, and it can be used to investigate the dose response to a specific food source[24]. In combination with the remarkable variety of techniques used to manipulate neuronal circuitry in *D. melanogaster*, this assay also allows researchers to investigate the role of reinforcement systems on feeding behavior[12, 17, 18].

Disclosures

The authors have nothing to disclose.

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