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The CApillary FEeder assay measures food intake in Drosophila melanogaster

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Corresponding Author:	Soeren Diegelmann Albertus-Magnus University Cologne Cologne, GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	sdiegelm@uni-koeln.de
Corresponding Author's Institution:	Albertus-Magnus University Cologne
Corresponding Author's Secondary Institution:	
First Author:	Soeren Diegelmann
First Author Secondary Information:	
Other Authors:	Annika Jansen
	Shreyas Jois
	Katharina Kastenholz
	Laura Velo Escarcena
	Nicole Strudthoff
	Henrike Scholz
Order of Authors Secondary Information:	
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.

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TITLE:
The CApillary FEeder assay measures food intake in *Drosophila melanogaster*

AUTHORS:
Soeren Diegelmann[1]
sdiegelm@uni-koeln.de

Annika Jansen[1]
ajansen@smail.uni-koeln.de

Shreyas Jois[2]
svj582@mail.usask.ca

Katharina Kastenholz[1]
kkastenh@smail.uni-koeln.de

Laura Velo Escarcena[1]
laura.velo@uni-koeln.de

Nicole Strudthoff[1]
nicole.strudthoff@web.de

Henrike Scholz[1]
henrike.scholz@uni-koeln.de

1) Institute of Zoology
Albertus-Magnus University Cologne
Cologne, Germany

2) Department of Veterinary Biomedical Sciences
Western College of Veterinary Medicine
University of Saskatchewan
Saskatoon, Canada

CORRESPONDING AUTHORS:
Soeren Diegelmann; sdiegelm@uni-koeln.de
Henrike Scholz; henrike.scholz@uni-koeln.de

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SHORT ABSTRACT:
The CApillary FEeder (CAFE) assay is a simple, budget-friendly, highly reliable method for

investigating mechanisms underlying food intake. Used with the highly versatile genetic model organism *Drosophila melanogaster*, it provides a powerful means of gaining new insights into regulatory mechanisms of food intake.

LONG 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.

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[1–3]. 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[4–6]. 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[7–9]. 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 minutes 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 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.

1.1. Use a *Drosophila melanogaster* culture plastic vial (optional 8 cm height, 3.3 cm diameter) as a tube for the assay.

1.2. 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.

1.3. 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.

1.4. To safely handle multiple prepared vials, place them into a plastic box with a gridded inlay (Figure 2A).

2. **Preparation of flies**

2.1. Keep flies on standard food at 25 °C, 60% relative humidity and a 12 h/12 h light–dark cycle.

2.2. 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.

2.3. 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.

2.3.1. 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).

2.4. 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.

2.5. 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**

3.1. 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.

3.2. 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.

3.3. Keep stock solutions at –20 °C and working solutions at 4 °C; use within 1 week.

3.4. 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**

4.1. 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.

4.2. 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.

4.3. 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.

4.4. 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.

4.5. 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).

4.6. 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).

4.7. 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.

4.8. 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.

4.9. 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.

4.10. Repeat experiments with the same genotypes on at least three different days.

5. **Data collection and analysis**

5.1. 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.

5.2. 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

5.3. 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.

5.4. 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.

5.5. To account for differences in body size, such as between male and female flies, normalize food consumption to body weight (µL food/mg fly).

5.6. 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[24–26]. 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.

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.

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. ***$P \leq 0.001$; $N = 27$. B) Flies significantly prefer a sucrose solution containing 23% EtOH during a 4-day trial. ***$P \leq 0.001$; **$P \leq 0.01$; $N = 16$.

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.

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).

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).

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.

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.

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).

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 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].

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

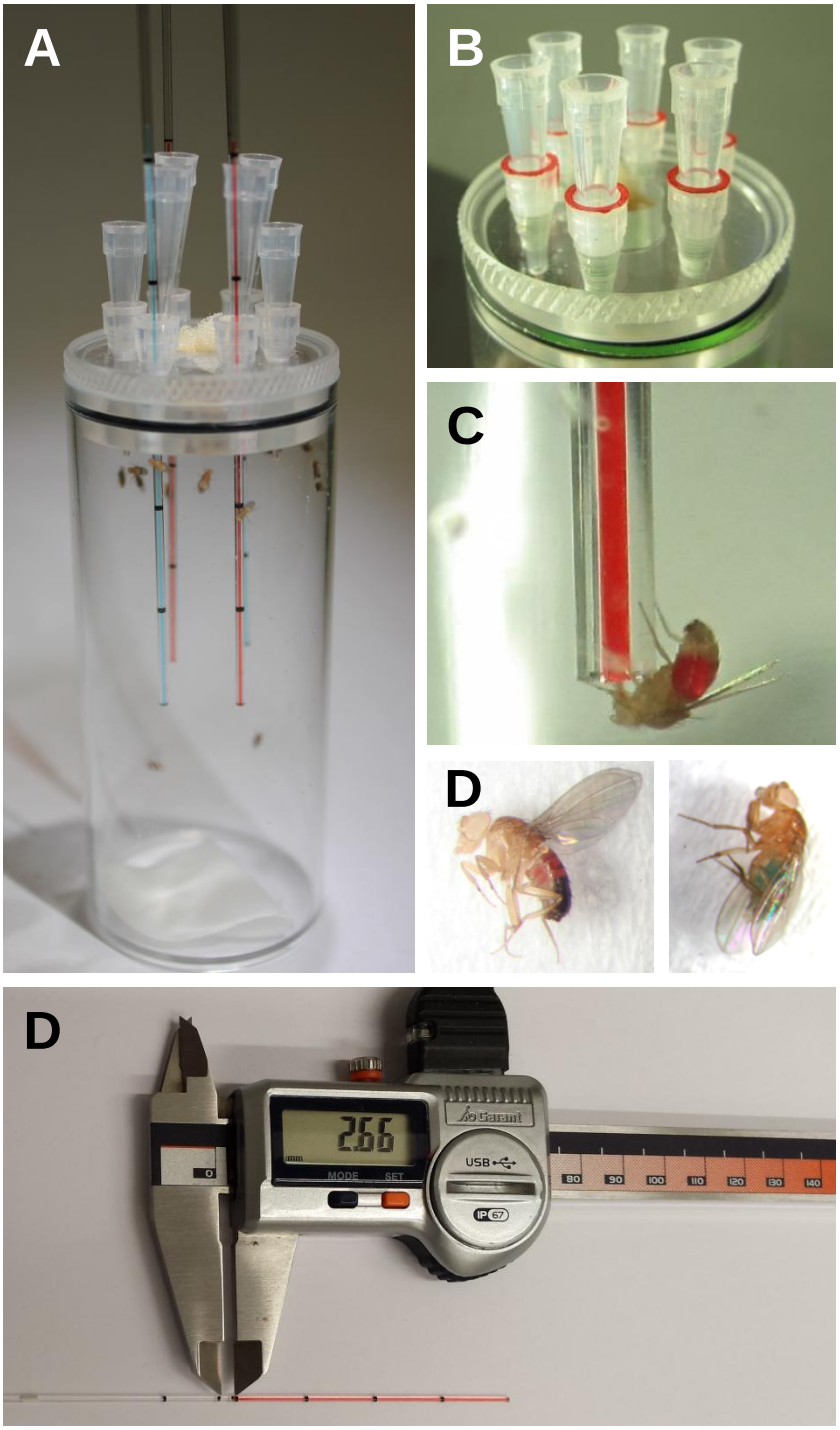

Figure 2

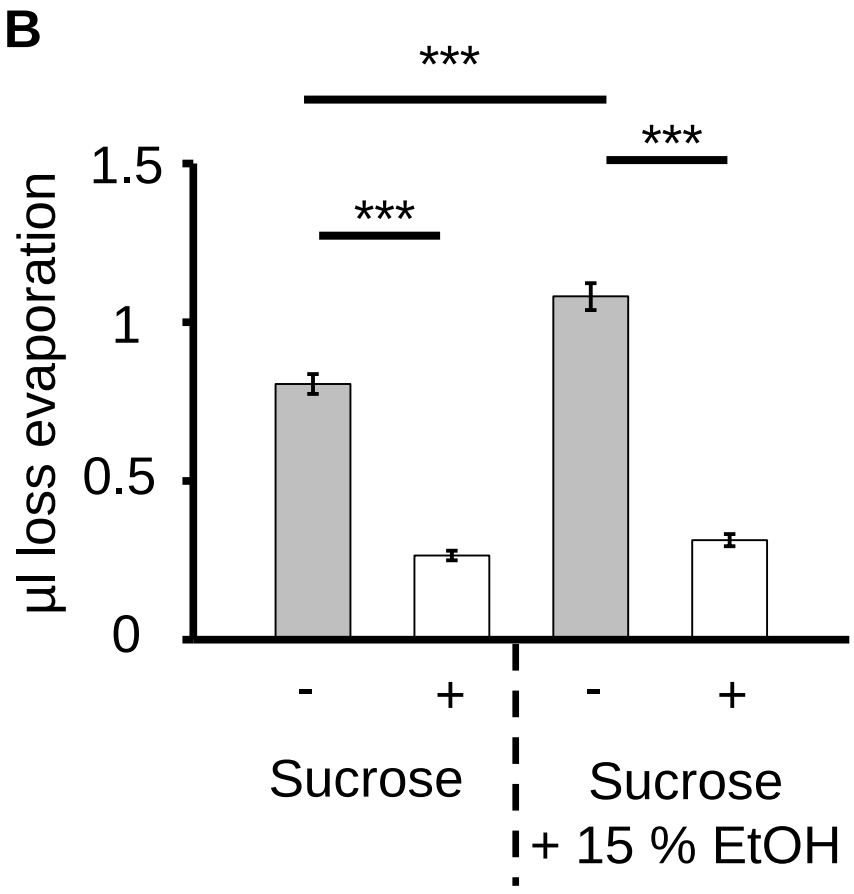

Figure 3

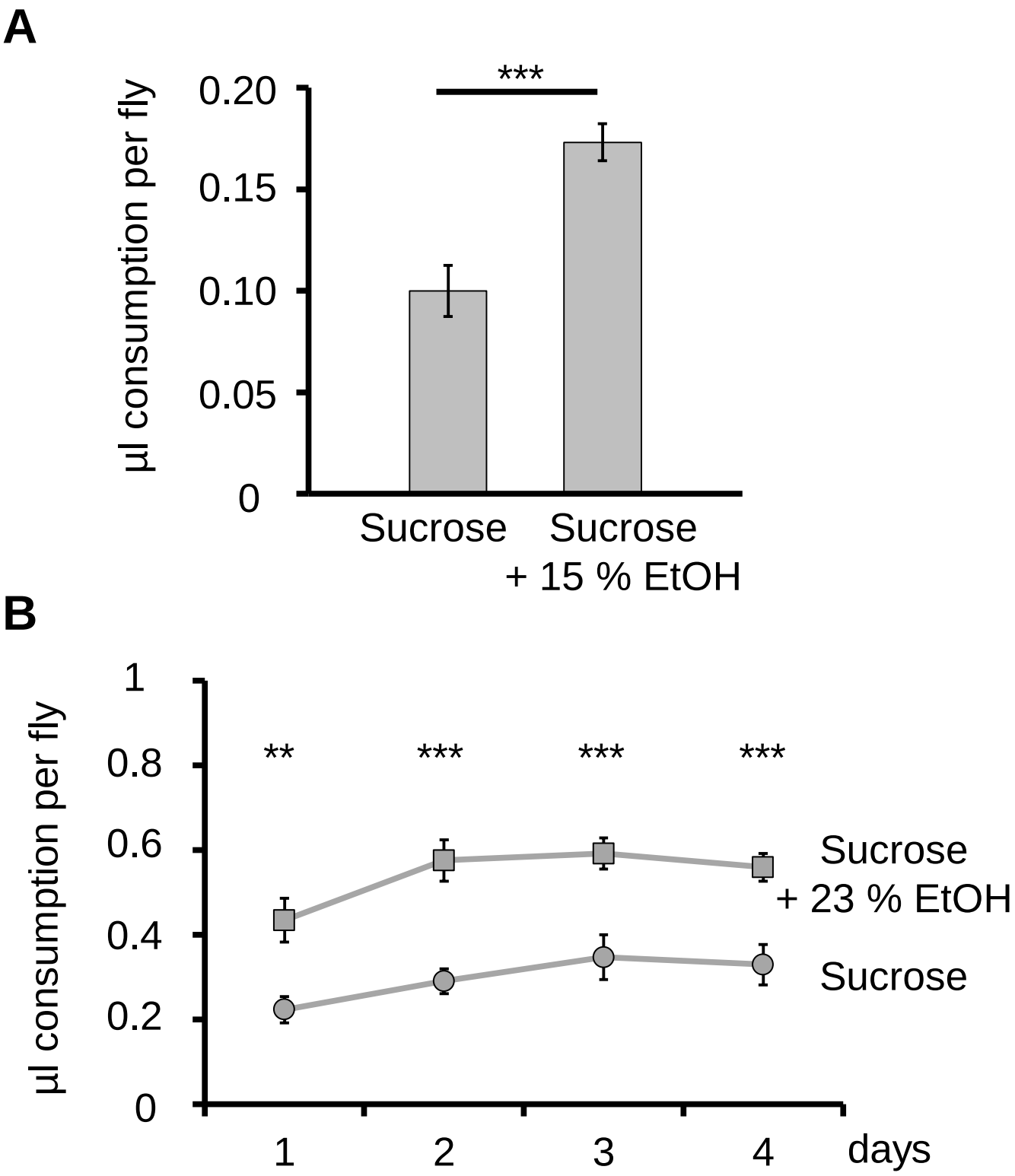

Figure 4

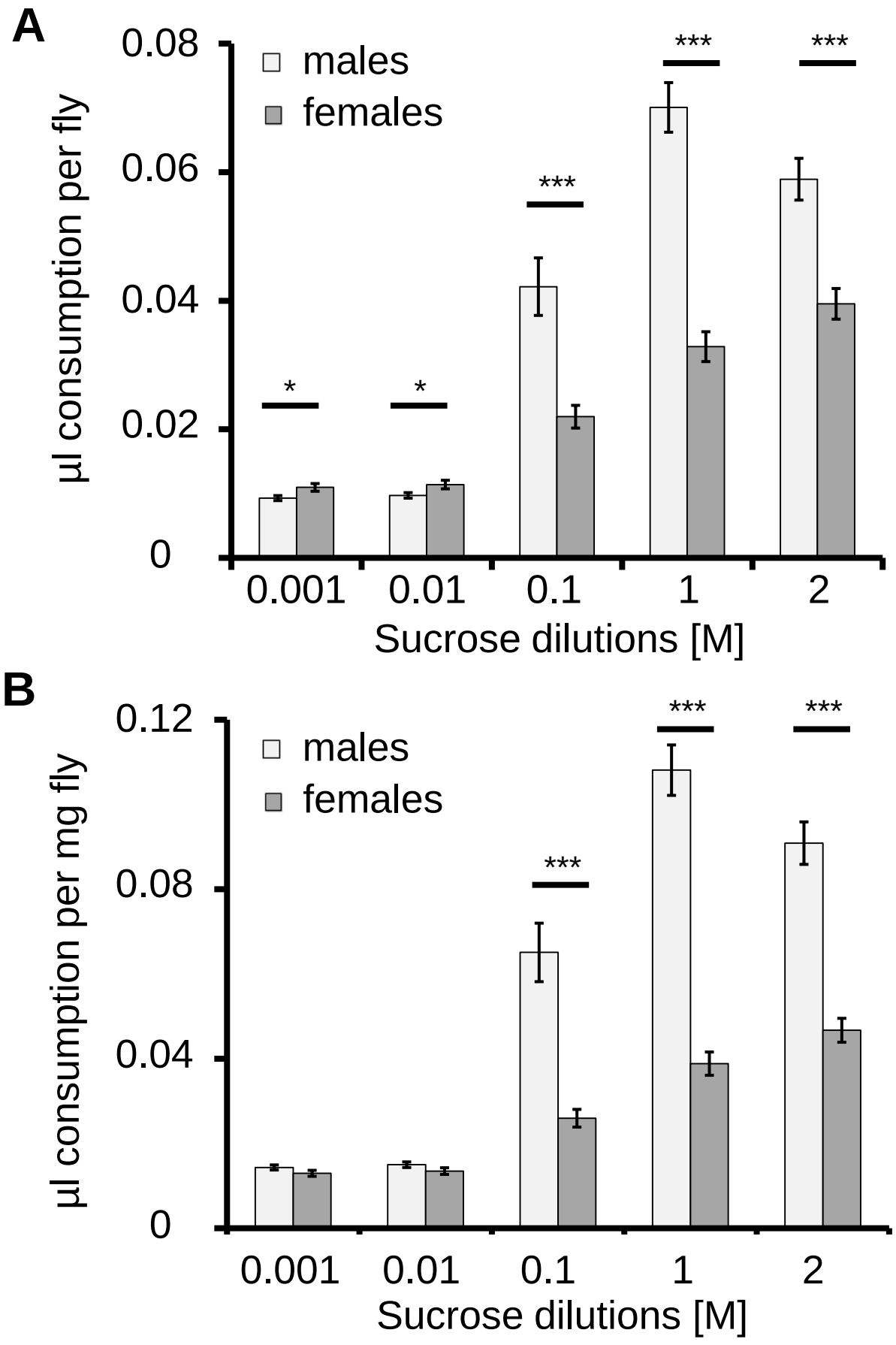

Table 1

Click here to download Table JoVE55024R1-Diegelmann-Table-1.xlsx

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 2

Click here to download Table JoVE55024R1-Diegelmann-Table-2.xlsx

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 3

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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 4

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Day	Genotype (sex)	Number of flies	µl [Suc] per fly	µl [Suc/EtOH] per fly	Prefrence Index	Day	Number of flies	µl [Suc] per fly	µl [Suc/EtOH] per fly	Preference Index	Day	Number of flies	µl [Suc] per fly	µl [Suc/EtOH] per fly	Preference Index	Day	Number of flies	µl [Suc] per fly	µl [Suc/EtOH] per fly	Preference Index
1	*white* ^{1118} (male)	8	0.25	0.57	0.39	2	8	0.38	0.57	0.20	3	8	0.29	0.61	0.35	4	8	0.46	0.51	0.05
1	*white* ^{1118} (male)	8	0.21	0.70	0.53	2	8	0.54	0.42	-0.13	3	8	0.27	0.85	0.52	4	8	0.22	0.60	0.47
1	*white* ^{1118} (male)	8	0.13	0.45	0.56	2	8	0.21	0.41	0.34	3	8	0.21	0.43	0.34	4	8	0.41	0.44	0.03
1	*white* ^{1118} (male)	8	0.44	0.59	0.15	2	8	0.35	0.40	0.07	3	8	0.18	0.71	0.59	4	8	0.16	0.51	0.53
1	*white* ^{1118} (male)	8	0.53	0.77	0.19	2	8	0.49	0.29	-0.26	3	8	0.23	0.79	0.55	4	8	0.34	0.59	0.26
1	*white* ^{1118} (male)	8	0.24	0.56	0.41	2	8	0.36	0.40	0.05	3	8	0.13	0.74	0.70	4	8	0.49	0.51	0.02
1	*white* ^{1118} (male)	8	0.06	0.35	0.71	2	8	0.21	0.32	0.22	3	8	0.71	0.64	-0.05	4	8	0.24	0.45	0.31
1	*white* ^{1118} (male)	8	0.09	0.79	0.80	2	8	0.26	0.60	0.39	3	8	0.24	0.78	0.53	4	8	0.57	0.65	0.07
1	*white* ^{1118} (male)	8	0.17	0.33	0.32	2	8	0.25	0.49	0.32	3	8	0.36	0.52	0.18	4	8	0.20	0.58	0.49
1	*white* ^{1118} (male)	8	0.24	0.14	-0.26	2	7	0.30	0.79	0.45	3	7	0.38	0.44	0.08	4	7	0.19	0.74	0.59
1	*white* ^{1118} (male)	8	0.25	0.16	-0.23	2	8	0.29	0.78	0.46	3	8	0.33	0.53	0.23	4	8	0.34	0.74	0.37
1	*white* ^{1118} (male)	8	0.29	0.39	0.14	2	8	0.16	0.61	0.59	3	8	0.29	0.56	0.31	4	8	0.26	0.61	0.41
1	*white* ^{1118} (male)	8	0.22	0.32	0.18	2	6	0.19	0.93	0.66	3	6	0.96	0.40	-0.41	4	6	0.23	0.63	0.46
1	*white* ^{1118} (male)	8	0.26	0.34	0.14	2	8	0.20	0.63	0.52	3	8	0.21	0.50	0.41	4	8	0.12	0.59	0.65
1	*white* ^{1118} (male)	8	0.09	0.28	0.52	2	7	0.34	0.83	0.42	3	7	0.39	0.37	-0.02	4	7	0.85	0.19	-0.64
1	*white* ^{1118} (male)	8	0.12	0.23	0.31	2	8	0.12	0.72	0.72	3	8	0.37	0.60	0.23	4	8	0.21	0.61	0.49
	MEAN	**0.22**	**0.43**	**0.30**		**MEAN**	**0.29**	**0.58**	**0.31**		**MEAN**	**0.35**	**0.59**	**0.28**		**MEAN**	**0.33**	**0.56**	**0.29**
	STDEV	**0.12**	**0.21**	**0.30**		**STDEV**	**0.12**	**0.19**	**0.28**		**STDEV**	**0.21**	**0.15**	**0.29**		**STDEV**	**0.19**	**0.13**	**0.32**
	STERROR	**0.03**	**0.05**	**0.07**		**STERROR**	**0.03**	**0.05**	**0.07**		**STERROR**	**0.05**	**0.04**	**0.07**		**STERROR**	**0.05**	**0.03**	**0.08**

Table 5

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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	0.008	0.050	0.065	0.030	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	0.061	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	0.153	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	0.019	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	0.139	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**	**0.003**	**0.003**	**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	0.049	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	0.166	0.020	0.023	0.038	0.040	0.075
3	w^{1118} (female)	0.011	0.011	0.009	0.023	0.043	0.096	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	0.009	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 of Materials

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Product	Company	Product number	Web address
Vials (breeding)	Greiner Bio-One	960177	www.greinerbioone.com
Vials (CAFE assay)	Greiner Bio-One	217101	www.greinerbioone.com
Lid-CAFE assay	Workshop	–	–
Plastic box, low wall	Plastime	353	www.plastime.it
Cover for the plastic box	Workshop	–	–
Capillaries	BLAUBRAND	REF 7087 07	www.brand.de
Pipette tips	Greiner Bio-One	771290	www.greinerbioone.com
Filter paper circles	Whatman	10 311 804	www.sigmaaldrich.com
D(+)-Sucrose	AppliChem	57-50-1	www.applichem.com
Ethanol absolute	VWR Chemicals	20,821,330	www.vwr.com
Food color (red, E124)	Backfun	10027	www.backfun.de
Food color (blue, E133)	Backfun	10030	www.backfun.de
Soap solution (CVK 8)	CVH	103220	www.cvh.de
Digital caliper	GARANT	412,616	www.hoffmann-group.com

Product	Comments
Vials (breeding)	Height 9.8 cm, diameter 4.8 cm
Vials (CAFE assay)	Height 8 cm, diameter 3.3 cm
Lid-CAFE assay	Produced in university workshop, technical drawing supplied
Plastic box, low wall	A plastic grid inlay was custom-made for 8 x 10 vial positions
Cover for the plastic box	Dimensions (37 x29 x18 cm)
Capillaries	DIN ISO 7550 norm, IVD-guideline 98/79 EG, ends polished
Pipette tips	Pipettes for the outer circle are cut according to the lid
Filter paper circles	45 mm diameter works nicely if folded for the vials used
D(+)-Sucrose	Not harmful
Ethanol absolute	Highly flammable liquid and vapor
Food color (red, E124)	Not stated
Food color (blue, E133)	Not stated
Soap solution (CVK 8)	Odor neutral soap
Digital caliper	

Standard fly food	(for 20 L)
Agar	160 g
Brewer`s Yeast	299.33 g
Cornmeal	1200 g
Molasses	1.6 L
Propionic acid	57.3 mL
Nipagin 30%	160 mL

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CORRESPONDING AUTHOR:

Name: Henrike Scholz

Department: Dep. of Animal Physiology

Institution: University of Cologne

Article Title: The CApillary FEede assay measures food uptake in Dros. mel.

Signature: H Scholz Date: 15.5.2016

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Cologne, 11th September 2016

Dear Dr. Nguyen,

We resubmit our revised version of JoVE55024R1 “The CApillary FEeder assay measures food intake in *Drosophila melanogaster*” for publication. We appreciated the constructive criticisms of the Associate Editor and the reviewers and addressed each of their concerns as outlined below.

We incorporated a new figure and supporting data into the manuscript. Both are addressing how the variability of the assay due to evaporation can be reduced, a concern two of the reviewers had. We believe that we have substantially revised and improved the manuscript and hope that is now ready to be turned into a JoVE video.

Sincerely yours,

Soeren Diegelmann

EDITOR COMMENTS:

1. Formatting:

-Do all authors have the same affiliation?
We provided an updated version.

-Please use the American standard for punctuation, with periods used for commas instead of commas, throughout the manuscript and in all figures and tables (including the materials table).
We updated all tables and figures with the American standard using periods instead of commas.

2. Grammar:

Line 115: “stereo foam plug”
We replaced the word “stereo foam plug” with the “sponge bung”.

Line 198: “to keep huminity constant inside the assay” - humidity
We corrected the misspelling.

3. Branding:

5.6 – “Statistica”
We replaced the term by “statistical software”.

REVIEWER COMMENTS:

REVIEWER #1

MAJOR COMMENTS

1) Since larval crowding during development can significantly affect adult body size (which correlates with absolute feeding), the authors might highlight the importance of careful development. That said, aren't the conditions used by the authors (35 females per vial with 3 days of egg-laying) quite crowded?

We agree with the reviewer that the development conditions play a significant role for adult body size. We set up the crosses for all genotypes tested at the same time and rear the animals under the same conditions (2.1). The vials for "breeding" the flies differ from the one for the "CAFE assay" by an over 2.5 time increase of volume. We clearly indicated in the retyped manuscript the dimensions of the "breeding" and "CAFE assay" vials. We included both vials also in the table of specific materials. We use the bigger vials to provide more space and food sources to avoid problems with overpopulation as the reviewer pointed out.

2) There's really no good scientific reason yet to normalize feeding by body weight. People just do it. The tone of the text could be adjusted to make this a suggestion, but there's no reason to indicate that it should be part of a definitive protocol. Hence, the entire third experiment (food demand between sexes and normalizing with body weight) doesn't seem scientifically relevant at this point. I'd recommend less "scientific" discussion and more focus on the technical protocol instead.

We reformulated the manuscript and stating that we suggest normalizing the consumption to the body weight. Normalization is essential if e.g. control and mutants differ significant in their body weight, otherwise low intake could be misinterpreted. We also rephrased the interpretation of the result in figure 3 and focus more on the technical protocol of the CAFE assay.

3) If flies consume 0.2-0.6 uL of food per day (which weighs approximately 0.2-0.6 mg), how much does this consumption contribute to the fly body weight measurements? Does it add significant variability to the results?

We agree with the reviewer that the intake of food lead to an increase in body weight, but at the same time the excretion system of the fly is lowering the weight potentially. Body weight therefore is a dynamic value, especially for long term experiment. We measure the body weight of the flies before the experiment and look for potential significant differences. The influence of consumption on body weight is therefore not addressed in the assay. We include this point in the discussion as one aspect to consider.

4) As mentioned above, there are many different iterations of the assay using different sized enclosures, different foods, different capillary sizes, etc. Highlighting some of the possibilities, or at least why the authors think their protocol should be the definitive one, or is better than others, might be valuable.

As the reviewer pointed out there are numerous variation of the CAFE assay setup.

Especially the lid range from simple sponge vial stopper with openings to placing the capillaries to plastic lids like in our setup, as the reviewer pointed out. We ourselves performed experiments using the sponge variation. We experienced that for example the secure positioning of the capillaries during the handling and the experiment period can be an issue. The usage of a custom-made lid avoids this drawback and we gained more reproducibility. It allowed us to perform the assay successfully during student practical courses.
We added a note at point 1.2 to highlight other possibilities for the lid and retyped the manuscript at specific point to guide the reader to alternatives.

5) One of the advantages of the CAFE assay is that supplements to the food, such as dyes, are unnecessary. These supplements can (and often do) affect feeding behavior. The food colorants that the authors use aren't necessary to visualizing the meniscus and shouldn't be the gold standard for the assay.

We agree that food dyes are not a necessity for the assay. Nevertheless the hard to see decline of the meniscus without this supplement makes data collection time consuming, which could be challenging if multiple assays are performed in parallel. We see a weak avoidance of food containing the dye. We included a note at point 3.1 to show that problem and to make clear that addition of food dyes is not mandatory for the assay. We also highlighted that if two dyes are used the supplemented dyes should be randomized.

6) Humidity within and outside of the CAFE chamber is important for minimizing evaporation and improving signal-to-noise. Applying water to the stereo foam plug (step 4.5) doesn't seem like it would work well. Not only is that a very limited amount of water that would evaporate quickly, but isn't that the only source of fresh air for the flies? For long-term experiments, I would suggest using agar in the vial itself as an alternative source of humidity and water, instead of the filter paper and water drop that probably dries out.

Indeed the humidity is a critical point for the assay. We included new additional devices we use to minimize the evaporation and to lower the signal to noisy ratio. This includes water filled vials inside the plastic box and box which can be placed above the ready to use CAFE assay vials to create an own humidity environment. We added the information of how much water is supplied to the central sponge bung daily in a long term experiment. The sponge bung is not the only source for fresh air for the vials. The six opening which can hold the capillaries can lead air through. The capillary is secured by the second pipette tip but there are still gaps for air exchange, but too small to let flies escape out of the vial.

7) Do the authors observe any fly deaths in their setup? If so, how are these handled? Any good set up should be usable for many days with young flies without mortality.

During our 3 h experiment we hardly see any death flies. Occasionally one fly dies during the fasting period before the experiment. In this situation we still perform the experiment and divide the consumption by for example 19 flies. For the long term assays we observe the survival of the flies every day. Depending on the food source provided we see 1-3 dead flies at the end of day 4. We always use the numbers of flies at the beginning of a 24 h period to estimate the consumption per fly in a vial.

We included a new point (4.6) and Note to make sure people notice death flies and to calculate the consumption accordingly.

8) There is no discussion of signal-to-noise. Namely, what percent of the "feeding" should background (evaporation) represent to be a good data set? Looking at Figure 3, each of the values can be multipled by the number of flies per chamber (20) to get the total consumption. One can then compare this to the evaporation presented in Table 2. On the lowest sucrose concentrations, feeding (0.2 uL) is far outweighed by evaporation (2-2.5 uL). Even on the higher concenrations (0.1 and 1M sucrose), evaporation represents 20-80% of the measurement! That's awful, and I believe for the publications that mention it, evaporation is best if it's less than 10% of the data. Lower would obviously be better. At these high levels of evaporation, one must also consider that the effective nutrient concentration in the capillaries has changed by quite a bit. There's zero evaporation with 2M sucrose, but this is not a commonly used food concentration.

We are thankful to the reviewer for this comment. By reviewing the data inside table 2 we noticed that the data shown are representing loss in mm not µl. The conversion of the values was not performed. The values shown were not from the actual experiment shown in figure 3. When the right data and the conversion were performed we saw the evaporation for the lower concentrations is around 28 %, the value for evaporation at 0.1 M solution, taking the females data set into account is around 15 %. All other comparisons show evaporation less than 10 %. We updated the table for the evaporation control and include data from a two choice assay performed in two ways. One time without further humidity approaches and one time in parallel with extra water filled vials and using a cover for the plastic box during the experiment (plus applying water to the central sponge bung every 24 h (new Figure 2 and new Table2).

9) Given that the assay has been used very successfully without custom parts, a "definitive" protocol that uses a custom-made lid seems like a poor choice. The lid and O-ring certainly look nice, but the authors should justify any significant advantages or, otherwise, highlight alternatives. I've seen some labs use a standard stopper that likely results in high evaporation. I've also seen the use of rubber septa.

We rephrased the usage of the lid and pointed out possible alternatives. We included a note (see 1.2) addressing alternative ways and advantages of our system. We show in the new figure 2 and table 2 that additional devices to control humidity lower the evaporation significantly and therefore help to perform the assay more reproducible.

REVIEWER #2

MINOR COMMENTS

Line 47: The authors mentioned already in the abstract that their report demonstrates using the assay with single or multiple flies, tho there are no data presented or modifications shown to successfully measure drinking of one fly in their setup.
We took out the term "single fly" as the reviewer mentioned correctly that we didn´t show data for a single fly. Our setup is indeed not useful for single flies and should be used with minimum of 8 flies.

Line 66: at the beginning of 21st century. just state the year.
We retyped the sentence.

Line67: Capillary drinking assay was described much earlier than by Ja et al. Dethier's Hungry fly from 1976 is a well known example. Benzer first published it in Drosophila and gave it the name.
We put the establishment of the CAFE assay in Drosophila in the right context.

Line 68: 1. meniscus decline also indicates evaporation. 2. "missing" is not the best word; it's not missing, it was consumed or evaporated. and it indicates food uptake too. Please, reformulate the sentence.
We reformulated the sentence and avoided the usage of the word "missing" and pointed out the influence of evaporation.

Line 83: should be "measuring food response", not consumption. Is there any reason to bring a new term - PE, instead of the widely used term - PER? Explanation or citation would be welcome. How would they measure consumption with either?
The more widely used term "PER" is used in the whole manuscript. The term "response" is used.

Line 83-85: these two sentences are exclusive of each other. with only the later being correct. PER doesn't measure uptake.
We retyped the sentence, see above.

Line 88: this is a very vague technical description of these two assays. Moreover, both of these assays measure not only PER/PE but also amount of time spent drinking which can be used to estimate volume.
We aware of the short description of the techniques, we believe that more detailed explanation is beyond the focus of our manuscript. The reader is guided to excellent references about those new developed techniques. We include an additional sentence pointing out the change to estimate food intake.

Line 89: Do authors understand under PE assay proboscis extension AND the following drinking?
We mean both possibilities.

Line 101: This sentence should be part of abstract instead of the one mentioning single fly experiment.
We retyped the abstract and took out the term "single fly".

Line 116: the conical openings do not fit the capillaries. They fit pipette tips.
We retyped the sentence pointing out the presumable usage of the pipette tips to hold the capillaries in the assay.

Line 117: there is no supplementary figure in the version I review. Cross section of the lid would be helpful.
We supplied a cross section within the technical drawing; visual information inside the media file should help the viewer to understand the usage of tips and capillaries.

Line 119: better description of capillaries needed. ID/OD, cut/polished, inner filament Y/N?
We provided more information for the capillaries within the comments at the table for specific materials.

Line 133: this is confusing. are flies allowed to lay eggs for 3 days in each vial or only in the first one, then 1 day in the second and two in the last one?
We reformulated the point to give a more clear time line. We also included when adult flies should be discarded.

Line 138: dry or wet weight?
We included the term "wet".

Line 149: Are capillaries refilled or discarded and always new fresh capillaries are used?
We included the handling of used capillaries after measurement of the meniscus (see also point 5.1).

Line 156: is there a difference between pre-starved and starved? It should be 'fasted' instead of 'starved' anyway.
We agree with the reviewer and used the term "fasted" instead.

Line 163: The stock solution is calculated wrong. 102.6g of sucrose should be FILLED to 100mL, not add to 100mL water.
We corrected the calculation of the solution.

Line 164: explaining serial dilution seems rather unnecessary. Moreover, the described method introduces potentially larger error than classical serial dilution.
From our experiences with students in the lab this explanation works fine and we therefore would like to keep it. We included the % w/v of the stock solution.

Line 168: should describe what the reason to do that is. What c of EtOH is being made?
We reformulated the point to state clearly the usage of the solution. We included the c for EtOH.

Line 173: not sure if 1 or 10 capillaries in a bundle is inserted. I assume it will be obvious on the video.
We rewrote the sentence to make clear that multiple capillaries are filled simultaneously. This point should be clear seeing the media file.

Line 179: Quantifying evaporation in empty vials is misleading; the evaporation is

higher in empty vials than in vials with flies. Using high c of bitter solution in one capillary with flies inside gives much more accurate values. Is wet filter paper always placed in the CAFÉ vials? If so, it should be mentioned.
We rephrased the usage of the filter paper and included a new figure showing the reduction in evaporation.

Line 183: I'm not sure what the authors mean with ".. placing the BACK of the pipette tip NEXT to…".
We rephrased the sentence. This point should be clear seeing the media file.

Line 187: Should not all ends be at the same height in all vials? Would be nice to specify which height it is/should be.
We exchanged the term "one" by all and stated at which height we normally let the capillaries end.

Line 188: This sentence suggests that filter paper is always in the tube. Is it? Is it wet? How is it kept wet in long running assays? Is it exchanged/wetted for days running assays?
The filter paper is indeed always in the prepared vial. It´s wet (see point 2.6). Fresh water can be applied via the sponge bung in the central opening of the lid (see point 4.5, which we retyped for gaining more clarity regarding the water supply).

Line 195: What's the point of the humidity controlled incubator? Or do the authors mean in case the assay is not in the incubator? In any case, the humidity inside of the tube is influenced by number of flies and the amount of drinking; and the following excretion.
We reduced the variability of our assay placing the prepared vials within a climate controlled chamber described in point 4.5.

Line 197: This sentence indicates that wet filter paper is not inside of the tube. Overall, the humidity control is not well specified and humidity (or lack of it) has a huge effect on drinking. It should be clearer and consistent.
We retyped point 4.5 stating more clearly that the bottom filter paper stays inside the vial and fresh water is applied via the centrally placed sponge bung.

Line 201: The note should be just the second sentence. The first sentence should be part of the protocol. And the variability is probably influenced more by humidity than temperature (within the room temperature range).
We eliminated the first sentence and pointed out the role of humidity for the assay.

Line 207: A care should be taken to note if the liquid is reaching the bottom of the capillary AND the markEND at the same time. Otherwise, the liquid probably shifted and was not reachable by the fly.
We included the problem if the liquid was not accessible to the flies within point 4.7.

Line 215: Authors probably mean "dissection" or "stereo" microscope.
We replaced "binocular" with "dissection microscope".

Line 220: This sentence should read: "To transfer data directly to a spreadsheet, use USB connected caliper (Figure 1E)." Normal digital caliper will not connect to computer
We retyped the sentence according to the reviewers' suggestion.

Line 226: The formula doesn't need "x 1uL" as 14.6mm is 1uL
We eliminated "x 1ul"

Line 228: As mentioned above, this is not very precise method. But depends on humidity control.
We included new evaporation data, figure and table showing the effect on evaporation.

Line 236: as mentioned earlier. is the calculation to dry weight or wet? If wet, is it gradually adjusted as flies drink in the assay? With higher concentration of sucrose and starved flies, the uptake can be very significant.
We included the term "wet weight" (point 2.3). The weight is measured before the experiment to see if significant differences exist. The influence of consumption on body weight during the experiment is not addressed with this assay.

Line 239: Is there any reason to use Statistica over another statistical program?
We deleted the branding and used the term "statistical software".

Line 251: rather sex difference than body size as will be mentioned later
We included this interpretation in the discussion section.

Line 262: Is it similar or the same food choice?
We used the term "similar" because we used a 23 % EtOH source instead of 15 % EtOH in the first experiment.

Line 264: It's just "Sucrose", not "Suc-EtOH"
We followed the reviewers' suggestion.

Line 272: This experiment is very inconclusive as it is not clear whether it is the effect of weight or sex. The flies of the same sex and of different weight should be used if the weight effect is being studied.
We included the interpretation of sex differences in the ability to perform the assay in the discussion section.

Line 274: statement is not reflected in the described experiment? Why is it mentioned then?
Mated female prefer protein-rich diets over sucrose containing solution. Therefore the reduction in sucrose intake (0.1-2M) in our 3 h experiment could reflect a prolonged search in females for their "right" food and less time spent in drinking the supplied food.

Line 276: So mated females AND males were used
We added the term "mated male and female".

Line 280: First, it is said that flies didn't feed "…appreciably on the lower concentrations…" but then big conclusion is drawn from those data?
We eliminated the term "appreciably" as it appears indeed too strong in the context.

Line 291: wet filter paper is mentioned here but not in the protocol. Still, how is it kept wet over days lasting experiments?
We retyped the protocol, stating now how the humidity device is kept wet during the period of the experiment.

Line 292: Are the food dyes always used or is it just for display in the figure? If the former, should be mentioned to randomize used dyes as they cause different aversive response.
In a two choice assay the use of the food dyes is randomized to avoid any bias because of aversive responses to the dye. We included this information within point as a note in point 3.1.

Line 295: A cross section of the lid would be helpful.
A cross section of the lid is provided as a technical drawing in a supplementary figure.

Line 298: In the picture, it seems like the liquid is too far for the fly to reach and the fly is only touching the capillary rim.
The fly in the picture reach the liquid which is at the end of the capillary. You can see actually that the fly is drinking because of the red staining inside the proboscis. This point will be also possible to illustrate in the media file showing a drinking Drosophila in close up.

Line 303: Should be ..."sucrose (suc)" and "...ethanol (EtOH).." Actually, the figure doesn't show any "Suc", so why to use it in the legend?
We followed the reviewers' suggestion.

Line 304: Be consistent with description of solutions. "solution containing 15% EtOH" vs "EtOh containg sucrose solution". I assume both are sucrose with EtOH but the description is unclear.
We retyped the legend for figure 2 and focused on consistency of naming the solutions.

Line 312/315: Is it 27 males or 27x20 males? Same for females
We included the term "xx trials with xx (fe)male flies each".

Line 318: mg per fly are shown in Table 1 but ug/fly are mentioned in the legend. Use of ug and mg is inconsistent few more times throughout the MS
We eliminated this confusion and used mg throughout the new manuscript version.

Line 334: again, not Suc-EtOH; just Suc
We eliminated "-EtOH".

Line 340: Is really intake of total sucrose calculated and shown or is it just the sum of all the solutions? I suspect it's the later.
We retyped the legend.

Line 350: As mentioned before, it was not invented decade ago; just published for use in D.m.
We eliminated the half sentence.

Line 357: The "pre-strvation" is rather confusing term. As opposed to "post-starvation'? The correct term should be "fasting" as we don't really know how and if the fly is actually starving when placed on water only.
We followed the use of the term "fasting" in the revised manuscript.

Line 361/363: Other versions of CAFE assay were described (Masek & Scott, 2010) that addressed this caveat and should be mentioned.
We included the citation.

Line 366: there is not such thing as "standard D food vial". Size or volume should be specified.
We changed the terms and use CAFE assay vial and breeding vials in the manuscript. More information is given in the table of specific materials.

Line 371: Pool et al 2014 can be cited here.
We included this citation.

Line 381: Spill also depends on viscosity of the liquid (EtOH for example) and should be mentioned.
We included the problem of viscosity (point 4.3), which is stated.

Line 385: Food accessibility should not be mentioned as pitfall but rather as a part of the main protocol. And it needs to be mention what this means in detail as it is a common problem. (moving the liquid away from the end of the capillary due to evaporation, moving of the assay or drinking)
We included the food accessibility in point 4.9 and guide the reader to discard the data if food was not available to the flies. We didn`t notice moving liquid from the end of the capillaries due to evaporation in our system.

Line 391: Vertical or horizontal position in MAFA?
The eating position of the fly in the MAFA assay is mentioned in the sentence.

Line 402: CAFÉ can be used for measuring robust drinking in as little as 3 mins.
We retyped the sentence.

Line 402: It's mentioned several times in the MS but proboscis extension is not a feeding assay.
We eliminated the PER assay here as it´s not a feeding assay.

Line 409: Yet, even in this MS, it is mostly not.
We suggest doing so.

Line 413: "…metabolic AND taste research…"
We included the term "taste".

Line 480: missing tab
We included the tab.

Fig 1 Cross section of the capillary/pipette tip arrangement is needed. C) The food is too far in the capillary for the fly to reach it or bad lighting. D) the two pictures (placement/brightness/shadows are too different

Figure1. We included a technical drawing in a supplementary figure, which shows a cross section. The media file will help to understand the arrangement of pipette tips and capillaries.
The food is reached by the fly in figure C, which can be seen in the red staining inside the proboscis. The media file can be used to visualize a drinking fly from the capillary end.
D) We adjusted the brightness and contrast of the picture and its orientation. The staining of the abdomen with red or blue food color is seen within the picture. The same placement/ shadow would not add any new information.

Fig 3 x-axis should be "Sucrose dilutions"; not a gradient. Why is w1118 sometimes shown in the figures and why it's in the tables when it is mentioned at the beginning that ALL flies shown are w1118. Seems unnecessary. And inconsistent.
We followed the suggestion of the reviewers for the x-axis and eliminated the genotype inside the figure. We keep the genotypes inside the figure legends and table (legend) as this helps the reader if the data is viewed without reading the manuscript first.

All tables must fit on one page. I see no reason for them to be so spread. They are very confusing the way they are now. And some, like the 3rd page of Table 5 is completely out of place.
We arranged all tables to fit on one page to eliminate a source of confusion if printed out. We simplified the table 1 by only showing the bodyweight per fly in mg.

Table 1 keep the naming consistent "/male vs (male)". It's not necessary to put "total weight" and "per fly" if # of flies is always 100. It's just moving decimal point.
We consistently use the term “(male)” or “(female)” in the revised version of the tables.

Table 2 The variability is rather large. Often around 2 x difference in different vials. What is the explanation?
We found two mistakes in table 2. The values shown were in mm and not converted to µL and not from the experiment shown in table 5 and figure 3. We corrected our mistake including the µL values for evaporation on three days. This also reduced the variability.

Table 3 The variability of the results is really large. 3.36vs0.14 or 2.67vs2.05 in the same day?
Table 4 Again so much variability. -0.26 to 0.8 under the same conditions?
That's a lot of variability. Maybe showing preference of two different concentrations of sucrose would better illustrate that CAFÉ assay can be very precise and highly reproducible assay. It would also show an experiment more relevant for other researchers besides those in EtOH field (where the authors are).
We believe the reviewer confused the two variants of solutions used in this experiment. 3.36 (Suc+EtOH) vs 0.14 (Suc). We therefore color labelled the different columns and also changed the order (to follow Figure 3A). The STDEV and STERROR for the solutions show a normal value and a listed under each column.

The differences among the solutions tested are statistical significant. We agree that showing results of data of preferences between two sucrose concentrations would result in less variation. In our tutorial we intend to show that the CAFE assay can be successfully used with different solutions and show the effect of food supplements. The experiment was already shown in the first publication of the assay in the fly and seem to us like a perfect representative result example.

Table 5 More ## after decimal point need to be shown for the low c of sucrose. It's meaningless as it is now
We included the third visual decimal point in all tables to avoid the rounded zero value.

REVIEWER #3

MAJOR COMMENTS

I have one general comment about the manuscript: I would like to see a clearer handling of the general discussion concerning how to measure Drosophila feeding. …….. This could be inserted in the introduction (perhaps at the start of the second paragraph) and once this has been established, the authors have the freedom to move past the discussion and describe the CAFÉ assay in isolation.

We rephrased the introduction and pointed out possible other assays in Drosophila. We wrote more clearly for which scientific question which assay would be an appropriate one. We believe that in combination with the discussion section the viewer should be able to choose an assay for their specific scientific question.

MINOR COMMENTS

Line 236: I am not entirely sure why the authors suggest to normalise intake to body mass. Without any knowledge about nutrient absorption this seems unnecessary and could even lead to over-interpretation of the data.
We reformulated the manuscript and stating that we suggest normalizing the consumption to the body weight. Normalization is essential if e.g. control and mutants differ significant in their body weight, otherwise low intake could be misinterpreted.

Line 261-266: there is also the possibility that males and females differ in their proficiency at feeding from the capillaries and therefore that the comparisons below reflect this difference.
We included the possibility of a difference in the ability to drink from the assay between male and females in the text.

Line 384: tling of solid particles is important and it is worth noting that this has lead researchers to use water soluble yeast extract in some assays that use capillary feeders. However, this is an incomplete source of nutrition for flies and can impose fitness costs. I feel this is an important point to make to further clarify the scope of uses for this assay.
We included the possibility and the potential downfall of the usage of water soluble yeast extract within the text.

REVIEWER #4

Line 62: 1. On line 62, there is a statement that few researchers in mammalian systems have examined the circuitry of eating disorders. This is too strong a statement, in fact there is a large community of mammalian researchers examining the circuitry of eating disorders studying systems and circuitries of the hypothalamus and gut, involving several signaling systems including ghrelin, NPY, and serotonin, to name a few.
We rephrased the sentence and highlighted the numerous efforts made in the mammalian field, including three new references.

Line 76: Change the text "already after" to "after only"
We followed the reviewers suggestion and replaced “already after” with “after only”.

Line 109, 124: The term "assay" is used to describe the final equipment setup, a more appropriate term such as "prepared assay vials" should be used.
We defined the use of the term “assay” more clearly and rephrase when vials are mentioned.

Line 115: What is a "stereo foam plug"?
Word replaced with “sponge bung”.

Section 2.3: line 136: Consider combining this with section 2.4, and emphasizing at the beginning of this section that it is important to measure the weight of the flies prior to feeding, and that this number (ug/fly) can be used to adjust for the total volume of food to be used in a given experiment to load the capillaries.
We combined the points 2.3 and 2.4 but leave the sub-points in place as they are important to setup the assay correctly for a 3 h or long-term experiment.

Section 3.1: Please include the % sucrose (w/v) final values, as many researchers use % sucrose instead of molar concentrations.
We included the % sucrose (w/v) for the stock solution, for clarity and readability we would like to not include the % value for each diluted working solution.

Section 4.5: line 194: The use of a humidification device within the assay tube is mentioned as being an option. This particular reviewer, however, has found that the addition of a humidification device to CAFÉ assay tubes appears to be necessary to prevent increased evaporation and confounded consumption measurements. This is especially important when the assays will be performed in a non-humidity controlled environment at 25°. I would advise adding stronger wording encouraging the use of a humidifying device in an assay under most conditions. That being said, the presence of such a device (foam plug, filter paper, etc. soaked with water) will introduce a confound into the system and flies will drink from it, therefore a mesh barrier is necessary to prevent flies from doing this.
We retyped the note for point 4.5 and highlighted the use of humidifying devices. The usage of a mesh barrier surrounding this water sources seems desirable but we haven´t used such a barrier in our assay yet.

Line 247: replace the word "a" with "the"
Word replaced

In Figure 1: it would be helpful to also show what the underside of the lids in 1A and 1B look like with the assembled tips and tubes present.
Figure 1: We included a technical drawing of the customized lid, including a cross section drawing in the supplementary figures. The assembled lid with tips and tubes should be shown in the media file; we believe that this way allows the viewer to understand the concept of the lid much easier as an additional picture of the underside of the lid.

General comments: How often and with what do you clean the lids with? Do you reuse your vials or capillary tubes?
We include additional sentences at point 4.7 and 5.1 showing the washing and the handling of the capillaries after the experiment. We include the soap which we use for cleaning in the table of specific materials.