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A High Throughput Microplate Feeder Assay for Quantification of Consumption in Drosophila

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TITLE:

A High Throughput Microplate Feeder Assay for Quantification of Consumption in *Drosophila*

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KEYWORDS:

feeding behavior, optic measurements, model organism, exposure, screening assay

SUMMARY:

The microplate feeder assay offers an economical, high throughput method for quantifying liquid food consumption in *Drosophila*. A 3D-printed device connects a 96-well microplate in which flies are housed to a 1536-well microplate from which flies consume a feeding solution with a tracer dye. The solution volume decline is measured spectrophotometrically.

ABSTRACT:

Quantifying food intake in *Drosophila* is used to study the genetic and physiological underpinnings of consumption-associated traits, their environmental factors, and the toxicological and pharmacological effects of numerous substances. Few methods currently implemented are amenable to high throughput measurement. The Microplate Feeder Assay (MFA) was developed for quantifying the consumption of liquid food for individual flies using absorbance. In this assay, flies consume liquid food medium from select wells of a 1536-well microplate. By incorporating a dilute tracer dye into the liquid food medium and loading a known volume into each well, absorbance measurements of the well acquired before and after consumption reflect the resulting change in volume (i.e., volume consumed). To enable high throughput analysis with this method, a 3D-printed coupler was designed that allows flies to be sorted individually into 96-well microplates. This device precisely orients 96- and 1536-well microplates to give each fly access to up to 4 wells for consumption, thus enabling food preference quantification in addition to regular consumption. Furthermore, the device has barrier strips that toggle between open and closed positions to allow for controlled containment and release of a column of samples at a time. This method enables high throughput

measurements of consumption of aqueous solutions by many flies simultaneously. It also has the potential to be adapted to other insects and to screen consumption of nutrients, toxins, or pharmaceuticals.

INTRODUCTION:

Drosophila melanogaster has seen wide use as a genetic model organism to study the biological underpinnings of food intake and traits associated with consumption[1]. It is estimated that 65% of human disease-causing genes have functional homologs in flies, with a significant proportion of those being expressed in functionally equivalent tissues between flies and humans[2]. Moreover, *D. melanogaster's* size, short intergenerational time, simple maintenance, and genetic tractability make it an attractive model for studies on the consumption of nutrients[3,4] and toxicological and pharmacological effects of a variety of substances, including insecticides[5], pollutants[6], pharmaceuticals[7], and drugs of abuse[8–10].

In many cases, the study of such traits requires precise quantification of consumption. Methods for quantifying consumption are diverse and include the CApillary FEeder (CAFE) assay[11], the MAnual FEeding (MAFE) assay[12], Proboscis Extension Response (PER) assay[13], tracer dye extraction[14,15], oligonucleotide tracer extraction[16], and radio-isotope extraction[5,17]. Recent efforts have focused on enhancing the throughput of these assays, as in the Expresso assay[18] or the plate-based Whole Animal Feeding FLat (WAFFL) system[19]. Despite their utility, these assays can be complicated, costly, or labor-intensive, hindering their use in high throughput studies.

[Place **Figure 1** and **Figure 2** here]

To overcome these hurdles, the Microplate Feeder Assay (MFA; **Figure 1**) was developed. In this assay, flies are housed individually in 96-well microplates. Each microplate is coupled to a 1536-well microplate using a custom, 3D-printed device. The device precisely orients the two plates such that each fly in its respective well of the 96-well plate has access to 4 wells of the 1536-well microplate. By using a bottomless 1536-well plate and sealing films, solutions are dispensed into select wells and perforated with precise 0.25 mm diameter needles to provide access to the flies. Critically, allowing consumption directly from a microplate allows for immediate absorbance-based measurements using a microplate reader. A dilute tracer dye is incorporated into the consumption medium, and the change in absorbance after exposure is used to determine the volume consumed (**Figure 2** and **Figure 3**). Since the liquid in each well approximates a column of fluid, volumetric differences will manifest as differences in the height of the column. (**Figure 3A**) According to the Beer-Lambert law[20]:

$$A = \varepsilon l c$$

where A is the absorbance, ε is the molar absorption coefficient for the attenuating analyte, l is the optical path length, and c is the concentration of the attenuating analyte. Thus, with constant molar absorption coefficient and concentration, changes in absorbance are due solely to changes in the optical light path, i.e., the fluid level within a given well. By measuring absorbance before and after exposure, the proportional change in absorbance reflects the proportional change in

volume (**Figure 3B**).

[Place **Figure 3** here]

Based on the change in volume, the amount of any ingested compound can be calculated from its known concentration in the feeding solution. The parts needed for the assay are low in cost and have a high degree of reusability, substantially reducing the recurring cost of the assay. Thus, this procedure offers an affordable, high throughput method of precisely quantifying consumption.

PROTOCOL:

1. Starvation plate preparation

1.1. Weigh out 1.5 g of agarose into a 250 mL glass beaker.

1.2. Add 100 mL of distilled H_2O to the beaker.

1.3. Microwave intermittently until agarose is fully molten.

NOTE: Observe the beaker since the agarose is prone to boiling over.

1.4. Pour the molten agarose into a reagent trough and dispense 80 µL of molten agarose into each well of a 96-well microplate using a multichannel pipette. Allow plates to cure while covered at room temperature. Refrigerate the leftover agarose for up to a week in a sealed bag and re-melt it for making additional plates.

2. Fly sorting and starvation

2.1. Prepare couplers by inserting barrier strips into the barrier strip channels. If barrier strips are too loose, coil them around the finger to give them curvature to hold them in the channels.

2.2. Affix the coupler to a starvation plate. Do not use the coupler to manipulate the plate since the coupler may slip off. Ensure that the couplers are correctly oriented (i.e., ensure the angled corner of the coupler matches the angled corner of the microplate).

2.3. Under CO_2 anesthesia (**Table of Materials**), sort 3–5-day old flies. Load individual flies by column into the starvation plate.

NOTE: Although flies are loaded by column, it is recommended to distribute groups of samples down rows of the plate rather than down columns (see **Figure 4** for plate layout example).

2.4. Close each column as it fills by adjusting its barrier strip to the closed position.

[Place **Figure 4** here]

2.5. Carefully record the sample layout within the microplate. Once the starvation plate is filled, allow the flies to recover spontaneously after removing the CO_2 and starve them for 6 h starting from their initial anesthetization time.

3. Liquid food preparation

NOTE: Make liquid food fresh every day.

3.1. Prepare a 10 mg/mL of dye stock solution of FD&C Blue #1 in distilled H_2O.

NOTE: This may be stored at room temperature for up to 6 months.

3.2. Prepare 10 mL of liquid food (4% sucrose, 1% yeast extract, 40 µg/mL of FD&C Blue #1) in a 15 mL conical tube by dissolving 0.4 g of sucrose and 0.1 g of yeast extract in 10 mL of distilled H_2O. Vortex the tube until the solids fully dissolve. Add 40 µL of dye stock solution and invert the tube repeatedly to homogenize the solution.

3.3. Transfer the liquid food into a 10 mL syringe tipped with a 0.45 µm filter. Filter ~1.5 mL of the solution at a time into a 1.7 mL microcentrifuge tube. Set aside the syringe containing the solution and filter the additional solution as needed during feeder plate preparation.

4. Feeder plate preparation

NOTE: Handle the feeder plates gently after filling to prevent the formation of bubbles or droplets in the well that could influence the absorbance readings.

4.1. Prepare a feeder plate by sealing the bottom of a 1536-well microplate with a sealing film. Use a sealing paddle to adhere to the film thoroughly. Trim excess film off the left and right edges with a razor blade.

4.2. Dispense 10 µL of the filtered liquid food column-wise (see **Figure 5** for illustration) into the appropriate wells of the 1536-well microplate. Dispense into the upper-left-hand well for each cluster of four wells (see **Figure 5** for illustration). [Place **Figure 5** here]

4.3. Once all the wells are filled, apply a sealing film to the top of the plate. Use a sealing paddle to adhere to the film thoroughly. Trim excess film off the left and right edges with a razor blade. Repeat for the desired number of plates.

4.4. Centrifuge the plates at 200 x *g* for 10 s to settle the fluid. Do not allow the plate to be chilled since this can cause condensation to build up in the wells, obscuring absorbance readings.

5. Exposure

5.1. Once the flies are ready for the consumption assay, perforate the wells on the top surface of the plate with the needle probe tool equipped with a 0.25 mm diameter needle. Use the same order to perforate as was used when dispensing the solutions. Flip the plate and perforate the wells on the bottom. Wipe off the needle between solutions to prevent cross-contamination. Be careful not to touch the perforations as this wicks the solution from the wells.

5.2. Read the plate's absorbance at 630 nm without a lid.

5.3. Place an internal lid on the top sealing film to ensure that the condensation rings encircle the perforated wells. Place the external lid on the plate.

5.4. Place the feeder plate face-up on the coupler such that the guides align the appropriate holes of the feeder plate and starvation plate. Ensure that the coupler and plates are correctly oriented (i.e., ensure the angled corner of the coupler and the plates match). Wrap elastic bands around the top and bottom plates to hold the coupler together. Check for alignment and gaps between the feeder plate and coupler.

5.5. Once all the feeder plates are loaded onto the couplers, open the wells for the plates by adjusting the barrier strips on the coupler. Place the coupler/plate assemblies in the secondary container. Each secondary container can accommodate up to six assemblies.

5.6. Place the lower half of a pipette box containing soaked paper towels into each secondary container to provide humidity. Close the lid of the secondary containers and transfer them to a controlled environment (25 °C, humidity controlled, 12 h light:dark cycles). Allow the flies to consume for 22 h.

5.7. After the 22 h exposure, check each plate for dead flies and update the plate layout accordingly. After all the plates are checked, anesthetize the flies *en masse* by pumping CO_2 inside the secondary container. After ~60 s, ensure that all the flies are immobilized. Gently tamp the flies into the starvation plate and replace the plastic barrier strips. Remove the feeder plates for reading.

5.8. Re-read the plate's absorbance at 630 nm. Repeat until all plates have been read.

6. Data analysis

NOTE: Analysis can be performed with the investigator's preferred software package.

6.1. Omit any flies that died during the 22 h exposure.

6.2. For each well, calculate the volume consumed as:

$$C = V * (\frac{ABS_0 - ABS_1}{ABS_0})$$

C – Volume Consumed (µL)
V– Initial Well Volume (i.e., 10 µL)
ABS_0– Pre-Exposure Absorbance
ABS_1– Post-Exposure Absorbance

NOTE: Consumption is denoted as a positive volume in the calculation.

6.3. To account for evaporation, subtract the mean evaporation volume from fly consumption values within respective plates. For 2-choice/preference testing, adjust every well by its respective solution, e.g., adjust "choice 1" wells by "choice 1" in the evaporation controls.

6.4. After adjusting for evaporation, drop any samples with a consumption value less than zero.

6.5. For 2-choice testing, calculate the preference for each well as:

$$P = \frac{F_A - F_N}{F_A + F_N}$$

P – Preference Index (positive direction indicates preference)
F_A – Volume of Liquid Food Consumed Containing Additive (Choice 2)
F_N – Volume of Normal Liquid Food Consumed (Choice 1)

7. Microplate and coupler washing protocol.

NOTE: Take care to prevent damage to the bottoms of the microplates, as damage can affect sealing.

7.1. Remove the films and labels from the 1536-well microplates. Separate the couplers and barrier strips. Place the barrier strips in a sealable container, such as a bottle. Wash the barrier strips by vigorously shaking in a series of warm tap water, mild detergent solution, warm tap water, and then distilled H_2O.

7.2. Rinse 1536-well microplates and couplers under warm tap water. For microplates, run tap water through each microplate's wells to clear as much solution and debris as possible. If needed, use a pipette tip to dislodge debris; do not use metal or glass utensils on the plates.

7.3. Cover each plate and coupler with a mild detergent solution (e.g., 1% v/v Aquet). For the plates, scrub the surfaces with a gloved hand. For the couplers, use a brush.

7.4. Rinse each plate thoroughly with tap water, and then with distilled H_2O. Ensure that the wells are specifically rinsed out under the water flow.

7.5. Allow the plates and couplers to air dry covered at room temperature. Store in a clean storage bin until use.

NOTE: Never handle the 1536-well microplates without gloves. Residual oils from the skin can hinder sealing, leading to well leakage and evaporation.

REPRESENTATIVE RESULTS:

To determine whether any correlations exist among the wells of individual plates, the evaporation was quantified for every well (n = 96 wells/plate for three plates). Evaporation was found to be -0.036 µL ± 0.003 µL (mean ± SEM throughout). (**Figure 6A**) Pearson correlations were calculated to evaluate trends between evaporation and well locations. The correlation coefficient (**Figure 6B,C**) for evaporation versus rows was -0.04 (p = 0.4949) and for evaporation vs columns was -0.23 (p = 0.0001). Groups were subsequently distributed among columns to mitigate the mild but statistically significant correlations across columns.

[Place **Figure 6** here]

To establish the validity of the protocol, consumption was quantified for 3–5-day old *Canton-S B* flies (n = 36/sex/plate and n = 24 evaporation controls/plate for three plates) (**Figure 7**). Evaporation among the control wells was significantly different from zero (-0.030 µL ± 0.006 µL, $p = 4.81 \times 10^{-6}$; one sample *t*-test vs zero). Two samples were omitted (both male) from the dataset, one due to death during the overnight exposure and the other due to negative consumption value following adjustment for evaporation. This yielded a > 99% sample retention rate.

[Place **Figure 7** here]

Subsequently, an Analysis of Variance (ANOVA) model was constructed as described by Y = µ + S + P + SxP + e, with Y as the group mean, µ as the overall mean, S as the fixed effect of sex, P as the fixed effect of plate, SxP as the interaction between Sex and Plate, and e as the residual variability. ANOVA showed no significant plate-to-plate variability (p = 0.671) or sex-specific interactions with plates (p = 0.104) for consumption, while sex alone significantly contributed to the observed variation in consumption ($p = 4.17 \times 10^{-18}$). A *post hoc t*-test showed that males consumed significantly less than females (0.500 µL ± 0.017 µL vs 0.811 µL ± 0.028 µL, $p = 1.13 \times 10^{-17}$, two sample *t*-test with unequal variance).

To demonstrate that the assay can be used for two-choice preference quantification, flies were given a choice between a 4% sucrose solution with 1% yeast extract and a 4% sucrose solution supplemented with 15% ethanol and 1% yeast extract. Both males and females showed overwhelming preference for the solution with ethanol and yeast extract with preference indices of 0.974 ± 0.026 for males and 0.876 ± 0.06 for females (average ± SEM) (**Figure 8**).

[Place **Figure 8** here].

FIGURE LEGENDS:

Figure 1: Components of the Microplate Feeder Assay. (**A**) 3D rendering of the assembled microplate feeder assay. The 1536-well microplate is oriented by the 3D-printed coupler such that each well of the lower 96-well microplate has access to four wells of the upper 1536-well microplate. Access to the wells can be controlled by adjusting the position of barrier strips slotted through the coupler. (**B**) A graphical representation of each well of the microplate feeder assay. Consumption solutions are retained in each well using a sealing film that has been perforated to allow access by the fly.

Figure 2: Overview of the procedures in the Microplate Feeder Assay. The figure shows a flow diagram that corresponds to steps 4.1–5.8 of the protocol.

Figure 3: Absorbance-based quantification of well volume. (**A**) Incident light of known input intensity (I_0) traverses each well. Attenuation of light at different fill volumes yields different output intensities (I), exhibiting a linear relationship between volume and absorbance. (**B**) Empirical measurement of absorbance vs. volume.

Figure 4: Representative Starvation Plate Layout. The diagram shows the organization of evaporation controls and male and female flies in a 96-well plate used in this study. Alternative configurations, including alternating rows of males and females with evaporation controls in rows A and H, can also be used.

Figure 5: Fill order and well location for the 1536-well feeder plate. The diagram illustrates step 4.2 of the protocol. Arrows show the order in which the feeding solution is introduced in the feeder plate one column at a time from column 1 through 12. Sample B1 is magnified to show an example of the location of feeding solutions for 1-choice and 2-choice assays.

Figure 6: Evaporation in the MFA. (**A**) Density distribution of evaporation changes with mean ± SD indicated by the dashed line. Correlations between evaporation and rows (**B**) or columns (**C**) with the Pearson correlation coefficient and p-value as indicated.

Figure 7: Consumption quantification using the MFA. (**A**) Consumption was visualized using a custom fabricated glass chamber. Flies were observed drinking from perforated wells and exhibited blue abdominal staining following ingestion of the dyed solution. See also **Supplementary Video S.1.** (**B**) Consumption values (mean ± SEM) among evaporation controls, male flies, and female flies. Pairwise *post hoc t*-test with unequal variance was performed for statistical comparisons, with significance indicated by bars.

Figure 8: Preference quantification using the MFA. Consumption of 4% sucrose versus 4% sucrose supplemented with 15% ethanol and yeast extract for male and female flies (n = 33 for each sex). Male flies consumed more of the ethanol solution than the control sucrose solution (0.511 µL ± 0.029 µL versus 0.00 µL ± 0.017 µL; $p = 4.06e^{-10}$; two-sample *t*-test). Female flies also

consumed more ethanol solution than the control sucrose solution (0.939 µL ± 0.044 µL versus 0.132 µL ± 0.044 µL; $p = 7.38e^{-17}$; two-sample *t*-test).

SUPPLEMENTARY FILES:

Supplementary Video S.1: **The video shows a fly feeding from the perforated well and accumulating blue abdominal staining while ingesting the dyed solution.** A still image is shown in **Figure 7A**.

Supplementary File S.2: Microplate Feeder Assay Coupler. This is a 3D-printable construct of the coupler used in the MFA. Printing material Nylon PA12 was used for the MFA.

Supplementary File S.3: Microplate Feeder Assay Barrier Strip. This contains the design of the plastic barrier strips used to toggle exposure of flies to the feeder plate. A single coupler can utilize up to twelve barrier strips.

Supplementary File S.4: Unpacking and fabrication instructions for the Microplate Feeder Assay. Instructions are included for unpacking the coupler and the barrier strips. Fabrication instructions are included for the inner lid, outer lid, and secondary container used to limit evaporation during exposure.

Supplementary File S.5: Cost comparison of the Microplate Feeder Assay (MFA) and a 1-choice single fly CApillary FEeder (CAFE) assay. Testing 72 flies/sex for a single line would require two sets of MFA equipment (couplers + plates + barrier strips), while the CAFE would only need 1 capillary for each culture vial. Despite the large difference in initial investment for the MFA, the large difference in recurring costs ($14.80 vs $46.08, respectively) would allow for the up-front costs to be recovered after testing only 4 lines (break-even point).

DISCUSSION:

The study describes a novel protocol for quantifying consumption in *Drosophila*: the Microplate Feeder Assay (MFA). In this assay, flies consume from sealed wells of a 1536-well microplate through controlled-size perforations (**Figure 1, Figure 2; Supplementary Video S.1**). Since liquid food is dyed and provided via microplate, measurements of the optical absorbance of the food can be obtained using a microplate spectrophotometer (**Figure 3**). In this manner, consumption is determined by comparing the absorbance before and after consumption, and then applying this proportion to the known volume dispensed prior to consumption. This was verified empirically by measuring the absorbance of different volumes of the dyed medium (**Figure 3B**).

To develop this assay, a device was needed that could leverage the absorbance-based quantification of consumption. Testing flies in a microplate format is appealing because it complements the microplate used to dispense food and allows for flexibility in selecting from multiple plate formats (e.g., 6-, 12-, 48-, or 96-well formats) by adjusting the coupler geometry. A 96-well microplate format was chosen to allow for individual fly culture.

The 3D printed device (**Figure 1**) precisely orients the 1536-well feeder plate with the 96-well

culture plate, giving each fly access to up to 4 wells of the feeder plate for consumption. Furthermore, to provide adequate time for distributing flies into the housing plate and to control assay initiation, the device includes toggling barrier strips containing the flies in their respective wells and preventing breaches. The files needed to procure or modify these parts are provided (**Supplementary Files S.2–S.3**), as well as the necessary fabrication instructions for the relevant pieces (**Supplementary File S.4**).

The MFA provides a simple high throughput method that complements more elaborate methods to monitor Drosophila feeding behavior[18,21,22]. The MFA offers multiple advantages over other methods used to quantify food intake. The throughput is increased by quantifying consumption using a plate reader. This eliminates manual measurements and obviates manual data entry. Data are also amenable to programmatic extraction and processing. In addition, the higher throughput increases the feasible number of biological replicates, particularly compared to communal feeder designs, which substantially increases the power to detect small differences in consumption. Using the MFA, a single experimenter can quantify the consumption or preference of over 500 flies per overnight run of the assay. By overlapping runs of the assay, over 2,000 flies can be tested in a 5-day period. Lastly, there are long-term cost savings due to the reusability of microplates and couplers (**Supplementary File S.5**). Using the MFA, the estimated cost per assay can be as low as $14.80, with a $127.60 up-front cost for the equipment. Using the classic CApillary FEeder (CAFE) assay, which requires costly precision microcapillaries, the estimated cost per assay for a comparable number of replicates is $46.08. Thus, while there is an upfront investment in acquiring the necessary equipment, the reduction in recurring costs can lead to substantial savings, particularly in instances where repeated testing is performed.

As with all assays, the MFA has certain limitations. Chiefly, it requires access to a microplate spectrophotometer capable of reading 1536-well microplates. Additionally, the reliance on absorbance measurements for quantification makes the method susceptible to optical interference. This manifests as negative consumption values for a small subset of samples tested. Nutrients, drugs, pharmaceuticals, or toxins of interest must also be water-soluble to be compatible with the assay.

Despite its limitations, this method offers a high throughput method of quantifying consumption behaviors in *Drosophila*. Furthermore, the coupling device could be easily modified to accept many plate formats, allowing it to accommodate a variety of insect species.

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DISCLOSURES:
The authors have no conflicts of interest to disclose.

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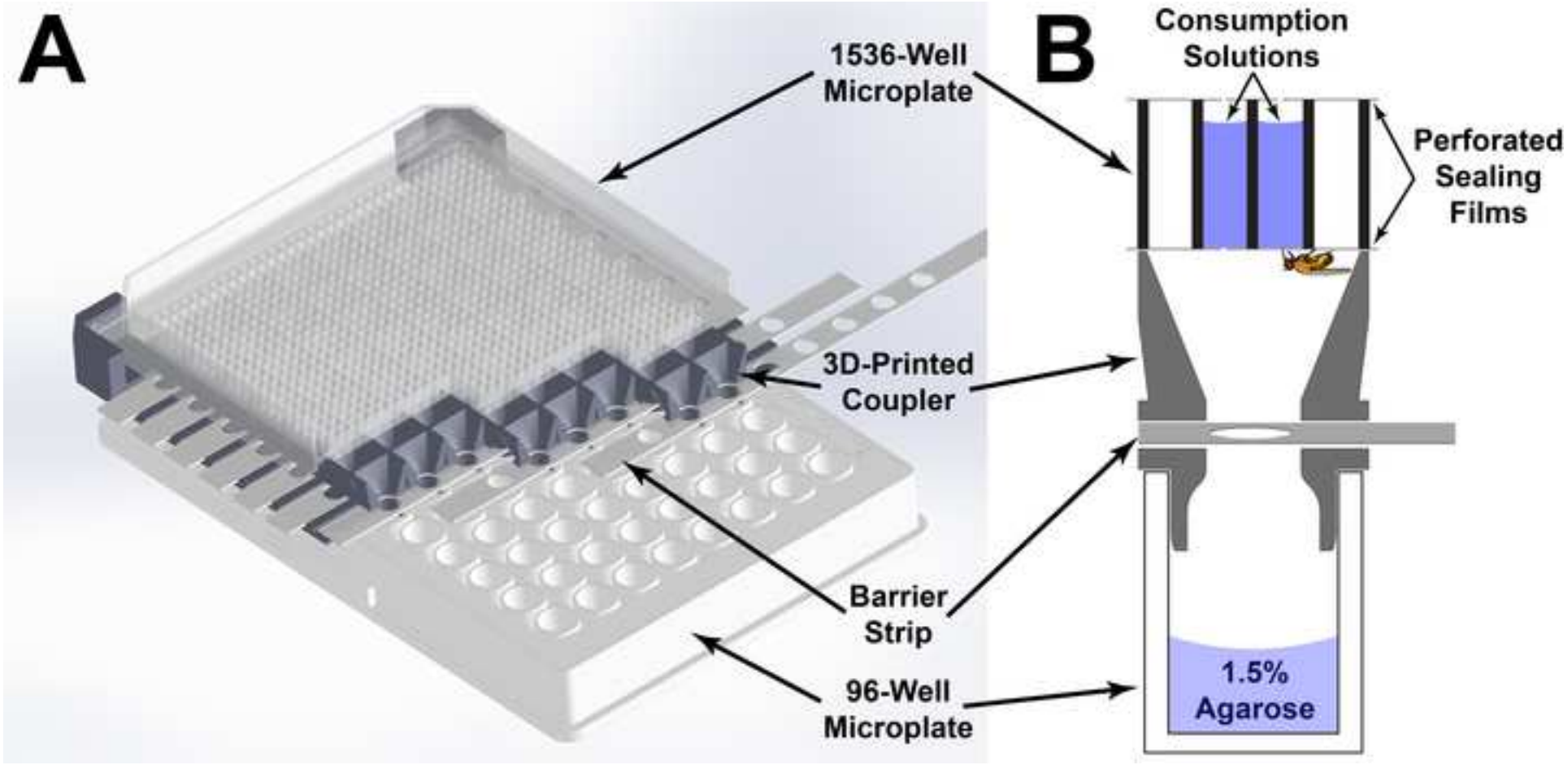
484

Figure 1



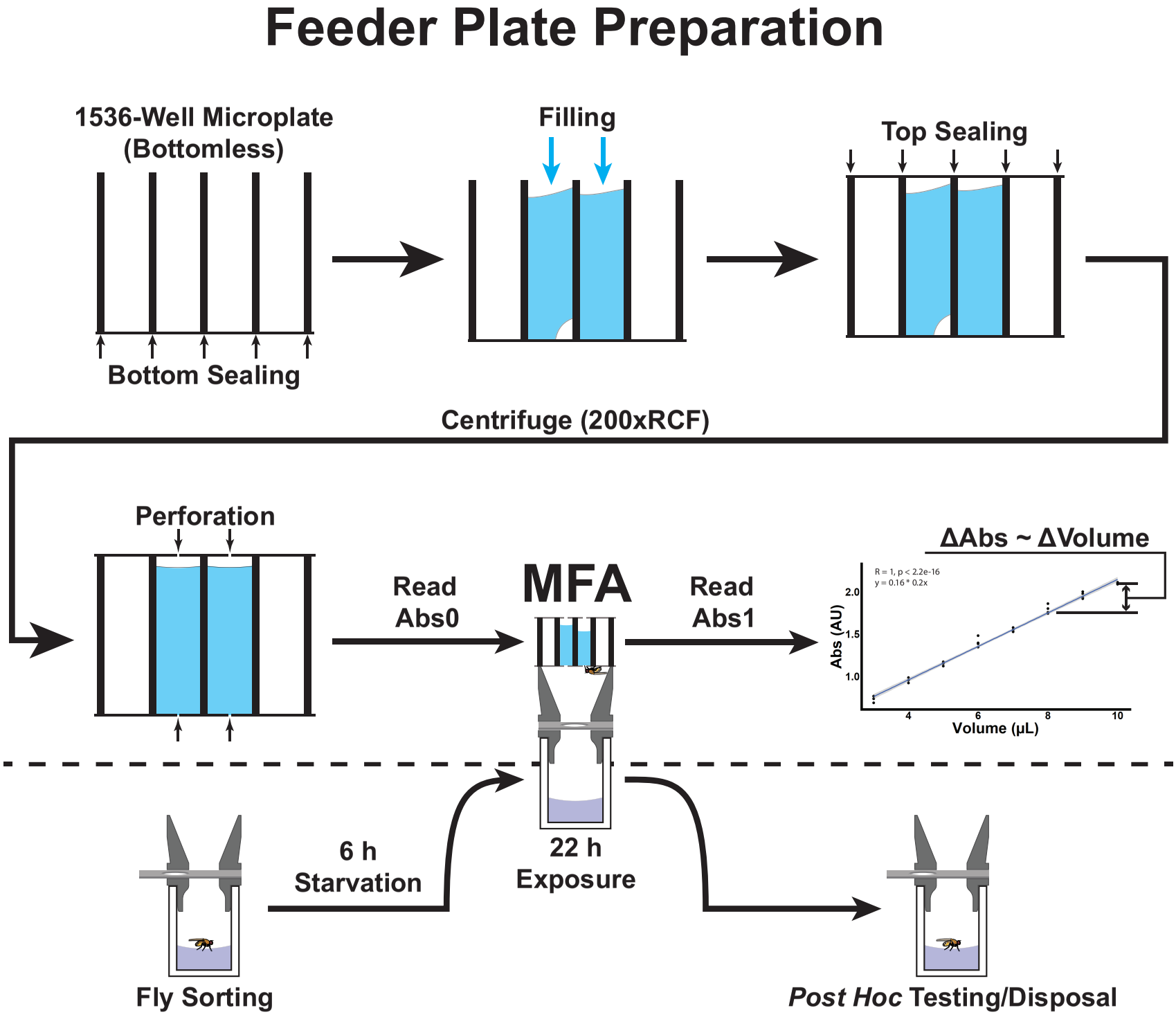
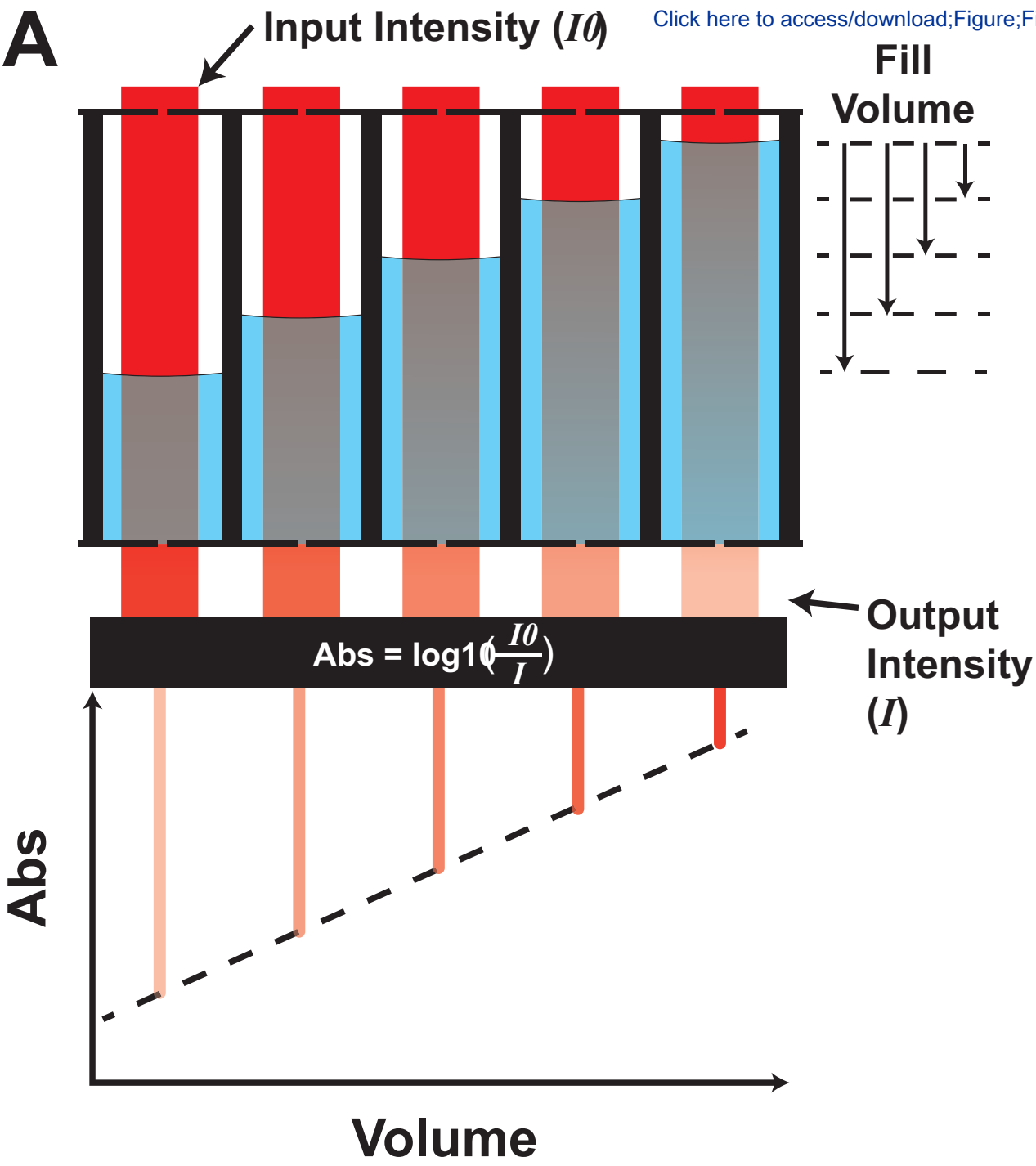
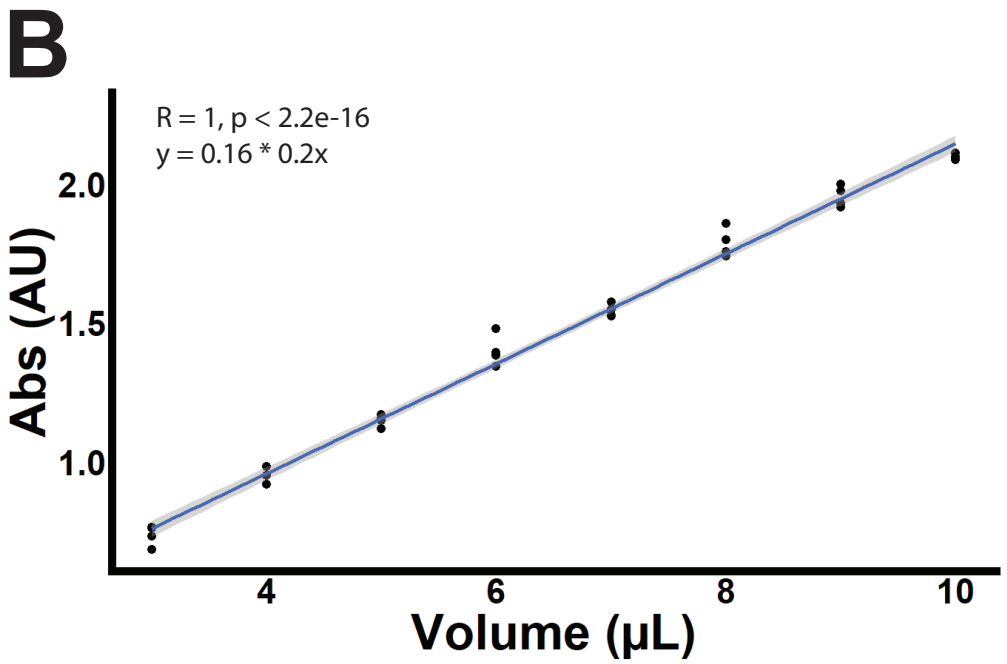


Figure 3





Starvation Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	E	E	E	E	E	E	E	E	E	E	E	E
B	E	E	E	E	E	E	E	E	E	E	E	E
C	M	M	M	M	M	M	M	M	M	M	M	M
D	M	M	M	M	M	M	M	M	M	M	M	M
E	M	M	M	M	M	M	M	M	M	M	M	M
F	F	F	F	F	F	F	F	F	F	F	F	F
G	F	F	F	F	F	F	F	F	F	F	F	F
H	F	F	F	F	F	F	F	F	F	F	F	F

E - Evaporation Control **M** - Male **F** - Female

Figure 4

Click here to access/download;Figure;Figure 4.eps

Figure 5

Figure 6

Click here to access/download;Figure;Figure 6.psd

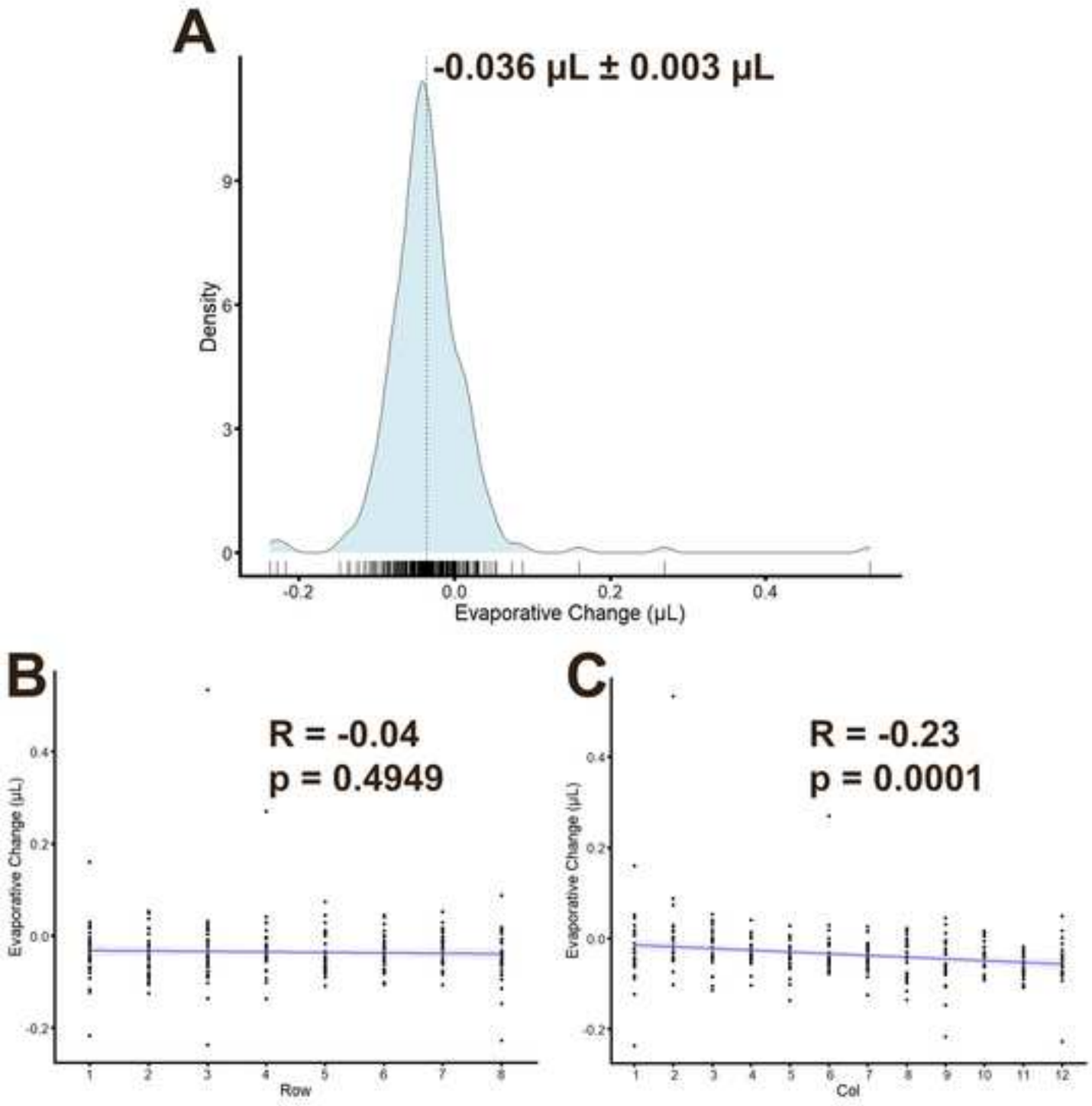


Figure 7

Click here to access/download;Figure;Figure 7.jpg

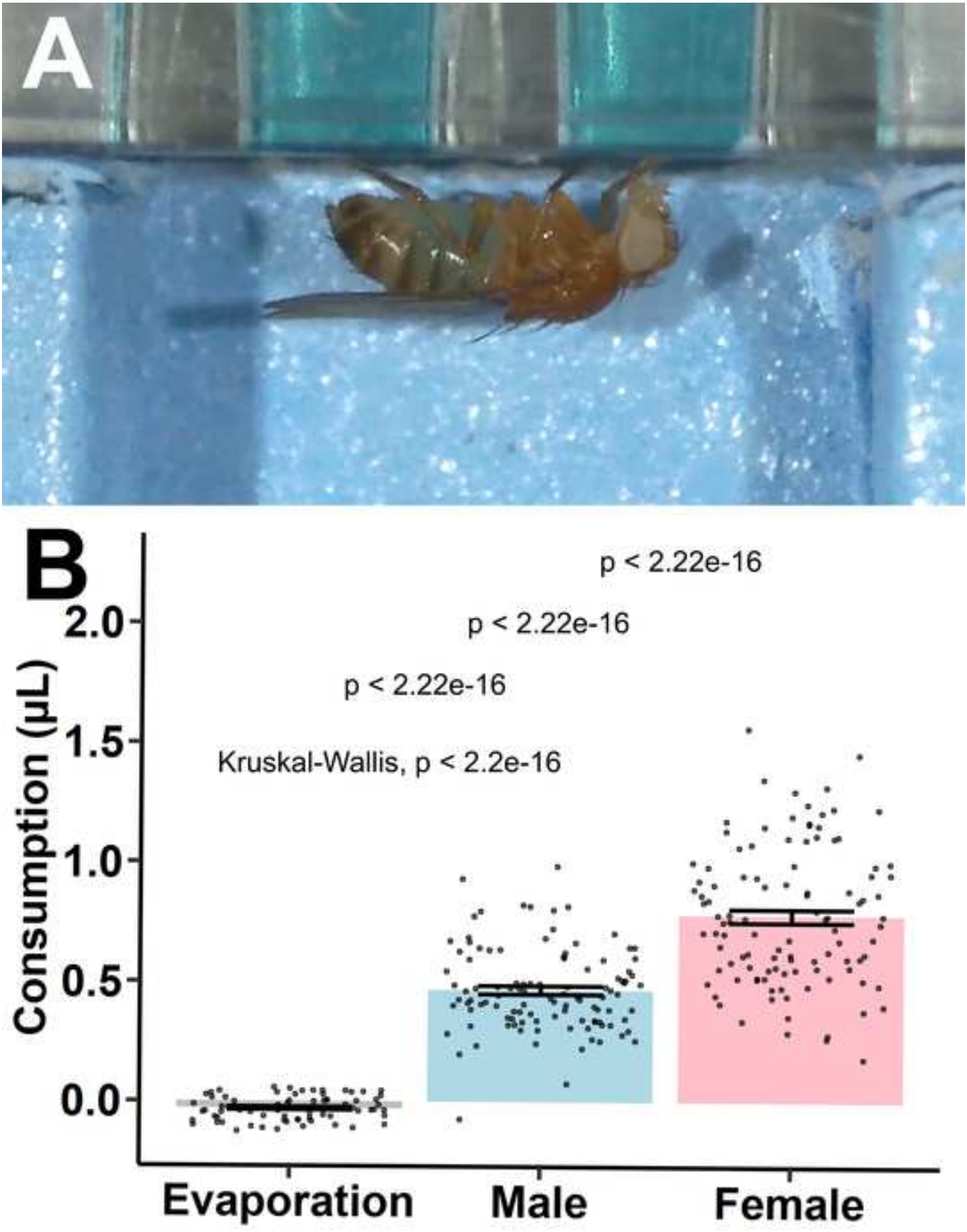
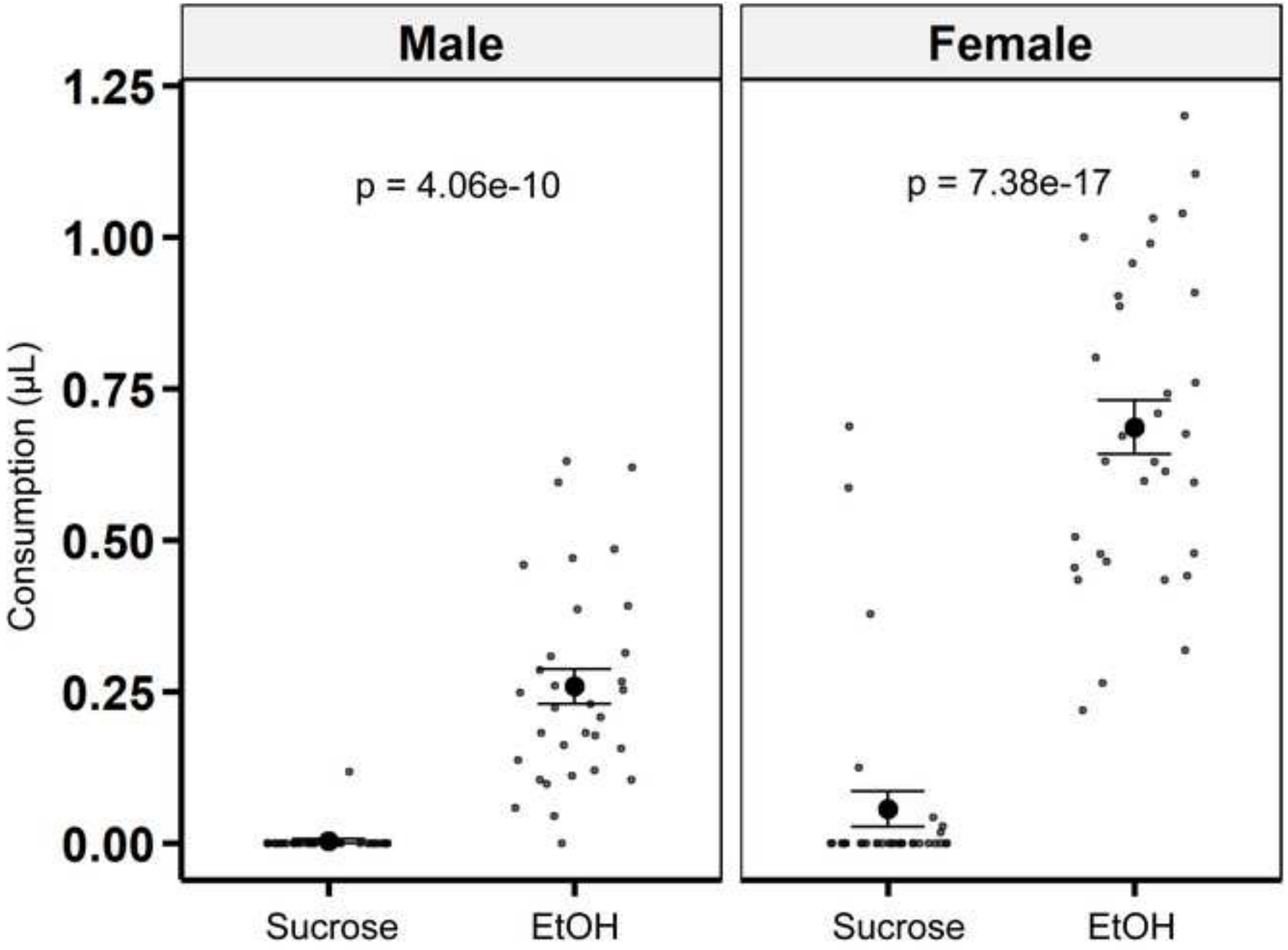


Figure 8

Click here to access/download;Figure;Figure 8.tif



Responses to Reviewers

We appreciate the reviewers' positive evaluation of our manuscript. We have carefully considered the editorial comments and the reviewer's' comments and made appropriate revisions in the manuscript. Below are our point-by-point responses:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We proofread the manuscript and there are no spelling or grammar issues.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents…"

We reviewed the summary statement and believe it is accurate.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

This instruction has been followed throughout the protocol.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We believe there is enough detail to enable replication of the protocol.

5. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

This instruction has been followed throughout the protocol.

6. 2.3: How many flies per column/per plate/per well is loaded?

We note to load individual flies by column into the starvation plate.

7. 2.4: How do you let the flies recover?

We indicate that flies recover spontaneously after removal of the CO_2.

8. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted elements of the protocol with yellow highlight that are essential steps for filming within a three-page limit.

9. Please ensure the results are described in the context of the presented technique, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Data from both successful and sub-optimal experiments can be included.

We believe that the description of the Results is appropriate.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All figures are originals; there are no copyright issues.

11. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Please include the legends for supplementary files/videos as well.

Figure legends have been added after the Representative Results.

12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We believe the discussion covers all necessary points.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes the construction and use of a Microplate Feeder Assay for Drosophila. It is an economical, high throughput method for quantifying liquid food consumption in Drosophila that could also be adapted for other small insects. This method is a welcome new addition to the large number of existing feeding assays, and will likely have an impact on the field given that extent assays are low throughput. The low cost of this system and its high-

throughput will also enable new labs to join this field and might even be used in education. It is a very nice new technique, and it is overall well described in this manuscript.

We appreciate the positive comments.

Major Concerns:
I have two main issues, (1) is to do with the size of the hole through which flies can feed, (2) is with accessing and understanding the supplementary information files.

1. Hole size: One aspect that is not well described is the hole through which the flies feed. The only explicit description of the process I could find is on line 167, and it does not seem right:

Line 167 "Once flies are ready for the consumption assay, perforate the wells on the top surface of the plate with the needle probe tool equipped with a 25mm diameter needle".

A 25mm diameter needle would pierce a hole that is larger than the size of a fly; could that be a typo? I do not understand how the liquid could stay in the wells if one pierces such large hole through it. The picture of figure 7A is very useful in showing a fly feeding through the film covering the food wells. However I cannot see a hole there and certainly not one of 25mm diameter.

Another place where the size of this hole is mentioned is on line 71: "By using a bottomless 1536-well plate and sealing films, solutions are dispensed into select wells and perforated with precise diameter needles to provide access to the flies". Here, the diameter is not specified.

Could the authors please clarify the size of this hole and explain clearly in the intro how the fly can feed through it and how the liquid is prevented from dripping into the chamber?

We apologize for the confusion which is due to a typographical error which we unfortunately missed. The diameter of the hole is 0.25 mm. We have corrected this in the text.

2.An important part of this manuscript is information on how to fabricate the assay. The authors state that the files needed to procure or modify these parts are provided (Supplementary Files S.2-S.4). I could however not find these files. There are several unlabelled figures on p23-27 of the PDF that seem to represent some of these figures (this appears to be an error stemming from the creating of the final PDF of this article). Some of them might be Supp files S2-S4, but it is hard to tell what they represent given that there are no figure legends. I especially do not see what to make of the strip with holes on page 26 and p 27. Could the authors ensure that the figure are properly labelled and provide legends for those figures (at least for the strips on page 26 and 27)

We apologize for this oversight. We have included proper references to the Supplementary Files in the text as well as legends.

I would expect a link to a 3D printing file for the coupler and a description of barrier strips. I could find a file labelled "Dual Plate Coupler V8.STL", which fits the description of the coupler and is a 3D printable file. This file is however not references in the text. I could not find any

explanation on how to fabricate the barrier strips. Are those the strips on page 26 and 27? Could the authors ensure that they describe how those strips are made? That they are laser-cut is mentioned on page 23, but that is not very helpful.

We updated the supplementary files and we removed the original Supplementary File S3, which the reviewer refers to, as it was outdated, redundant, and could not be opened without specific software. More importantly, a .STL file is industry-standard for 3D printing, so the .SLDPRT file is unnecessary.

Finally, I could not review the following file since they were neither on the submission portal, nor in the submission file: Supplementary Video S.1

The Supplementary Video S.1 File was too large to be uploaded in the JoVE website. We will send it as a Google Drive attachment to the editor.

Minor Concerns:
In the intro, the authors reference a series of existing feeding assays. Maybe mention the FlyPad, "Automated monitoring and quantitative analysis of feeding behaviour in Drosophila" ((doi:10.1038/ncomms5560).

We have added this reference.

Lines 92-312 of the discussion seem more appropriate for the introduction. I find that it provides the best overall description of the assay. Please consider moving this section to the introduction to replace the description on lines 66-72

It is not clear what section the reviewer refers to. Lines 92-312 cover a vast amount of the manuscript. We believe both the Introduction and Discussion are appropriate.

Line 109 "2.1. Prepare couplers". Why is couplers plural? In figure 1, there is only one coupler per assay?

Investigators can prepare multiple couplers to enable several assays to be run in parallel.

Line 329. "Species of interest must also be water-soluble to be compatible with the assay". The word "species" is very vague. Can the authors use a more specific word or words; such as molecules, nutrients, drugs or at least put examples between brackets (e.g. nutrients, drugs.)

We have revised the sentence to read: Nutrients, drugs, pharmaceuticals, or toxins of interest must also be water-soluble to be compatible with the assay.

Reviewer #2:
Manuscript Summary:
The authors describe a novel microplate-based feeding assay for quantifying liquid food consumption in Drosophila. This assay offers advantages that allow for performing flexible and high-throughput experiments at low cost. At its core, the assay uses perforated sealing chambers that contain liquids. The actual quantification of intake is done by calculating the

consumed volume by optical measurements. The manuscript is well written and describes rigorously the protocol for using the methods, making this assay an important step for moving towards reproducible science across labs. However, I am concerned that the authors have not fully verified the assay's capabilities to correctly and accurately quantify food intake in flies. I would consider the manuscript for acceptance after these major concerns are addressed.

Major Concerns:
- The authors do not quantify the actual food intake of the liquids in the flies. The authors could provide experiments where the actual intake could be marked and quantified by bioluminescence. A similar quantification has been performed in Itskov et al. (2014) (see below).

Food intake is evident from absorption of the dye as shown in Figure 7A and since each well contains an individual fly we know exactly how much of the feeding solution the fly has consumed and if necessary the amount of any ingested compound can be calculated from its known concentration in the feeding solution. We have indicated this in the Introduction.

- Furthermore, it would be great to see that this feeding assay can quantify more subtle preferences such as different concentrations of sucrose, instead of comparing sucrose to ethanol. Another possibility could be to test flies with different starvation times, as it is known that with increasing starvation time, flies will increase their intake of sucrose.

We agree that there are many interesting experiments that can be done with this assay. However, the data we present are sufficient to document the usefulness of the assay. Differences between males and females provide an indication of the resolution that can be obtained with the assay.

Minor Concerns:
- I would quantify and highlight the high throughput of this method, specifically
- How long does each step in the experimentation protocol take?
- How many flies could be tested in parallel?
- How many flies could be tested in an hour or a day?

We have indicated in the Discussion the following sentence: "Using the MFA, a single experimenter can quantify consumption or preference of over 500 flies per overnight run of the assay. By overlapping runs of the assay, over 2000 flies can be tested in a 5-day period."

- In Fig. 6, it is not clear to me which liquid has been used to quantify evaporation. I would expect that different solution, eg. sucrose and ethanol, would lead to different evaporation profiles.

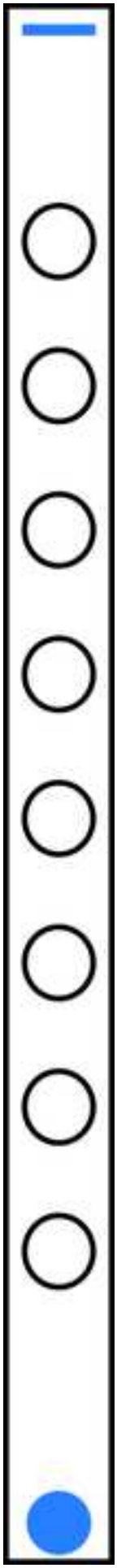
Evaporation rate is quantified for each experimental solution.

- some of the figures could be combined and scaled down (mainly fig. 1-5)

We prefer to leave the figures as they are, since combining and scaling down would reduce clarity.

- the authors discussed existing literature and methods for quantifying feeding and food intake in a very limited way. The authors should consider discussing the following papers:
- Itskov, Pavel M., et al. "Automated monitoring and quantitative analysis of feeding behaviour in Drosophila." Nature communications 5.1 (2014): 1-10.
- Ro, Jennifer, Zachary M. Harvanek, and Scott D. Pletcher. "FLIC: high-throughput, continuous analysis of feeding behaviors in Drosophila." PloS one 9.6 (2014): e101107.
- Yapici, Nilay, et al. "A taste circuit that regulates ingestion by integrating food and hunger signals." Cell 165.3 (2016): 715-729.

We have included references to the papers suggested by the reviewer.

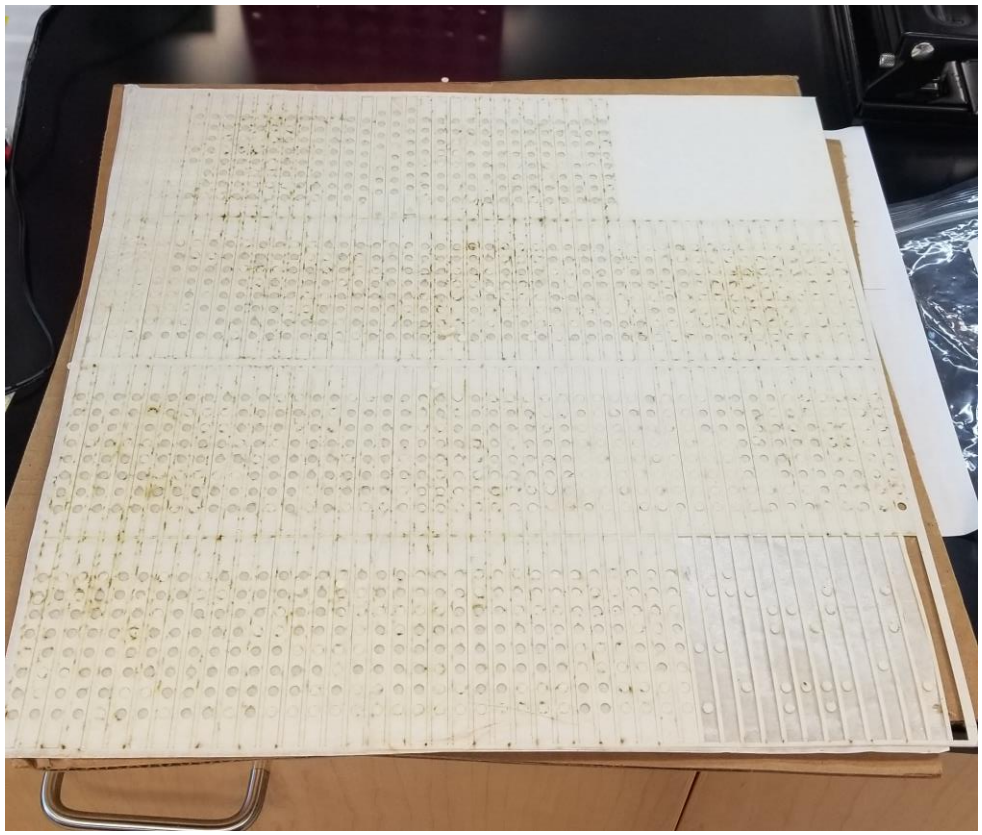


COUPLER UNPACKING INSTRUCTIONS

Upon receipt, the couplers will have residual sediment in the channels from the fabrication process. Clear out each channel using a barrier strip. There may be resistance until the sediment is fully removed. Wash couplers with a mild detergent solution prior to first use.

BARRIER STRIP UNPACKING INSTRUCTIONS

Barrier strips are received as a laser-cut sheet of parts covered by a protective paper backing (image below). To prepare the strips for use, remove each piece from the sheet and peel off the protective backing. Wash with a mild detergent solution prior to first use. For loose-fitting strips that slide too freely, coil them around your finger to impart curvature, which will help to hold the strip in the channel of the coupler.

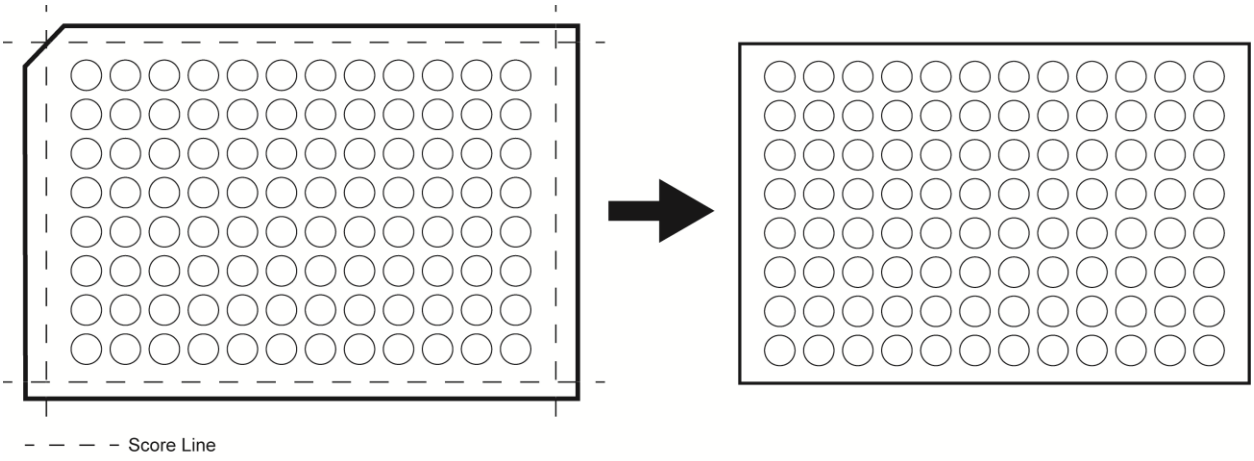


EVAPORATION-RESISTANT LIDS

Note: An inner lid and outer lid are needed for each MFA plate.

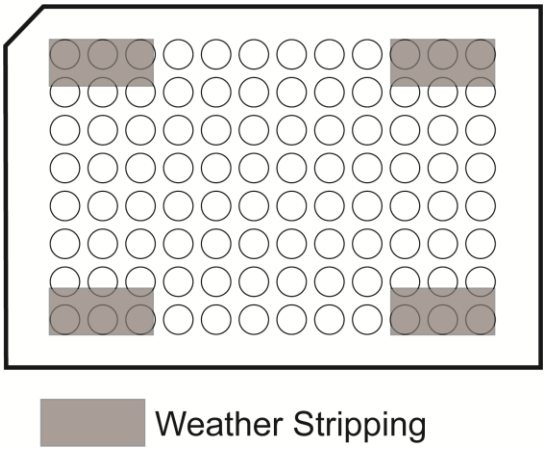
Inner Lid Fabrication

Using a razor blade and a straight-edge, carefully score a straight line ~4mm from each edge of the lid as indicated below. Using a pair of pliers, carefully snap off the excess plastic by bending along each scored line. Ensure that protective eyewear is used, as fragments can be projected during breaking.



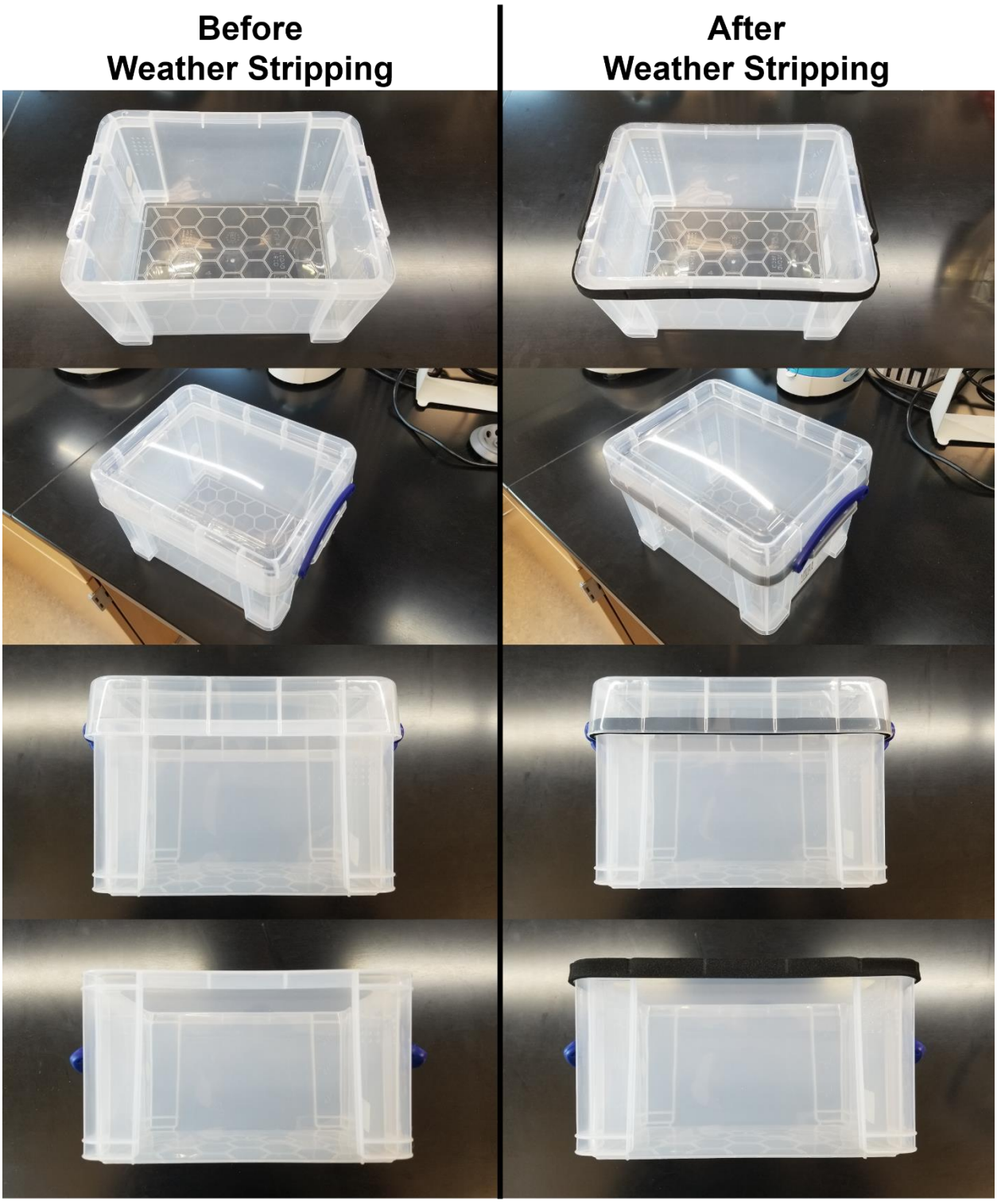
Outer Lid Fabrication

Cut small strips (~20mm long) of weather stripping, 4 strips per lid. Peal the adhesive backing and apply to the inside of the lids as indicated in the diagram below.



EVAPORATION-RESISTANT SECONDARY CONTAINERS

At the surface where the container and its lid overlap, wrap weather stripping along the edge to fully seal the box (image below).



Supplementary File S.6 Cost Comparison of the Microplate Feeder Assay (MFA) and a CApillary FEeder (CAFE) assay. Testing 72 flies/sex for a single line would require two sets (couplers + plates + barrier strips), while the CAFE would only need 1 capillary for each cultu difference in initial investment for the MFA, the large difference in recurring costs ($14.80 vs allow for the up-front costs to be recovered after testing only 4 lines (Break Even Point).

Testing Constraints

Number of Sexes	2
Total Lines	1
Replicates/Line/Sex	72
Total Flies	**144**

Microplate Feeder Assay	**Quantity Needed**	**One-Time Costs**
Couplers (reusable; $36/item*)	2	$72.00
Plates (reusable; $950/60 plates)	2	$31.60
Barrier Strips	24	$24.00
Sealing Films ($35/100 films)	4	--
96-well Microplates ($6/plate)	2	--
Total		**$127.60**

***Includes economic processing cost and standard shipping and handling.

CAFE	**Number per Line**	**One-Time Costs**
Capillaries/Vial	1	--
Capillaries ($250/1000)	144	--
Culture Vials ($0.07/vial)	144	--
Total		**$0.00**

Recurring Savings from MFA (per line)	
Break Even Point	

1-Choice Single Fly
s of MFA equipment
ıre vial. Despite the large
$46.08, resp.) would

Recurring Costs
--
--
--
$2.80
$12.00
$14.80

Recurring Costs
--
$36.00
$10.08
$46.08
$31.28
4 lines