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Knowledge Creation

Pedro M. Domingos, PhD

Principal Investigator, ITQB-UNL
Av da Republica, 2780-157 Oeiras
Portugal
Phone: 351 21 115 7781
Cell: 351 91 119 6164
domingp@itqb.unl.pt

Oeiras, 15th April 2020

Subject: Manuscript Revision JoVE61323

Dear Editor of *JoVE*,

Many thanks for your email and for the news about our manuscript. We are very pleased that the reviewers liked our manuscript and are also thankful for their specific comments, which we address in this revised version. We hope you find this version satisfactory for publication without further delays. Below we detail our response to each specific comment.

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We proofread the whole manuscript, hopefully there are no spelling or grammar errors.

2. Please use American English throughout.

Response: We used American English.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Response: We followed these format settings.

4. Please revise the text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Response: We eliminated all of these words in the protocol section.

5. 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.”

Response: We did the necessary changes to the manuscript in order to follow the recommended style.

6. Please ensure all centrifuge speed is converted to centrifugal force (x g) instead of revolutions per minute (rpm).

Response: We didn’t use rpm to indicate centrifugations speed.

7. The Protocol should contain only action items that direct the reader to do something in complete sentences.

Response: All the steps in the protocol are action items in complete sentences.

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

Response: All the individual steps in the protocol have a maximum of 3 action sentences.

9. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Response: We rearranged some of the information we were providing as notes in the protocol section, according to your suggestions. Part of this information was moved to the steps, other to the discussion and we also tried to simplify or eliminate bits of the notes that were not crucial.

10. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: We believe all our steps have the needed information of how to perform the actions.

11. 1.2: Volume of water needed?

Response: The volume of water needed in this step is dependent on the volume of food needed to prepare by the experimenter, as mentioned in the previous step. We cannot give a specific volume here, but added that is recommended to use around 50% of the total volume of food to be prepared. Additionally, in step 1.4, we added the following: "complete the total volume with distilled water." (lines 132-133).

12. 5.4: how is this done?

Response: In order to improve the understanding of the step 5.4, we changed the text and we added some extra information.

"The mechanical lysis is obtained by using a tissue lyzer and the glass beads added in step 5.1."

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 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.

Response: We took into consideration the guidelines you provided here to select the steps of the Protocol that should be filmed. The totality of the highlighted text in the protocol at this reviewed version is less than 2.5 pages.

14. Please ensure the result is described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

Response: In order to attain this suggestion, we added the following information to the third paragraph of the Representative Results section (lines 338-340).

"With the methods described in this protocol, we were able to quantify the relative amount of macronutrients consumed, in terms of P:C ratios, for animals under thermogenetic activation of specific neuronal populations in the larval nervous system."

15. 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 Figure Legend, i.e. “This figure has been modified from [citation].”

Response: Concerning Figure 3, this is an original ilustration done by Christen K. Mirth and not Marisa Oliveira, as previously stated by mistake.

16. Please do not include bullets in the reference section. Please ensure that the references are numbered as it is cited in the text. Please do not abbreviate the journal titles.

Response: We did all the changes needed to the reference section in order to follow your recommendations.

17. Please include table 1 as a table in .xlsx format and not a supplementary file. Please include a legend in the Figure/ table legend section.

Response: We edited table 1 so it is now in .xlsx format. We included as a table instead of a supplementary file.

Reviewers' comments:

- Reviewer #1:

Manuscript Summary:

The present protocol describes a high-throughput method to screen for variation in feeding behavior by adapting a well-stablished color-feeding method in the field. The use of larvae, facilitates the preparation of the experiment and its quantification. The method also allowes the researcher to measure the average individual food consumption, a type of information very difficult to obtain through other methods.

I consider that the protocol is clearly explained and providing a new method to study the neurogenetic basis of feeding behavior using fast and low-cost method adapting available techniques.

Response: We thank the positive evaluation of our manuscript and the excellent specific comments which we address below.

Major Concerns:

- Figure 1B. It shows a detailed description of the composition of the different

macronutrient diets, but the order is confusing, there is too much information and some of it is redundant. For example, protein and carbohydrate in 100g is the same for all diets but is repeated 4 times.

Authors could make just a single table with the next rows: Agar, Sucrose, Yeast, Protein in 1L, Carbohydrates in 1L, P+C and Calories in 1L.

As columns, Protein in 100g, Carbohydrates in 100g and the next four columns the corresponding, 1:1, 1:4, 1:16 and L3 diets. In fact, I think that the rows protein and carbohydrates in 1L and P+C are necessary as they do not provide any essential information.

Response: We changed Figure 1 following your suggestions.

- Section 2.1. Embryo collection cages. Could the authors provide a better description of the system they used. After the adults are removed, the larvae growing in the plates are kept in the cages or the petri dishes are removed from the cages, covered with a plastic lid and put in an incubator at 18C?

Response: This description and detailed action steps were, actually, missing. Thanks for pointing it out! We added two extra steps (2.3 and a new 3.2) and we added an associated note.

“2.3 In the end of the mating period, remove and discard the L3 rearing diet plates used in the genetic crosses. Substitute them for fresh L3 rearing diet plates, in order to perform the egg lays and larval staging.”

“3.2 In the end of the egg laying period, remove the plates from the cages and cover them with plastic lids.”

“NOTE: The mated adults can be transferred to fresh L3 rearing diet plates, so you can perform more egg lays and obtain more experimental larvae. Consecutive egg-lays can be performed with the same adults during one entire working week.”

Minor Concerns:

- Could the absorbance be used to measure feeding in adults with the same efficacy as with larvae? Could the cuticle affect the reader?

Response: Yes, it is a common method used in adults, but extra steps of centrifugation must be done to make sure the extracts are clean enough so that cuticle debris do not interfere with absorbance readings.

- Could the authors briefly described if the system could be used in a two-choice color assay using larvae?

Response: we actually mention the possibility of adapting our protocol to food choice assays. In paragraph of lines 501-502.

"…and setting up food choice assays as previously described [30]".

- Could the authors recommend any alternative method to lyse the larvae in case a tissue lyser is not available?

Response: we added the requested recommendation at the step 5.4.

"(if a tissue lyser is not available, use a homogenizing pestle)".

Reviewer #2:

Manuscript Summary:

This article describes a valuable protocol employing a simple colorimetric method to quantify food intake in third instar Drosophila larvae. It is a handy method that can be used in primary screens to understand larval feeding behavior. In this protocol third instar larvae are grown on medium incorporated with colored food dye for an hour. The food ingested is then quantified by crushing the larvae and measuring the dye content colorimetrically. To test for the neural mechanisms that control feeding behavior using this method, a primary pilot screen was carried out. The screen was set up to assess the role of neuronal populations on nutritional content of larval food intake . Using Drosophila genetics, various neuronal populations were activated in a temperature dependent manner in the larvae. Cohorts of these larvae were fed on different macronutrient media with varying protein to carbohydrate ratio compositions. Based on these pilot quantification of media consumption they could classify the neuronal expression lines into different phenotypic categories. In discussion they highlight the advantages and limitations of this method and the changes that can be brought about in the basic protocol to make it more sensitive and robust. This method, though simple, is an improvement from the previous attempts at quantifying larval food intake. It allows for more interesting feeding behavior assays using larvae to explore areas such as nutrient choice, foraging, satiety and starvation. With the power of Drosophila genetics to manipulate temporal and spatial gene expression one can perform precise measurements and explore different nutrient sensing mechanisms. It will certainly

be useful to have a video protocol for a larval protocol which should guide people working on various aspects of behavior and nutrition.

Response: Thanks for the positive appraisal of our work and the helpful comments.

Major Concerns:

A. 3.1 First egg lay after mating has to be discarded to maintain homogeneity and obtain better synchronized larval development. Females have fertilized eggs in oviduct and lay them in varying stages of development making it hard to maintain uniformity among larval collection. It is imperative that at least the first hour egg collection plate be discarded before the final collection.

Response: To fix this point, we added two extra steps (2.3 and a new 3.2 with an associated note) and an extra paragraph in the Discussion section.

"2.3 In the end of the mating period, remove and discard the L3 rearing diet plates, used in the genetic crosses, from the collection cages. Substitute them for fresh L3 rearing diet plates, in order to perform the egg lays and larval staging."

"3.2 In the end of the egg laying period, remove the plates from the cages and cover them with plastic lids."

"NOTE: The mated adults can be transferred to fresh L3 rearing diet plates, so more egg lays are performed, and more experimental larvae can be obtained. Consecutive egg-lays can be performed with the same adults during one entire working week."

Lines 472-476 "Please, note that he first egg lay after mating, has to be discarded to maintain homogeneity and obtain better synchronized larval development. Females have fertilized eggs in oviduct and lay them in varying stages of development making it hard to maintain uniformity among larval collection. It is imperative that at least the first hour egg collection plate be discarded before the final collection."

B. The reliability of the pilot study could be made stronger if

1. In the proof of principle experiment, some Gal4 lines were included that were known effectors (both positive and negative) of change in feeding behavior. For example hugin neurons that modulate feeding behavior (Melcher and Pankratz 2005, Schoofs et al 2014).

OR

2. While the utility of a ShibireTS line to suppress neuronal function is highlighted as

a suggestion three times (line 110,147,466), It would have been interesting to show the opposing effect of at least one or two Gal4 lines tested in the study. If it was possible to show the reversal of the phenotype through suppression it would reflect better on the method and be a testament to reliability of the protocol.

However, the above suggested experiments do not affect the utility of the dye estimation method itself, but rather a suggestion to improve the macronutrient feeding part of the paper.

Response: This is a very legitimate point and we agree that the reliability of the pilot study would be enhanced with additional tests. However, we do not have a complete data set to address this point. Further, since we are currently under an imposed lockdown policy, due to Covid-19 pandemia, it is impossible for us to access the lab and complete the assays in a timely manner.

Minor Concerns:

A. Corrections by section

3.2 Is the residual yeast cleaned off the egg laying plates when continuing to grow the larvae until the ninth day. If not done so the varying nutritional content leaves the sets of larvae non-uniformly fed before transferring them to test on macronutrient media.

Response: You are right, this is a very important observation. In fact, we only seed the plates with yeast extract during the mating period. In the 3-4 hours egg lays there's no any yeast supplementation, so we avoid that non-uniform feeding before the macronutrient balancing assays. To make that point clear, we added the following information in the note of step 3.2.

"In case yeast extract was used to supplement the L3 rearing plates, make sure to remove all the residual yeast in the end of the egg laying. This is important to avoid non-uniform feeding during the larval growth."

3.4 Mention After Egg Laying (AEL) times for staged collection (L2, L3) and also suggest what time the larvae must be harvested with respect to when the egg was collected. For example, if the egg collection was between 1-4 pm, after 9 days what time should the larvae be collected? Which is recommended 2.30 pm or 4 pm or some other point?

Response: Typically, what we do is to perform the egg lays in the morning (10am-2pm) and the collection of experimental larvae is done 9 days AEL at approximately same times in the morning, so that the rest of the protocol can be performed during the rest of the day.

In order to address this comment, we added the following information to step 3.5 (lines 196-198). “The larvae collection should be done during equivalent time periods of the day used to do the egg lay (for example, if the egg laying occurred between 10am-2pm, collect the larvae during the same period of time 9 days AEL)”.

4.4 To avoid confusion about multiple timers please change the first part of sentence to "Keep multiple timers set to 1 hour ready."

Response: Thank you for making this step clearer. We changed the sentence as you suggested.

4.5 End the paragraph with "start a timer for each plate to maintain accurate one hour feeding sessions".

Response: This is also a good suggestion. We added the requested information to the end of the paragraph.

4.5/5.2 Why are two sensitive methods used to transfer L3 larvae. Is one better than the other specifically for these steps.

Response: Yes, they are specific for each step. In 4.5, it is advantageous to use the plastic boats as they float for the heat-shock (optional 4.3) and also since they are small, make it easier and faster to transfer the larvae to the macronutrient assay plates. In 5.2, the use of the plates’ lids is more convenient as they are bigger and allow for a better washing of the larvae bodies to remove debris. The lids are also more stable than the boats, contributing to an easier and more efficient larval handling. Concerning the use of the forceps at 5.2 and not at 4.5: this is done only to recover the larvae that are burrowed in the food substrate of the macronutrient assay plates.

5.4 Is it possible to improve the dye extraction by dissecting the gut out to extract the dye if the screen is not too large?

Response: In the context of a small-scale screen, gut dissection would be possible, and we believe it would eventually increase the efficiency of the dye extraction, especially for the genotypes eating small amounts of food. However, this extra dissection step would be much more laborious, and would certainly slow down large-scale screens.

6.1/ line 466 Mention the range obtained in your pilot screen quantification as a reference as to what quantities can be expected.

Response: We have added the requested information in the note part from step 6.1. “In the case of the pilot screen here presented, as the dye concentrations obtained for the larval extracts ranged from 0.02 to 1.93 µL/mL, it was used a standard curve

obtained by measuring the absorbances of 8 serial dilutions of a 2 µL/mL blue-dye solution in methanol. If needed, increase or decrease the concentration of these solutions, depending on the dye concentration of the experimental samples obtained."

6.2 Though suggested in a note later it is imperative that 'zero dye food' control should be used to quantify the dye ingestion especially as entire larvae are crushed to quantify the contents though colorimetry. These controls should be recommended at section 3.4. This is in addition to the attp control for the Gal4 lines.

Response: Please note that because of other additions/alterations, the step 3.4 in the submitted version is now the step 3.5. We added the requested information as an action step and also as a note.

"Additionally, collect groups of 10 L3 for the 'zero-dye food' control."

"The 'zero-dye food' control animals are larvae that in the feeding assay are given food without blue-dye. This control is imperative to remove the background absorbance of the larval extracts."

Also, we split part of the information into a new step, 3.6.

"3.6 Transfer the collected experimental larvae to plastic dish weight boats containing 1 mL of water. Make sure L3 are collected, and not L2, by following the directions given in Figure 3."

Finally, we added "Also, always run the control experiments (the progeny of the "empty Gal4" line crossed to UAS dTRPA1 and the "zero-dye food" larvae) …" to the note in step 4.

B. Corrections by Line number

Line 62-63 'namely mammals' is out of place in the sentence

Response: We substituted "namely" for "including". We hope it sounds better and not misplaced now.

Line 129 Please label L3 diet as "Standard or Regular" diet to distinguish it from the macronutrient diets.

Response: Given the corrections made according to the next comment, we removed parts of the text that we believe were confusing to you and that we agree that would probably generate confusion to the reader too.

“To the standard food, used as L3 rearing diet, and after allowing the medium to cool down, add nipagin and propionic acid solutions to the diets, at a final concentration (v/v) of 3% and 0.3%, respectively.”

Line 129-130 Was Nipagin and propionic acid added only to L3 medium or did the macronutrient diet have them as well. As 'limited shelf life' is not one of the reasons mentioned (in line 135-138) for preparing the macronutrient media fresh.

Response: Thank you so much for noticing this issue in our text. We did add nipagin and propionic acid to the macronutrient diets. This is extremely recommended, especially if the diets are not used in the same day of preparation. We changed the text in step 1.4 so we could make this clear.

“After allowing the mediums to cool down, add nipagin and propionic acid solutions to a final concentration (v/v) of 3% and 0.3%, respectively. To the macronutrient balancing diets, add blue food dye to a final concentration (v/v) of 1%.”

Line 299 Please add reference "Raubenheimer and Simpson 1997" along with reference-23. 23 is a review that describes the protein leveraging effect and mathematical modeling of it, however it is based on data on foraging in insects from this earlier work of the same group. Referring to the latter helps understand the foraging habits and protein content in insects.

Response: Thank you pointing this out. We added the suggested reference, which is now the reference 25.

“25 Raubenheimer, D. & Simpson, S. J. Integrative models of nutrient balancing: application to insects and vertebrates. Nutr Res Rev. 10 (1), 151-179, doi:10.1079/NRR19970009, (1997).”

Line 322-323 What basis was used to consider the 'hits' for lines. How many lines were tested and how many were used for the analysis? How many neuronal gal4 lines are present in the fly-light /Rubin Gal4 database, how many were considered and how many finally are represented in plots (10). On what basis were these 10 chosen. Please add the number of lines used, tested and finally tabulated.

Response: In order to clarify how we selected the lines to use as representative data, we added a paragraph with this information to the Representative Results section.

Lines 355-360 “In the activation screen we tested, in total, 36 Janelia Gal4 lines known to be sparsely expressed in the larval nervous system. Using linear regression models, we determined which genotypes exhibited significantly different

food intake with reference to the genetic control animals. These differences included either differences in the absolute amount of food eaten across all diets, or differences in the macronutrient balancing response (slope of the response to the different P:C ratios of the diets).“

Line 369 Change to "Mechanical lysis of larvae" as the gut is not dissected out to be homogenized.

Response: We changed “Mechanical lysis and homogenization of larval guts’ content” to “Mechanical lysis of larvae”, as suggested (now line 409).

Line 435 What factors were considered when the feeding duration was decided as 60 min? What range of time duration can be quantified using this method? Was egestion after feeding considered as a confounding factor?

Response: The duration we decided to use in this protocol (60 min) was chosen taking into account the following factors.

- Keep a duration of the feeding assay compatible with high-throughput screens, keeping the total duration of the protocol as short as possible to allow to complete all the steps in sections 4, 5 and 6 in one working day;
- Avoid the possible confounding effect of egestion and exposure to a high temperature. We didn´t test if (and when) these effects actually happen, though;
- In our hands and from our experience, assay’s duration ranging from 1 to 2 hours should allow the quantification and observation of relative differences among genotypes tested.

Therefore, and considering your comment, we decided to add the following text to the discussion section (lines 489-494) and also an additional reference:

“This duration is convenient and compatible with high-throughput screen. Also, keeping the total duration of the protocol as short as possible allows the completion of all the steps in sections 4, 5 and 6 in one working day. If it is necessary to change the duration of the feeding assay, assay durations ranging from 60 to 120 minutes should allow an efficient quantification of food intake across genotypes, as previously demonstrated [29].”

"29 Almeida-Carvalho, M. J. et al. The Ol1mpiad: concordance of behavioural faculties of stage 1 and stage 3 Drosophila larvae. J Exp Biol. 220 (Pt 13), 2452-2475, doi:10.1242/jeb.156646, (2017)."

Line 480 Kindly provide Bloomington (BDSC) IDs of all lines used in this study.

Response: In order to provide this information, we decided to elaborate an additional table (Table 2). We hope you find this an adequate solution.

"**Table 2: *Drosophila* lines used in this work.** Detailed information of all the lines used: code name, genotype, associated gene, origin and the Bloomington Drosophila Stock Center (BDSC) number."

C. Figures and Tables

Figure 1 Remove the first two columns as values are the same across all samples. Sample applies to Agar content (uniformly 10 g/L). This will help focus the table on the Protein:Carbohydrate ratios and that the media are all isocaloric.

Response: Thank you for this suggestion, we agree that the table had redundant information. We changed this figure taking into account the comments of the two reviewers. Hope you find it clearer now.

Figure 2 Correct the number of larvae on the plate schematics (Column B and C) to be 10 at all times.

Response: We corrected the number of larvae to 10 in all the schematics of columns B and C.

Figure 3/line 378-379 Add bright field images of II and III instar larvae with scale bar along with the schematic. Another magnified image showing the orange ring tips of third vs second instar larvae should help as identification of stages unambiguously is a challenge.

Response: We agree on how difficult it is to unambiguously distinguish second and third instars larvae, especially without training. Unfortunately, we don't have these images, so we would have to generate them. Because of the imposed lockdown due to covid-19, we are currently not allowed to perform experiments in our institutions. To address this, we suggest the possibility of doing it during the video production phase of the article. We can add this information either by producing a

new Figure 3 with an additional panel for the bright field images, as you suggested, or by showing it in the video for the step 3.6.

Figure 4 Why does the figure have more distance between 1:1 and 1:4 than 1:4 and 1:16 on the X axis. The axis either needs to be equidistant just showing three macronutrient conditions or it should represent the protein:carbohydrate ratios proportionally.

Response: This is because they are represented as scalar values and not categories (the scalar values are not equidistant as this corresponds to 1, 0.25, 0.0625). Plotting them in this way allows us to make inferences about slope. We could plot the ratios as categories, but then our plots differ from our statistical analyses.

Table 1

A. Provide a legend explaining the table.

B. ANOVA: Provide details of the way ANOVA was performed and what is the interpretation of table 1. Please include the method, result and discussion of the ANOVA performed.

Response to A and B: We now provide a legend to the table where we explain what is shown and the method used.

"A linear model was fitted in order to determine the genotypes exhibiting a feeding behaviour significantly different than the control animals."

C. Is there a reason why Food:Genotype attp control is not included in the ANOVA calculations? It would be a useful reference value in the table.

Response to A and B: The control genotype is included in the linear model. All genotypes are compared to the attp control, which means that significant differences shown in Table 1 are significant relative to the control genotype attp.

Additional changes, done by the authors, to the reviewed version of the manuscript

1. We rearranged the order of the authors: CKM is now the last co-senior author, PMD the second co-senior author, and GMP is the corresponding author.

2. We added the following reference to the introduction section (lines 93-96), where we explain how specific P:C ratios of interest can be generated using Yeast-Sucrose diets:

16. Pocas, G. M., Crosbie, A. E. & Mirth, C. K. When does diet matter? The roles of larval and adult nutrition in regulating adult size traits in Drosophila melanogaster. Journal of Insect Physiology. 104051, doi:10.1016/j.jinsphys.2020.104051, (2020).

Please contact us if you require any further information.

Thank you for your consideration.

Sincerely,

Pedro M. Domingos, PhD